Sunday, December 3
Science Education 1

P1001
Board Number: B1
Biomedical postdocs: Salaries and population in the US.
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The number of postdocs in the U.S. is elusive. Estimates of the postdoctoral population, of which the largest area of study is the biomedical sciences, are made difficult by the range of institutional policies and tracking efforts made. We have undertaken a study of the postdoctoral salaries, and therefore the population of postdocs at the time, at U.S. public institutions in response to recent changes to postdoctoral salary recommendations. This has been compared to the NSF’s Survey of Graduate Students and Postdocs (the “GSS”), which was itself evaluated to determine its ability to document postdoc population trends. In summary, while some institutions report postdoc data quite well, there are many caveats with the data that prevent making very clear pronouncements about postdoc data in general. However, it is clear that there are postdocs paid at a wide range of salaries, from the legal minimum of $23,660 to four times as much. We hope this evaluation will cause postdocs and institutions alike to reflect on the postdoctoral experience.

P1002
Board Number: B2
Outcomes From a Novel Online Course, Planning Your Scientific Journey.
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Formulating a scientific question is an essential first step in doing research. So, how does one begin the daunting task of coming up with one? And, what makes a scientific question “good?” These are some of the complex questions we try to answer in a free online course called “Planning Your Scientific Journey.” The goals of the course are to have students (1) evaluate potential research questions and experimental approach with a set of criteria, (2) develop a plan for their research, and (3) prepare for a meeting to discuss their research project and plan with their mentor.
The course is run on the Open edX massive open online course (MOOC) platform. It is aimed at graduate students and postdoctoral researchers, but could be useful for undergraduates and other researchers. Designed with busy schedules in mind, it is 6-weeks long and is meant to take 1-3 hours of work a week.
Our online course is innovative in both curriculum design and video production. Because the topics are complex and somewhat subjective, we applied a unique approach to developing and presenting the content. Videos with “one lecture by one expert” wouldn’t sufficiently convey the diversity of ways to ask scientific questions and plan research. And so, we interviewed a variety of scientists on these topics. We edited the interviews into short documentary-style videos that each focus on one key learning objective with multiple perspectives represented.
We launched “Planning Your Scientific Journey” this past June (2017) and then ran the course a second time in October. Here we present course outcomes, examining both quantitative and qualitative measures to answer five important questions: (1) did we meet our course goals, (2) what was the level of engagement with the course, (3) what did students learn, (4) what is the level of course satisfaction, and, (5) what needs to be improved? Our evaluation includes the analysis of pre-, post- and follow-up course surveys, discussion forum posts, and quantitative data regarding student enrollment, completion rates, video analytics, and satisfactory completion of assignments.

P1003
Board Number: B3
Providing resources and strategies to enhance career training for junior scientists.
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Academia became the minority destination for junior U.S. biomedical scientists in the 1980s. In spite of this, early career researchers (such as graduate students and postdocs) are not being adequately equipped with the tools and resources needed to transition away from the bench into a non-academic job, and are not sufficiently trained in the mentoring and management skills required for academic positions.

Future of Research (FoR) is a nonprofit that helps early career researchers to organize local meetings to discuss problems they experience with science, and propose solutions to advocate for changes. Our aim is to improve the scientific research endeavor and scientists’ holistic careers. We advocate for improved training for early career researchers to help them achieve their desired career goals, as this has been an area of concern highlighted in our first junior scientist-organized conference in Boston in 2014 (see Shaping the Future of Research: A Perspective From Junior Scientists by GS McDowell et al. in F1000Research, 2015).

Our organization discussed this topic during recent workshops held at the American Society for Cell Biology Annual Meeting (ASCB 2016) and NatureJobs Career Expos in Boston (2015 and 2016). In this poster, we present a summary of the data we gathered at these meetings, including barriers to training identified by early career researchers; career development resources that they are aware of; and those resources which they have identified as still needed.

There is a clear gap in the training that early career researchers want, and what training they are able to receive. We call for collaboration in terms of career programming and resources among various stakeholders, for example by centralizing such information in an online database as suggested at the American Society for Biochemistry and Molecular Biology (ASBMB) Sustainability Summit (see Biomedical research workforce summit makes strides in implementing change for postdocs by Viviane Callier in Nature Biotechnology, 2016). Properly training the next generation of scientists for the roles they will play as future scientific leaders is critical to the future of the scientific enterprise.
P1004

Board Number: B4

‘Planting the seed of research in the undergraduate mind’: Conducting mentoring sessions on research opportunities and graduate school by INSPIRE/IRACDA fellows.
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A core mission of the IRACDA NIH-funded postdoctoral training program is to develop a diverse group of trained scientists in the fields of biomedical research by broadening participation at the undergraduate level. What better way to encourage participation of diverse groups than to have undergraduates experience first-hand early in their careers what science is all about? Student engagement in actual research improves their understanding of concepts, retention of information, problem solving skills and ability to apply knowledge to real world problems. However, many students are unaware of the research opportunities available to undergraduates or are intimidated by the application process. To encourage undergraduates to participate in research and pursue careers in biomedical science, we, fellows of INSPIRE (IRACDA program at Rutgers University), have developed mentoring sessions at our minority-serving partner institutions focused on summer research opportunities and graduate school. We provide students with information about local and national opportunities, answer questions, and mentor students through the application process. Our mentoring sessions are different from the conventional information sessions. We tailored the format of the session to foster conversations with students based on our and their personal experiences. These conversations promote development of mentor-mentee relationships between the fellows and the students at MSIs (minority-serving institutions). Here we present how we conduct mentoring sessions at our partner institutions, share student feedback, outcomes, reflections of the fellows and partner school coordinators. We hope that we will inspire others to conduct similar sessions at their institutions to enhance participation of diverse scientists in research.

P1005

Board Number: B5

Sustained teaching mentoring works—and benefits mentors as well as those mentored. An update on the Promoting Active Learning and Mentoring (PALM) Network.
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Many instructors and instructors-to-be have heard of the value of using active learning to guide students to deep learning of course material. Some have attended workshops or several-day immersion experiences to learn how to employ effective active learning activities. In spite of best intentions, plans to actually implement active learning techniques often collapse once an instructor gets caught up in the academic year. To address this situation, ASCB, in cooperation with other professional societies and academic groups and with NSF funding, established the Promoting Active Learning and Mentoring (PALM) Network. This program provides PALM Fellows with a one-on-one teaching mentoring relationship for at least one semester with a mentor experienced in active learning. Participants to date have come from various partner societies within the Network. Analysis of teaching behavior before and after mentoring provides evidence that Fellows have been able to increase their use of active learning in
One faculty member and one graduate student at different institutions were paired by the Mentoring Active Learning and Teaching (MALT) program of the American Society for Cell Biology (ASCB). They worked together to create a Course-based Undergraduate Research Experience (CURE) centered on the research of the graduate student in the field of hematologic development, specifically exploring early embryonic red blood cell maturation. The faculty member had taught undergraduate courses in a flipped format threaded with active learning for years. However, one of the limitations of teaching at a primarily undergraduate institution (PUI) is the lack of access to cutting edge technology. The graduate student works in a large research lab at the forefront of imaging flow cytometry, a technology that combines flow cytometry with cell imaging to allow for analysis of immunophenotype and morphology simultaneously. Together, the faculty member and graduate student centered the class and lab on the use of this and other techniques to compare the maturation of primitive red blood cells to apoptosis in an established cell line, an important research question relevant to the graduate student. Importantly, this faculty member had expertise in adherent cell culture that the graduate student did not. As part of the MALT program, the graduate student travels to the faculty member’s institution, or uses assistive digital technology, to teach several lectures and labs. The faculty member worked with the graduate student on incorporating active learning techniques into his lectures and labs. Undergraduate students became research collaborators with the graduate student, conducting experiments they designed on adherent cell cultures they grew and maintained during the lab. In addition to conducting experiments at their PUI, the students traveled to the graduate student’s institution to conduct experiments at the flow cytometry core facility. Internal funding sources (faculty development funds, departmental funds, NSF funds, private endowments, course fees, and lab fees) from the both the faculty member and the graduate student paid for travel, accommodations, meals, equipment and supplies. Student comments from end of course evaluations revealed that they were motivated to and engaged in learning course content. This experience invigorated the faculty member’s teaching and gave the graduate student hands-on teaching experience in a PUI class and lab. Students presented a poster they created on their research at local and regional student conferences. The faculty member and graduate student were invited speakers for seminars and research panel discussions at each other’s institution. Overall, this MALT and CURE experience proved to be truly collaborative for all involved.
**P1007**

**Board Number: B7**

Mentorship for Developing course-based undergraduate research experiences (CUREs): The CUR/ASCB Mentorship for Integrating Research Into the Classroom (MIRIC) program.

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The life science education community has responded to the recommendations of the American Association for the Advancement of Science (AAAS) Vision and Change document with several initiatives designed to improve the way in which undergraduates learn science. These initiatives have often taken the form of one-time workshops that generate awareness of and interest in developing authentic research experiences for undergraduate STEM classrooms. However, they have been less successful with respect to generating the sustainable change necessary to bring real reform to undergraduate science education. To create sustainable change, long-term faculty development initiatives focused on mentorship are needed so that instructors seasoned in developing and implementing course-based undergraduate research experiences (CUREs) can convey their experiences to mentees interested in using these pedagogical techniques as the centerpiece of their own teaching. The Council on Undergraduate Research (CUR) Biology Division and American Society for Cell Biology (ASCB) Education Committee are creating the Mentorship for Integrating Research Into the Classroom (MIRIC) program to provide a means for members with an interest in developing improved and sustainable active learning techniques to gain experience in this style of teaching through close, long-term interaction with a veteran teaching mentor.

Developed from the former ASCB Mentorship in Active Learning and Teaching (MALT) program, MIRIC focuses on the development of instructors who wish to develop a dynamic CURE. Current and future life science instructors pair themselves up with seasoned veterans of CURE development and work with them and their students over the course of a semester or longer to develop a CURE that will allow the mentee to bring authentic research into his or her classes.

In our pilot studies, we collected qualitative and quantitative data based on participant interviews and coding videos of student and instructor actions during classroom activity (Smith et al., 2013), respectively, that suggest that MIRIC/MALT mentorships have made positive gains in promoting sustainable active learning techniques among participants. Going forward, we wish to use instruments like the Laboratory Course Assessment Survey (Corwin et al., 2015) and Experimental Design Ability Test (Sirum and Humburg 2011) to assess the effectiveness of the MIRIC laboratory intervention.

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**P1008**

**Board Number: B8**

It’s a small world: a cell biology outreach program for young learners.

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In the Cimini lab, we strive to understand the intricate details that drive accurate cell division and what happens to a cell population when these details are perturbed. It is easy for us to see how important and beautiful cell division is, but this can be difficult to portray to a younger audience; therefore, we decided to develop an engaging and inspiring outreach program focused on cell division. In order to learn about cell division, one must first understand the cell, and to understand the cell, one must first comprehend the microscopic scale. Therefore, our program has two parts. The first part is designed to

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encourage visitors to explore the microscopic scale using various types of microscopes, such as a dissecting 'scope or a light microscope, and challenge them to “think small.” The second part of the exhibit consists of an animated tutorial that takes participants through a “microscope wheel”. It starts from something they can see with their eyes in nature, such as a beaver (or other leaving creatures they may be familiar with), and then moves to what is invisible to the eye (e.g., the beaver’s retinal cells). Next, they view videos of dividing cells to learn why cell division is important and discover how something as small as a single cell can create and sustain something as large as an adult animal! This outreach program includes a permanent exhibit housed at the local nature center, as well as a moving exhibit for science festivals or classroom settings. The goal of our outreach program is to provide activities for participants to see with their own eyes what the world looks like under a microscope and to help them understand how important cells and cell division are to perpetuating life. With this program we hope to impart excitement for cell biology onto the future generation.

P1009
Board Number: B9
Share your expertise and enthusiasm with teachers: Organize a workshop to build cheap, homemade microscopes.
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To get low-cost microscopes in front of as many children as possible, we have (1) shared freely a design for a homemade microscope (K.Y.) and (2) have run several workshops for elementary school teachers to build and take away their own microscopes (B.G.). The original microscope design was shared freely online in a 2013 Instructable entitled "$10 Smartphone to digital microscope conversion!" This is a design for a do-it-yourself microscope that takes advantage of ubiquitous smartphone cameras. We envision that like other inexpensive microscopes, these microscopes are likely to encourage children to explore microscopic worlds around them, encouraging curiosity-based learning within and beyond classrooms. This microscope design is relatively unique in that the microscope parts are mostly transparent, making usage intuitive to teachers and children without a need for written instructions. And the microscope parts for this design are readily available, mostly from hardware stores, so teachers or kids can build more microscopes when they want. Based on feedback from teachers, we’ve enlarged the original design to accommodate iPads and other tablets, which are available in many schools and which make it possible for groups of children to interact with each other around each microscope—a significant change from older, traditional microscopes in which only each individual child might know what he/she saw through an eyepiece. For the workshops with teachers, we prepare enough materials for each teacher to build one microscope to take away and use in a classroom. Each such workshop typically lasts 1.5 to 2 hours, during which three things happen: (1) each teacher assemblies a microscope and learns how to build more on their own if they’d like, (2) the teachers use the microscopes to take photos of objects they brought with them (for example, bugs, table salt, cloth, leaves, as well as petals at a resolution sufficient to see individual cells), and they email the photos directly into an online gallery that we project to everyone in the room and that remains online as a picture archive, and (3) teachers brainstorm about how they will use the microscopes with children to best match specific curriculum needs as well as children’s own interests. Current work includes development of outcome assessments with a collaborator, and work to expand the workshops to underfunded school districts. In this poster, we will share a protocol for anyone who would consider running your own workshop(s) with teachers at local schools or museums, and we will bring some microscopes for anyone to try.
P1010

Board Number: B10
Citizen scientists detect pathogens associated with tick-borne illnesses in Ixodes scapularis.
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The *Ixodes scapularis* tick has been expanding its range across the upper Midwest and New England, which is likely to be increasing the risk to individuals of developing tick-borne illnesses. Ticks acquire pathogens from primary reservoir hosts like the white-footed mouse, *Peromyscus maniculatus*, and depending on the prevalence of the primary host in the environment there can be variability in the percentage of ticks carrying the pathogens associated with Lyme disease and Anaplasmosis. Public health data is limited by the number of samples that can be collected; however, with the advent of mobile technologies there are growing opportunities for the public to engage in citizen science. As part of a public education campaign to promote the adoption of behaviors that protect against Lyme disease, we have partnered with local middle school students in the extraction of tick DNA using Biomeme field sample preparation kits combined with portable real-time PCR analysis to test for the presence of pathogens. We have found that the students are able to successfully extract DNA that can be used in RT-PCR analyze and have found that 25% of ticks in Central Wisconsin carry *Borrelia burgdorferi* and 10% carry *Anaplasma phagocytophilum*. Importantly, we found a higher than random coincidence of the bacteria, which may impact disease transmission. Future work will involve pathogen strain analysis and a more in depth understanding of the benefits and challenges of engaging middle students in science.

P1011

Board Number: B11
A Five Week Cell Biology Laboratory Exercise: Characterization of Head and Neck Cancer Cell Growth and Invasiveness.
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The complex nature of cell matrix interactions is a foundational concept of cell biology that proves challenging to teach in a traditional lecture setting. To give students real-world experience with studying this phenomenon, a laboratory exercise was designed utilizing current methods. One key concept is the interaction with and modification of the extracellular matrix (ECM) by cells. Cellular invasion into tissues is an important process for development and organismal homeostasis; this process is critical for the progression of disease states such as inflammation and tumor cell metastasis. In tumor cells, ECM degradation is accomplished by ventral actin-rich membrane protrusive structures termed invadopodia, the formation of which directly correlates with the ability to invade into local stroma. Having real world experience with the formation of invadopodia and corresponding ECM degradation helps learners connect these complex cellular phenomena to the basic principles of cell migration presented in lecture. Over five weeks lab pairs characterized the growth rate and invasiveness of several head and neck cancer cell lines. This laboratory exercise takes advantage of the highly invasive nature of these cell lines to introduce several techniques to undergraduate students: 1) culturing human tissue culture cells; 2) fluorescence microscopy; 3) establishment of a growth curve; and 4) data analysis. Students then presented posters with their results to their peers and two guest judges. Final exam
questions linked to laboratory material showed a significantly higher average score (p< 0.01) than questions that did not have accompanying laboratory exercises.

P1012
Board Number: B12
The yeast orphan gene project: Undergraduates finding a place for ORFans to GO.
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When the genomic sequence of the model eukaryote Saccharomyces cerevisiae was completed in 1996, the expectation was that an understanding of the integrated functioning of the collection of genes in this single-celled eukaryote would shortly follow. Despite almost 20 years of intense collaborative effort among yeast researchers, nearly 10% of open reading frames (ORFs) are considered uncharacterized. Determining the function of these orphan genes (ORFans) will require mining the current yeast genomic data, compiled in the Saccharomyces genome database (SGD), to most effectively design ORF-specific experiments in cell and molecular biology, and comparative genomics. A network of yeast researchers/educators with a focus on teaching experimental design could help overcome the challenges and absorb the risks of researching individual ORFans. The goal of the proposed Yeast Orphan Gene Project is to organize a consortium of undergraduate researchers and faculty at primarily undergraduate institutions (PUIs) to coordinate resources and design strategies to assign molecular functions to S. cerevisiae ORFan. Ultimately, the yeast orphan gene project aims to use the process of determining orphan gene function as a tool to teach undergraduate students key concepts in bioinformatics, genomics, molecular biology, and genetics and impart valuable experience in scientific collaboration and leadership. Based on analysis of SGD information, students will design and execute ORF specific experiments for defining gene function. The network will facilitate collaboration between students at different institutions so that students can share strategies and technique solutions while developing experience in on-line collaborations. These skills, including the ability to (1) apply the process of science, (2) use modeling and simulation, and (3) communicate and collaborate, are core competencies in “Vision and Change”. The proposed network will prepare undergraduates for careers in STEM, provide tools for faculty at PUIs to expand research experience for undergraduates, and incorporate research experiences into undergraduate courses. It will also be a mechanism to distribute a tested model of an authentic course-based research experience to a diverse set of institutions by providing workshop and assessment support. The networking activities will expand the expertise of faculty, as well as provide undergraduates tools and resources for collaboration. Defining the function of yeast ORFans will advance the goal of the yeast community to determine the function of the entire set of annotated yeast ORFs.

P1013
Board Number: B13
Steel City Blues: Leveraging a Legacy of Pollution for Research and Reflection in Introductory and Advanced Undergraduate Biology Courses.
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Place-based education (PBE) is a form of “in situ” science pedagogy that helps cultivate a sense of place among students as they interact with their local environment and community. PBE engages students
more effectively than traditional science curricula, promotes self-efficacy, and may play a role in identity formation. Despite these benefits, use of PBE has largely been confined to K-12 environmental education in rural settings. The goal of this project is to test the efficacy of PBE in three undergraduate biology courses at an urban liberal arts college. Specifically, heavy metal pollution from local industries is used to frame course-based undergraduate research and critical reflection. In an introductory cell and molecular biology course, students isolate and characterize bacteria from contaminated soils, looking for evidence of co-evolution of resistance to heavy metals and antibiotics. Quantification of interactions between microbes primes students for a subsequent course on evolutionary ecology. In an introductory organismal biology course, students use *Drosophila melanogaster* as a model organism to study the effects of heavy metals on development and behavior. They also develop and test hypotheses regarding the ability of diet to mitigate these effects. In an upper-level course on genetic engineering, students apply concepts from user-centered design to construct novel whole-cell bioreporters for chromate. Whereas the introductory labs will be implemented in Fall 2017, the 2016 pilot of the genetic engineering course suggests that this version of PBE is effective at engaging students. In pre-/post-surveys, 80% of students self-reported gains in “understanding how scientists work on real world problems” and 60% of students self-reported increased “ability to tolerate obstacles in the research process.” These gains are particularly notable, given that these students were all seniors, many of whom had prior undergraduate research experience. This cohort (*n*=10) also reported statistically significant gains in their readiness for more challenging research, their ability to contribute to science, and their understanding of the process of science (*p*<0.05). At the beginning and end of the term, students completed critical reflections addressing civic engagement. Rubric-driven analyses of these reflections suggest a possible increase in students’ abilities to articulate the relationship between their research and public interests, as the average score increased from marginal to proficient, and this difference approached statistical significance (*p*=0.1). Consistent with a limited number of reports in the literature, these preliminary data suggest that PBE can be adapted to engage urban undergraduates in STEM.

**P1014**

**Board Number: B14**

A semester-long cell biology research experience: from novice to project owner.

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Participation in authentic research experiences has been shown to increase student persistence, knowledge, scientific thinking and correlate with long-term career satisfaction. Incorporating authentic research into the undergraduate curriculum via course-based undergraduate research experiences (CUREs) is one mechanism for reaching a wider audience of students than classic apprenticeship models. Metrics for assessing student gains in research experiences include measures of experimental design ability and sense of project ownership. In this project, the lab component of a cell biology course was designed to introduce students to common methods used in molecular cell biology, and to encourage gains in scientific thought, understanding of the process of research and critical thinking through an extended independent project. In the first section of the course, students were introduced to key cell biology techniques via 1-2 week long modules, including mammalian tissue culture, transfection, immunofluorescence, Western blotting and data analysis and quantification. Each of these modules was designed as a scaffolded experience, with increasing independence as skills were gained and experimental design was understood to a greater degree. In the second section of the course, students worked in small groups to identify an extension of their preliminary data from the skills section, or to identify a new question related to course material, and to design and execute experiments to address this hypothesis. At the conclusion of the course, students presented their work in both oral and written
formats. To assess gains made in this research experience, the Experimental Design Ability Test (EDAT) and the Project Ownership Survey (POS) were used. Students who completed the course made large gains in their experimental design abilities, with EDAT scores of 3.7 (pretest) and 7.2 (posttest) on a 10-point scale. Additionally, students reported high degrees of ownership and satisfaction on both the POS and in written evaluations of the course. Together, these data indicate that this course improved scientific thought/experimental design abilities of students, and gave students a sense of place within the community, both of which are positive outcomes associated with undergraduate research experiences. Ideas for future iterations and improvements of the course will also be discussed.

P1015
Board Number: B15
CUREs for Everyone: Introductory Biology Lab Course Converted to Research Experience.
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The seminal educational report Vision and Change calls upon scientists and educators to provide all their students with authentic research experiences in teaching laboratories. However, most laboratory courses do not simulate how real scientists conduct research. To provide students with a more genuine research experience in their first laboratory course, we have incorporated principles of iteration and relevance into an introductory course. We have expanded on our previous use of pClone (Campbell et al., 2014) so that students can design and test potential promoter sequences, ribosomal binding sites (rClone; Eckdahl et al., 2017), or modify promoters regulated by a transcriptional activator (actClone) or a transcriptional repressor (repClone). Students read research papers and design DNA sequences to test in one of four plasmids (pClone, rClone, actClone, or repClone). Each group clones an engineered DNA fragment and determines its function. Student groups present their results to the other three groups who have not worked with their particular plasmid. Based on the first round of results, all four groups can choose any plasmid for the second iteration and revise the DNA sequence to be tested. The students repeat the cloning and measuring procedures and then confirm the DNA by sequencing. To determine the effects of this course and the potential benefits of iteration on learning, student’s scientific self-efficacy, identity (Chemers et al., 2011), and career intentions (Estrada et al., 2011) will be measured before and after the laboratory course. Additionally, the Project Ownership Survey (Hanauer and Dolan, 2014) and Laboratory Course Assessment Survey (Corwin et al., 2015) will be administered at the end of the course to determine if potential gains are similar to those of Course Based Undergraduate Research Experiences (CUREs) that are usually restricted to upper level courses. We will present the results of this novel educational experiment that will be conducted in the fall of 2017 with first-term, first-year students. If successful, this model for introductory laboratory courses could be scaled to a national level at no additional costs to institutions.

P1016
Board Number: B16
Evaluating Cures as a Model for Persistence and Success in Science Majors and as a Model for Accelerating Departmental Curriculum Change.
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Research based learning taking place in biology laboratories had been identified an efficient way to engage students and to teach undergraduates about scientific research. Scientists make good teachers

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when they can guide student learning through discovery of information rather than its direct transmission (Boyer Commission, 1998). However, it is a challenge to provide laboratory access to a large number of students owing to the intense time commitment of mentored research and limited seats available across various institutions (Olson & Riordan, 2012; Wood WB, 2009). CUREs (Course Based Undergraduate Experiences) offer a solution to this issue by incorporating authentic research experiences in a course-based format accessible to large populations of undergraduates (Auchincloss et al., 2014; Brownell & Kloer, 2015, Rodenbusch et al., 2016). In this work, we present three CURE-based outcomes. First, we highlight efforts to implement semester-long CUREs with model organisms (Zebrafish, C. elegans) to study human disease and genomics (Sarmah et al., 2016). Starting first with freshman biology labs, we are now developing CURE modules using model organisms into cell biology, genetics and microbiology labs. Second, we present a plan to develop, coordinate, and build connections between lab courses to produce a comprehensive CURE experience across the curriculum via multiple biology courses from freshmen to senior level. This will include revising existing courses with CURE based learning outcomes using the participant perception inventory as well as measuring student learning gains with robust experimental design assessments. Third, we share a blueprint for implementing departmental transformation to enhance teaching and learning via CUREs. Creating integrated CURE experiences between courses supports a departmental teaching model where both faculty and students understand the benefits of developing a “researcher mindset” in undergraduate students, where key concepts, techniques, and modules introduced in one course become the foundation for additional learning and skill development in successive courses, from freshman year through the senior capstone. This department wide effort to progressively implement CUREs carries the potential to contribute significantly to the knowledge of the educational effects of CUREs as well as provides original research findings of interest to the scientific community.

P1017
Board Number: B17
Investigating the Role of Authentic Research Experiences on Science Identity of African American women.
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The President’s Council of Advisors on Science and Technology (PCAST) calls for a million more STEM professionals by 2022 to meet growing economic and global demands in the STEM fields (PCAST, 2012). In 2012, African Americans (AAs) accounted for only 7.5 percent of all STEM undergraduate degrees and only 4.5 percent of doctoral degrees awarded in STEM (NCES, 2014). In order to meet this need and to encourage diversity in these efforts, underrepresented populations that include women and AAs must be supported in their progress toward attaining STEM degrees. Women and (AAs) students are already underrepresented, and continue to leave STEM majors. Therefore, it is important to understand factors contributing toward under-representation and also to determine what elements help to promote attainment among under-represented students in order to create a strong and diverse workforce (National Science Board, 2007; Griffith, 2010). Previous literature shows that participation in authentic research experiences has positive influence on students’ level of engagement, interest in science, and retention in STEM majors and careers (Elaine, 2004; Eagen, 2013, Lopatto, 2007;Russel, 2007). Therefore, we have introduced undergraduate biology AAs female students to authentic research experiences in matrix biology. Their research involves investigating the role of the animal heme-peroxidase, Peroxidasin, in the cross linking of collagen IV molecules within the basement membranes. The goal of our research study is to identify factors within exposure to authentic, research experiences
that influence the career decision-making of AA women in the sciences. We will investigate the intersectionality of these factors between institutional cultures of predominantly white institutions (PWIs) and historically black colleges and universities (HBCUs).

**P1018**  
**Board Number: B18**  
**Student Retention using a Forensic Science Based Approach.**  
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¹Biology, Lane College, Jackson, TN

Lane College is a historically black college committed to educating underserved minority students. My goal is to give students a diverse teaching and research experience that includes implementing course-embedded undergraduate research to aid the students in understanding the scientific process and incorporating a major active learning/inquiry based learning component and improve retention by engaging students through forensic sciences. Through the popularity of television shows like The First 48, CSI, NCIS, and Cold Case Files the study of forensic science has flourished and given teachers a method to teach biology, chemistry, and physics. To implement forensic science in General Biology I in the Spring 2018 semester first students will study and determine the items that need to be analyzed from a mock crime scene the first week of laboratory. Each additional week the students will participate in microscopic hair analysis, comparison of animal and human hair, fabric analysis, soil analysis, synthetic blood tests, and DNA fingerprinting. Secondly, the students will have active learning/inquiry based learning exercises in class with a forensic science focus. My objective is that in this environment it will foster the students to ask questions, research background information, design experiments, gather and analyze the data obtained leading them to understand the method of scientific discovery. Hopefully through this process the students will learn to collaborate and build their critical thinking skills while becoming self-learners. I will develop an assessment strategy and rubric to evaluate student learning outcomes that will include testing of hypotheses, recording observation in a laboratory notebook, and communication of results through written reports and oral presentations.

**P1019**  
**Board Number: B19**  
**A Peer-Led Team Learning Strategy for Course-based Undergraduate Research in General Biology.**  
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¹Biology, Lane College, Jackson, TN

Lane College is a historically black college with a commitment to educating underserved minority students. Our overall goal for this project is to improve student retention in STEM through student-centered activities within our first year foundational courses. These approaches include course-based undergraduate research experiences (CURE) within the General Biology II laboratory course. The instructors who teach this course are developing a CURE project based on their research interests, including environmental microbiology and plant genomics. For our model, we are assigning a more experienced student to assist the instructor and serve as a peer mentor for students enrolled in each section of the course. During the Spring 2017 semester, we began training our first cohort of peer mentors through the traditional apprenticeship research model. Six students majoring in biology worked on research projects either in environmental microbiology or genomics. These students assisted with the development of protocols and obtained baseline data for the CUREs to be implemented in the Fall.
2017 semester. For our environmental microbiology project, each student cultured bacteria from the Lane College campus, performed gram staining, and completed initial antibiotic resistance screens. They isolated three different bacteria strains that are resistant to penicillin, ampicillin, and amoxicillin. These strains included a gram-negative coccius, a gram-negative bacillus, and a gram-positive bacillus. In addition, the students optimized the amplification of the 16s rDNA gene and the beta-lactamase TEM1 gene from Escherichia coli transformed with the pUC19 plasmid using polymerase chain reaction (PCR). For our plant genomics project, our students will amplify and sequence the GAPC gene from Tennessee native plants, as well as the model organisms Arabidopsis thaliana and Ceratopteris richardii. We are currently developing assessment strategies and rubrics to evaluate student learning outcomes of the process of science, such as development and testing of hypotheses, recording observations within a laboratory notebook, and communication of results through oral presentations and written reports. We plan to fully implement these CURE laboratory exercises in General Biology II laboratory courses during the 2017-2018 academic year.

P1020
Board Number: B20
Assessment of Mapping the Brain, a research and neurotechnology based approach for the modern neuroscience classroom.
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Neuroscience research is changing at an incredible pace due to technological innovation and recent national and global initiatives such as the BRAIN initiative. The BRAIN initiative, launched by the White House in 2013, rivals the Human Genome Project in scale and is propelling the field forward through the development of novel neurotechnologies. This transformation of modern neuroscience requires that we reflect on our current approach to neuroscience education, identify best practices, and consider new strategies in the classroom. Given the wealth of data supporting the value of course-based research experiences for students, we developed and assessed a neurotechnology- and inquiry-based course: Mapping the Brain. The goal of the course is to immerse undergraduate and graduate students in research and to explore emerging technologies in neuroscience. In the laboratory portion of the course, students pursue a hypothesis-driven, collaborative National Institutes of Health research project. Using chemogenetic technology (Designer Receptors Exclusively Activated by Designer Drugs-DREADDs) and a recombinase-based intersectional genetic strategy, students map norepinephrine neurons and their projections, and explore the effects of activating these neurons in vivo. In lecture, students compare traditional and cutting-edge neuroscience methodology, analyze primary literature, design hypothesis-based experiments, and discuss technological limitations of studying the brain. We evaluated this course over two consecutive years in the Biotechnology Program at North Carolina State University, assessing achievement of student learning outcomes and knowledge of Society for Neuroscience’s (SfN) core concepts and essential principals of neuroscience. Using analysis of student assignments and pre/post content- and perception-based course surveys, we found student’s attained the course-learning outcomes. Next we assessed whether the course improved student primary literature analysis and neurotechnology assessment. Our analysis revealed the course increased student confidence in their ability to analyze primary research articles and methodologies. Students also report greater focus on data while reading articles post course. In summary, through the integration of authentic research and a neurotechnology focus, Mapping the Brain provides a unique model for an introductory
neuroscience course. Students finish the course with new technical and intellectual skills along with knowledge of SfN’s core concepts and essential principles of neuroscience. We hope that Mapping the Brain and similarly designed courses, which actively engage students in the scientific process and cutting edge research, will inspire a new cohort of innovators in the field of neuroscience.

P1021
Board Number: B21
Integrating Cell Biology Concepts: Comparing Learning Gains And Self-Efficacy In Live And Virtual Undergraduate Lab Experiences.
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Multiple pedagogical approaches, such as experimental experiences or computer-based activities, have been shown to increase student learning and engagement. We have developed a laboratory module that includes both a traditional “live” experimental component and a student-designed “virtual” computer simulation component. This laboratory employs the mating pathway of the model yeast Saccharomyces cerevisiae to demonstrate four fundamental cell biology concepts: cell signaling, cytoskeleton, cell cycle, and cell cycle checkpoints.

In the live laboratory, students add mating pheromone to yeast cultures, then measure the rate of cell division and changes in morphology characteristic of the S. cerevisiae mating response. The “virtual” complement to this laboratory was designed using the principles of Design Thinking in collaboration with an undergraduate Computer Science course. The students generated two computer simulations which can support the live laboratory or provide a virtual laboratory experience.

We assessed the live and virtual laboratories at two undergraduate institutions by measuring student learning gains for three learning objectives: applying fundamental cell biology content, developing laboratory skills, and predicting novel experimental outcomes. Students who completed the live lab and the simulation, or the simulation alone, demonstrated gains across all three objectives, with greater gains for students who completed the live lab. Students who performed neither the live nor virtual lab had no significant learning gains. In a student attitudinal survey, students reported positive perceptions of their learning gains, engagement, and self-efficacy after performing the live and virtual labs.

Our data demonstrate that students at both campuses achieved learning gains in key cell biology topics and acquired an improved sense of self-efficacy as a result of performing this laboratory. We believe that the successful implementation of this laboratory at two institutions of varying selectivity indicates that this exercise has a broad appeal for a wide variety of students. The virtual laboratory simulation provides an opportunity for students not enrolled in a laboratory course to achieve some of the learning gains observed with the live laboratory; however, our data underscore the value of a hands-on experimental experience.
P1022
Board Number: B22
Fine-tuning summer research programs to increase underrepresented students’ scientific identity.
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The Leadership Alliance, a consortium of leading PhD-granting and minority-serving institutions (MSIs), leverages partnerships to place underrepresented (UR) students from MSI and non-MSI institutions in competitive research environments through its national Summer Research Early Identification Program (SR-EIP). Here, we sought to identify components of this summer program that increased students’ awareness of and commitment to research careers and enhanced research knowledge and skills. Using longitudinal pre/post data collected from student surveys, we applied social cognitive career theory as a conceptual framework to examine how research engagement, skill development, and mentorship aspects of this program affect students’ pursuit of research careers. Self-reported knowledge of research skills, time engaged in research activity, and students’ understanding of and attitudes toward pursuing graduate study were measured in relation to the classification of students’ home undergraduate institution, level of students’ pre-existing research experience, and demographic factors. All research skills knowledge items evidenced significant positive post-program change. The highest degree of mean rating increase change included bibliographic software (49.8%), statistics software (30.2%), and approaches to quantitative data analysis (26.7%). Pre-survey respondents were most likely to agree that they understood the careers available in their discipline (72.9%), and least likely to agree that they had an understanding of graduate school life (60.4%) or the graduate school application process (62.0%). The majority of post-survey respondents (96.0%) agreed that they understood the graduate school application process, understood graduate school life (91.8%), and understood the careers available in their discipline (90.4%). Regression analyses show that time spent on research preparation, learning activities, professional development, data gathering, and analysis was positively associated with significant gains on career pathway variables and the research skills index. Mentor quality was positively associated with significant gains in research skills and knowledge of graduate school life index. While student demographics and institutional characteristics were not significantly associated with pre-program measures, modest significant differences were observed post-program with regard to research skill development and academic career planning. Our results provide evidence of specific programmatic components that positively impact students’ research skills and career planning. The acquisition of research skills and increased awareness of graduate programs and careers contribute to the ongoing formation of motivational behaviors that shape subsequent career pursuits.
P1023
Board Number: B23
A short authentic research module increases complexity in student thinking about research without sacrificing student experience or content knowledge.
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The AAAS Vision and Change (2011) for undergraduate biology students identified the Ability to Apply the Process of Science as a core competency. We embedded an authentic research module in an existing introductory Cell and Molecular Biology course to provide practice in experimental design, experimentation, and analysis to a large number of students. The three-week laboratory module addresses the fundamental cell biological question of how cell cycle is regulated using the model organism Tetrahymena thermophila. The protein CDT1 is required for licensing origins of replication, and its mutation has clear human health impacts. Students used a strain of Tetrahymena that can overexpress the protein CDT1 to determine what role CDT1 plays might play in Tetrahymena. Our research module runs during part of an academic quarter, and students draw on their existing knowledge from the classroom and from previous laboratory sessions to engage with the process of science during the relatively short timeframe. In-class and take-home activities prompt students to actively reflect upon the information they are using to design their experiments and to draw their conclusions. This short authentic research module serves as a model for introducing authentic research into large, diverse, introductory courses.

We used pre-post surveys, task-based questions, and focus group discussions to assess how the authentic research module affected the student experience. Pre-post survey responses of students who did and did not participate in the authentic research module suggest that participation did not change student opinions of their engagement with the process of science, and in particular with problem solving in Biology. However, students who participated in the authentic research module gave more, and more sophisticated, responses to a task-based question that required practice in self-direction and self-reflection. Focus group interviews with students at a variety stages of their undergraduate Biology education support our assertion that short authentic research module succeeded in providing choice to students, which helped them feel engaged and invested in the research experience. These findings will help inform the how we study students’ engagement with the process of science, and how best to enhance course-based research at the undergraduate level.

P1024
Board Number: B24
Design-based and Interdisciplinary Strategies for Learning in Laboratory Sciences.
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We developed a model curriculum for interdisciplinary STEM teaching and learning utilizing a collaborative and inquiry-based approach to Cell Biology laboratory. The pedagogical goals were to develop, adapt, and refine design-based inquiry approaches to laboratory experiences using real-world student-developed research studies; incorporate learning technologies -- such as interactive and video tutorials -- into the curriculum; and assess the impact of this approach on student understanding of scientific concepts and processes and attitudes toward science and research. To most effectively model
active learning and the collaborative nature of science, students in Cell Biology worked alongside students in Biochemistry and Mathematics courses throughout the semester. The differences between pre- and post-Participant Perception Indicator (PPI) survey responses in each question in the categories understanding, skills, attitudes, integration of learning, and meeting learning objectives (relative to the baseline semester) indicated that, when paired with like students, high-performing students are more likely to report improvements in the areas of skills, integration of learning, meeting learning objectives, and understanding, while mid-level performing students report significant improvements in the categories of understanding, attitudes, and meeting learning objectives. Low-level performing students report regressions in these areas. Performance task quiz data reveal improvements in critical thinking through our pedagogical approach to teaching laboratory sciences.

P1025

Board Number: B25

Inquiry-based cell culture course improves student conceptual and practical understanding of biomedical research.

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In order to develop strong scientific thinking abilities in the context of cell biology experimentation, we have developed a laboratory-intensive undergraduate cell culture course for acquisition of a broad technical skill set along with inquiry skills necessary to conduct scientific research. Our “Fundamentals in Cell Culture Technology” course engages students in hands-on projects that require them to develop hypotheses, design and conduct experiments, analyze their results, and present their conclusions to their peers. Proliferation, viability, and differentiation modules promote the gaining of cell culture skills including aseptic technique, mammalian cell line maintenance, cryopreservation, condition optimization, and critical thinking in the scientific method. We hypothesized that the course would produce technically competent students who are confident in conceptual knowledge, capable of scientific inquiry, and interested in pursuing areas of biomedical research. To assess the students’ growth in these key areas, we administered pre- and post-course self-efficacy and career aspirations surveys and conducted the Experimental Design Ability Test (EDAT) for each of the three semesters that the course has been offered. Our analyses show a positive effect on the students’ confidence in their ability to design and conduct experiments and to present their conclusions from those experiments. Students indicate a better understanding of biomedical research and a slight increase in their interest in biomedical research above the already high level observed at the beginning of the course. Post-course evaluation of experimental design ability did not demonstrate significant improvement. Our future work includes assessment of experimental design ability in the specific context of cell biology as well as the development and assessment of new modules to teach flow cytometry, gene expression analysis, and primary explant culture.
P1026
Board Number: B26
Implementation and assessment of an intensive 7 week summer CURE designed for incoming freshmen.
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MOL152 is an intensive summer course-based undergraduate research experience (CURE) that is part of the Freshman Scholars Institute (FSI) program at Princeton University. The program allows first generation and low-income students an opportunity to take two courses during the summer preceding their freshman year. This CURE was designed with the goals to increase the conceptual knowledge and technical and analytic skills of the students, while also promoting a sense of belonging, identity and self-efficacy in research. The specific research project was designed to allow for a parallel project structure which gives the students project ownership. Working in the C. elegans model system, the roles of serotonin receptors in modulating behaviors such as pharyngeal pumping, egg laying and locomotion were investigated. Student pairs were assigned a specific serotonin receptor gene target and used feeding RNAi to knockdown expression of their target gene in either neuron cells or all tissues except neurons through the use of tissue specific RNAi sensitive strains. The curriculum is designed so that students have the chance to critically evaluate experimental data, formulate hypotheses, and design follow up experiments. Integrated into the course are opportunities for guided reading of primary literature related to the project. Students write lab reports and deliver oral presentations to report their results and the course culminates with the publication of a Princeton version of the Worm Breeder’s Gazette. Pre and post surveys were implemented to the measure the impact of the CURE on student concept knowledge, skills, attitudes and mindset. Analysis of the survey results indicates that the course has enhanced student content knowledge and writing and data analysis skills based on their own assessment of their learning. The course also resulted in changes in student attitudes and mindsets. The course has had a positive impact on student learning and identity as a STEM major. Further analysis of the survey data will help determine the ability of this course to impact the success and satisfaction of first generation and low-income students interested in pursuing STEM majors.

P1027
Board Number: B27
An Inquiry-Driven Optical Tweezer Experiment for Upper Division Physics Lab.
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Medical physics and biophysics graduate programs have become attractive next steps for physics undergraduates. To better serve such students, an inquiry-driven optical tweezer experiment was developed as part of an upper division biophysics lab course offered by the physics department at the University of Illinois. We supplemented the inexpensive and well-supported kit from Thorlabs with an E. Coli mutant strain (KAF95) acquired from Howard Berg at Harvard University. The eight hour lab was taught in two four-hour parts. In the first, students learned basics on the biology, capabilities of the instrument, and analysis techniques. During the second, students came up with and conducted their own experiments in groups of four. While the lack of experiment time meant none of the datasets were large enough to draw clear conclusions, students received points on clarity of their hypotheses and
effective use of the instrument. Post-lab surveys were extremely positive: nearly all students indicated a preference for more labs in the course to be taught in a similar style.

**P1028**

**Board Number: B28**

*A bioinformatics curriculum that teaches gene structure is associated with learning gains for beginning college students.*

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The related features of eukaryotic gene structure, transcription, alternative splicing, reading frames, and translation are complex molecular biology concepts that are often taught to beginning biology college students using a lecture format in a traditional classroom setting. Computer-based bioinformatics tools, such as a genome browser, can be used in an active learning strategy to provide a hands-on approach for students to visualize and understand these challenging concepts. One of the major barriers to using such an approach has been the lack of available curriculum for faculty who are not experts in bioinformatics. We have created a series of six teaching modules that make use of a UCSC Genome Browser mirror maintained by the Genomics Education Partnership (GEP) to illustrate these biological concepts. The genome browser allows one to see the “big picture” for multi-exon genes, provides translation to exhibit the alternative reading frames, and lets the student zoom in to find specific signals such as the start codon. These teaching modules and associated videos are available through the “Introducing genes” section of the GEP website (http://gep.wustl.edu). The modules have been used in a variety of classroom settings at diverse institutions, and have been shown to be associated with learning gains for this molecular biology content, as well as helping students develop familiarity with a genome browser, a basic bioinformatics tool. The knowledge gains achieved through the use of this introductory curriculum provide students with the skills needed to participate in classroom-based research experiences in genomics that build on careful gene annotation. The GEP is collaborating with Galaxy to create G-OnRamp, a tool that creates a genome browser (in UCSC or JBrowse formats) for newly sequenced genomes, thereby allowing students to participate in a variety of genomics research projects. [The GEP is currently supported by NSF IUSE grant #1431407, while development of G-OnRamp is support by NIH grant R25 GM119157, both to SCRE.]
P1029
Board Number: B29
Bioinformatics Core Competencies for Undergraduate Life Scientists.
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Bioinformatics is becoming increasingly central to research in the life sciences. Despite its increasing importance, however, there is a general lack of integration of bioinformatics skills and knowledge into undergraduate biology education. This curricular gap prevents biology students from harnessing the full potential of their education and limits their career opportunities. In order to advance the integration of bioinformatics, a framework of evidence-based core bioinformatics competencies is needed. To that end, the Network for Integration of Bioinformatics in Life Sciences Education (NIBLSE; “nibbles”), an NSF Research Coordination Network for Undergraduate Biology Education, recently conducted a survey about the teaching of bioinformatics to undergraduates. The survey was targeted to life sciences faculty in the United States (n = 1,260). Results indicate widespread agreement that bioinformatics knowledge and skills are critical for undergraduate life scientists and considerable agreement about what specific bioinformatics skills are necessary. Perceptions of the importance of some skills varied with the respondent’s degree of training, time since degree earned, and/or the Carnegie classification of the respondent’s institution. To assess which skills are currently being covered in curricula nationwide, we analyzed syllabi submitted by survey respondents. Finally, we used the survey results, the analysis of the syllabi, and our collective expertise and experience in both teaching bioinformatics and applying it to research to develop a set of bioinformatics core competencies for undergraduate life sciences students. NIBLSE is supported by a Research Coordination Network for Undergraduate Biology Education grant from the National Science Foundation (DBI 1539900).

P1030
Board Number: B30
Application of DNA Sequence Analysis Software in Undergraduate Biology Curriculum.
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Providing inquiry-based tools to students as they actively learn to link concepts in molecular biology and genetics is an important way to help students develop critical thinking and analyses skills. The challenge in undergraduate curriculum at a primarily undergraduate research institute, where resources are limited, is developing easily accessible tools that are both authentic and engaging for students, all while being applicable to both lecture and laboratory components of the course. Here, we describe how open source DNA sequence analysis software, such as A Plasmid Editor (ApE), which is used by many Research-1 labs, is integrated into Genetics and Molecular Biology courses at two different PUs. The learning objectives for using molecular biology software as an integrated instructional tool is for students to become competent in the functionality and applications of such tools, and then use these tools when applying genetic and molecular biology principles in research-based setting. To achieve
these learning objectives, we have developed exercises where students use open source software in both class and lab work, in both guided instruction and inquiry-based experiences. During the instruction phase students are taught to use this software in lab, using these tools to build plasmid maps and annotate features, determine restriction enzyme sites, and DNA fragment sizes, while concurrently performing the corresponding guided experiment. Once familiarity is gained, students then apply these skills and tools to inquiry-based experiments including the analysis of single nucleotide polymorphisms in alleles for a gene isolated from their own DNA and or generating homology directed repair templates for CRISPR-based gene edits. In class, these software platforms become an active instructional tool where students use molecular biology software as a complement to traditional didactic instruction. Using such software allows for more inquiry-based assessment such as analysis of DNA sequencing data, identification of mutations, and determining how the molecular lesion affects the reading frame and translation. Pre-and post-assessment of utilization of the application of open source molecular biology software in coursework reveals the effectiveness of this approach in promoting higher order thinking in undergraduate biology students and confidence in ability to understand experimental design and analyze data.

P1031

Board Number: B31

Incubators: A community based model for improving the usability of bioinformatics learning resources.


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The Network for Integrating Bioinformatics into Life Sciences Education (NIBLSE) is an NSF-funded Research Coordination Network that aims to establish bioinformatics as an essential component of undergraduate life sciences education. As part of that effort, NIBLSE is working to make existing bioinformatics learning resources more accessible to non-specialists and to increase their use across undergraduate biology courses. To this end, NIBLSE has partnered with the Quantitative Undergraduate Biology Education and Synthesis (QUBES) project and CourseSource to develop and implement a novel model for supporting the refinement, publication, and dissemination of high quality bioinformatics teaching resources. NIBLSE Incubators are small, short-lived, online communities that work with an existing learning resource to (1) improve its usability across diverse life sciences classrooms, (2) introduce and teach important bioinformatics learning outcomes, and (3) move the learning resource toward publication and broader dissemination. Incubator pilots have highlighted the opportunities and challenges of this approach and led to the refinement of the development process. NIBLSE and QUBES are supported by grants from the National Science Foundation (DBI 1539900 and DUE 1446269, respectively). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.
P1032
Board Number: B32
New NIGMS Funding Opportunities: Collaborative Program Grants for Multidisciplinary Teams (RM1); Maximizing Investigators' Research Award (MIRA) (R35); Technology Development (R21, R01).

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1National Institute of General Medical Sciences, National Institutes of Health, Bethesda, MD

The National Institute of General Medical Sciences (NIGMS) has initiated several research grant funding announcements Collaborative Program Grant for Multidisciplinary Teams (RM1). Contact: Paul Sammak The Collaborative Program Grant (PAR-17-340), has replaced the Program Project Grant (P01). The first receipt date is January 25th, 2018. It is intended to support complex and challenging research within the NIGMS mission that require multidisciplinary teams from 3 to 6 investigators and between $700,000 and $1,500,000 direct costs. Research questions should need the integrated efforts from different areas of intellectual and technical expertise. Projects should have a unified scientific goal that will produce convergent, lasting scientific benefits or produce major advances in their fields. The description of the research program is limited to 30 pages. A separate 6-page section on team management is required. Leadership structures should be designed to match the research program goals and to enhance Interdisciplinary team performance. An additional $250,000 for an early stage investigator pilot program may optionally be included. Maximizing Investigators' Research Award (MIRA) (R35). Contact Kris Willis The MIRA (PAR-17-094, and for Early Stage Investigators, PAR-17-190) provides support for the NIGMS mission-relevant research and provides support for the research program rather than a project with defined specific aims. The goal of the MIRA program is to increase the efficiency of NIGMS funding by providing investigators with greater stability and flexibility, thereby enhancing scientific productivity and the chances for important breakthroughs. Another aim of the program is to help distribute funding more widely among the nation’s highly talented and promising investigators. Technology Development without Biomedical Hypotheses. Contact Paul Sammak Projects should be focused on technical questions that aim to develop and validate novel research tool including instruments, devices, algorithms and software, chemical reagents and processes for modified molecule production, and the manipulation of biological systems. The eventual biomedical significance of the technology should be apparent, but the work plan must not include testing of unknown biological questions. The R21(PAR-17-046: Exploratory Research for Technology Development) is a high-risk feasibility test of a concept that has not been evaluated with preliminary data. The R01 (PAR-17-045: Focused Technology Research and Development) is for later stage project that are feasible but where technological obstacles remain.

New Technologies in Light and Electron Microscopy

P1033
Board Number: B34
Applying Machine Learning and Pattern Recognition for Accurately and Rapidly Determining Cellular Signaling Status.

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Two frequently used tools to acquire high-resolution images of cells are scanning electron microscopy (SEM) and atomic force microscopy (AFM). Many of those images have been used to determine cellular
P1034

Board Number: B35

The comparison of 3D imaging methods of electron microscopy for phages.

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After the infection, viruses plunder the system of the replication, transcription and translation, and grow proliferously in host cells. Finally, host cells were burst by viruses were grown in cells. Therefore, some viruses are cause of lethal diseases. Knowledge of the 3D structure and distribution of viruses in cells play very important role of elucidation of mechanism of these diseases and developments of their therapies. However, the size of viruses is too small to observe by the optical microscopy. Scanning electron microscopy (SEM) can observe only surface structure, and Transmission electron microscopy (TEM) can observe only thin 2D area. Thus, little is known about the 3D structure and distribution of viruses in cells. Recently, some 3D imaging methods with SEM were developed, and some study reported that these methods were applied to biological sample. In this study, we analyzed the 3D structure and distribution of phages (T4, λ, Φ174) in E.coli with these new SEM methods. We used four methods in this study, and compared these methods. All methods required for samples that were stained by heavy metals and embedded by resin. First method is Serial block-face SEM (SBF-SEM) method. This method uses the SEM that has ultra-microtome in the specimen chamber; samples are able to slice in specimen chamber. This method repeats to slice the sample and observe the new surface. Finally, we can obtain 3D image to stack serial slice images. Second method is FIB-SEM. This method uses the instrument that combine with focus ion beam (FIB) and SEM. This method ablates the sample surface by FIB, and new surface is observed too by the SEM. In this result, we can obtain serial image series, and reconstruct the 3D image. Third method is array tomography. In this method, we prepare the serial section ribbons by ultra-microtome, and these ribbons are mounted on the conductive base like a silicon wafer. Finally, we obtain image series in order, and stack these images on to one another. Forth method is TEM tomography. In this method, we obtain the serial tilt projection image by TEM, and 3D image was calculated by Computerized-tomography. In these results, SBF-SEM, FIB-SEM, and array tomography can observe the 3D morphology of infected E.coli, and 3D distribution of
phages in cells. On the other hand, TEM tomography is required to observe the 3D morphology of phages in cells.

P1035
Board Number: B36
Unlocking the distribution of fluorescent-labeled albumin in zebrafish through correlative microscopy.
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In the past decades, the zebrafish model has become increasingly popular in many research disciplines for their unique genetic and functional resemblances with that of humans. The animal small size, translucent nature and ease of manipulation and observation as a whole, make them an attractive experimental model. However, only a few publications related to drug delivery into the digestive system of zebrafish, and particularly the liver, have been reported, resulting in limited information on the ultrastructural architecture and drug behavior/pathways. The efficacy of a therapeutic drug relies on the accumulation of the drug at the site of action at therapeutic levels. Hence, it is important to understand the drug behavior, rate of uptake and site of accumulation of prospective drugs to better tailor drug targeting studies and set a limit of confidence in the use of the zebrafish as a model for drug delivery studies. The study presented herein reports on the widely-used protein-based drug carrier, albumin. Fluorescent-tagged albumin was injected into the liver of 12 days post-fertilisation zebrafish. At this age, previous studies have shown that the zebrafish presented a mature digestive system, resembling closely in structure and function to that of human. By applying the concept of correlative microscopy, functional and structural information were provided on the distribution of albumin in the zebrafish larvae liver. Live confocal imaging was used to follow fluorescent-labelled albumin. At different time points, the zebrafish were fixed and processed for electron microscopy using a protocol whereby the fluorescence is retained. Sections across and throughout the liver were then produced and imaged using both fluorescence-and electron microscopy. Correlation of the acquired data provided insights on the site of accumulation, being the liver sinusoidal endothelial cells, the space of Disse and the hepatocytes as well as respective rates of accumulation. Albumin was found to be taken up within the liver sinusoidal endothelial cells as early as 5 min after injection. After 10 min, the accumulation rate stagnated and further incubation up to 15 min indicated post-uptake processing taking place at the cellular level. The workflow established herein allowed to demonstrate the albumin pathway in zebrafish liver which is in line with that in rodents and human. Not only can this experiment form the basis for future albumin-based drug-complexes experiments, it also validates the zebrafish as a suitable animal model for drug delivery studies.
P1036
Board Number: B37
Development of a new type of low-voltage cryo-electron microscope enabling simultaneous imaging of STEM and SEM in biological samples.
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We developed a new type of cryo-electron microscope by attaching a newly developed cryo-transfer holder and anti-contamination trap to a scanning electron microscope (SEM: SU9000) equipped with a cold field emission gun (FEG: Acceleration voltage of 30 kV). The new cryo-transfer holder and anti-contamination trap were cooled with liquid nitrogen slash, resulting in ultimate temperatures of −190 °C and −210 °C, respectively. The SEM also works as a scanning transmission electron microscope (STEM) because it is equipped with a detector that catches transmitted electrons. For this reason, simultaneous measurement of a transmission electron image and the secondary electron image is possible. In STEM, an image is formed as a bit map of an electron, and is therefore not affected by contrast transfer function. Moreover, low acceleration voltage acts to increase contrast. When enough contrast is created, the images are produced without defocus, meaning that the original resolution of the microscope is maintained. These characteristics in a low-acceleration-voltage STEM (LVSTEM) are very useful for single particle analysis. Practically, when compared with a conventional 100-kV TEM, LVSTEM showed more detailed structures in single particle analysis of actin filaments. In the case of cells, cells cultured on carbon-coated grids were unroofed by weak sonication and used as samples. Because some soluble components were eluted upon unroofing, the spatial architecture of the cytoskeletal actin filaments and microtubules were observed at extremely high contrast. Many ribosomes and an endoplasmic reticulum were found in the cytoplasm despite the cells being unroofed.

P1037
Board Number: B38
Graphene-oxide as a substrate for high-resolution single-particle cryo-EM.
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Specimen preparation is a substantial remaining challenge for single-particle cryo-EM. While elegant, the traditional blot-and-plunge procedure for creating thin, free-standing films of vitrified buffer is actually quite harsh to macromolecules and requires orders of magnitude more sample than theoretically necessary. Adsorbing the specimen to a substrate film on the grid before vitrification can concentrate and protect fragile macromolecular complexes. Monolayer sheets of graphene oxide (GO) are a promising substrate, but we found that available methods for coating grids with GO were hard to reproduce.

In this study, we developed a simple and robust method for coating EM grids with thin films of GO and then evaluated the suitability of such GO grids for high-resolution single-particle cryo-EM. We report that GO grids produced by a simple Langmuir-Blodgett technique can be used for high-resolution single-particle cryo-EM and that background images of the GO lattice may serve as high-resolution fiducial markers for image quality assessment and motion correction.
P1038

Board Number: B39

An adaptive optical, structured illumination, lattice light sheet microscope for isotropic 100 nm resolution imaging of living specimens.

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I will discuss recent efforts to push both spatial resolution and imaging depth in light sheet microscopy. We have designed and constructed a new 5-objective system to obtain a 2X isotropic resolution improvement via structured illumination through a triad of excitation lenses mounted at 120 degree angles, increase imaging depth via adaptive optics and direct wavefront sensing, and double the collection efficiency and triple the axial resolution by combining lattice light sheet excitation with widefield interferometric imaging through opposed detection objectives. Axially structured light sheet excitation patterns fill the gaps in the optical transfer function that are associated with interferometric detection of fluorescence emission. Our ultimate goal is to perform isotropic 100 nm spatial resolution imaging in living specimens at high speed and with minimal phototoxicity.

P1039

Board Number: B40

A high resolution, tomography-compatible electron microscopic method for assessing subcellular distribution of membrane proteins.

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Localization of membrane proteins via electron microscopy (EM) at high resolution is dependent on a robust detection technology and a sample preparation method that confers superior ultrastructural preservation of membranes. Current methods for precise localization rely on chemical fixation of the sample followed by alcohol dehydration at room temperature. Such traditional methods, however, exhibit poor preservation of the membrane, induce membrane artifacts and diminish resolution. In contrast, high pressure freezing (HPF) of cells followed by heavy metal staining via freeze substitution (FS) in acetone at sub-zero temperatures offers the best possible membrane preservation, but this method has not been exploited for protein localization at subcellular resolution. Here, we report the development of a hybrid method that combines high pressure freezing with an existing peroxide-based detection technique typically used with chemical fixation methods. As proof of concept, we assessed the localization of a fusion protein consisting of the N-terminus of HMG CO-A fused with a peroxidase tag that faces the cytosol. We exploited the induction of OSERs (organized smooth endoplasmic reticulum) in cells by the over-expression of this N-terminal fragment as a metric for specific staining and membrane preservation. We next extended our studies to assess the localization of the sole human Fic (filament induced by cyclic AMP) protein, HYPE/FicD. HYPE resides in the ER lumen, but global proteomic studies have identified cytosolic proteins that interact with HYPE, suggesting alternate cellular locations for HYPE. Using our hybrid methodology, we provide strong evidence that HYPE is compartmentalized within various subdomains of the ER only. We further demonstrate via serial sectioning that our technique enables the localization of membrane proteins through a large volume of the cell. Our work, thus, aligns well with the three-dimensional mapping of membrane proteins in large organelles using EM-tomography in a near-native cellular context.
P1040
Board Number: B41
Imaging Live Uterine Smooth Muscle Modulation.
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The use of immunohistochemistry or histological staining to identify localization of specific receptors and general morphology in sectioned tissue are the most widely used imaging techniques in endocrinology and reproduction. A major drawback of these methods is that physiological events, such as smooth muscle contraction, is a complex system that contains dynamic feedback mechanisms. Without observing the live tissue, the researcher would be making interpretations based on random snapshots in time. We are interested in expanding our imaging capabilities to visualize uterine smooth muscle contractions using confocal and light sheet microscopy in order to observe live tissue receptor mediated modulation. In this study, uterine tissue from GFP Lifeact mice enabled visualization of F-actin during live smooth muscle contraction. Antibodies to specific receptors, conjugated to a fluorescent probe, were co-incubated with the tissue to visualize the actual response and timing of receptor recruitment during tissue stimulation. Initial imaging was conducted using a dipping lens on a Leica SP5 confocal microscope. This technique allowed the tissue to be imaged while alive and as a complete functional unit so that contractile activity could be observed. This technique was further explored using the Leica SP8 Lightsheet system. This system utilizes TwinFlect mirrors that align on both sides of the tissue expanding the observable area to greater than 180 degrees around the uterine horn. By imaging live tissue and stimulating the tissue with various hormones, peptides and amines, we were able to observe the dynamic response of both agonist and antagonist receptor recruitment. Utilizing this whole tissue-imaging technique has proved to be very useful in understanding the modulation and feedback mechanisms in response to various biological stimuli and helps in correlating the results found using standard techniques on fixed tissues.

P1041
Board Number: B42
LITE imaging: a high numerical aperture, low photobleaching fluorescence imaging technology.
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The dynamics of cellular processes demand visualization with fluorescence microscopy at high spatial and temporal resolution with; however, conventional fluorescence microscopy techniques are very light-intensive and introduce unwanted photobleaching, phototoxicity, and out-of-focus fluorophore excitation. Light sheet fluorescence microscopy decreases these issues by selectively illuminating the focal plane of a detection objective using an orthogonal excitation objective.1 However, existing light sheet microscopes are physically limited in the numerical aperture (NA) of the detection objective, which decreases both the microscope’s efficiency and the native image resolution.1-4 We present a novel light sheet illumination method: Lateral Interference Tilted Excitation (LITE), in which a sheet of light is generated at the focal plane of the imaging objective without a sterically limiting illumination objective. This allows the use of practically any detection objective, including oil immersion, with no upper NA limit. Thus, LITE achieves the low photodamage of light sheet imaging while exploiting the
benefits of high spatial resolution, high efficiency, coverslip-based objectives. We demonstrate the efficacy of LITE in imaging animal, fungal, and plant model organisms over many hours at high spatiotemporal resolution. Specifically, we are now able to generate multigenerational nuclear pedigrees in Ashbya gossypii over the course of more than 7 hours of continuous imaging, allowing us to study nuclear asynchrony at high spatiotemporal resolution over long developmental times.

P1042
Board Number: B43
A new method for large-volume high-resolution intravital imaging using multiphoton microscopy identifies microenvironment-driven tumor cell phenotypes leading to metastasis. D. Entenberg1,2,3, Y. Wang1,2,3, J. Pastoriza4, M.H. Oktay1,2,3, J.S. Condeelis1,2,3;
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We report, for the first time, a new approach to intravital imaging that successfully stabilizes tumors in living animals and isolates them from imaging artifacts associated with living tissue. This allows the capture, over days, of many high-magnification, sub-cellular resolution images in a mosaic pattern that maintains their spatial relationships. These images are then stitched together to produce a low-magnification, but high resolution, large volume image over time: a process we call Large-Volume, High-Resolution Intravital Imaging (LVHR-IVI). LVHR-IVI does not suffer from the magnification and spatial resolution artifacts inherent with mesoscopic and light sheet intravital imaging. LVHR-IVI is applicable to many different tissues, which includes soft tissues such as the mammary fat-pad, lymph nodes, and salivary gland which are particularly challenging as they are extremely compliant and easily transmit motion and vibrations from the animal's involuntary movements. Furthermore, using permanent imaging windows in mice in combination with LVHR-IVI extends imaging sessions from hours to multiple days over several weeks.

We have used LVHR-IVI to identify and quantitate modes of tumor cell motility, invasion and systemic dissemination in transgenic breast tumors which maintain the clinically observed histology presented by patients. High-resolution time-lapse imaging of these tumors identify the tumor microenvironment of metastasis (TMEM) - mediated transient vascular permeability events as the mechanism of tumor cell intravasation and hematogenous dissemination.

Furthermore, we used LVHR-IVI to evaluate the percent contribution of each of the proposed origins of circulating tumor cells and tumor cell clusters in vivo. Potential mechanisms of migration toward blood vessels include: 1) single cell migration; 2) streaming migration; 3) collective migration. Potential mechanisms of intravasation of blood vessels include: 1) Cell crowding of individually arriving tumor cells at sites of intravasation; 2) Collective vascular invasion by collectively migrating tumor cells; 3) Single cell intravasation followed by cluster formation in the blood.

The results indicate streaming migration resulting in cell crowding at TMEM intravasation sites are the major mechanisms associated with intravasation. Furthermore, the arrival of single tumor cells at distant sites in close temporal proximity can result in cluster formation within blood vessels there. Finally, the survival of single cells during metastatic seeding is 10-100 fold greater than previously believed. These new insights narrow the targets associated with metastatic progression and allow for better prognostic markers, treatment strategies and associated companion diagnostics.
P1043
Board Number: B44
Innovative measuring methods of optical transparency of the cleared brains by various newest tissue clearing technique.
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The advent of methods for achieving optical transparency in neuronal tissue, in conjunction with optical imaging techniques, has allowed for a refined, 3D visualization of structures and cellular networks in the brain with great resolution. Numerous efforts have been made to improve clearing by reducing light scattering and absorption in the tissue; however, little has been done to quantify the efficacy of clearing.

In this study, we designed a novel PACA (Punching-Assisted Clarity Analysis) assay, which quantifies levels of light attenuation in order to analyze the efficiency of tissue clearing. PACA was used to assess regional transparencies in the rat brain, as well as the efficacies of previously reported tissue-clearing techniques including PACT (PAssive Clarity Technique). Results were then confirmed by imaging luminous-light permeation. Compared to standard methods that measure refractive indices as a proxy for tissue clarity, PACA yielded results with significantly greater precision and accuracy. With its innovative approach and superiority over existing quantification methods, PACA shows great promise in future analyses of tissue transparency.

P1044
Board Number: B45
Detection of PI(3,4,5)P3 on cellular endomembranes using fluorescence correlation spectroscopy.
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Methods for the detection of cellular phosphoinositide lipids are based on using protein lipid-binding domains, antibodies or LC/MS. While the latter method is arguably the most sensitive, it cannot be used in the context of live cells, whereas the other reagents provide little quantitative information. Here, we report use of fluorescence correlation spectroscopy to monitor the concentration and dynamics of phosphoinositide lipid PI(3,4,5)P3 on cellular endomembranes.

Pleckstrin homology (PH) domain of Akt1 is widely used as a genetically encoded probe to detect accumulation of PI(3,4,5)P3 at the plasma membrane. Detection of PI(3,4,5)P3 on the endomembranes, however, is non-trivial. The surface area of endomembranes has been estimated to be as much as 200 times larger than that of the plasma membrane (Schmick et al, 2014), indicating that internal membranes occupy much of the perceived cytoplasm. Combined with the low steady-state fraction of the membrane-bound PH domain, these factors make it difficult to discriminate between the membrane-bound and cytosolic probe using conventional, diffraction-limited imaging.

As an alternative approach, we monitored the diffusive behavior of eGFP-tagged PH domain in live cells using fluorescence correlation spectroscopy with pulse-interleaved excitation (PIE-FCS). FCS allows discriminating between cytosolic and membrane-bound eGFP-PHWT pools by their respective fast (8.21±1.10 μm²/s) and slow (0.06±0.01 μm²/s) diffusion. In contrast, the mCherry-PH¹⁴Δ point mutant
that does not bind PI(3,4,5)P₃, displayed a single fast diffusive component (7.83±0.58 μm²/s), demonstrating that the slower component corresponds to PI(3,4,5)P₃-bound PH domain on cellular endomembranes. Additionally, FCS allows the precise determination of concentration of membrane-bound eGFP-PHWT domain in different cell compartments. To avoid overestimation of the slow-diffusing population, we used a de-trending algorithm (Wachsmuth et al, 2015), benchmarked using a small GTPase Rab7. We show that ~45% of PI(3,4,5)P₃-bound PH domain is found at the plasma membrane, whereas endomembranes account for up to 15% of cellular PI(3,4,5)P₃ (or PI(3,4)P₂), as measured by FCS. PI3K inhibition by GDC-0941 resulted in rapid decrease of the membrane-bound PHWT both at the plasma membrane and in the cell interior. Our results demonstrate the utility of FCS for detecting lipids and intracellular dynamics of peripheral membrane proteins within the complex environment of live cells.

P1045
Board Number: B46
Methodology to uncover a 100-year-old mystery, how does ploidy affect cell volume?
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How cells coordinate their growth and division are fundamental homeostatic cell biology questions impacting normal and pathological cellular functions. For the latter, one well established phenomenon that has remained poorly understood and often ignored, is that which correlates ploidy with cell volume. To interrogate such fundamental biological processes, we utilized real time ptychographic imaging to determine the volumes of karyotypically abnormal (A549) and normal (NIH-3T3) cell lines. Ptychographic imaging (Livecyte, Phasefocus, UK) is a label free quantitative phase imaging (QPI) technology ideally suited to cellular segmentation which enables label free, low toxicity, real time imaging of cellular dynamics. QPI permits the acquisition of many parameters including cell size, shape, roughness, dry mass with the potential to derive a measure of cell volume by measuring the phase delay of coherent light. This phase delay is dependent on intrinsic properties of the cell including thickness and refractive index. Utilising the average cell RI, the optical volume can be translated into actual cell volume. Correlative QPI and confocal fluorescence image datasets of dye-loaded cells demonstrated a one-to-one relationship between the average cell volume derived via each method. This minimally perturbing multiparametric live cell imaging methodology could help unlock the mechanisms of the one-hundred-year-old fundamental mystery of why ploidy correlates with cell volume and what effect it has on cells thereby contributing to a fundamental understanding of how cancer develops.

P1046
Board Number: B47
Dual-Color Metal-Induced and Förster Resonance Energy Transfer for Cell Nanoscopy.
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We report a novel method, dual color axial nanometric localization by Metal Induced Energy Transfer (dcMIET), and combine it with Förster Resonant Energy Transfer (FRET) for resolving structural details in cells on the molecular level. We demonstrate the capability of this method on cytoskeletal elements and adhesions in human mesenchymal stem cells (hMSCs). Our approach is based on Fluorescence-Lifetime-
Imaging Microscopy (FLIM), and allows for precise determination of the 3D architecture of intracellular structures, here in particular, stress fibers anchoring at focal adhesions, thus yielding crucial information to understand cell-matrix mechanics. In addition to resolving nanometric structural details along the z-axis, we use FRET to gain precise information on the distance between actin and vinculin at focal adhesions. Both measurements combined can give an unprecedented insight into the nano-structure of the linkage between stress fibers and focal adhesions. Here, we will present data where we use our method to follow the maturation of focal adhesions and acto-myosin fibers in hMSCs over time and show that actin fibers are nearly parallel to the substrate yielding an inclination angle of only 0.2°.

P1047
Board Number: B48
Hybrid phasor unmixing for hyperspectral fluorescence imaging.
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Fluorescence labels enable the visualization of proteins or peptides as indicators for biological dynamics. With growing needs from fluorescent biological imaging, multicolor labeling techniques have been widely proposed. However, the spectra of fluorescence labels are often overlapping, making the analysis more complicated. Hyperspectral fluorescence imaging adds a new dimension to microscopy imaging and enables unmixing of the overlapping spectra. With the continuously growing size of datasets, there is a need for fast and robust software to analyze the complicated data. In our previous study, we proposed an algorithm that tackles this problem based on the Fourier transform method, Hyper-Spectral Phasors (HySP). In this research, we provide a new solution hybrid unmixing combining traditional linear unmixing and HySP. This method combines both techniques’ advantages—it can denoise data, reduce computational time and solve spatially overlapping spectra with more than three labels. In this preliminary test, we apply this method’s enhanced sensitivity and robustness to observe liver metabolism reporters in zebrafish. Fluorescence signals could be separated easily, allowing observation of dynamic cellular interaction. In addition, we identify multiple autofluorescence signals spectra and extract their contribution from the biological activity.

P1048
Board Number: B49
Enhancing visualization of hyperspectral data with Phasor-Maps.
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Hyperspectral fluorescence imaging is gaining popularity, for it enables spectral separation of fluorophores whose spectra overlap by extending the acquisition dimension into spectral domain. This imaging modality produces large datasets generally complex and lengthy to analyze. In this work we present a visualization tool based on Hyperspectral Phasors, that provides a quantitative insight on the dataset prior to performing the full-scale analysis. Hyperspectral Phasors approach is a powerful tool for separating different fluorescent labels based on their spectral signature. The fluorescent spectral cube is
Fourier-transformed into a 2D plot where vector logic and histogram statistics provide an enriched and simplified insight into the data. However, visualization of hyperspectral images into tristimulus displays still proves to be a complex dimensionality reduction problem, where numerous hyperspectral bands are reduced to only three bands. Here we propose a fast and comprehensive approach to interpret hyperspectral fluorescent dataset based on similarity of spectral properties by designing multiple spectral maps aim at preserving much spectral information as well as enhancing spectral properties of the relevant pixels. We apply these phasor maps to multiple transgenic fluorescent zebrafish embryos of ubi:zebrabow, H2B-venus as well as wildtype AB/TL. Our results show enhanced visualization of spectral properties, separating fluorescent signal from autofluorescence and highlighting differences within intrinsic signals during live imaging. Phasor-Maps provide an image representation that is easy to interpret for human eyes and that quantitatively highlights different spectral characteristics of the sample.

P1049

Board Number: B50

Higher spatial resolution of overlapping gene expression achieved through a combination of multiplexing in situ Hairpin Chain Reaction and Hyperspectral Phasor analysis.

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Development requires integration of distinct gene regulatory networks (GRNs) that coordinate patterning, growth, and morphogenesis. GRNs are defined by overlapping expression patterns of key cell fate determinants, forming a molecular code that specifies different populations of cells for unique roles during development. The ability to spatially resolve overlapping gene expression at single cell resolution in the context of the whole embryo is critical for dissecting GRNs. However, spatially resolving multiple overlapping gene expression remains a significant challenge. The recent development of multiplexed in situ Hybridization Chain Reaction (HCR) has met some of these challenges by allowing the simultaneous detection of multiple genes within a single sample. The approach uses sequence-specific DNA probes coupled to a hairpin-based amplification system that are labeled with Alexa-fluors for fluorescent detection of target genes. However, the spectral overlap of Alexa-fluors limits the multiplexing capability of in situ HCR. Here, we combine multiplexing in situ HCR with Hyperspectral imaging and Phasor (HySP) analysis to characterize the overlapping expression of transcription factors critical for establishing the molecular code within the lateral plate mesoderm (LPM) for cardiac fate specification. HySP imaging and analysis allows the identification of multiple fluorescence spectra by capturing the full spectrum of wavelengths and segmenting the pixel data by Fourier transformation provides an efficient representation of the hyperspectral data without data loss. The combination of these approaches allows for better multiplexing capabilities of in situ HCR, leading to the identification of novel non-overlapping patterns of hand2 and gata5 gene expression in the LPM. Importantly, the higher signal-to-noise allows for more accurate modeling of the GRNs controlling early cardiac specification. Our results show that multiplexing in situ HCR in combination with HySP imaging analysis provides the resolution needed to better understand the dynamics of GRNs, shedding light on the molecular mechanisms of development.
P1050
Board Number: B51
Unraveling combinatorial labels in vivo with Voronoi Hyper-Spectral Phasor.
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Increasing the number of labels in samples is becoming a common step in biology for distinguishing, tracking and revealing function of cells complexes, bundles of neurons or cells and perform lineage analysis. The recent blooming of transgenic strategies for combinatorial expression of fluorescent proteins provides formidable tools for tackling the problem of multicolor labeling such as Brainbow. This multicolor toolbox, however, shifts the problem from biology to image acquisition and analysis, providing a complex problem of stochastic combination of fluorescent labels to be acquired first and then unraveled after imaging. Multiple approaches have been used for tackling these issues, mostly using standard confocal microscopy approaches in combination with tristimulus color image processing. However, these image analysis approaches face difficulties when translated to fluorescence data. Limiting factors such as bleed-through, instrument noise, photo-bleaching and toxicity greatly compromise signal quality and consequently the quantitative results. In this work, we report a method for rapidly acquiring and analyzing Zebrabow samples base on parallel multispectral confocal fluorescence and Hyperspectral Phasors. We extend acquisition in the spectral dimension, acquiring a full spectrum for each pixel to overcome bleed-through effects. Using Hyperspectral Phasors, we enable lossless denoising and obtain a graphical representation of the dataset that can be easily interpreted. The linearity of phasors proves ideal for detecting linear combination of fluorescent labels. We then apply Voronoi segmentation in phasor space to successfully quantify and segment 3D and 4D datasets of ubi-Zebrabow samples at multiple developmental stages.

P1051
Board Number: B52
Nanophotonics and Optogenetics – A Novel Combination towards Precise Stem Cell Regulation.
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Major breakthroughs in the field of genomics, stem cell biology, optogenetics, and biophotonics are enabling the control and monitoring of biological processes through light. By incorporating light-actuated/light-emitting proteins into cells, key biological processes at the sub-cellular level can be controlled and monitored in real time. In this direction, we will be taking the first steps towards the control of stem cell regulation processes with real-time operation through the development of novel nanophotonic devices. More specifically, the objective of this study is to demonstrate the feasibility of controlling genomic functions in multipotent Neural Progenitor Cells (NPCs) by activation and deactivation of protein expression/interaction using novel nano-actuators. The first stage of the project is to develop nano-actuators at 480 nm (blue) that allow light-controlled molecular toggle switch to control the nuclear fibroblast growth factor receptor-1 (nFGFR1). As an intermediate step prior to the
design of blue-light nano-lasers, we have designed and fabricated an opaque mask with an array of micro-hole openings on a blue-light laser diode to mimic an array of micrometric lasers able to illuminate individual neurons. Each opening is 5 micrometer long and wide, and the spacing between holes is also 5 micrometers. A 3D printed support was designed and built to facilitate the positioning of the laser infinitesimally close to the NPCs (to limit the amount of light diffraction), but not in contact (to prevent electrical damage to the laser). Secondly, we are developing optogenomic constructs that can be activated and deactivated using nano-actuators. Preliminary experiments were performed by transfecting NPC’s with the channelrhodopsin-Chr2 (green) plasmid, and observing the effects of exposing it to 480 nm light with a flash of light occurring every 2.5 min for 15 min. NPCs were then allowed to recover for 90 min and were immunostained. We observed light-induced Chr2-dependent upregulation of cFos, nuclear FGFR1 and FGF2. In addition, we have also performed double transfection of Chr2 and CMV-R-GECO1 (GECO) (red), a Ca2+ indicator plasmid. Initial experiments were performed using KCl to activate GECO and observe Ca2+ release to test the efficacy of the plasmid. Currently, we are in the process of using We have also successfully used the 480 nm light to activate Chr2 plasmid which in-turn will activated GECO resulting in Ca2+ release. This is the first steps towards using nano-actuators for precise control of optogenetic system. The eventual goal of the project is to develop a nanophotonic - optogenetic system that will function as light-based switches for creating or decoupling protein-protein interactions for photo-regulation of stem cells.

P1052
Board Number: B53
Liquid Tunable Microscopy to study Chromatin-DNA.
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Taking inspiration from the philosophical and sociological speculation by Zygmunt Bauman (Z. Bauman, Liquid Modernity, Polity Press, Cambridge, 2000) a new paradigm for optical microscopy is proposed regarding design, implementation, and applications. The current advances in optical microscopy related to the accessibility of data at the nanoscale in living systems or matter physics studies make super-resolved microscopy, label-free approaches, time and space encoding and decoding strategies, single molecule imaging and tracking approaches impregnated with a liquidity capable of condensing in itself the most significant aspects of the status of the art: a dimension in which the lasting gives way to the transient, the need to the discovery, and the necessity to the utility. This approach appears suitable to study chromatin-DNA at different temporal and spatial resolution scales. Phase contrast, confocal and multiphoton microscopy were a prelude for the super-resolved approaches. Today, in the era of super-resolution, we have a continuous growth of variations on the theme (Diaspro A. and van Zandvoort M.A.M.J. (eds) 2016. Superresolution Imaging in Biomedicine. CRC Press). Super-resolved Fluorescence Microscopy offers incredible performances and unlimited spatial resolution. (Diaspro A. 2014. Il Nuovo Saggiatore). Moreover, the modern methods allow tuning on the needed resolution in a direct, simple and efficient way. (Viciodini G. et al. 2014. Methods.) (Lanzanò et al. 2015 Nat. Com.). Different optical platforms can be used (Scipioni et al. 2016 Biophysical Journal; Castello et al. 2017. Rev.Sci.Instruments), and label-free methods (Bianchini and Diaspro, J.Biophotonics, 2008). (Mazumder et al. 2017 J.Optics) (Diaspro et al. 1990 IEEE Trans.Biomed. Eng.) become integrated modules of the modern liquid tuneable microscope. The microscope of the future, combining different converging technologies, is a liquid tunable microscope able to provide a scalable insight in the sample. It is liquid because it overlaps in an efficient and optimised way different mechanisms of contrast and it is tunable because it offers a real time tunability regarding spatial and temporal resolution like a radio tuned to the preferred radio station. It is smart because can adapt its architecture to the current scientific question

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and is open to additional light-matter interaction modules. The case study of interest here is related to study high order chromatin-DNA organisation in a scalable way with special attention to label free approaches coupled to high precision fluorescence mapping.

New Technologies in Cell Biology: CRISPR, Biosensors and Machine Learning Platforms

P1053
Board Number: B54
CRISPR/Cas 9 generated knockout cell lines for antibody screening and validation.
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While antibodies are among most commonly used reagents in biomedical research, they have been blamed for the main causes of reproducibility crisis of biological experiments. To find a solution to improve the specificity, sensitivity and reproducibility of antibodies, we tested CRISPR/Cas 9 generated knockout (KO) cell lines for antibody screening and validation of different antibodies. First, we used PITPNB KO cell lysate to screen different hybridoma clones and the Western blot (WB) results showed three clones had specific bands in parental cell lysates but two of them also showed bands in KO cell lysates, which indicated potential cross-reactivity with similar size protein(s). Second, we cross tested KRT7 and KRT8 KO antibodies against KRT7 or KRT8 KO cell lysates, the WB results indicated that the KO lysates could clearly identify the specificity of KRT7 and KRT8 KO antibodies. Finally, we compared HER2 knockout cell line and its isotype control for HER2 antibody specificity analysis in flow cytometry, the results indicated that the KO cell line is a better control than traditional isotype control for antibody specificity analysis. The study concludes that KO cell lines and their lysates are extremely useful tools for antibody screening and validation.

P1054
Board Number: B55
Pooled screening in an insect cell-line.
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Forward genetic pooled screening using shRNA or genome-editing approaches in mammalian cultured cells is a powerful method for the systematic identification of fitness-essential genes and genes conferring resistance to drugs. Despite the existence of numerous established insect cell-lines, a pooled screening method had not been possible due the absence of high-diversity gene delivery techniques. First, we report the development of a \( \phi C31 \)-based method allowing diverse pool delivery in insect cells. Next, we combine the method with CRISPR-Cas9 knockout screening and identify approximately 50 known and novel genes conferring resistance to the mTor-inhibitor rapamycin (positive selection). Finally, we use the technique to identify \(~1450\) fitness-essential genes (negative selection) with a false-discovery rate of 15%. Of these, 30% are uncharacterized in the fly; 85-95% have human orthologs; and 70% of the orthologous genes map to essential pathways in mammalian CRISPR knockout screens. Together, these results extend pooled screening to insect cell-lines as a rich source for discovering novel genes and pathways regulating basal and context-specific cell proliferation control.
P1055
Board Number: B56
Designing an imaging pipeline for gene edited hiPSC-derived cardiomyocytes.
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The Allen Institute for Cell Science is creating an imaging pipeline to study the changes in cellular organization during the differentiation of human induced pluripotent stem cells (hiPSCs) into cardiomyocytes. Using CRISPR/Cas9 we have fluorescently tagged ~15 target genes which localize to and thereby identify key cellular organelles, including adhesions, actin and microtubule cytoskeleton, mitochondria, nuclear envelope, desmosomes, endoplasmic reticulum, and the Golgi. In addition, we have initiated editing of several cardiomyocyte-specific genes including ACTN2, ssTNNI1, TNNI3, MYL2, MYL7, and TTN to study the organization of the cardiomyocyte contractile apparatus. To study these structures in iPSC-derived cardiomyocytes we have developed an optimized, scalable, and robust protocol for both cardiac differentiation and for live, high-resolution 3D fluorescence imaging. This was achieved by evaluating established cardiomyocyte differentiation protocols that use either small molecules or a combination of cytokines and small molecules, and optimizing parameters such as cellular density, dissociation enzyme, and substrates for imaging the edited cardiomyocytes. Here, we present our results along with the quantitative and qualitative assays used to determine the efficacy of differentiation, including cellular morphology, myofibril contraction, cardiac protein expression, transcriptome profiling, and localization of cardiac proteins such as troponin T and alpha-actinin to the myofibrils. We will also present images of the structural organization of cardiomyocytes using these gene edited iPSC-derived cardiomyocytes. This imaging pipeline will be used to perform high-resolution live cell fluorescent imaging of these gene-edited cardiac-specific structures in addition to the major intracellular organelles to build a predictive and interactive model of the cell from pluripotency and extending throughout the cardiac differentiation process.

P1056
Board Number: B57
Systematic gene tagging to illuminate stem cell organization.
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We are generating a collection of genome-edited clonal hiPS cell lines (WTC line) for studying cellular organization and dynamics. At present, we have ~15 GFP-tagged structures (www.allencell.org) that include major organelles, like the nucleus and mitochondria, as well as other key organizational structures that include microtubules, actin bundles, and cell-cell and cell-matrix adhesions. We have validated these cell lines for genomic, cell biological, and stem cell integrity and made them available to the research community. Here we present our newest iPSC lines with GFP-tagged structures; they include the ER, golgi, centrosomes, lysosomes and endosomes, along with some key observations of these structures in hiPS cells using live 3D microscopy. Our approach uses CRISPR/Cas9 gene editing to introduce fluorescent tags via homology driven repair (HDR) into genomic loci whose expressed products localize to specific organelles. Editing yields isogenic hiPSC lines expressing fusion proteins unique to each cell line under endogenous regulation. Most often these lines contain mono-allelic tags; but we have now also generated two tagged lines containing both mono- and bi-allelic tags. These gene-edited diploid cells display great consistency in GFP-tagged protein levels and cell behavior, greatly facilitating imaging and analysis. We have also developed strategies and workflows for successfully
tagging structures with red fluorescent proteins, generating dual structure tagged lines, and introducing fluorescent tags at the safe harbor (AAVS1 locus) for cytoplasmic and membrane localization. We present genomic and imaging data representing these newest gene-editing efforts and discuss the utility of these cell lines for generating image-based integrated models of cell organization and dynamics.

P1057
Board Number: B58
Eyes in the cell: Visualizing active kinases using genetically encodable fluorescent biosensors.
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Cell fate and physiology are regulated by cell signaling wherein signaling proteins play a central role. On receiving cues (internal and/or external) proteins are known to form complex interactomes thereby regulating many processes with immense specificity. Very often the same protein/family of proteins gives rise to very different cellular outcomes and responses downstream of these cues. Therefore, to understand a given cellular pathway, understanding the stringent spatio-temporal regulation of these signaling proteins becomes essential. One such approach is to selectively detect and report the "active" state of the protein. Using this approach, our lab has generated a toolbox of fluorescent biosensors for the Src family of kinases. Src kinases are a family of non-receptor tyrosine kinases and signals emanating from these kinases are known to feed into multiple pathways. Fyn kinase (an SFK member) is ubiquitously expressed in all cell types wherein it controls a myriad of functions ranging from control of myelination of neurons by oligodendrocytes to regulation of adhesion structures in keratinocytes making it an interesting candidate protein. However, a better understanding of its role in this process has been lacking due to the paucity of specific tools available owing to the high degree of homology amongst the Src kinases. By adapting a stable scaffold based high through-put screening regime we have been able to generate a very specific FRET based "biosensor" for Fyn kinase. Using this sensor we find that cells plated on fibronectin (an integrin ligand) are highly polarized with respect to Fyn activity and that on addition of Platelet-derived growth factor (PDGF), an amplification in this polarized signal is achieved. This is the first conclusive evidence showing involvement of Fyn in mediating cross-talk between different types of receptors and such an approach could help uncover a general mechanism for Fyn's biological action.

P1058
Board Number: B59
Sensitive biosensor imaging based on membrane-permeant, environment-sensing dyes.
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Biosensors with environment-sensitive fluorescent dyes have proven useful to study the spatio-temporal dynamics of protein activity in living cells. When dyes are attached to proteins or protein-binding reagents, they can report protein conformational changes or posttranslational modifications. The utility of dye-protein conjugates has been limited by the difficulty of loading them into living cells. They are
unusually are unable to pass through cellular plasma membranes so must be mechanically loaded or introduced using protein translocation approaches that can generate fluorescent vesicles and obscure fluorescent signals. To take advantage of recent techniques enabling protein labeling within living cells, we have developed solvent-sensitive dyes that are water soluble, use acetoxymethyl esters to pass through the plasma membranes, and show minimal staining of internal cellular structures. These dyes are bright (ε > 100,000 M-1cm-1, φ = 0.23), reducing the amount of labeled protein required in cells, and fluoresce at long wavelengths (excitation ~560 nm, emission ~590 nm), avoiding cellular autofluorescence. Reactive groups were incorporated for attachment of the dyes to either expressed proteins or small molecules. We utilized an unnatural amino acid (UAA) incorporated in the WASP CRIB domain to successfully extend the application of the novel dye to biosensor imaging of endogenous Cdc42 activity in living cells. When the dye mero166 was conjugated to an inhibitor of histone methyl transferase, the dye-conjugate translocated into nuclei, consistent with the biological functions of the transferase. The novel environment-sensitive dye with in cell labeling by UAA provides a generalizable strategy to access dye-based biosensors for a variety of protein or signaling components in living cells, and to trace the intracellular behavior of small molecules.

P1059
Board Number: B60
The development of non-FRET ratiometric ATP indicator “QUEEN-37C” and its application for single-cell metabolism analysis.
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Adenosine triphosphate (ATP) provides energy to intracellular reactions. It is still unclear how the ATP concentration is maintained. Two pathways are known to supply the ATP: glycolysis and oxidative phosphorylation (OxPhos). Recent studies have clarified that some cells such as ES cells and cancer cells are rather dependent on glycolysis, but some other cells are more dependent on OxPhos. The relations between this metabolic switching and the cell differentiation are actively studied. FRET-based ATP indicators have been developed to measure concentration of ATP at single cell level. These probes enabled to visualize the relative temporal or spatial changes within in a same cell. However, the apparent FRET efficiency is biased by the heterogenous maturation of fluorescent proteins that constitutes the FRET pair, which has been the hurdle for the quantification of the absolute concentration of ATP. To circumvent this problem, we have developed “QUEEN”, a non-FRET ratiometric fluorescent ATP indicator protein for quantification of ATP inside bacterial cells. Here we developed an improved version, “QUEEN-37C”, for visualization of ATP concentrations in mammalian cells at 37°C. We first validated the accuracy of ATP concentration measurement by using QUEEN. The ATP concentration was measured from the ratio image of Madin-Darby canine kidney (MDCK) cells stably expressing QUEEN-37C. Then, the average concentration of ATP of the same cells was determined by using luciferase. The results agreed within the experimental errors, demonstrating that QUEEN-37C can be used for quantitative analysis of absolute ATP concentrations in mammalian cells. In this presentation, we will show our latest results using QUEEN-37C. For example, we expressed QUEEN-37C in co-cultured neuron and astrocytes. Perturbations with inhibitors of glycolysis or OxPhos showed that neurons are heavily dependent on OxPhos, but glial cells can maintain ATP concentration by glycolysis alone. More interestingly, we have noticed that metabolic switching stochastically and transiently occurs in MDCK cells when cultured at high densities.
P1060

Board Number: B61

Tool-box of Fluorescent Biosensors for Visualizing Protein Kinase Activation Dynamics in Live Cells.

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Cell behavior is governed by complex interconnected networks of signaling proteins that tightly regulate whether on the cellular context. Classical biochemical and protein depletion approaches are limited in deciphering spatio-temporal regulation of these molecules. Therefore, fluorescent biosensors and probes that can report on cellular dynamics of specific-signaling proteins are extremely valuable. Here, we describe generally applicable, high throughput strategies to build new biosensors for the activity of Src family kinases (SFKs), using tailored protein mono body libraries. Protein activity biosensors are limited by the availability of reagents that specifically recognize the active forms of signaling proteins. We use high throughput, yeast surface display screening to generate protein mono body binders that recognize the active form of Src family kinases. In this approach, the surface residues of DNA-binding protein Sso7d (~7 kDa protein from Sulfolobus solfataricus) has been randomized to generate combinatorial libraries of scaffold proteins, which can express the mutant proteins as fusions to a yeast cell-wall protein. We screened the yeast surface display library to fish-out the specific binder for Fyn SH3 domain using magnetic selection followed by FACS. The isolated Fyn binder ORF was sub-cloned in to expression vector and purified using biochemical methods. We performed in-vitro binding analysis as well as pull down assays to determine its specificity towards Fyn SH3 domain. The binder has a strong affinity towards the Fyn SH3 domain and is capable of pulling-down the full-length kinase from mammalian-cell lysate. The structure of binder-SH3 domain interacting complex was resolved using solid state NMR and further validated using structure-function studies. Using this binder we built a genetically encodable FRET-biosensor that could help in visualizing Fyn kinase activity in living cells. The new biosensor reveals that Fyn, downstream of integrin receptor engagement, has a highly polarized and pulsatile activity pattern. Growth factor stimulation experiments further provide a dramatic illustration of how signalling via multiple receptor classes can be modulated by spatio-temporally localized Fyn action. These results not only provide the first visual insights into Fyn activation dynamics in cells, but also provide a cellular framework to address the role of Fyn kinase in cell migration and metastasis. We will also showcase exciting new strategies to make highly sensitive, fluorogenic, genetically encoded sensors for endogenous, unmodified kinases. These strategies can be used to study signaling targets that have been difficult to study using conventional techniques due to limitations of size, post-translational modification etc.
P1061
Board Number: B62
Use of orthogonal binding interfaces to develop small GTPase biosensors with greatly reduced cellular perturbation.
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Specificity of cellular signaling pathways can be achieved in part through precise spatio-temporal control and recent advances in biosensors have enabled an unprecedented view of these mechanisms in vivo. While these tools have proven invaluable in capturing the spatio-temporal dynamics of protein activation, most are based on protein fragments, inhibitors and/or use overexpression methods that can perturb the cell physiology being studied. To overcome these significant limitations in biosensor development, we have used multistate interface design to engineer biosensors for the small GTPase Rac1 which bind to upstream regulators (and therefore properly reflect Rac activation) but do not interact with downstream components. These dual-chain biosensors consist of a GTPase fused to a donor fluorophore and an acceptor fluorophore fused to a polypeptide that binds specifically to the activated form of the GTPase. Taking binding domains from endogenous effector proteins as a starting point we used Rosetta to computationally randomize the binding interface between the GTPase and polypeptides and then scored the mutations with three objectives: 1) to stabilize the interaction between biosensor components, 2) to destabilize the interaction between sensor components and endogenous downstream binding partners and 3) to minimize perturbation of interactions with upstream regulators. We selected approximately 25 computationally predicted orthogonal pairs for experimental screening and, through high-content screening, identified two mutational pairs that confer tight binding between sensor components and show greatly reduced interactions with endogenous binding partners. In fibroblasts we found a substantial decrease in perturbation of edge motility dynamics, and confirmed modeling results indicating that the use of orthogonal interfaces could increase the concentration range over which biosensors can be expressed without significant cell perturbation. Using this approach can lead to biosensors that minimally perturb normal signaling, opening a window to the behavior of low abundance proteins and processes affected by subtle changes in activation.

P1062
Board Number: B63
Luminescent metabolite detection assays to monitor cancer cell metabolism in real-time.
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Studying the role of metabolism in cancer has presented challenges in developing reliable rapid and miniaturized homogeneous methods for measuring changing levels of metabolites to understand the changes in metabolic pathways. We have developed highly sensitive luminescent assays to detect metabolites such as glucose, lactate, glutamine, glutamate, and NAD(P)/NAD(P)H and have applied them to monitor changes in levels of metabolites over time in vitro. The assays are based on a common core technology that uses selective dehydrogenases and a coupled enzymatic assay approach to generate a luminescent signal from luciferase. Sample deproteinization is not required thus enabling the
measurement of kinetic changes in metabolite levels by removing very small aliquots of culture medium at defined time intervals from the same population of cells. We demonstrate the utility of the assays to study glycolysis and glutaminolysis using two ovarian cancer cell lines: OVCAR-3 (considered to be low invasive) and SKOV-3 (considered to be highly invasive and glutamine addicted). Both cell lines showed comparable glucose consumption (0.5pmol/h/cell) and lactate secretion (0.9pmol/h/cell) rates. However, differences in glutamine metabolism were observed. The glutamine consumption rate was higher in the more invasive SKOV-3 cells (0.2 pmol/h/cell versus 0.15 pmol/h/cell for the OVCAR-3 cells and the glutamate secretion rate was 20-fold higher. A difference was also reflected in intracellular metabolite levels. Intracellular glutamine was not detected in lysates of the more invasive SKOV-3 cells while OVCAR-3 lysates had 28 mM glutamine. Both cell lines contained significant amounts of glutamate (20 mM for SKOV-3 and 41 mM for OVCAR-3). In addition, we have applied these luminescent assays to validate an approach for screening for modulators of lactate and glutamate production utilizing known inhibitors (2DG and BPTES). These simple homogeneous luminescent assays will enable greater understanding of how various treatments affect the metabolic state of tumor cells in real-time.

**P1063**

**Board Number: B64**

**Deep Cell: Deep Learning in Biological Image Analysis and Phenotypic Profiling.**

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Advances in fluorescent probes and light microscopy have revolutionized imaging in biological research, generating large, multidimensional datasets. However, image analysis tools accessible to biologists have not scaled with the increased volume of data, creating a bottleneck. Large image datasets require automated, data-driven analysis. We are developing machine learning tools that use deep neural networks or ‘deep learning’ to fill this need. We have developed convolutional neural network (CNN) based pipelines for biological applications including unsupervised phenotypic profiling for high-throughput microscopy and image segmentation. The hypotheses driving our study are: 1) pre-trained CNNs can be used as feature extractors to discover novel relationships in biological data and 2) CNNs will outperform conventional machine learning at segmenting objects in complex biological assays. We trained our networks on a large, diverse set of fluorescent micrographs of >600,000 cells from 22 cell lines labeled for 20 compartments, curated from The Human Protein Atlas and our lab. We use this dataset to train deep residual networks for phenotypic profiling. First, we trained our networks to classify 22 different cell lines based on staining for DNA, ER, and microtubules (94% accuracy). After training, we removed the classification layer to extract a high-dimensional vector representing image phenotypes, which can be visualized with dimensionality reduction techniques. We show that these “deep features” can be applied to new datasets with different acquisition settings, cell types, and fluorescent labels while retaining useful semantic information about biological phenotypes. We find that image features extracted from a network trained to classify cell type accurately cluster subcellular organelles without additional training, supporting the strength and flexibility of this approach. Future applications include phenotypic profiling in microscopy screens, where clustering genetic or drug treatments by image phenotypes may reveal novel relationships among genetic or pharmacologic pathways.

Next, we develop image segmentation pipelines for assays that require specific and precise measurements. We compared the performance of CNNs to a Random Forest classifier in the task of segmenting GFP-LC3 engulfed mitochondria to quantify mitochondrial autophagy. The CNN classifier is...
highly accurate (95% sensitivity, 83% specificity) and significantly outperforms the Random Forest classifier (93% sensitivity, 5% specificity). Together, these results demonstrate the power and promise of computer vision and deep learning in biological image analysis. We will open source and distribute code and trained networks to the biological community to expedite application and discovery.

P1064
Board Number: B65
A machine learning framework for kinetic phenotypic prediction of neurological disease states in patient-derived cell models.

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Patient-derived cell models coupled with long term imaging microscopes and state-of-the-art live cell fluorescent reporters provide a powerful platform to study dynamic morphological and biomolecular events and infer kinetic phenotypes to predict the states of the neurological diseases.

We are developing a machine learning based kinetic informatics discovery (KID) framework. It is applied to induced motor neurons (iMN) culture of amyotrophic lateral sclerosis (ALS) patient populations, to detect subtle differences underlying disease states. A machine learning enabled soft-matching detects somas and neuritis of iMNs. A tracking function tracks all iMN-like objects. Trajectory features for tracked objects are used to filter out non-iMN tracks. The states of the remaining true iMNs are inferred by measuring the responses of fluorescent reporters. A field inference stage uses random-forests classifier to infer the disease states at the field level. Like the field inference step, a patient inference stage uses random-forests classifier to infer the disease states at the patient level.

The KID tool is validated by rigorous tests at each stage using testing movies of iMN conversion and degeneration processes acquired on a Nikon BioStation CT. Both motor neuron reporter and neuronal firing reporter channels were imaged every 6 hours for 27 days, each patient in triplicate in 96 well plates. Neurotrophic factors were withdrawn and iMN survival was then imaged for 20 days.

The detection, tracking and state inference accuracies were tested by 12 movies, 4 from healthy controls and 8 from diseased patient lines. The detection accuracy is 94.6% ± 0.287% and the tracking accuracy is 94.5% ± 1.387%. The state inference accuracy is 87.9% ± 0.408%. The field inference accuracy was tested using 10 movies, 5 from healthy controls and 5 from diseased patient lines. The field inference accuracy is 90%. The patient inference accuracy was tested using 8 movies, 3 from healthy controls and 5 from diseased patient lines. The patient inference accuracy is 87.5%.

To further validate the KID tool, we used a masked test set consisting of 5 healthy and 5 patient lines to compare Z factor between KID score and survival alone. The KID tool showed Z factor of 0.5056 (good separation). The Z factor of the conventional survival assay using iMN survival metrics was -19.43 (very poor separation). These results provide a strong evidence of analyzing dynamic molecular events using time-lapse microscopy for discovering the pathogenic mechanisms underlying disease phenotypes. We will multiplex additional biomolecular reporters for spatial-temporal functional dynamics and cell fate readouts in the future.
P1065

Board Number: B66

Automated novel neuronal type discoveries by machine learning.
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The structures of neuronal dendrites and axons play fundamental roles in synaptic integration and network connectivity. The neuron morphological characterization enables comparative anatomical investigations, morphometric analysis of cells, or brain modeling. Advancement in neurobiology, microscopy, and imaging software are rapidly transforming the three-dimensional (3D) reconstruction of neuronal morphology into a mainstream technique. However, classification and quantitative characterization of morphologies from 3D microscopy neuronal reconstruction is challenging since it is still unclear how to delineate a neuronal cell types and the best features to define them. There is a critical need for analytical tools to enable the discovery of novel neuronal patterns and cell types automatically.

We developed a machine learning framework for robust automatic object classification. The machine learning-enabled classifier allows the teaching of classification algorithms to look for specific phenotypes (e.g. developmental stages, disease states and cell types.) present in the data. The framework supports neuron classification and classification of individual dendrite segments, dendrite branches, spines, and other 3D objects such as cells and nuclei.

We are extending the machine learning framework for novelty detection to identify new or unknown types that the framework has not been taught. Three different methods are supported: one class random forest (OCHR), one class support vector machines (OCSVM) and kernel null Foley-Sammon (KNFS).

The novelty detection tool is applied to neuronal type discoveries. It is tested on several data sets to validate the tool and to help direct improvement and compare strengths of different methods. The test data sets include a human set (101 neurons, 4 types), a mouse set (287 neurons, 6 types) and a rat set (354 neurons, 6 types). The tests were performed by training different number of types and evaluating the untaught types as novel types. 80% of the data were used for training and 20% were used for testing. We evaluated detection accuracy, precision and recall. In the novelty detection, the precision is the most important metric. For one type training and detection, the achieved precisions are 88% (human), 97% (mouse) and 94% (rat) for OCRF. They are 75% (human), 91% (mouse) and 90% (rat) for OCSVM. Finally, they are 84% (human), 92% (mouse) and 94% (rat) for KNFS.

These results indicate that OCRF is a good machine learning method for the neuron applications. The results also provide a promising direction of automated novel neuronal type discoveries using machine learning. We will further test and improve the methods using additional data sets including dendritic spine classifications.

P1066

Board Number: B67

Identification of genes involved in CAR-T therapy using CRISPR screening.
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Chimeric antigen receptor (CAR)-engineered T cells (CAR-T cells) have yielded unprecedented efficacy in B cell malignancies. However, somatic gene mutations can alter the vulnerability of cancer cells to T-cell-based immunotherapies. Here we describe a rapid high-throughput CRISPR-based screening
approach that allows a comprehensive identification of genes in human cancer cells that involved in CAR-mediated tumor cell killing. A membrane specific CRISPR/Cas9 library that consisted of 3279 genes was used in Raji cells. After co-cultured with CD19-CAR-T, genes involved in CAR-T resistance were profiled by deep sequencing. A set of genes whose loss impaired CD19-CAR-T efficacy, including CD19, were identified. This method allows a rapid and comprehensive determination of immune-modulatory genes in CD19 and any other target based CAR-T therapy.

P1067  
**Board Number: B68**  
Potent transcriptional activation using CRISPRa and synthetic crRNA:tracrRNA.  
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The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated 9 proteins) system derived from Streptococcus pyogenes has been adapted to upregulate any gene in its endogenous context, enabling gain-of-function or gene activation experiments while avoiding the use of exogenous over-expression plasmids. For CRISPR activation (CRISPRa), the guide RNA forms a complex with a nuclease-deactivated Cas9 (dCas9, D10A and H840A), which is in turn fused to transcriptional activators. The machinery then acts upstream of the transcription start site to up-regulate expression of a target gene. CRISPRa provides new tools to identify gene functions that might otherwise go undetected using loss-of-function studies down through-regulation of gene expression or gene knockout. Here we demonstrate a strategy to conduct CRISPRa experiments using chemically synthesized crRNA and tracrRNA molecules. We examine the functionality and advantages of using the synthetic approach for CRISPRa in multiple cell lines. Additionally, we show how the transcriptional activation leads to a significant increase in the target protein level, which in turn causes phenotypic effects by inhibiting or activating downstream genes. The methods presented are broadly applicable as a strategy to up-regulate any gene including systematic functional gene analysis in arrayed screening format.

P1068  
**Board Number: B69**  
Establishing Ubiquitylation Patterns in Cells: Efficient Monitoring of Cancer Biomarkers and Drug Activity.  
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Cells regulate their functions by altering the ubiquitylation status of many key proteins, especially oncoproteins. As a result, ubiquitin proteasome system (UPS) substrates and the relevant UPS enzymes have been identified as attractive anti-cancer targets. However, to date, the only effective ways to measure ubiquitylation patterns has been by immunoprecipitation coupled with immunoblot analysis (IP/IB) or by mass spectrometry methods, which are either insensitive, low throughput, or highly intensive. Here, we describe two Tandem Ubiquitin Binding Entity (TUBE) based assays, UbiQuant S and UbiTest, which provide a platform to efficiently detect ubiquitylation of anti-cancer targets. UbiQuant S is a high throughput assay to quantify ubiquitylation status in a cells or tissue. Ubiquitylated proteins are identified by energy transfer between AlphaLISA bead-labeled TUBE and antibody against the endogenous protein of interest in a homogenous plate-based assay. In the case of extremely low-abundance or otherwise challenging target proteins, a TUBE-based isolation followed by two-antibody
While applications published multicellular prominent treatment (CRISPR), immunoblot We has shown important K48 ubiquitin linked vessels). Engineering, 1Molecular P1070 Inc., 1Cellecta, 1Genetic 1Genome loss of function screening is a fundamental method to identify genes responsible for driving biological processes. Complex pooled lentiviral-based libraries expressing large numbers of genetic disruptors, such as shRNAs (RNAi) or sgRNAs (CRISPR), make large-scale cell screening practical. While RNAi-based approaches have proven to be an effective strategy for these screens, recent work has showed CRISPR-based technologies offer not only an effective alternative, but distinct advantage. We will present unpublished data from genetic screens in human Cancer cell lines using CRISPR-KO, CRISPRi and CRISPRa technologies, as well as RNAi. Although loss-of-function shRNA and sgRNA pooled library screens are similar in concept, the gene loss of function is achieved by different mechanisms (mRNA degradation with RNAi, full gene disruption with CRISPR-KO, transcriptional inhibition with CRISPRi), so some divergences are expected and indeed observed when comparing results obtained using one method versus the other. Furthermore, contrary to RNAi, CRISPR technology can be modified to activate gene expression (CRISPRa), thus enabling the use of genome-wide gain-of-function screening in gene function studies.

**Cellular Functions of the Actin Cytoskeleton**

**P1070**

**Board Number: B72**

Metastatic tumor cells exit circulation as multicellular clusters augmenting secondary tumor formation ability in melanoma cancer.

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Metastasis remains the leading cause of cancer-related mortality worldwide. Accumulating data suggests metastatic primary tumor cells disseminate through the circulation and seed distant sites as multicellular clusters. However, whether these clusters must first dissociate to extravasate (exit blood vessels) and form secondary tumors remains largely unknown. We hypothesized that circulating tumor cells (CTCs) could maintain adherence to each other while exiting blood vessels through a recently published alternative method of cell extravasation, termed angiopelosis.
To test this, we used an in vivo transgenic zebrafish larvae model, tg(fli1a:egfp), in which blood vessels exclusively fluoresce. We infused fluorescent human and mouse melanoma tumor cells directly into the circulation of the larvae and used intravital imaging to observe the circulating tumor cells in real-time. Next, using an RNA silencing approach, we knocked down the cell adhesion-related protein plakoglobin (gamma-catenin), and determined the effect of varying conditions on CTC cluster migration and extravasation in vivo. Additionally, we used a mouse metastasis model to evaluate whether circulating tumor cell clusters formed metastatic nodes more efficiently than individual circulating tumor cells. Here, by studying metastasis in murine and transgenic zebrafish larvae models, we show circulating tumor cell clusters possess the ability to exit blood vessels while maintaining a multicellular phenotype, through the recently identified mechanism of extravasation, angiopeliosis. We discovered tumor cells which extravasate as multicellular clusters exhibit an augmented ability to proliferate while individually-extravasating cells remain characteristically dormant. Furthermore, we found that silencing of cell adhesion molecule plakoglobin was sufficient to decrease the clustering ability of tumor cells in vivo, diminishing their ability to extravasate and establish secondary foci. Our results challenge the hypothesis that circulating tumor cell clusters must first disassociate to exit the circulation, and posits an alternative model. We propose tumor cells both travel and exit the circulation while maintaining a multicellular phenotype. Our results suggest CTCs’ ability to form secondary tumors and speed at which these tumors arise is directly proportionate to the number of extravasating cells in an aggregate; this is known collectively as the Cancer Exodus Hypothesis. We anticipate our findings to provide a starting point for more complex in vivo studies understanding the dynamics of group extravasation on tumor clusters ability to establish secondary tumors. We also expect the results to prompt development of clinical targets specifically to mitigate tumor cell clusters in circulation.

P1071
Board Number: B73
Pseudophosphatase MK-STYX regulates neurite outgrowth and alters the morphology of primary neurons.
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Mitogen-activated protein kinases (MAPKs) are essential players in important neuronal signaling pathways including neuronal development, plasticity, survival, learning, and memory. The inactivation of MAPKs is tightly controlled by MAPK phosphatases (MKPs), which also are important regulators of these neuronal processes. There is compelling evidence that a unique catalytically inactive member, MK-STYX (mitogen activated kinase phosphoserine/threonine/tyrosine binding protein), is a regulator in neuronal differentiation such as neurite outgrowth. We previously reported that MK-STYX dramatically increases the number of primary neurites in rat pheochromocytoma (PC-12) cells through the RhoA signaling pathway. MK-STYX decreases RhoA activation, whereas knockdown of MK-STYX increases RhoA activation. Furthermore, MK-STYX increases phosphorylation of coflin, a RhoA downstream effector, whereas coflin phosphorylation decreases when MK-STYX is knocked down with shRNA. Throughout the course of these experiments, we noticed that cells overexpressing MK-STYX developed more neurites, which often branched. Here, we report that microtubules and actin, components of the cytoskeleton that are involved in the formation of neurites, are present in MK-STYX-induced outgrowths. In addition, in response to nerve growth factor (NGF), MK-STYX-producing cells produced more actin growth cones.
than non-MK-STYX-expressing cells. Transmission electron microscopy confirmed that MK-STYX-induced neurites form synapses. Furthermore, the expression of Tau-1 and MAP2 (microtubule associated protein) in MK-STYX-induced neurites suggests that they have both axonal and dendritic properties. Further studies in hippocampal primary neurons demonstrated that MK-STYX altered their morphology. A significant number of primary neurons overexpressing MK-STYX had more than the control number of primary neurites. Taken together these data provide evidence that MK-STYX causes cytoskeletal rearrangement. It also highlights that this unique member of the MKP subfamily has the potential to have a major role in neuronal signaling.

P1072
Board Number: B74
Evolutionarily Conserved Mechanisms Drive Sarcomere Assembly in Cardiomyocytes.
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The sarcomere is the basic contractile unit within cardiomyocytes driving heart muscle contraction. Mechanisms governing sarcomere assembly, however, are poorly understood. Complicating this, there are no manipulable assays to test mechanisms of de novo sarcomere assembly. Here, we develop a novel assay using human cardiomyocytes to test de novo sarcomere assembly. Using this assay, we report non-muscle like stress fibers (NMLSFs) are essential sarcomere precursors, and evolutionarily conserved mechanisms drive sarcomere assembly in human cardiomyocytes. We show sarcomeric actin filaments arise directly from NMLSFs, which requires formin-mediated actin polymerization and non-muscle myosin IIB (NMIIB). We demonstrate formin-mediated sarcomere assembly is driven by the formin FHOD3, knockdown of which stops the NMLSF to sarcomere transition. Interestingly, we show NMIIB is required for the assembly of de novo sarcomeres but not maintenance of existing sarcomeres. We confirmed NMIIB is required in vivo for sarcomere assembly, as Zebrafish NMIIB knockdown animals fail to form sarcomeres. Furthermore, we show muscle myosin II filaments concatenate to form the A-band through a mechanism that is completely dependent on organized actin filaments. Thus, we provide a robust model of de novo cardiac sarcomere formation based on evolutionarily conserved mechanisms between non-muscle and muscle cells. This model offers new insight into the mechanisms governing sarcomere formation in human cardiomyocytes, and provides a testable platform to investigate normal sarcomere assembly during development or aberrant sarcomere assembly during disease states, such as cardiomyopathies.
P1073  
Board Number: B75  

**EhRho1 regulates cell motility and phagocytosis in *Entamoeba histolytica***.

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*Entamoeba histolytica*, a protozoan parasite causes amoebic dysentery or amoebiasis by invading colonic mucosa of host tissue. Phagocytosis and motility of parasite have often been associated with virulence behavior of *E. histolytica* and are essential factors responsible for pathogenesis. During tissue invasion, *E. histolytica* cells display high degree of blebbing due to high friction surfaces of gut epithelial cell lining which tend to employ blebbing as a mode of migration over pseudopods. After invasion, parasites may reach to liver, brain or lung and cause abscess in the tissues. Rho GTPases have been shown to play an important role in regulating actin polymerization and phosphoinositide kinases to regulate cytoskeleton dynamics in phagocytosis and cell motility in many model systems. In the present study, an attempt has been made to understand the role of EhRho1 in blebbing dependent motility and phagocytosis in *E. histolytica*. EhRho1 is a small GTPase that resides in the plasma membrane and enriches in the membrane, forming blebs. We have shown a novel mechanism for regulation of bleb formation in *E. histolytica* by EhRho1 through PI3 kinase pathway. Overexpression of mutant EhRho1 defective for GTP-binding or lowering expression by anti-sense RNA, in trophozoites results in reduced blebbing and motility. Also, Serum replenishment or LPA (Lysophosphatidic Acid) treatment was observed to induce high levels of GTP-EhRho1 in trophozoites resulting in increased blebbing while treatment with wortmannin, a known PI3K inhibitor reduced the bleb formation by reducing the PIP2 (Phosphatidylinositol 4,5-bisphosphate) levels in plasma membrane. Our study indicates the role of EhRho1 in bleb formation by amoebic cells that may contribute to the motility during host cell invasion. On the other side, our results show that EhRho1 regulates actin dynamics and localizes in the phagocytic cups during erythrophagocytosis and remain associated with the newly formed phagosome. Binding of activated EhRho1 (GTP bound) with Rho bind domain of EhFormin1 releases it from intermolecular autoinhibitory state and recruits to phagocytic cups. Expression of dominant negative mutant or lowering expression of EhRho1 by anti-sense RNA in trophozoites cause mislocalization of EhFormin1 from phagocytic cups which results in impairment of phagocytic processes and decrease F-actin content. The overall results concluded that EhRho1 regulates bleb based motility of parasite by regulating PIP2 level in plasma membrane through PI3 kinases pathway. Activation of EhFormin1 is required for progressions of phagocytic processes through the EhRho1 pathway in *E. histolytica*.

P1074  
Board Number: B76  

**XMAP215 is important for coordination between actin filaments and microtubules in embryonic neuronal growth cones.**

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During neuronal development, neurons coordinates the actin and microtubule (MT) cytoskeletons present in the axonal growth cone to determine growth cone shape and generate an axonal morphological response. The family of plus-end tracking proteins (+TIPs) binds to and regulates MT behaviors, and can couple MTs to F-actin dynamics. Our lab has demonstrated that the +TIP and MT
polymerase, XMAP215, participates in certain MT behaviors that appear to be dependent on F-actin regulation, yet the mechanism by which this occurs is unknown. Our goal is to determine the XMAP215 contribution to growth cone morphology and to MT/F-actin coupling. We used genetically-manipulated \textit{Xenopus laevis} spinal cord explants, in addition to high and super resolution microscopy. We find that knockdown of XMAP215 results in: 1) larger growth cones with longer filopodia; 2) an increase in the looped morphology of MTs; 3) MTs that are more spread within the growth cone; and 4) an increase of dynamic MTs in the growth cone. Finally, even though we find that the MT/F-actin co-alignment is not altered, we observe an increase in the number, orientation and length of MTs advancing into the periphery that are not coupled to F-actin bundles. These results show that XMAP215 could have a role in the regulation of spatiotemporal guidance of MTs, a function that is attributed to the actin cytoskeleton. Thus, XMAP215 could be an important participant in MT/F-actin coupling within embryonic growth cones.

**P1075**

**Board Number: B77**

**Functional Behavior of Overexpressed Fusion Proteins in Melanoma Cells Under Confinement Mediating Leader Bleb Based Motility.**

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Cell movement is mediated by a remarkably plastic array of morphologies. Cancer cells migrating in confined 3D environments mimicking tissue microenvironment can switch between modes of migration depending on the degree of confinement and the availability of adhesive ligand. Under high confinement and low adhesion, cells undergo a mesenchymal-to-amoeboïd switch and take on a highly stereotypical morphology with fast, persistent movement termed ‘leader bleb-based migration’ (LBBM). The morphology is characterized by formation of a large ($\sim 20.5$ um) sausage-shaped bleb that points in the direction of movement (the “leader bleb”) separated from a smaller ($\sim 12.5$ um) spherical cell body by a contractile neck. LBBM has been shown to be driven by actin and myosin II assembly and retrograde flow along the bleb, however, how actin binding proteins organization mediate motility is not elucidated, nor has the fundamental organization of cellular organelles necessary for various functionalities of cancer cell motility in this unusual cellular morphology. We performed a survey of the localization of 16 actin associated proteins including nucleators, bundlers, crosslinkers, and stabilizers, as well as various organelles in metastatic human A375 melanoma cells undergoing LBBM. We expressed fluorescent fusion proteins in cells confined to a 3um space under an agar pad and imaged by time-lapse spinning-disk confocal microscopy, and analyzed the spatial distribution within the cell body and leader bleb, as well as the effect of overexpression on the frequency of mesenchymal-to-amoeboïd transition and cell motility parameters. Our results indicate that nucleators Arp2/3 and mDia2 localizes towards the leader bleb tip, while $\alpha/\beta$-spectrin and FMN2 are confined to the cell body and $\alpha$-actinin and filamin are found exclusively in the bleb. All membranous organelles examined are localized in the cell body; however, cisternal endoplasmic reticulum and some Golgi is also found in the bleb; peroxisomes and mitochondria are found along the base of the bleb closer to the neck; and the nucleus and centrosome translocate between body and bleb. Of all proteins examined, only over-expression of $\alpha$-actinin or filamin induces alterations in leader bleb morphology. Our study provides the first description of a group of actin binding proteins and organelle dynamics during LBBM, and suggests an important possible role of actin crosslinkers in establishing LBBM morphology, thus enhancing our
understanding of cancer cell morphology and migration, which may lead to better treatment options for inhibiting cancer metastasis.

P1076
Board Number: B78
Uncovering the developmental functions of nuclear actin.
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Several decades of nuclear actin research implicate it in the regulation of transcription, chromatin remodeling, nuclear organization, replication, cell cycle, and the DNA damage response. However, what functions nuclear actin play in development and how the structure of nuclear actin impacts its functions remain largely unexplored. We have identified three reagents to examine nuclear actin during Drosophila oogenesis, i.e. follicle development. Follicles are composed of roughly 1000 somatic follicle cells and 16 germline cells, including 15 nurse or support cells and a single oocyte. Follicles progress through a series of 14 morphological stages, from the germanium to Stage 14 (S14). We find that monomeric actin (G-actin) is present in all cells and fairly uniform levels throughout oogenesis. Conversely, one actin antibody (C4) recognizes nuclear actin during early oogenesis (germarium-S9), including germline and somatic stem cells, mitotic follicle cells, and in transcriptionally silent nurse cells. These data suggests C4 nuclear actin promotes stemness, regulates the cell cycle, and inhibits transcription. This nuclear actin appears to be largely monomeric, and does not localize to the chromatin. As C4 nuclear actin decreases with follicle development, another actin antibody (AC15) begins to label nuclear actin. Specifically, the nurse cells exhibit AC15 nuclear actin starting at S8 and this increases with development; the follicle cells exhibit AC15 nuclear actin weakly at S9 and highly starting at S10. AC15 nuclear actin appears to be polymeric and localizes to the DNA; this and other data suggest it promotes transcription in both the nurse cells and the follicle cells. To further explore the roles of nuclear actin, we manipulate nuclear actin levels by blocking its nuclear import (Importin 9) and export (Exportin 6). Knockdown of Importin 9, results in female sterility and defects within the germarium, supporting a role for nuclear actin in stemness. Additionally, reduced Importin 9 levels cause chromatin defects. Loss or knockdown of Exportin 6 results reduced female fertility, distinct chromatin defects, heterochromatin issues, and alterations in the nuclear envelope. These data indicate nuclear actin regulates nuclear organization, and we predict such a function impacts transcription. Together these studies reveal nuclear actin plays critical roles in Drosophila follicle development. Considering the evolutionary conservation of actin structure and function, these studies will also provide insight into the roles of actin in the nucleus in the development of other tissues and organisms, and during tissue homeostasis and disease.

P1077
Board Number: B79
Nuclear actin interactome links actin to novel functions inside the nucleus.
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Although actin is still best known from its actions in the cytoplasm, current studies have strengthen the importance of nuclear actin. Inside the nucleus actin has been linked in various functions from gene expression to chromatin remodeling, but still the molecular mechanisms behind these functions remain unclear. To tackle this problem, we have used two mass spectrometry (MS) based techniques: Strep-HA
Thus, Fabrogates DNA degranulation modifier. Here, cell actin checkpoint interactions. As remodeling down complexes. Our requirement for Manchester, Pediatrics and Human Immunobiology, Baylor College of Medicine, Houston, TX, MCCIR, University of Manchester, Manchester, United Kingdom.

As essential players of the innate immune system, Natural Killer (NK) cells are responsible for the targeted lysis of virally infected or transformed cells. Upon activation, they establish a specialized cell-cell interface called the immunological synapse (IS). Activation-induced F-actin remodeling at the lytic IS is a critical requirement for cytotoxic function but neither the dynamic regulation of synaptic actin nor its specific function have been determined at a nanoscale level. Here, live cell super-resolution microscopy is used to define nanoscale F-actin dynamism as well as its requirement for NK cell lytic granule secretion. Although total branched F-actin content at the mature lytic synapse is stable, the displacement of individual filaments enables stochastic formation and disappearance of clearances. And while we have previously shown that lytic granules undergo degranulation within minimally sized regions of actin hypodensity, we found here that clearance formation occurs independently of lytic granule positioning. This F-actin dynamism depends upon branched network formation by Arp2/3 and contractility by myosin IIA. Inhibition of F-actin dynamism abrogates lytic granule exocytosis, underscoring the importance of this previously undefined nanoscopic F-actin architecture.

Thus, NK cell lytic secretion is dependent upon the multimodal generation of nanoscale F-actin dynamism at the immunological synapse, which is thereby delineated as a complex and novel regulatory checkpoint for cytotoxic activity.
P1079
Board Number: B81
Differential effect of M-CSF vs. GM-CSF on macrophage morphology and phagocytic ability.
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Macrophages are innate immune sentinels, capable of eliminating pathogens while also maintaining homeostasis by clearing apoptotic cells. Phagocytosis is central to both of these processes. Not all macrophages are equally competent in the performance of these roles, displaying specialized phenotypes that favor anti-microbial or anti-inflammatory homeostatic functions. Acquisition of such phenotypes is influenced by exposure to defined cytokines, notably macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF). M-CSF is abundant in healthy human serum and is constitutively secreted by a variety of cell types including epithelia, fibroblasts, endothelial cells and even by the macrophages themselves. It is needed for the proper development of most tissue resident macrophages. In contrast, GM-CSF is rarely detectable in healthy tissues and is upregulated during injury, inflammation and in certain tumors. In-addition, GM-CSF is expressed in the lung, where it is necessary for the proper development of alveolar macrophages. In vitro, exposure to these cytokines differentially affects the appearance of macrophages: M-CSF-treated cells appear elongated and ruffle extensively, while GM-CSF-treated cells adhere more tightly to the substratum and show minimal dorsal ruffling. Because these morphological features suggest differential cytoskeletal organization, we compared also the phagocytic ability of the cells polarized with either M-CSF or GM-CSF. M-CSF-treated cells engulfed phagocytic targets much more effectively than did GM-CSF-treated cells. Differential engulfment was observed using bacteria, zymosan particles, as well as red cells coated with either IgG or complement, suggesting an overall attenuation of phagocytosis in the GM-CSF-treated macrophages, regardless of the receptors involved. The molecular basis of these differences is under investigation and will be discussed in detail.

P1080
Board Number: B82
Targeting mechanoresponsive cytoskeletal proteins to inhibit pancreatic cancer cell metastasis.
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Metastatic disease is characterized by increased cellular contractility and deformability, lending cells and groups of cells, the flexibility to navigate through different microenvironments. This ability to alter cell shape is predicated on the structural elements of the mechanobiome – the cytoskeletal proteins that sense and respond to mechanical stimuli. Here, we present findings that the expression of known mechanosensory proteins in the mechanobiome, specifically non-muscle myosin IIA, IIB, and IIC, α-actinin-1 and 4, as well as filamin A and B, are altered in pancreatic ductal adenocarcinoma (PDAC) patients. We demonstrate that these proteins directly impact cellular mechanics using knock-down and overexpression cell lines. We further quantify the non-muscle myosin II family members in patient-derived cell lines and identify a role for myosin IIC in actin-band formation, using tissue spheroids and
computer-assisted image analysis. Additionally, we have previously shown that the targeting of myosin IIC by the small molecule mechanical modulator 4-hydroxyacetophenone (4-HAP) affects PDAC mechanics. 4-HAP decreases dissemination and induces cortical actin belts in spheroids, likely affecting retrograde flow. Mice treated with 4-HAP show a reduction in metastasis to the liver, suggesting that the mechanobiome is an effective, new drug space that can be exploited to improve the 5-year survival rate of pancreatic cancer patients, currently at ~6%, with a median survival time of <6 months.

P1081
Board Number: B83
Structure-based virtual screening to identify first-generation inhibitor of profilin1:actin interaction with anti-angiogenic property.
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Profilin1 (Pfn1) is an important regulator of actin cytoskeleton that plays a vital role in many actin-based cellular processes. Previous studies have shown that profilin1’s interaction with actin is important for endothelial cell (EC) migration and angiogenesis, suggesting that this interaction is a suitable target for an anti-angiogenic strategy. In this study, we performed structure-based virtual screening of small molecules that can disrupt the hot spots of the Pfn1:actin interaction followed by a biochemical assay to identify two hit compounds with similar structures that can reverse Pfn1’s effect on actin polymerization in vitro. A proximity-ligation assay further confirmed compound-induced inhibition of direct Pfn1:actin interaction in EC. Treatment with these compounds had potent anti-angiogenic effects on EC both in vitro and ex vivo. Consistent with the importance of Pfn1:actin interaction in the regulation of actin polymerization and cell migration, treatment of these compounds also led to a reduced overall level of cellular F-actin and slower EC migration. In summary, this study provides the first proof-of-principle of small-molecule-mediated interference of Pfn1 function that may have a potential to serve as a novel anti-angiogenic strategy in the future.

P1082
Board Number: B84
ARAP2 suppresses Akt signaling through APPL1.
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ARAP2 is an Arf GAP that controls the dynamics of focal adhesions. The Arf GAP activity is necessary but not sufficient to explain the effects. Because ARAP2 binds APPL1, a regulator of Akt and Akt has been reported to regulate FA dynamics, we hypothesized that ARAP2 regulates FAs in part by controlling Akt. We discovered that ARAP2 can affect Akt levels through interaction with APPL1, but the effect was independent of effects on FAs. Reducing ARAP2 increased pAkt and overexpressing ARAP2 decreased pAkt in U118 cells; however, ARAP2 affected FAs but had no effect on Akt in MDA-MB-231 cells. Furthermore, an Akt inhibitor did not block the effect of reduced ARAP2 on FAs in U118. Altering FAs by other means also did not affect Akt in U118 cells. Thus, ARAP2 induced changes in Akt were not directly linked to FAs in U118 cells. The effect of ARAP2 on pAkt may be mediated by ARAP2 binding to APPL1. APPL1 had similar effects as ARAP2 on pAkt in U118. The effect of ARAP2 knockdown on pAkt was not
reversed by overexpression of APPL1 whereas the effect of APPL1 knockdown was reversed by overexpressing ARAP2. The effect of ARAP2 on Akt required binding to APPL1 but not Arf GAP activity. We conclude that Akt signaling is not involved in ARAP2 regulation of FA and that ARAP2 may have function outside of FA regulation in the regulation of Akt signaling in some cells.

P1083
Board Number: B85
Nuclear actin and actin regulated transcription factors in heat shock responses.
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Actin is an essential protein both in the cytoplasm as part of the cytoskeleton and in the nucleus, where it has been linked to transcription, RNA processing, and chromatin remodeling. Interestingly, actin also seems to play a role in different cellular stress responses, including the evolutionarily conserved heat shock response (HSR). It is well established that HSR is essential for preventing proteotoxic or cytotoxic damage to cell during stress, and it requires transcriptional activation of heat shock protein (hsp) genes for this process. Previous studies have shown that in rat fibroblast cells, the actin cytoskeleton is altered and actin is localized inside nuclei as actin/stress bars upon heat shock. MAL (also known as MRTF-A/MKL1) is an actin-regulated transcription coactivator of serum response factor (SRF), and heat shock has been shown to induce expression of MAL/SRF target genes. On the other hand, MAL seems to bind to hsp genes at least in *Drosophila* ovaries. However, the molecular mechanism and functional significance of actin accumulation in nuclei, MAL/SRF transcriptional activation upon heat shock or binding of MAL to hsp genes upon heat shock are unknown. Here we have investigated the role of actin and MAL in transcription using heat shock responses in *Drosophila* and mammalian cell lines as model systems. By using chromatin immunoprecipitation followed by deep sequencing (ChIP-seq), we have shown that in *Drosophila* ovaries, actin co-occupies most transcription start sites together with RNA polymerase II. Interestingly, actin is also highly enriched on hsp genes, binding throughout the gene body. Also, ChIP-seq in a *Drosophila* cell line showed high accumulation of actin on the hsp70Aa gene upon heat shock. Further manipulation of nuclear actin and MAL activities will reveal their relevance and molecular interplay in hsp gene transcription under heat shock conditions.

P1084
Board Number: B86
Optogenetic stimulation of Ras-RalGEF-Ral axis promotes cell migration through recruitment of Exocyst-Wave complexes at the plasma membrane.
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The Ral GTPases (RalA and RalB) are key players in the transduction of oncogenic signals down-stream Ras. Direct activators of Ral, the RalGEFs (Guanine nucleotide Exchange Factors), are direct effectors of Ras. Even though it is well known that activation of Ras-RalGEF-Ral signaling is crucial for human oncogenesis, it remains very difficult to establish how at molecular level a particular event (for example a Ras mutation) causes the acquisition of a particular malignant phenotype (for example invasiveness). To dissect these cause-effect mechanisms we took advantage of an innovative approach, optogenetics, which is a novel discipline that combines genetics and optics to control well-defined events in living cells. We developed an Opto Ral system, based on a light-gated dimerization system that upon
illuminated recruits the GEF domain of Ral to a specific subcellular domain of the plasma membrane, leading to a localized and specific activation of Ral signaling pathway with no crosstalk activation of other Rho GTPases. Coupling Opto Ral system with TIRF microscopy technique, we observed that a primary consequence of Ral activation at the plasma membrane is the stimulation of cell edge protrusion events. A possible mechanistically explanation of this phenotype came from previous work in our lab (Biondini et al, J Cell Sci. 2016, 129:3756-3769) where we established a direct link between the Ral-exocyst pathway and the Wave Regulatory Complex (WRC), a crucial regulator of actin polymerization and protrusion formations. Indeed, we found that the optogenetic activation of Ral leads to a subsequent recruitment at the plasma membrane of WRC complex, supporting the following mechanistic model: under the control of Ral, exocyst and WRC complexes associate and exocyst works as "molecular taxi" physically transporting WRC from cytoplasm to leading edge. We are currently using an exocyst point mutant, deficient in WRC-exocyst association, to evaluate the impact on protrusion efficiency upon uncoupling Ral, exocyst and WRC recruitment and . This novel optogenetic approach revealed the mechanistic relationship between Ral activation, "the cause", and WRC recruitment to the front, "the effect", and provided an example of the power of optogenetics in untangling the molecular mechanisms at the base of cellular processes.

P1085
Board Number: B87
Matricellular Protein Cysteine-Rich Angiogenic Inducer 61 (CCN1/CYR61) – A Potential Therapeutic Target to Lower Intraocular Pressure.
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Purpose: Well-documented corroborations link actin cytoskeleton and extracellular matrix (ECM) proteins in the trabecular meshwork (TM) of aqueous humor (AH) outflow pathway and the regulation of intraocular pressure (IOP). Decreasing cell-cytoskeleton and -matrix interactions decrease the tissue stiffness to decrease IOP, which is a major risk factor for glaucoma. This study investigates the role of CCN1, a secreted matricellular protein that binds to and signal via integrins, in the regulation of the contractile properties of TM and IOP homeostasis. Methods: Using immunofluorescence and immunoblotting analyses, we assessed - a) cellular distribution, basal expression and secretion of CCN1 in primary human TM (HTM) cells and culture media, b) regulation of CCN1 by factors that regulate TM cytoskeleton and IOP including lysophosphatidic acid (LPA), dexamethasone, transforming growth factor-β2 (TGFβ2), and vascular endothelial growth factor (VEGF), c) effects of recombinant human CCN1 (rhCCN1) alone or in the presence of TGFβ2 on the changes in actin cytoskeletal organization and fibrogenic markers including α-smooth muscle actin (α-SMA) and collagen1A, and d) changes in cell stiffness after treatment with rhCCN1 using atomic force microscopy. All experiments described had sample size >3. Paired Student's t-test used to calculate statistical significance and results were significant if p<0.05. Results: Immunoblotting analysis confirmed CCN1 (40kDa) expression in HTM cells and its secretion in conditioned media. Cellular distribution by immunofluorescence showed predominantly cytoplasmic localization. Serum-starved HTM cells when treated with TGFβ2 (10ng/ml) and VEGF (20ng/ml) significantly increased intracellular CCN1 expression (≥1.5 folds) within 5h whereas LPA (10μM) for 24h and dexamethasone (500nM) for 5 days did not. Further, treatment with rhCCN1 (20ng/ml) for 5h led to decreased actin stress fibers and expression of the fibrotic markers - α-SMA and collagen1A as visualized by immunofluorescence and a 2.6 fold decrease in α-SMA compared to 0.1% BSA treated control HTM cells as detected by immunoblotting. Treatment of TM cells with rhCCN1 significantly attenuated TGFβ2-mediated induction of α-SMA and Collagen1A (>1.5 fold). On the other
hand, knockdown of CCN1 using shCCN1 led to a significant increase in α-SMA levels (1.7 fold). Atomic force microscopy studies revealed significant decrease in Young’s Modulus (cell stiffness) 1h post CCN1 treatment and reversed back to baseline by 4h. Conclusions: Taken together, CCN1 decreased the cytoskeletal integrity and the cell stiffness and negatively regulated the fibrogenic responses in AH outflow pathway making it an attractive and a novel mechanism-based therapeutic target to lower the IOP.

P1086

Board Number: B88
Anillin regulates epithelial cell mechanics by structuring the medial-apical actin network.
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Anillin is a scaffolding protein with a well-characterized role in cytokinesis and a novel role in regulating cell-cell junction structure. Anillin knockdown in the Xenopus laevis epithelium results in domed cells with wavy cell-cell junctions and reduced phospho-Myosin II light chain at junctions, hallmarks of reduced junctional tension. Additionally, Vinculin, which is recruited to adherens junctions under tension, is reduced when Anillin is knocked down and increased when Anillin is overexpressed. Contractility of the epithelium after the addition of ATP is also reduced when Anillin is knocked down and increased when Anillin is overexpressed. Based on these findings, we hypothesized that junction recoil following laser ablation would correlate with Anillin expression levels – that is, increased recoil when Anillin is overexpressed and reduced recoil when Anillin is knocked down – indicating that Anillin promotes line tension parallel to the junction. Surprisingly, recoil inversely correlates with Anillin expression: when Anillin is knocked down, cell vertices recoil faster than controls, and when Anillin is overexpressed, the vertices separate more slowly. We attribute this result to the changes in the medial-apical actin network that spans the apical surface of the cell. In control cells, the medial-apical actin network is dense and dynamic. Anillin knockdown reduces medial-apical actin accumulation, while Anillin overexpression results in a stable network of thick actin cables. We propose that loss of Anillin results in reduced medial-apical actin integrity, and cells are not able to maintain their shape after ablation causing the vertices separate rapidly. In contrast, when Anillin is overexpressed, the robust actin cables provide an apical support network that helps maintain cell shape after laser ablation of the junction. This work reveals a novel role for Anillin in regulating epithelial mechanics through the medial-apical actin network and could be important for understanding Anillin’s potential roles in tissue homeostasis, morphogenesis, and disease processes such as cancer metastasis.

P1087

Board Number: B89
Asymmetrical distribution of actin and endocytic proteins provide differential mechanical forces to propel myoblast fusion.
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Cell-cell fusion is critical for the development of syncyial tissues in multicellular organisms, including muscle, trophoblasts and osteoclasts. Although actin cytoskeleton has been known as the main driving force to overcome the energy barrier of intercellular membrane fusion, recently endocytic machinery and the membrane fission GTPase dynamin are reported to be essential for myoblast and osteoclast fusion. To understand the detailed roles of actin and endocytic proteins in cell-cell fusion, we utilized
mouse myoblast C2C12 to recapitulate myogenesis and observe the distribution of actin and endocytic proteins during myoblast fusion. We found that actin, but not tubulin, is asymmetrically coordinated in two fusing myoblasts that one cell protrudes actin-rich, protrusive structure to attack, and the other cell possess prominent cortical actin and stress fiber to receive. Furthermore, we found both actin and endocytic proteins are asymmetrically distributed in two fusing myoblasts that they are enriched at the fusion site of attacking cell. Intriguingly, the actin- and dynamin-enriched membrane protrusion can be observed in both of first phase myoblast fusion, fusion between two single nuclear myoblasts, and second phase fusion, fusion between two multinuclear myoblasts. Together, our results suggest that intercellular membrane fusion is orchestrated by actin and endocytic machineries in attacking and receiving myoblasts to provide invasive and resistance forces respectively.

P1088
Board Number: B90
Differential regulation of the axonal cytoskeleton by glycolysis and mitochondrial respiration. 
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The bioenergetics of axon extension are poorly understood. Actin is an ATPase and ATP loading of actin is required for filament polymerization. We used chicken embryonic neurons to address how glycolysis and mitochondrial respiration regulate the axonal actin cytoskeleton. While mitochondria distribution in axons and growth cones has been previously detailed, the distribution of glycolytic enzymes has not been addressed. Through immunocytochemical analysis, we observed glycolytic enzymes for both the preparatory and pay off phases in axons and growth cones. Acute inhibition of respiration and glycolysis with Antimycin A and glucose free medium with 2-deoxy glucose, respectively, both blocked axon extension. However, inhibition of glycolysis also resulted in axon retraction, which is an active process requiring actomyosin contractility. Measurements of ATP levels in growth cones using the fluorescent reporter PercevalHR indicate that inhibition of glycolysis or respiration results in a decrease of about 25% and in combination in a 50% decrease. Inhibition of glycolysis, but not respiration, resulted in the formation of axonal filament bundles. Similarly, the growth cone also exhibited bundles through the central domain at the expense of lamellipodial structures. In contrast, inhibition of respiration resulted in greatly simplified and collapsed growth cones. Moreover, the density of growth cone actin puncta/foci, structures considered to be reflective of substratum attachment points, was decreased by inhibition of glycolysis but not respiration. Collectively, these data suggest the hypothesis that glycolysis may provide energetic support for growth cone attachment mechanisms and a mechanism that counteractomyosin driven contractility.

P1089
Board Number: B91
FRAP Simulations and Computational Modeling show that F-Actin Meditated Focusing of Vesicles at the Cell Tip Is Essential for Polarized Growth. 
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Filamentous actin has been shown to be essential for tip growth in an array of plant models, including Physcomitrella patens. One hypothesis is that diffusion can transport secretory vesicles, while actin
plays a regulatory role during secretion. Alternatively, it is possible that actin-based transport is necessary to overcome vesicle transport limitations to sustain secretion. Therefore, a quantitative analysis of diffusion, secretion kinetics and cellular geometry is necessary to clarify the role of actin in polarized growth. Using FRAP analysis, we first show that secretory vesicles move toward and accumulate at the tip in an actin-dependent manner. We then depolymerized F-actin to decouple vesicle diffusion from actin-mediated transport, and measured the diffusion coefficient and concentration of vesicles. To measure vesicle diffusion and concentrations, we developed a three-dimensional computational model of the FRAP process that incorporates particle diffusion, cell boundary effects, and the optical properties of the scanning confocal microscope. Using the measured vesicle concentration and diffusion coefficient, and experimentally extrapolated vesicle fusion kinetics, we constructed another model this time for diffusion-mediated cell growth. This model predicts that diffusion-mediated growth is dependent on the size of the region of exocytosis at the tip, and that diffusion-based growth would be significantly slower than normal cell growth. To further explore the size of the secretion zone, we used a cell wall-degradation enzyme cocktail, and determined that the secretion zone is smaller than 6 μm in diameter at the tip. These results highlight the requirement for active transport in polarized growth and provide important insight into vesicle secretion during tip growth. Additionally, our computational FRAP model can easily be extended to other cellular geometries and other fluorescence dynamics assays, via the graphical Java-based application which we also provide—the Digital Confocal Microscopy Suite, DCMS.

Higher-Order Actin-Based Structures

P1090  
Board Number: B92  
Function of Dynamin-2 in Postsynaptic Neuromuscular Junction.  
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A neuromuscular junction (NMJ) is a chemical synapse equipped with complex membrane and cytoskeleton structures designed for neurotransmission and recycling at presynaptic membrane, as well as neurotransmitter receptors clustering at postsynaptic membrane folds. Recently, an actin-rich adhesion and invasion structure, podosome, has been reported to be present in aneurally formed postsynaptic apparatus and participate in postsynaptic maturation. Moreover, the large GTPase dynamin, long known for catalyzing membrane fission during synaptic vesicle recycling, was found enriched at the synaptic podosomes. As yet, the function of dynamin in postsynaptic membrane remains to be investigated. Here we found that during the lifetime of podosomes, the ubiquitously expressed dynamin isoform, dynamin-2 (Dyn2), forms a ring-like structure around the actin core for a short period of time, and this structure strongly correlates with the maturation and turnover of podosomes in C2C12 myotubes. Defective or exaggerated podosome morphologies were found when we knocked-down Dyn2 or treated myotubes with a GTPase inhibitor dynasore. From in vitro analysis, we found that Dyn2 has promising bundling activity towards both filamentous and branched actin, and Dyn2 mutations with altered assembly ability have abnormal actin bundling efficiency. Overexpression of these mutants also lead to abnormal podosome morphologies in myotubes. Furthermore, we demonstrated that expression of the hyper-assembly Dyn2 resulted in disturbance to the postsynaptic NMJ cytoskeleton in Drosophila. Taken together, our findings suggest that Dyn2 plays a structural role in podosome maturation through actin bundling activity, and therefore affect the cytoskeleton organization in synaptic podosome-
regulated postsynaptic NMJ maturation. We are going to further examine our hypothesis in the mammalian NMJ with myotubes and primary neurons co-culture system.

P1091
Board Number: B93
Investigating cytoskeleton-mediated mechanotransduction using a stem cell model of oogenesis.
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The cytoskeleton serves a crucial role at several stages of mammalian reproductive biology, such as meiotic maturation, fertilization, and the cleavage of early-stage embryos. However, the role of cytoskeletal dynamics in the differentiating mammalian female germ line is understudied. The isolation of oogonial stem cells (OSCs), and propagation of isolated OSC cultures in vitro, provides a new model to investigate the role played by the cytoskeleton during oogenesis. This work focuses on the organization and distribution of F-actin filaments throughout the differentiation of OSCs, starting with mitotically dividing progenitor cells, ending with the terminally differentiated oocyte-like cells (OLCs) that spontaneously differentiate in vitro.

OSCs maintained as pure germ cell cultures were stained with rhodamine phalloidin. Overall, OSCs exhibited fibrillar anisotropic actin filaments, as expected in adherent cultured cells. However, a small percentage of these cells, thought to be entering meiosis to form OLCs (thus differentiating from mitotically proliferating cell to OLC), showed a dramatic shift in cell morphology toward a large spherical OLC. With rounding, the actin network became highly radial, comprised of filaments flowing around the nucleus. After completing differentiation, OLCs were collected and found to have a dense cortical layer of actin, similar to the well-characterized cortical actin of mature oocytes. These three phases of actin organization: anisotropic, radial, and cortical, correlated with distinct patterns in the localization of YAP, a downstream mediator of the Hippo signaling network frequently associated with mechanotransduction. YAP was seen to be distributed between the both nucleus and cytoplasm in cells with randomly distributed F-actin, however, in cells with radial actin YAP was strongly localized within the nucleus, and distinctly cytoplasmic in OLCs, suggesting a previously unknown role for YAP during oogenesis. Treatment with the YAP inhibitor Verteporfin decreased basal rates of oogenesis (75 nM, -40%, n=3 P<0.01), with no negative impact on cell proliferation. Inhibiting F-actin stress fibers formation through treatment with the ROCK inhibitor Y-27632 did not impact basal differentiation of OSCs to OLCs, however treatment with Y-27632 prior to applying mechanical strain (10.5% elongation) inhibited both mechanical induction of oogenesis (Strain: 1.23-fold increase, Y-27632+Strain: 0.96, n=3; P<0.01) as well as strain-dependent decreases in phosphorylated YAP.

Taken together, these data indicate that OSCs are highly sensitive to cytoskeleton-mediated mechanotransduction mediated through the effector, YAP.
P1092
Board Number: B94
Src-mediated cortactin tyrosine phosphorylation regulates filopodia formation in neuronal growth cones.
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Proper wiring of neurons is key to the functionality of nervous system. This is achieved by a highly motile structure referred to as the neuronal growth cone located at the tip of axons and dendrites during both development and regeneration. Sensing of extracellular cues and motility are main functions of filopodia and lamellipodia, two distinct actin-based structures in the peripheral domain of neuronal growth cones. Whereas the regulation of actin networks in lamellipodia has been extensively characterized, little is known about the signaling pathways that control the formation, dynamics, and stability of growth cone filopodia. Previously, we reported that cortactin mediates the effects of Src tyrosine kinase in regulating actin organization and dynamics in both lamellipodia and filopodia of Aplysia bag cell neuronal growth cones. Here, we identified a single cortactin tyrosine phosphorylation site (Y499) to be important for the formation of filopodia. By comparing mutants of the three putative Src tyrosine phosphorylation sites of Aplysia cortactin (Y499, Y505, Y509), we found that Y499 is the only phosphorylation site relevant for regulating filopodia formation. Overexpression of the 499F phospho-deficient cortactin mutant decreased filopodia length and density, whereas overexpression of the 499E phospho-mimetic mutant increased filopodia length, regardless of the phosphorylation state of Y505 or Y509. Using a custom-made antibody against cortactin phosphorylated at Y499, we showed that phosphorylated cortactin is enriched in the peripheral domain, specifically along the leading edge. We found that treatment with the Src inhibitor PP2 decreased cortactin phosphorylation, while overexpression of Src2 increased cortactin phosphorylation. In order to test the hypothesis that the Arp2/3 complex is a key effector of Src/cortactin-mediated filopodia formation, we expressed the cortactin NTA domain as dominant negative Arp2/3 inhibitor either alone or together with a cortactin triple tyrosine phosphorylation mutant FFF. Overexpression of the NTA domain together with the cortactin FFF mutant didn’t further decrease filopodia density or length when compared to neurons expressing either construct alone, suggesting that cortactin acts upstream of Arp2/3 complex in filopodia formation. In conclusion, we have identified a tyrosine phosphorylation site in Aplysia cortactin that plays a major role in the Src/cortactin/Arp2/3 signaling pathway controlling filopodia formation.

P1093
Board Number: B95
Actin crosslinking ACD toxin is a universal inhibitor of tandem-organized actin-regulatory proteins in living cells.
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Delivery of bacterial toxins to host cells is efficiently hindered by a potency of the host protective barriers. This obstruction dictates remarkable efficiency of the toxins, a single copy of which can be sufficient to compromise a host cell. The efficiency of actin-targeting toxins is further hampered by an overwhelming abundance of the target: actin is one of the most abundant proteins in eukaryotic cells
 (>100 µM). To circumvent this barrier, toxins employ sophisticated mechanisms of toxicity amplification. One such mechanism is demonstrated by the actin crosslinking domain (ACD)-containing toxins of V. cholerae, V. vulnificus, and A. hydrophila, which catalyzes the formation of covalently crosslinked actin oligomers with actin subunits connected by side chain-amide bonds. Since these ACD-produced actin oligomers are non-polymerizable, crosslinking bulk amounts of actin eventually leads to failure of most of its functions and cell rounding, however, this mechanism requires high doses of the toxin to be effective. Conversely, our recent data imply that ACD-conferred cytotoxic effects are evidenced when only 2-6% of actin is crosslinked, suggesting that low doses of the actin oligomers are highly toxic. We recently reported a “gain-of-function” mechanism of toxicity amplification whereby the actin oligomers act as potent secondary toxins that directly inhibit the formin family of proteins with an abnormally high affinity, taking advantage of their unique geometry and tandem organization. Here we examined and verified a hypothesis that the toxicity of the oligomers extends towards WASP-homology motifs 2 (WH2) proteins involved in nucleation, elongation, severing, branching, and bundling of actin filaments. Many of these proteins contain G-actin-binding WH2-motifs in close proximity and may act as a multivalent platform for high-affinity interaction with the ACD-crosslinked actin oligomers. Using live-cell single-molecule speckle (SiMS) microscopy analysis, total internal reflection fluorescence (TIRF) microscopy, and bulk actin polymerization assays, we found that the actin oligomers potently inhibit Ena/VASP, Spire, and nucleation promoting factors (NPFs) of the Arp2/3 complex, albeit with different efficiencies. In live cells, SiMS microscopy confirmed potent inhibition of formins and Arp2/3 complex and halted dynamics of Ena/VASP and WAVE leading to a massive disarray of the host cytoskeleton. This study redefines ACD as an indirect, universal inhibitor of tandem-organized G-actin-binding proteins that overcomes the abundance of actin by redirecting the toxicity cascade towards less abundant targets whose inhibition by actin oligomers leads to disorganization of actin cytoskeleton and halts its dynamics disabling normal cellular functions.

P1094
Board Number: B96
Cryo electron tomography of the actomyosin cortex in isolated blebs.
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Cell shape changes are key to cell physiology and underlie fundamental processes such as cell migration, cell division and tissue morphogenesis. Cell shape changes are precisely controlled by cell surface mechanics. Cell surface mechanics is primarily determined by the cellular cortex, a thin network of actin, myosin and associated proteins underlying the plasma membrane. Cortex mechanical properties are determined not only by the network composition but also by its nano-scale architecture. Yet, very little is known about cortical network organisation. This gap in understanding is mostly due to the small dimensions of the cortex, which thickness is close to the diffraction limit of light microscopy. To circumvent this issue, we have developed a method to image actin filaments in the cortex in three dimensions using cryo electron tomography. We use isolated blebs, which have been shown to repolymerise a cortex similar to that of intact cells (Biro et al., 2013) and which have dimensions compatible with cryo electron microscopy (Medalia et al., 2002). We first characterised isolated blebs using super resolution microscopy and observed that repolymerised cortices have dimensions, morphology and dynamics comparable to intact cells cortex. A first round of cryo electron tomography demonstrated the feasibility of our approach, allowing us to visualise actin filaments in isolated blebs. We have developed a protocol to automatically segment actin filaments and reconstruct the cortical network, focusing on crossings between filaments and branching points. These quantifications allow for
a quantitative description of cortical architecture. We are currently implementing this approach to investigate how cortex architecture changes during the cell cycle.

**P1095**

**Board Number: B97**

**Mechanisms regulating actin cortex architecture in embryonic stem cells.**

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Embryonic stem cells (ESCs) are uniquely valuable cell types due to their pluripotency, the ability to differentiate into any specialized lineage. However, although their applications in regenerative medicine and tissue engineering have been intensively investigated, several fundamental aspects of their cell biology remain not well understood. For instance, recent studies suggest that the cytomechanical properties and mechanical cue responses of ESCs may differ significantly from differentiated cells. The actin cortex is an active network of actin cytoskeleton underlying the plasma membrane, and a major determinant of animal cell mechanics. We hypothesize that the cortical organization of ESCs may provide a structural basis accounting for their cytomechanical properties. Here, using super-resolution, live-cell, and atomic force microscopy to characterize mouse ESCs (mESCs), we observed that the mESC actin cortex comprises a low-density isotropic actin network of ~50-nm thickness, interspersed with high-density structures we termed “asters”. We found that the global architecture and the elasticity of the cortical networks are maintained by a fine balance between the activities of formin and Arp2/3 complex, with minimal contribution from Myosin II contractility. We identified the actin capping protein (CapZB) as a key regulator of both the network density and cortical elasticity, primarily via its activity in the transient asters. The asters also contain Arp2/3 complex and actin associated proteins such as a-actinin, and apparently serve as actin polymerization hotspots to regenerate the cortex upon local disruption. The isotropic nature of the mESC cortex largely excludes integrin-based adhesions, restring them to the cell periphery. However, these adhesions are compositionally mature, exhibit conserved multilayer nanoscale architecture, and are dependent on myosin contractility. Our results indicate that mESCs possess a distinctive and highly regulated cortical architecture which we suggest could represent a minimal undifferentiated state of the actin cortex that may be necessary for maintaining their nuclear softness and pluripotency. In addition, the mESC cortex is highly amenable to imaging and thus could serve as a useful model system for investigating the structure, dynamics, and mechanics of cellular actin networks.

**P1096**

**Board Number: B98**

**IRTKS elongates brush border microvilli using EPS8-dependent and -independent mechanisms.**

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The intestinal brush border lines the apical surface of enterocytes and is composed of thousands of actin-supported protrusions called microvilli, which extend into the intestinal lumen. Microvilli function to increase intestinal surface area and a properly formed brush border is critical for maintaining
intestinal homeostasis. However, the mechanisms underlying the formation of microvillar protrusions remain unclear. Here we provide evidence that the I-BAR domain containing protein, insulin receptor tyrosine kinase substrate (IRTKS), is essential for normal microvillar growth and elongation. IRTKS targets to the tips of actively growing microvilli, elongates microvilli when overexpressed, and is required for microvilli to achieve their normal length during BB formation. Our experiments reveal that the SH3 domain of IRTKS binds to the actin capping and bundling protein epithelial growth factor receptor pathway substance 8 (EPS8), and the two proteins colocalize at the distal tips of microvilli. Similar to IRTKS, knockdown of EPS8 impairs microvillar elongation. Interestingly, when IRTKS is knocked down, EPS8 levels at microvillar tips are reduced. This latter finding suggests that IRTKS promotes tip enrichment of EPS8 and provides at least one explanation for the short microvillar phenotype upon IRTKS knockdown. We also found that an IRTKS construct lacking its C-terminal WH2 domain generates microvilli that are significantly shorter; however the length of these protrusions can be rescued to WT levels when EPS8 is simultaneously overexpressed. Together these results suggest that IRTKS elongates microvilli in two ways: directly using its WH2 domain, and indirectly using its SH3 domain to promote the tip localization of EPS8, which in turn promotes microvillar elongation using a separate mechanism. Thus, an IRTKS/EPS8 complex targets to the tips of microvilli where it controls the length of these protrusions during brush border formation.

P1097
Board Number: B99
Impact of tip-enriched adhesion on the morphology and dynamics of actin-based protrusions.
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During intestinal epithelial differentiation, apical microvillar packing and organization are driven by adhesion complexes formed between two protocadherins, CDHR2 and CDHR5, that localize to the distal tips of microvilli, where they drive physical interactions between neighboring protrusions. CDHR2 is also capable of weak homophilic interactions. We recently reported that the actin-based motor myosin-7b (Myo7b) promotes the accumulation of the intermicrovillar adhesion complex (IMAC) components at microvillar tips, which is essential for the proper function of the complex. Additionally, two scaffolding proteins, USH1C and ANKS4B, play critical roles in complex formation and function. However, many questions still remain about how Myo7b, a monomeric motor, is capable of localizing these proteins and why there are so many seemingly redundant interactions. To address these questions, we developed an in-cell reconstitution assay using filopodial protrusions and their myosin transporter, myosin-10 (Myo10). The use of filopodia provides better spatial and temporal resolution, and heterologous expression allows us to control which IMAC components are present and able to interact. To allow us to take advantage of filopodia, we generated a chimera containing the motor domain of Myo10 and the cargo-binding tail domain of Myo7b, which localizes to the distal tips of filopodia. IMAC components that can interact with Myo7b will become enriched at filopodial tips when coexpressed with the chimeric motor. Using this assay, we found that the chimeric motor alone can target CDHR2 and CDHR5 to the distal tips of filopodia. Tip enrichment of CDHR2 results in increased filopodial number and stability. Additionally, CDHR2 forms homophilic interactions between adjacent filopodia, resulting in interfilopodial adhesion and tipi-like clusters of dorsal filopodia which resemble the clusters of microvilli found on differentiating epithelial cells. The chimeric motor can also transport both USH1C and ANKS4B individually to filopodial tips. Future studies will focus on determining how the adaptors promote the transport efficiency, stability, and lifetime of the IMAC.
P1098
Board Number: B100
A node organization generates tension and promotes stability in the fission yeast contractile ring.
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During cytokinesis in fungi, amoeba, and animals, cells divide physically into two using the tension generated by a contractile actomyosin ring. Ring tension is generated by myosin-II that pulls on actin filaments, but how these forces are marshalled to generate tension is unclear. Recently the ultrastructure of fission yeast constricting rings was probed by super-resolution microscopy (FPALM), showing that myosin-II and formin-capped barbed-ends of actin filaments colocalize in membrane-anchored protein complexes called nodes (Laplante et al., 2016). Using this information, a mathematical model showed there exists two contra-rotating families of nodes. Actomyosin forces operate both within and between families, and we show both contribute equally to ring tension. Anchoring ensures that nodes aggregate slowly such that turnover can protect the ring from these instabilities.
We incorporated this nodes ultrastructure in a mathematical model of the fission yeast ring that uses a coarse-grained, continuous representation of the nodes, and is severely constrained by the ample experimental data about the fission yeast ring. We find there are two classes of node, stochastically determined by the polarity of the actin filaments associated with the node: nodes that move clockwise, or nodes that move counterclockwise around the ring, due to myosin-II pulling forces. Thus, two node families contra-rotate with respect to one another. This agrees with the bidirectional motion measured in FPALM.
We find that nodes of opposite polarity are pulled towards one another, and this sliding filament mechanism generates 50% of ring tension due to the resistance provided by anchor drag that resists motion. Pulling forces between nodes of the same polarity contributes another 50% and involves no relative motion. The predicted ring tension agrees with experiment (390 pN), with a best-fit force per Myo2 head of ~1 pN, close to previous measurements.
To test for the effects of turnover, we ran the simulation with turnover switched off. Without turnover, an intrinsic contractile instability of catastrophic proportions sets in. Small density fluctuations are amplified and fracture the ring. Restoring turnover reduced instabilities as turnover time is smaller than the aggregation time.
In conclusion, long ago a sliding filament mechanism of ring tension similar to striated muscle was proposed (Schroeder 1968). Our model shows that a sliding filament mechanism is at work, of a highly stochastic and transient nature due to turnover. In addition to the mutual sliding of apposing filaments originating in the contra-rotating families, we find an equal contribution from a fixed filament mechanism, arising from interactions between nodes of the same family that rotate in the same sense.
Two isoforms of myosin-II cooperate to organize the fission yeast cytokinetic ring for maximal tension production.

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Cytokinesis of animal, fungal and amoeboid cells is driven by an actomyosin contractile ring that generates tension. How ring components organize to generate tension, and how the organization is maintained are unanswered questions. Here we show that the two isoforms of myosin-II in the fission yeast ring not only exert force, but have equally important roles as crosslinking agents that bundle actin filaments and organize the ring, cooperating to boost ring tension while avoiding fracture. Recent FPALM super-resolution fluorescence microscopy measurements of the fission yeast ring showed that protein complexes (“nodes”) from which the ring is assembled persist into constricting rings (Laplanche et al., 2016). Nodes, anchored to the plasma membrane, contain several components including myosin-II Myo2. A second myosin-II isoform, Myp2, may be unanchored: fluorescence images show a Myp2 ring of smaller radius than the Myo2 ring (Laplanche et al., 2015), and Myp2 vanishes from the ring following Latrunculin A-mediated disassembly (Takaine et al., 2015). Here we measured ring tension in fission yeast protoplasts. We found ~650 pN tension in wild type cells, ~65% the normal tension in mpy2 deletion mutants and ~40% normal tension in myo2-E1 mutant cells with negligible ATPase activity and reduced actin binding.

To understand the relation between organization and tension, we developed a molecularly explicit simulation of the fission yeast ring with the above organization. Our simulations revealed a clear division of labor between the 2 myosin-II isoforms, which maintains organization and maximal tension. (1) Myo2 anchors the ring to the plasma membrane, and transmits ring tension to the membrane. (2) Myo2, extending ~100 nm away from the membrane, bundles half (~25) of the actin filaments in the cross-section due to filament packing constraints, as only ~25 filaments are within reach. (3) To increase tension requires that the ring be thickened, as tensions in the ~25 membrane-proximal filaments are close to fracture. (4) Unanchored Myp2 indeed enables thickening, by bundling an additional ~25 filaments and doubling tension. Anchoring of these filaments to the membrane is indirect, via filaments shared with the anchored Myo2.

In simulated myo2-E1 rings ~20% of the actin filaments peeled away from the ring and formed Myp2-dressed bridges, as observed experimentally in myo2-E1 cells. The organization in simulated Δmyp2 rings was highly disrupted, with ~50% of the actin filaments unbundled.

In summary, beyond their widely recognized job to pull actin and generate tension, myosin-II isoforms are vital crosslinking organizational elements of the ring. Two isoforms in the ring cooperate to organize the ring for maximal actomyosin interaction and tension.
P1100
Board Number: B102
Self organization in liquid droplets of cross-linked actin filaments.
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Soft materials constructed from biomolecules self-assemble into a myriad of structures that work in concert to support cell physiology. One critical soft material is the actin cytoskeleton, a viscoelastic gel composed of cross-linked actin filaments. Although actin networks are primarily known for their elastic properties, which are crucial to regulating cell mechanics, the viscous behavior has been theorized to enable shape changes and flows. We experimentally demonstrate a liquid phase of cross-linked actin using a model system where cross-linker condenses dilute short actin filaments into spindle-shaped droplets, called tactoids. By theoretically describing the tactoid shape dynamics with a liquid crystal continuum model, we find that the cross-linker provides a tunable parameter to modulate the liquid interfacial tension and viscosity. In cells, the molecular motor myosin II binds to and translocates actin filaments, creating contractile actomyosin structures. In liquid actin tactoids, we find that myosin II filaments self-organize at the equator, reminiscent of organization observed in mitotic spindles. The motors also cluster, eventually creating distortions in the actin liquid crystal resulting in tactoid deformation. Our results demonstrate self-organization in cytoskeletal assemblies and provide insight to how anisotropy influences structures formed from macromolecular liquid phases.

P1101
Board Number: B103
A-Band Assembly in Vertebrate Cardiac and Skeletal Muscles Observed with Super-resolution Microscopy.
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Myofibrils in vertebrate cardiac and skeletal muscle are organized in aligned arrays of filaments formed from multiple protein components. Despite considerable functional information on individual proteins, determining how they assemble de novo into mature myofibrils is still an imposing challenge. Studies of sarcomeric protein localization during myofibril assembly in our lab has led us to propose that assembly proceeds in a three-step progression from premyofibrils to nascent myofibrils, culminating in the formation of mature myofibrils. This is accompanied by transitions in myosin isoforms and maturation in structure (reviewed in Sanger et al., 2017). Premyofibrils, forming initially at the spreading edges of muscle cells, are composed of minisarcomeres that contain small bands of non-muscle myosin II filaments alternating with muscle-specific alpha-actinin-enriched Z-Bodies attached to thin actin filaments. Titin and muscle myosin molecules first appear in nascent myofibrils, and the Z-Bodies in adjacent fibrils align to form beaded Z-Bands. In addition to non-muscle myosin II, muscle-specific myosin filaments in nascent myofibrils localize in an overlapping arrangement when viewed in widefield and confocal microscopes. In the mature myofibrils, non-muscle myosin II is absent and M-Band proteins localize to the muscle-specific myosin filaments, presumably aiding their alignment by cross-linking them into A-Bands. Super-resolution microscopes (SIM and STED) were used in this study to uncover details of the arrangement of the two different types of myosins in nascent myofibrils. With
this technology, filaments of non-muscle myosin II in nascent myofibrils appear to be flanked by mini-A-bands of muscle-specific myosin. Furthermore, in contrast to decades-old statements that vertebrate muscle myosin thick filaments form at their final 1.6 micron lengths, mini-A-bands are first detected at a length of about 0.4 microns. The bands gradually increase four-fold in length to 1.6 microns in elongating myotubes and spreading cardiomyocytes. These new discoveries in vertebrate muscle cells share a common characteristic with nonvertebrate muscles where some A-Bands can grow to lengths reaching 25 microns.

P1102
Board Number: B104
Actin rod formation and cytoskeletal dysregulation in the neurodegenerative motoneuron disease Spinal Muscular Atrophy (SMA).
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Cytoskeletal dysregulation and impaired maintenance of neuromuscular junctions (NMJs) partially lead to muscle denervation followed by muscle atrophy in SMA. We and others showed that the RhoA/ROCK pathway, a key pathway in the control of the actin cytoskeleton, is hyperactive in SMA. Nonetheless, responsible receptor-ligand interactions upstream of RhoA/ROCK are poorly understood. Based on transcriptome analysis in SMN-depleted NSC34 cells, we identified the guidance receptor PlexinD1 (PLXND1) as a potential upstream effector. Due to its role in axonal guidance, NMJ maintenance, control of monosynaptic specificity of reflex arcs, and vascularization, we analyzed the role of PlexinD1 in different in vitro and in vivo SMA models with regard to SMA pathology. PLXND1 was differentially expressed in SMN-depleted NSC34 cells, but the protein level was unchanged compared to control cells. Interestingly, PLXND1 formed predominantly intranuclear aggregates in about 50% of the cells upon SMN knockdown and in 10% of the control cells. Those structures were also detected in lumbar spinal cord sections of SMA model mice starting pre-symptomatically at P3. Density gradient enrichment of the structures from NSC34 cells revealed them as actin rods (actin-containing, aggregate-like pathologic structures known from other neurodegenerative diseases and congenital myopathies) which were here decorated with an intracellular PLXND1 cleavage product. Additionally, we found that PLXND1 is processed by a metalloprotease (MP) and that its cleavage altered the cellular response to PLXND1’s specific ligand Semaphorin3E (SEMA3E) as assessed by quantification of growth cone collapse. Actin rods are initially protective to prevent ATP decline upon oxidative stress. When the stress persists, rods become permanent and develop pathologic features. Since we detected rods prior to onset of symptoms in SMA mice, our data suggest that actin rods contribute to SMA pathogenesis even upon ASO-mediated SMN increase. Additionally, a PLXND1 fragment binds to rods and its cleavage as well as signal transduction were restored by knockdown or inhibition of MPs. Therefore, the responsible MP displays a putative novel target for a combinatorial treatment of SMA. Whether MP inhibition in vivo prevents rod formation and PLXND1 cleavage as a molecular link to vascularization defects, altered NMJ maturation and malformed monosynaptic reflex arcs as seen in SMA, will be part of future investigations.
Dynamic interplay between filopodia, focal adhesions and stress fibers.

Actin mediates migration both by providing a protrusive or “pushing” force against the plasma membrane, as well as a contractile or “pulling” force in combination with myosin II. Filopodia are protrusive structures at the leading edge, whereas stress fibers (SF) are contractile structures. Here, we use dynamic Airyscan microscopy to examine the coordination of these two structures at focal adhesions (FA) in U2OS cells, and find an interesting reciprocal relationship in both actively spreading and stably spread cells. In spreading cells, the majority of new FAs (72%) are assembled directly at the sites of filopodial protrusion, with two main locations of new adhesion: at the filopodial base, and within the filopodial shaft. These nascent FA not homogenous, but are composed of parallel linear units of 0.3 micron width. As the cell spreads, the FA and their associated SF undergo a progressive series of longitudinal “splitting” events, resulting in mature FA of single linear units. These structures further differentiate to the three SF classes previously identified in stably spread cells: ventral SF, dorsal SF and transverse arcs. A different relationship between filopodia and SF exists in stably spread cells, whereby a disproportionate percentage of filopodia (84%) arise directly from FA. We have quantitatively analyzed the roles of four actin-regulators (Arp2/3 complex, FMNL3, mDia1, and VASP) in the assembly and dynamics of filopodia, FA, and the three SF sub-types, with each regulator having distinct effects. From these studies, we propose that FAs can assemble either filopodia or SF, depending on the complement of assembly factors. Arp2/3 complex, mDia1 and VASP are necessary for FA-based actin assembly in general, whereas FMNL3 is crucial for coordinating filopodia assembly. VASP’s role in FA-mediated filopodial assembly is at the FA itself, not at the filopodial tip. Little detectable VASP is present at filopodia in U2OS cells, in contrast to past studies showing abundant VASP at filopodia tips in neuronal growth cones. Together, our findings highlight a striking coordination between filopodia and SF assembly in migrating cells.

Filamentation in *Schizosaccharomyces japonicus* in response to natural stimuli.

*Schizosaccharomyces japonicus* is a dimorphic fungus related to the well-established fission yeast *Schizosaccharomyces pombe*. It is the only fission yeast able to undergo hyphal growth, which it does in response to nutritional, nitrogen and DNA damage stresses. We identified a new inducer of vegetative hyphal formation that is independent of nutrient stress: grape extract. The unique ability of *S. japonicus* to undergo such fast cell shape modification provides an ideal and fascinating model to study dimorphism in this distant ascomycete clade. A first part of the project aims to describe and characterize *S. japonicus* morphological transition from yeast to filamentous growth. Interestingly, we found that it does not assemble a classical Spitzenkörper, a well-described spherical structure guiding hyphal growth observed in other filamentous organisms such as *Neurospora crassa* or *Candida albicans*. We also observed that *S. japonicus* does not form multi-septated filaments and appears to stay mononuclear during its life cycle therefore underlining again its distinctness from other well studied filamentous fungi. We also described cytoskeleton behavior and vesicular transport associated with the change in
type of growth. Interestingly, we noticed that actin and microtubule cables were partially colocalizing in both the yeast form and the hyphal form suggesting cooperation in delivery of cargo to the tips. A second part of the project aims to identify the genes involved in the transition. To do so, we used RNA sequencing to study gene expression changes, over time, upon addition of the hyphal inducer. This work will help us understand what genes and metabolic pathways are involved in filamentation and shed light on how S. japonicus can achieve such a dramatic change in growth.

P1105
Board Number: B107
Intranuclear and cytoplasmic actin rod assembly in Dictyostelium discoideum.
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Intranuclear and cytoplasmic rods are aggregates consisting of actin and coflin that are formed in consequence of chemical or mechanical stress. The formation of actin rods is associated with a variety of pathological conditions in humans such as Nemaline myopathy and certain neurological disorders. It is still not well understood what exactly triggers the formation of rods, and what the underlying mechanisms of rod assembly or disassembly are. Furthermore, it is unclear whether other proteins contribute to the formation of rods. We have used Dictyostelium discoideum as a model to study appearance, stages of assembly, composition, stability, and dismantling of intranuclear rods. We have employed in-vivo microscopy of cells expressing fluorescently labeled proteins as well as immunolabeling, and have analyzed wild-type or mutant cells lacking specific actin-binding proteins under different conditions that provoke the formation of actin rods. Our data show that intranuclear rods, in addition to actin and coflin, are composed of a distinct set of other proteins comprising actin-interacting protein 1 (Aip1), coronin (CorA), filactin (Fia), and the 34 kDa actin-bundling protein B (AbpB). A finely tuned spatio-temporal pattern of protein recruitment was found during formation of rods. Aip1 is important for the final state of rod compaction indicating that Aip1 plays a major role in shaping the intranuclear rods. In the absence of both Aip1 and CorA, rods are not formed in the nucleus, suggesting that a sufficient supply of monomeric actin is a prerequisite for rod formation. Furthermore, we have explored the conditions that lead to the induction of cytoplasmic actin-cofilin rods, and show that rods formed in the cytoplasm are characterized by a greater variability in appearance and shape.

P1106
Board Number: B108
Identifying actin cytoskeletal components required for actin-based motility in the frog-killing chytrid fungus: Batrachochytrium dendrobatidis (Bd).
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Chytrids are a polyphyletic clade of fungi categorized by their flagellated zoospores. They represent an ancient lineage diverging over 800 million years ago from their most recent common ancestor. The chytrid species Batrachochytrium dendrobatidis (Bd) has a 2-stage life cycle; a flagellated non-substrate
dependent zoospore, and a substrate-dependent zoosporangium. The swimming zoospore attaches to the substrate and forms a cell wall, transitioning to a zoosporangium. Immediately after release from the zoosporangium, we have observed zoospores swimming with flagella but also creating dynamic actin-filled structures used to crawl. The Bd cytoskeleton includes polymerized branched actin networks and activated Arp 2/3 complexes, creating pseudopods within the first 6-10 hours. We are identifying the components that differentiate branched actin networks used for motility, rather than other cellular functions (i.e. cytokinesis, endocytosis, etc.), by identifying gene orthologs of components and regulators of the actin cytoskeleton in Bd and other chyrid species, some of which appear to crawl, and others that do not. We expect motility-specific genes to be conserved exclusively in crawling chyrid species.

**P1107**

**Board Number: B109**

Anti-Inflammatory effects of annonacin in vascular endothelium in response to TNF-α-induced cell stress.

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Annonacin, an acetogenic compound present in certain tropical fruits, like the pawpaw fruit, has been noted to have anti-inflammatory properties; however, the mechanisms underlying these anti-inflammatory properties are still being characterized. The actin cytoskeleton has been shown to reorganize into stress fibers following treatment with various forms of mechanical and chemical cell stress. An established model of cell stress in vascular endothelial cells uses TNFα to induce an accumulation of actin stress fibers. We hypothesize that pretreatment with annonacin prior to TNF-α will prevent cell stress in vascular endothelial cells. Annonacin was extracted from the North American pawpaw fruit using Soxhlet Extraction, and verified using IR, NMR, and UV spectroscopy. Pretreatment of vascular endothelial cells with annonacin prior to TNF-α treatment resulted in a marked decrease in actin stress fiber number and intensity. These data suggest that annonacin may be involved in preventing inflammation at the cellular level.

**P1108**

**Board Number: B110**

Anti-Inflammatory effects of resveratrol in vascular endothelium in response to TNF-α-induced cell stress.

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Resveratrol, an antioxidant found in high concentrations in red grapes and wine, is thought to have beneficial effects on cardiovascular disease and incisional wound healing. Studies of resveratrol have shown that it possesses anti-inflammatory properties, which may help reduce the TNF-α-induced inflammation upregulated in cardiovascular disease. The actin cytoskeleton has been shown to reorganize into stress fibers following treatment with various forms of mechanical and chemical cell stress. An established model of cell stress in vascular endothelial cells uses TNFα to induce an accumulation of actin stress fibers. We hypothesize that pretreatment with resveratrol prior to TNF-α will reduce cell stress in vascular endothelial cells. Resveratrol was extracted from red wine (Merlot) using liquid-liquid extraction, and verified using IR, NMR, and UV spectroscopy. Preliminary data suggest that pretreatment of vascular endothelial cells with resveratrol prior to TNF-α treatment resulted in an
apparent increase in actin stress fiber number and intensity. These data suggest that resveratrol in higher concentrations may be involved in promoting inflammation at the cellular level.

P1109

Board Number: B111

Evolution and interplay of axonal actin assemblies.
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Actin dynamics are fundamental to neuronal survival, growth, and differentiation. Most studies on neuronal actin have focused on terminal growth cones and synapses, and actin along axon shafts has been relatively unexplored. More recent experiments have revealed three distinct types of cytoskeletal structures in axons: actin waves, actin rings, and actin trails. While actin waves are growth cone-like structures that originate near the cell body and move towards the neurite tips; actin rings are stable, circumferential, and periodic cytoskeletal structures that are separated by spectrin tetramers. Actin trails are dynamic filaments that elongate bidirectionally, originating from discrete actin foci inside the axon shaft. Though all three assemblies organize into very different structures, they are fundamentally composed of the same cytoskeletal protein, and may have underlying commonalities that can lead to new biological insight. Working on this premise, we examined various axonal actin assemblies in cultured hippocampal neurons as they mature. We found that the actin rings are visible in the putative axon as early as two days after plating, predominantly as nascent patches of loosely organized “ring-like” structures. As the axon develops, the discrete patches become continuous, probably by coalescing. Interestingly, although the frequency of actin waves diminishes over time, actin rings and trails become more numerous and prominent as the neurons develop. While perturbation of actin rings – by knockdown of βII-spectrin – had no effect on actin waves, there was a striking attenuation of actin trails; suggesting mechanistic links between the actin rings and trails. Current experiments in the lab are focused on investigating the nature of this relationship, and underlying mechanisms.

P1110

Board Number: B112

Autonomous structure formation and contraction of actomyosin regulated by contractile ring related cross-linking proteins (CRCPs).
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While actin makes various structures, which play important roles in eukaryotic cells such as morphogenesis and cytokinesis, it is not that these functions are achieved both by only actin dynamics. A variety of ordered structures are formed and changed by both ATP-hydrolyzing motor, myosin that drives F-actin and crosslinking proteins that connect F-actin to each other in an ATP independent manner. Currently, collaborative structure formation and contraction by these three kinds of proteins are attracting attention. However detailed mechanisms are not yet understood. In this study, we reconstruct actin network using actin, myosin and contractile ring related cross-linking protein (CRCP) to characterize CRCP function in autonomous structure formation and contraction. In the solution of G-actin and myosin, the minimum clusters, which F-actin were contracted by myosins in minimum-scale, were formed immediately after the start of the assay in the presence of ATP. When CRCP was added to
the solution, the F-actin networks were first formed and shrunk locally. The size of this network and contraction expanded as the concentration of CRCP (CCRCP) increased. We also found that the contraction initiation time (CIT) was constant with a range of ATP 1 – 6 mM (CATP) when CRCP was absent or low concentration. Conversely, when the CCRCP was high, CIT was delayed as the CATP increased. It was also found that myosin was able to cleave F-actin crosslinked by CRCP at low CATP.

These results suggested that (i) the actin network formed by crosslinking of CRCP is not able to contract owing to the balance of forces across the network, (ii) a trigger element, which is considered to be dissociation of CRCP from F-actin in the case of low CCRCP and severing of F-actin in the case of high CCRCP, is necessary to contract the network.

P1111
Board Number: B113
Shootin1-Mediated Dendritic Spine Formation in Hippocampal Neurons.
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Dendritic spines are actin-enriched protrusions emanating from dendritic shafts and provide contact sites for axons to form synapses. They are thought to play key roles in learning and memory. Dynamic filopodia-like spines are precursors of dendritic spines, which are thin and long, and commonly observed in the early developmental stages of neurons. As neurons mature, filopodia-like spines change into mushroom-shaped spines and form stable synapses. The morphological changes of dendritic spines require a dynamic network of actin. It has been demonstrated that actin filament (F-actin) polymerizes at the tip of filopodia-like spines and undergoes retrograde movement (Tatavarty et al., Mol. Biol. Cell, 2012). Here we analyzed a possibility that the spine formation is regulated by a mechanical linkage between F-actin retrograde flow and cell adhesion through molecular clutch. Shootin1a is a brain-specific clutch molecule involved in the axon outgrowth and neuronal polarity formation (Toriyama et al., J. Cell. Biol., 2006; Shimada et al., J. Cell. Biol., 2008). Shootin1a mechanically couples F-actin and cell adhesion molecule, L1-CAM, through actin-binding protein, cortactin, in order to generate traction force for axon outgrowth (Kubo et al., J. Cell. Biol., 2015). In this study, we show that shootin1a was detected not only in early stage (DIV 3) but also in the later stages (DIV 7, DIV14 and DIV 28) of cultured hippocampal neurons. Our immunocytochemistry assay showed that shootin1a is co-localized with PSD-95, a postsynaptic marker protein. Additionally, shootin1a is also co-localized with F-actin, cortactin and L1-CAM in dendritic spines and highly accumulated at the tip of filopodia-like spines. Suppression of shootin1a by RNAi led to a significant decrease in number of postsynapses and presynapses, which are stained with PSD-95 and synaptophysin, respectively. Similar data were obtained with hippocampal neurons cultured from shootin1 knockout mice. Furthermore, our in vivo data showed that hippocampal neurons in shootin1-KO mice brain exhibited a low number of dendritic spines compared to those in wildtype. The numbers of mushroom-shaped spines were also lower in the shootin1-KO mice brain. Taken together, these data suggest that shootin1a is involved in the dendritic spine formation, probably mediating the linkage between F-actin retrograde flow and L1-CAM as a clutch molecule.
The Sharpin interactome reveals a role for Sharpin in lamellipodium formation via the Arp2/3 complex.

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Sharpin, a multifunctional adaptor protein, regulates several signalling pathways. For example, Sharpin enhances signal-induced NF-kB signalling as part of the linear ubiquitin assembly complex (LUBAC) and inhibits integrins, the T cell receptor, caspase1 and PTEN. However, despite recent insights into Sharpin and LUBAC function, a systematic approach to identify signalling pathways regulated by Sharpin has not been reported. Here, we present the first ‘Sharpin interactome’, which identifies a large amount of novel potential Sharpin interactors in addition to several known ones. These data suggest that Sharpin and LUBAC might regulate a larger number of biological processes than previously identified, such as endosomal trafficking, RNA processing, metabolism and cytoskeleton regulation. Importantly, using the Sharpin interactome we have identified a novel role for Sharpin in lamellipodium formation. We demonstrate that Sharpin interacts with Arp2/3, a protein complex that catalyses actin filament branching. We identified the Arp2/3-binding site in Sharpin and demonstrate using a specific Arp2/3-binding deficient mutant that the Sharpin-Arp2/3 interaction promotes lamellipodium formation in a LUBAC-independent fashion.

Three-dimensional simulation of remodeling lamellipodial actin filament network.

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The dense actin network of branched filaments provides the driving force for lamellipodial protrusions. Within this network, filaments undergo structural network changes such as polymerization, depolymerization, branching, capping, and severing. Electron microscopy of the lamellipodia has revealed that the structure of filaments vary with distance to the leading edge. Near the leading edge filaments are short within a dense brushwork. Density decreases with distance from the leading edge and filaments in the rear are longer and appear more linear. The mechanisms behind this remodeling have yet to be determined. We created a three-dimensional stochastic model at the filament level to understand how size and structural properties are regulated within lamellipodia. We found the size of the lamellipodia depends on polymerization, depolymerization, branching, capping, and severing. Larger severing rates resulted in lamellipodia with smaller depths. Lamellipodia with branching throughout caused an increase in the depth. Our simulation also reproduces orientation patterns similar to the two-dimensional simulations. These mechanisms are being studied to better understand the process of lamellipodial remodeling.
Myosins 1

P1114
Board Number: B117
Mutations in Non-muscle Myosin 2A Disrupt Actomyosin-Microtubule Dynamics Resulting in Male Infertility.
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Mutations in non-muscle myosin 2A (NM2A) encompass a wide spectrum of anomalies collectively known as MYH9-Related Disease (MYH9-RD) that can include cataracts, glomerulosclerosis, macrothrombocytopenia, and deafness. We previously created mouse models of the three mutations most frequently found in humans: R702C, D1424N, and E1841K. While homozygous R702C and D1424N mutations are embryonic lethal, we found homozygous mutant E1841K mice to be viable, but male, and not female, mice were infertile. Here, we report that E1841K homozygous males have reduced testes size with defects in Sertoli-Sertoli and Sertoli-germ cell junctions, resulting in loss of blood-testis barrier integrity and premature germ cell loss. Furthermore, excessive acetylation of tubulin in mutant testes and Sertoli cells are correlated with actin disorganization indicating potential disruptions in actomyosin-microtubule dynamics. Analysis of NM2A and tubulin expression in primary Sertoli cells in culture shows that mutant cells have abnormal NM2A distribution, form thicker filaments, and fail to coordinate spatial localization of NM2A and tubulin. Together, these results identify a previously unreported characteristic of NM2A mutations in MYH9-RD mouse models and provide further insight into the role of NM2A in regulating actomyosin-microtubule dynamics in Sertoli cells to support germ cell maturation during postnatal testes development.

P1115
Board Number: B118
Nonmuscle Myosin 2 Is Important for Atrioventricular Endocardial Cushion Remodeling.
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Anomalies in endocardial cushion formation lead to the most common congenital cardiac defects including septal defects (atria, ventricular and atrioventricular septal defects) and cardiac valve disease. We have previously shown that mice globally ablated for nonmuscle myosin 2B (NM 2B) developed cardiac structural defects such as double outlet of the right ventricle (DORV) and ventricular septal defects (VSD). Moreover, global introduction of the NM 2B motor-impairing mutation (R709C) into mice resulted in an additional cardiac defect, the abnormal enlargement of atrioventricular (AV) endocardial cushions. This study is designed to understand the role of NM 2 in regulating cardiac cushion development by conditional ablation of NM 2A and/or 2B from endocardial cells in mice using Nfatc1-cre. Ablation of either NM 2A (A\(^{f\text{natc}1}/A^{\text{f\text{natc}1}}\)) or 2B (B\(^{f\text{natc}1}/B^{\text{f\text{natc}1}}\)) alone in endocardial cells does not affect AV cushion remodeling. However compound ablation of NM 2B together with one allele of NM 2A (A\(^{f\text{natc}1}/A^{\text{f\text{natc}1}}\); B\(^{\text{f\text{natc}1}}/B^{\text{f\text{natc}1}}\)) results in markedly increasing the thickness of AV cushions at E14.5 and E17.5. A\(^{+/f\text{natc}1}\); B\(^{f\text{natc}1}/B^{\text{f\text{natc}1}}\) AV cushions are less compact with a reduced cell density compared to control cushions. A\(^{+/f\text{natc}1}\); B\(^{f\text{natc}1}/B^{\text{f\text{natc}1}}\) cushions also show reduced mesenchymal cell apoptotic activity, but no changes in their proliferative activity compared to controls. Compound A\(^{f\text{natc}1}/A^{\text{f\text{natc}1}}\); B\(^{f\text{natc}1}/B^{\text{f\text{natc}1}}\) cushions show a moderate increase in cushion thickness, but not as severe as A\(^{+/f\text{natc}1}\); B\(^{f\text{natc}1}/B^{\text{f\text{natc}1}}\) cushions, compared to control cushions. The data demonstrate that NM 2 (2A and 2B) is required for AV cushion
remodeling to form thin and elongated normal cardiac AV valves. Since mice expressing motor-impaired R709C-NM 2B showed similar defects in cushion remodeling compared to compound endocardial NM 2A/2B ablated mice, these results provide further direct evidence that mutant NM 2B interferes with NM 2A in cardiac cushion remodeling.

P1116
Board Number: B119
A distinct role of non-muscle myosin 2B and microtubules in control of cell contact guidance and polarization.
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The term of contact guidance describes a phenomena of microenvironment geometry dependent orientation and motility of cells. In vivo contact guidance is responsible for both proper positioning and migration of cells inside the growing embryo and during maintenance of adult tissues. As non-muscle myosin 2A and 2B proteins were shown to be crucial for embryonic development and cell migration we addressed their functions in contact guidance using a micropatterning technique to allow precise control over the topography of microenvironment in order to dissect out possible non-redundant functions of these isoforms. We found that absence of NM 2B altered contact guidance of fibroblasts manifesting in substantial loss of cellular projections and uniaxial directional polarization, increased contractility and elasticity of cells. Using cytoskeleton targeting compounds and series of genetic transformations we verified a possible role of 2B isoform in contact guidance and proposed a model in which roles of NM 2A and 2B isoforms diverge in control over structure of actin cytoskeleton and its crosstalk with microtubules.

P1117
Board Number: B120
Nonmuscle Myosin 2 isoforms are uniquely expressed in mouse renal epithelial cells and play a critical role in renal tubular function in adult mice.
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Nonmuscle Myosin 2 (NM2) is an actin associated motor protein that is essential for cellular processes like adhesion, migration, polarity, and division. The NM2 family is composed of three isoforms, NM2A, NM2B, and NM2C. Each of these isoform has unique and redundant functions in cells due to differences in their affinity for actin and ATPase activity. Our previous work demonstrated that NM2 function (isoforms NM2A and NM2B) is critical for both compensatory and constitutive clathrin mediated endocytosis. Previous studies have shown that point mutations in the NM2A gene, MYH9, are associated with human diseases involving blood, eye, ear and kidney disorders. We hypothesized that
the underlying mechanism in NM2 related kidney disorders is compromised renal tubular endocytosis resulting in decreased protein reabsorption. Consistent with this hypothesis all three isoforms are expressed in the renal tubules with NM2B and NM2C being the predominant isoforms expressed in the renal tubules. Although NM2A is expressed to a similar degree as the other isoforms in whole kidney lysates, its distribution in the renal tubules is limited to a single segment, the loop of Henle. The distinct localization of the NM2 isoforms within the epithelial cells of the renal tubules suggests that each isoform has a unique function and loss of each isoform may lead to specific pathophysiology. Inducible conditional inactivation of NM2A, NM2B, and both NM2A/NM2B within the renal tubules of adult mice using the doxycycline inducible Pax8-rtTA; Tet-O-Cre system revealed critical roles of NM2A and NM2B in the renal tubules. Histological evaluation showed that both NM2A and NM2B single knockout mice have mild tubular dilation by 20 weeks of age with infiltrating cells that progressed over time especially in the NM2A knockout mice. The NM2A/NM2B double knockout mice show decreased urine pH and increased blood urea nitrogen and serum creatinine levels by 9 weeks of age. Histological analysis revealed major tubular dilation as well as glomerular sclerosis by 12 weeks of age and mice were moribund by 13 weeks of age. Our data demonstrates the critical role of NM2 in maintaining normal renal tubular function in the adult mouse kidney. In addition, these inducible conditional knockout models will be excellent models to study the progression of kidney disease.

P1118
Board Number: B121
The role of nonmuscle myosin 2A in early embryonic development.
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Nonmuscle myosin 2A (NM 2A) not only plays numerous and diverse roles in vertebrate development, but also functions as a negative regulator for the formation of squamous cell carcinoma (SSC). Our previous research reported that ablation of NM 2A in mice (A-/A-) led to abnormal formation of the visceral endoderm and embryonic lethality by E7.5. However, the molecular mechanisms responsible for A-/A- embryonic lethality were not fully characterized. Since in vitro differentiation of embryonic stem cells (ESCs) has been shown to faithfully mimic in vivo early embryonic development, we first established mouse ESC differentiation systems, including a spontaneous differentiation system and a hematopoietic differentiation system. The morphology of A-/A- ESCs was less organized and the surface cells were frequently scattered, in contrast to the tightly clustered A+/A+ ESCs. A-/A- ESCs significantly increased Sox2 and Nanog expression by almost 30 percent. Moreover, spontaneous differentiation of NM 2A deleted ESCs inhibited endoderm expression of genes, such as Sox17, Foxa2, and HNF4a. Analysis of the cell cycle with propidium iodide revealed that the peaks of G1 and G2/M shifted to the right in A-/A- embryonic body (EB) cells, indicating the increased binding of the propidium iodide to the DNA in the A-/A- cells compared to controls, by day 6 of EB formation. These data suggest that NM 2A deletion could affect chromatin structures. Using a nucleosomal DNA prep kit, it was determined that the genomic DNA of A-/A- ESCs or EBs was more easily digested into nucleosomes than control counterparts. Moreover, the levels of tri-methylation of K9 and K27 on histone H3 (H3K27me3 and H3K9me3, markers for repressive signals controlling development regulators), were significantly decreased in A-/A- EBs. These data indicate that there is a difference in chromatin structures between the wild-type and the A-/A- EBs. Because chromatin change has been demonstrated to be critical in early embryonic development, we hypothesize that ablation of NM 2A induces chromatin changes which consequently affect early embryonic development. Hematopoietic differentiation of ESCs in vitro is a useful method for investigating the molecular mechanisms controlling hematopoietic fate decisions. The ESC hematopoietic differentiation system has been widely used to study primitive hematopoiesis,
including hematopoietic progenitors, primitive erythropoiesis, and primitive macrophage development. Flow cytometry analyses showed that A-/A- EBs yielded a 50 percent reduction in hematopoietic progenitors by day 6. Since primitive hematopoiesis occurs in mouse embryogenesis about E7.5, these data suggest that abnormal primitive hematopoiesis might be one cause of embryonic lethality of NM 2A knockout mice.

P1119
Board Number: B122
Intestinal enteroids: A 3D model system to study the role of cytoskeletal dynamics and motor proteins in epithelial cell extrusion.
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Epithelia are a composed of highly-specialized cells that come together to form barriers between different compartments within an organism. Importantly, epithelia perform their barrier function while at the same time being highly regulated and dynamic. This way, epithelial cells regulate the transport of solutes, fluids, macromolecules, and immune cells/pathogens through the space between them, while preserving epithelial integrity. Epithelia also maintain their integrity in the face of a constant removal of damaged cells and addition of fresh cells. This process is very pronounced in mammalian gut, where the shedding of cells at the villus tip in a process known as apical cell extrusion is counterbalanced by recurring cell divisions in the stem cell population located in the villus crypt. Cell extrusion is essential for epithelial homeostasis because cells are constantly being damaged by exposure to toxins and infectious agents. Two striking features of apical cell extrusion are that both tight and adherens junctions remain functional during extrusion events, and that these junctions are intimately coupled to an underlying actomyosin cytoskeleton. Until now, most studies that looked at cytoskeletal dynamics during epithelial cell extrusion have focused on model epithelia (e.g. MDCK cells) growing on porous substrates. In this study, we adapted the 3D intestinal enteroid culture model to study the involvement of nonmuscle myosin 2 isoforms and their close relative myosin 18 in apical cell extrusion in a setting more closely reflecting the environment in the gut. To this end, we prepared enteroids from GFP myosin 2 A/B/C knock-in mice, as well as from mice expressing fluorescent reporter proteins. In addition, we introduced genes into epithelial stem cells using lenti virus. Immunostaining and live cell imaging revealed an interesting glimpse into how the different myosin 2 isoforms contribute to epithelial cell extrusion in the gut. First, the expression level and steady state localization of the three myosin 2 isoforms in intestinal enteroids is rather different. Myosin 2A is expressed at high levels and consistently localizes to cell:cell contacts, the apical domain and more diffusely in the cytoplasm. 2B, on the other hand, is expressed at very low levels without any striking localization pattern. Expression level of myosin 2C falls in between that of 2A and 2B and it is predominantly localized to the apical domain. Secondly both, myosin 2A and 2C dynamically change their localization pattern during extrusion events. Interestingly, myosin 2A is strongly enriched at the basal domain within the cell that is about to exit the epithelial layer, whereas myosin 2C seems to redistribute along the entire cell:cell contact area of the neighboring cells.
P1120
Board Number: B123
Identification and characterization of a putative myosin-mediated contractile activity in sponges.
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A relatively uncharted field of study concerns the evolution of muscles and muscle-like cells in the earliest animals (e.g., Porifera, Cnidaria). Freshwater and marine sponges display coordinated contractile ability in response to the presence of particular environmental cues. These simple animals offer the opportunity to explore the cellular origins of contraction in models absent of neurons or true muscle cells. Little is known about the cellular characteristics of contraction in sponges as traditional muscle-like tissues have not been identified or described. We have shown in field studies that sponges living in the Florida Keys (Cliona varians) contract in response to exposure to the neurotransmitter GABA. We measured contraction by analyzing the size of the oscular opening through quantitative video analysis. Laboratory-cultured Ephydatia fluviatilis, a fresh-water sponge, also contracted in response to GABA exposure. To determine if this contraction was acto-myosin mediated, we treated the E. fluviatilis sponges with blebbistatin prior to GABA exposure. Contraction was measured via quantitative analysis of time-lapse microscopy. We observed that blebbistatin pretreatment resulted in a delay in the onset of GABA-induced contraction. With the increase in availability of sequence databases, we also approached the question of contractility in sponges using an informatics. We took to the task of uncovering the muscle-like components by searching sponge databases for genes within the myosin superfamily. We searched within the genome of the Pacific Ocean sponge, Amphimedon queenslandica and within the transcriptome of Cliona varians. We found evidence for multiple myosin classes within both datasets, including multiple class II myosins in each species. We then compared the motor domains of the class II myosins to known striated muscle, smooth muscle, and non-muscle myosins from other species. Sequence alignment and phylogenetic analysis revealed that the majority of the C. varians myosin II like sequences grouped with the striated muscle myosin sequences. We then searched the A. queenslandica genome for other components present in the minimal muscle-like contractile unit, the sarcomere. Many of the sarcomere components were present in the sponge genome except for Troponin T. We also searched for evidence of the dystrophin complex, and the G-protein regulatory machinery. Few dystrophin complex components could be easily identified and strong evidence exists for G-protein mediated regulation. We would next like to understand the structure of contractile tissues as well as the mechanisms that lead to regulated tissue contraction. In doing so, we hope to further our understanding of the evolution of muscle and of contractility in general.

P1121
Board Number: B124
Myosin II A controls red blood cell membrane morphology and mechanical properties.
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Nonmuscle myosin II (NMII) is an F-actin-activated Mg++ATPase that assembles into bipolar filaments and binds a variety of F-actin cytoskeletal structures to generate force to mediate diverse cellular processes such as migration, division, and shape changes. In mammalian red blood cells (RBCs) and other differentiated cell types, F-actin forms a network of short, membrane-associated filaments cross-
linked by spectrin tetramers. Previous studies have identified NMII in mature human RBCs, but whether and how NMII interacts with F-actin in the RBC spectrin-actin network, or if NMII controls RBC membrane properties and function, remain completely unknown. Here, we show that NMIIA is the NMII isoform present in RBCs and that it associates with the spectrin-actin network in an ATP-dependent manner, suggesting that this interaction involves NMIIA motor domains and the short F-actins in the network. Indeed, results from epifluorescence and TIRF microscopy reveal NMIIA puncta, identified as bipolar filaments by super-resolution microscopy, localized near the RBC membrane. The NMIIA heavy and light chains are both phosphorylated in vivo in RBCs, indicating that NMIIA motor activity and filament assembly are actively regulated. To test the function of NMIIA in RBC membrane morphology and mechanical properties, we treated RBCs with blebbistatin, an inhibitor of NMII motor activity. The results show that treatment with the active enantiomer of blebbistatin resulted in an altered distribution of NMIIA and F-actin at the RBC membrane, suggesting rearrangements in the long-range organization of the spectrin-actin network. In addition, RBCs treated with active blebbistatin exhibit elongated cell shapes and reduced biconcavity, as well as changes in membrane oscillations and increased membrane deformability in microfluidic assays. Together, this data characterizes a novel function for NMII in regulating cell membrane morphology and mechanical properties through interactions with the spectrin-actin network.

P1122
Board Number: B125
A Novel Role for Nonmuscle Myosin II Monomers in Regulation of Focal Adhesion Dynamics.
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The role for nonmuscle myosin II (NMII) in positive regulation of actin stress fiber contraction, cell cortex tension and focal adhesion (FA) maturation is well established. In order to perform these functions, NMII must be activated by myosin light chain (MLC) phosphorylation and, according to the classic model, be polymerized into bipolar filaments. However, bipolar filament assembly and disassembly is regulated separately from NMII activation through MLC. A combination of these regulatory mechanisms, in principle, can produce motor-active but unpolymerized NMII molecules (monomers), which can represent a physiologically relevant functional NMII species in cells. Using methods of molecular cloning, biochemistry, and light and electron microscopy, we provide evidence that NMII monomers can regulate FA dynamics in cells. We show that endogenous MLC-activated NMII monomers exist in the cell and often associate with FAs through their tail domain. To explore their function, we created monomeric mutants of NMIIA and NMIIIB isoforms by deleting both Assembly Competence Domains (ACD1 and ACD2) from the respective heavy chains. The monomeric NMIIA and NMIIIB mutants neither formed bipolar filaments nor incorporated into stress fibers; however, TIRF microscopy revealed their increased accumulation in active cell protrusions and at the front and sides of the mature FAs. When expressed in cells, monomeric NMIIIB mutant produced a strong phenotype on cell adhesion. Specifically, cells overexpressing monomeric NMIIIB exhibited more dynamic protrusions, smaller FAs, thinner stress fibers, decreased spreading area, and eventually detached. Quantitative analysis of FA dynamics showed that overexpression of monomeric NMIIIB increased rates of both FA assembly and disassembly, but with greater enhancement of the disassembly rate. Time-lapse imaging revealed a correlation between the accumulation of monomeric NMIIIB at the front of a FA and subsequent FA disassembly. Monomeric NMIIIB mutant lacking the actin-binding motor domain also localized to the vicinity of FAs, whereas the isolated short C-terminal nonhelical tailpiece did not. We propose a model in which MLC-activated monomeric NMII is targeted to FAs through its tail domain and enhances FA dynamics. The mechanisms of FA regulation by monomeric NMII and NMII tail interaction partners are currently under investigation.

Sunday-79
P1123
Board Number: B126
Myosin IIB mechanoresponsive accumulation is determined by relative assembly into bipolar filaments.
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Mechanoresponse, the ability of cells to sense and respond to mechanical stimuli, drives essential processes such as cell division, tissue morphogenesis, cell migration, and metastasis. However, the direct mechanism by which the components of the cortical cytoskeleton assemble and interact to exert force and change cell shape in response to mechanical perturbations remains unclear. To quantify and characterize the contributions of the non-muscle myosin II paralogs to a responsive shape-change process, we perturbed cells by aspiration and defined the kinetics of fluorescent myosin IIA, IIB, or IIC accumulation to a region of high strain. We discovered that in a number mammalian of cell types, myosin IIA and IIC display similar kinetics and magnitudes of accumulation. However, myosin IIB displayed different magnitudes of accumulation in different cells types and in different phases of the cell cycle, indicating a unique regulatory system for myosin IIB mechanoresponse. In fact, the relative assembly of myosin IIB into bipolar thick filaments in these different situations, i.e. the ratio of assembled to unassembled myosin, reveals an optimal zone where myosin IIB mechanoresponse is highest. This is found where ~18% of the total myosin IIB is in the filament state, very similar to historical observations in Dictyostelium. Myosin IIB mechanoresponse can be perturbed by altering the assembly of myosin IIB by mimicking a phosphorylation event at S1935 in the heavy chain tail. Additionally, myosin IIA can influence the assembly state of myosin IIB and alter IIB mechanoresponse. Therefore, the cell can respond to network strain by accumulating myosin II to contract in opposition, and can regulate its response to this strain by tuning the relative assembly state of myosin IIB.

P1124
Board Number: B127
The role of cAMP and protein kinase A (PKA) in regulation of myosin II-dependent pigment granule aggregation in RPE of sunfish, Lepomis spp.
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Retinomotor movements in eyes of fish and other lower invertebrate include long-distance migration of RPE pigment granules in response to light or dark. While it has been established that actin filaments and myosin motors, including myosin II and other unidentified plus-end myosins, are involved in pigment granule motility, the mechanisms and regulation of motility remain elusive. The benefits using fish RPE as a model system is that pigment granule movement can be chemically triggered, manipulated, and observed in isolated cells using relatively simple techniques, making this an extremely useful system for the study of organelle transport. In dissociated, cultured RPE cells, centripetal movement of pigment granules, which has been shown to be dependent on myosin II and ROCK activation, can be triggered by application of underivatized cAMP. We sought to confirm that cAMP stimulation of aggregation occurs through a canonical pathway involving activation of protein kinase A (PKA), and characterized targets of PKA phosphorylation in RPE to better understand the myosin II dependence and regulation of pigment granule motility. Isolated RPE cells from sunfish (Lepomis spp.) were microinjected with PKA catalytic subunit and the position of pigment granules (aggregated vs. dispersed) was compared that of un-
injected cells. PKA catalytic subunit stimulated pigment granule aggregation (N=6 cells) while pigment granules in uninjected cells remained dispersed. Previous results have demonstrated that pigment granules in sham-injected cells punctured with a microneedle similarly remain dispersed. Thus, cAMP-dependent activation of aggregation occurs via PKA phosphorylation. To identify PKA targets, RPE tissue was treated with phosphatase inhibitors and cAMP (stimulates pigment granule aggregation) or dopamine (stimulates pigment granule dispersion). PKA-phosphorylated proteins were identified by immunoblotting and immunoprecipitation using an anti-Ser/Thr PKA phosphorylated PKA substrate antibody. PKA-phosphorylated proteins in cAMP-treated samples were identified as 112, 82, and 42-48 kD proteins. Potential candidates for PKA-phosphorylated targets include proteins associated with the activation of myosin II.

P1125

**Board Number: B128**

**Differential actomyosin contractility in tumorigenecity.**

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Bleb formation has been correlated with nonmuscle myosin II (NM-II) activity. Whether three isoforms of NM-II (NM-IIA, -IIB and -IIC) have the same or differential roles in bleb formation is not well understood. Here we report that ectopically expressed, GFP-tagged NM-II isoforms exhibit different types of membrane protrusions, such as NM-IIA induces multiple bleb protrusion and NM-IIC1 induces lamellipodia, whereas multiple blebs or lamellipodia formation was not affected by over expression of NM–IIB in tumor MCF-7 cells (Dey and Singh et al., MBoC 2017, 28:1034-1042). Of interest, NM-IIB has an almost 50% lower rate of dissociation from actin filament than NM-IIA and –IIC1 as determined by FRET analysis both at cell and bleb cortices. Interestingly, when cortex was ablated in metastasis tumor MDA MB 231 cells, we find majority of cells (88%) show multiple blebs formation whereas only 42% of normal MCF-10A cells shows multiple blebs formation upon cortex ablation, suggesting the presence of differential actomyosin contractility in the different degree of tumor cells. We correlate that differential actomyosin contractility is dependent on the type of NM II isoform present in the actomyosin complex. This study also suggests tumorigenecity can be evolved by changing the actomyosin contractility.

P1126

**Board Number: B129**

**Characterization of MYO19 knockdown phenotype in a cultured neuron-like cell line.**

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Myosin-XIX (MYO19) is an actin-based motor protein implicated in normal mitochondrial dynamics and distribution. Mitochondrial movement and positioning is thought to be essential in order for energy intensive processes (such as cell division, cell motility, and morphogenesis) to occur efficiently. We hypothesized that MYO19 aids in mitochondrial dynamics and positioning during neuronal differentiation, a complex and energy intensive process resulting in long cellular extensions protruding from the cell body. During this cellular event MYO19 may be involved in supporting cell motility (growth cone or cell body motility) as well as overall morphogenesis (neurite establishment). In order to test this hypothesis, we used the murine, neuron-like CAD cell line and lentiviral infection to generate new lines stably-expressing short-hairpin RNA (shRNA) that target the MYO19 message. We generated multiple non-clonal CAD lines that each expressed a different shRNA, and verified knockdown via western
Surprisingly, quantitative Morris, and consisting actin localize motor lines R.T. plasmids. We made a delay may differentiation-IQ1-IQ2 domain, actin, and mitochondria. We observed actin filaments were destabilized, thereby interfering with the linkage between actin and mitochondria. These results suggest that decreased levels of MYO19 may be influencing CAD cell differentiation by altering the cell's ability to move and position mitochondria via the actin network.

**P1127**

**Board Number: B130**

**Contributions of Myo1 motor and tail domains to Myo1 localization and function at the endocytic sites in fission yeast.**

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Myo1, the only class-1 myosin in Schizosaccharomyces pombe, is recruited to sites of endocytosis called actin patches just before a burst of branched actin assembly. Myo1 plays a role in endocytic internalization since without Myo1, internalization is reduced. Yet, how Myo1 is recruited to and functions at actin patches is not fully understood. Myo1 has an N-terminal motor domain which binds F-actin, a lever arm with two light-chain-binding motifs (IQ1 and IQ2), and a C-terminal cargo-binding tail consisting of a phospholipid-binding TH1 domain, a proline-rich TH2 domain, an SH3 domain and an Arp2/3 complex-binding CA domain. To dissect the contribution of each domain to Myo1 localization, we made a nested series of deletions starting from either the N- or C- terminus of Myo1. Loss of the motor domain or its activity resulted in re-localization of Myo1 from actin patches to the plasma membrane (PM) and eisosomes. However, disruption of F-actin with Lat A was not sufficient to re-localize Myo1 to eisosomes, resulting in Myo1 at stable cortical puncta with other endocytic proteins. Surprisingly, while Myo1 tail (TH1-TH2-SH3-CA) alone localized to the cytoplasm, deletion of SH3-CA, TH2-SH3-CA and internal deletion of TH2 reduced Myo1 accumulation in actin patches and upon treatment with Lat A resulted in complete cytoplasmic localization of these mutants. To further understand how the tail regulates localization, we expressed GFP-tagged tail domain constructs from plasmids. We found that TH2-SH3-CA but not full Myo1 tail could weakly localize to patches suggesting TH1 may inhibit TH2- and SH3- mediated interactions. We propose that while the motor-IQ1-IQ2 and TH1 function in Myo1 recruitment, TH2 and SH3 domains help retain Myo1 at patches. We tested the functional impacts of Myo1 truncation mutations by tracking the internalization of actin patches. We found that the motor-IQ1-IQ2-TH1 portion of Myo1 is minimally sufficient to support endocytic internalization. We then tested whether attaching motor-IQ1-IQ2 tagged with a GFP binding protein to GFP-tagged tail, which alone is cytoplasmic, or IQ2-tail, which alone is in eisosomes, would rescue Myo1 patch localization. Surprisingly, while heterodimerization of the motor–IQ1-IQ2 and tail restored Myo1
actin patch localization and rescued internalization, heterodimerization of the motor-IQ1-IQ2 and IQ2-tail failed to restore patch localization as both fragments co-localized in eisosomes. This suggests that the state of the tail dominates Myo1 localization and we propose that the functional motor portion of Myo1 is able to suppress the state of IQ2-tail that drives eisosome localization in the context of the full length Myo1 but not when the two halves are heterodimerized.

**Dynein**

**P1128**

**Board Number: B131**

The dynein activator Hook1 is required for long-distance trafficking of BDNF-signaling endosomes in neurons.

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In neurons, neurotrophic signals are transported long distances along the polarized microtubules of the axon via signaling endosomes, which promotes neuronal development and survival. The retrograde transport of signaling endosomes is driven by the molecular motor cytoplasmic dynein, yet it remains unclear how dynein is spatially and temporally regulated in this context. We hypothesize that adaptor proteins mediate precise cargo transport by differentially regulating dynein motors associated with distinct cargos. To assess this model, we focused on coiled-coil proteins in the human Hook family, Hook1 and Hook3. Using single molecule TIRF assays and inducible dimerization assays, we found that Hook proteins act as dynein adaptors that induce superprocessive motility. Hook proteins induced both longer run lengths and higher velocities than the previously characterized dynein activator, bicaudal D2 (BICD2). These results indicate that dynein adaptors can differentially regulate dynein to allow for organelle-specific tuning of the motor. Next, we used live cell imaging to investigate the role of Hook proteins in endosomal transport in primary hippocampal neurons. We found that Hook1 co-migrates with a subpopulation of Rab5-positive endosomal vesicles, which display more retrograde-directed motility and faster velocities than Hook1-negative early endosomes. While Hook1 was also associated with a subpopulation of Rab7-positive endosomes, Hook1 did not appear to enhance the motility of this subpopulation. Knockdown of Hook1 produced modest effects on the overall motility of Rab5- and Rab7-positive endosomes, but significantly reduced the motility of BDNF-positive signaling endosomes. This inhibition of signaling endosome motility was specific to depletion of Hook1 but not Hook3, and was rescued by expression of siRNA-resistant Hook1. To specifically model signaling endosome transport from the axon terminal to the nucleus, we used microfluidic chambers to control addition of BDNF-bound Quantum dots to axonal tips only. Hook1 knockdown resulted in a significant decrease in the frequency of BDNF-Qdot motility along the mid-axon suggesting that Hook1 may affect both the generation and motility of signaling endosomes. Together, this work suggests that Hook1 acts as a specific dynein adaptor for BDNF uptake and signaling endosome transport from the distal axon back to the soma, and supports the model that differential association with cargo-specific adaptors efficiently regulates dynein function in neurons.
P1129

Board Number: B132

Distribution of Cortical Dynein Attachment Molecule Regulates Dynein-Mediated Spindle Pulling Mechanism in Budding Yeast.

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In budding yeast, dynein-dependent microtubule sliding along the bud cortex mediates spindle movement into the mother-bud neck. Here we show that spatial distribution of the dynein-anchoring protein, Num1, plays a role in regulating dynein-dependent cortical pulling mechanisms. We found that Num1 accumulated at the bud tip in cells lacking cortical ER attachment molecules Scs2 and Scs22. Three-dimensional time-lapse imaging of astral microtubule behavior revealed that bud tip-localized Num1 mediated capture-shrinkage of astral microtubule plus ends, pulling the mitotic spindle into the mother-bud neck. Disrupting dynein anchorage or dynein motor activity at the bud tip, but not deleting Kar9, Kar3 or Kip3 (the canonical components mediating capture-shrinkage pathway), abolished the observed end-on capture-shrinkage of microtubule plus ends. Intriguingly, a NUM1-CAAX allele restored Num1 distribution throughout the cell cortex, rescuing dynein-dependent cortical pulling via microtubule sliding. Our findings revealed a novel mechanism of dynein-dependent spindle pulling in budding yeast that is regulated by the distribution of the cortical attachment molecule Num1.

P1130

Board Number: B133

Investigating the mechanism by which the dynein cortical receptor Num1 activates dynein motility.

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Cytoplasmic dynein is the major minus-end directed microtubule motor in cells. To regulate its myriad functions, dynein requires numerous regulatory proteins including LIS1 (Pac1 in yeast), NudE/NudEL (Nd1 in yeast), and the growing family of adaptor proteins (e.g., BicD2, Spindly, Hook) that link dynein to dynactin. The potential interplay between these various regulatory proteins has been difficult to parse out, and may depend on the specific dynein function (e.g., cargo transport vs. spindle positioning). For instance, LIS1 reduces dynein velocity while dynactin promotes its processivity and it is unclear whether their activities are mutually exclusive. We are utilizing budding yeast as a model to dissect the various roles of several such regulators, and to understand how these molecules individually and in concert contribute to dynein regulation. Specifically, we have previously shown that dynein motility is activated in cells by Num1, the dynein cortical receptor. Our in vivo data supported a model in which Num1 activates dynein motility by relieving inhibition by Pac1/LIS1. To further examine the mechanism by which Num1 activates dynein, we have reconstituted Num1-dynein-dynactin motility in vitro, and are examining the effects of Pac1 and Nd1 on this motility. Despite Num1 exhibiting structural similarity with adaptor proteins, we find that it has no appreciable effect on the processivity of single molecules of dynein-dynactin. Our data also demonstrate that Pac1/LIS1 can reduce the velocity of dynein-dynactin to an extent similar to that of dynein alone, indicating that Pac1/LIS1 and dynactin binding to dynein is not mutually exclusive. We find that Pac1/LIS1 has a similar effect on the motility of single Num1-dynein-dynactin complexes, indicating that Num1 is not sufficient to disengage Pac1 from dynein. Given that human NudE has been shown to compete with dynactin for a binding site on the dynein tail, we are
currently investigating what role, if any, Ndl1 has in affecting the motility of Pac1-Num1-dynein-dynactin complexes. Our work will provide an unambiguous dissection of many of the effector proteins involved in the regulation of cellular dynein activity.

**P1131**

**Board Number: B134**

**Cell cycle regulation of dynein activity prevents DNA damage.**

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How do cells balance active, motor driven, transport to position the mitotic spindle while retaining genome integrity? Dynein is a molecular motor responsible for positioning the mitotic spindle, which is critical for proper cell division and cell fate determination. Understanding how dynein activity is controlled during the cell cycle is an important step of regulation. In budding yeast, dynein activity is restricted to positioning the spindle during anaphase onset by using astral microtubules to pull the nucleus into the daughter cell. We hypothesize dynein activity is tightly controlled during cell division to limit mechanical damage exerted on the genome. We are testing this hypothesis in two ways: 1) investigating whether hyper-activating dynein is sufficient to cause DNA damage, and 2) identifying cell cycle regulators that are mechanistically linked to dynein regulation. We have shown several mutations that increase dynein-dependent spindle movement also increase DNA damage. Using confocal imaging of living budding yeast cells, we measured the accumulation of DNA double stranded breaks by tracking fluorescently labeled Rad52 foci and we measured a 10-fold increase in Rad52 foci accumulation through anaphase in the mutants that hyper-activate dynein, compared to wild type control cells. We are now testing other mutants that increase or decrease dynein activity to investigate how DNA damage is generated, and elucidate the mechanism that normally restricts dynein to specific phases of the cell cycle. These results will give insights into the mechanisms that regulate dynein during the cell cycle and why dynein regulation is important for the integrity of DNA.

**P1132**

**Board Number: B135**

**Mechanism for G2 phase-specific nuclear export of the kinetochore protein CENP-F.**


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Centromere protein F (CENP-F) is a component of the kinetochore and a regulator of cell cycle progression. CENP-F recruits the dynein transport machinery and orchestrates several cell cycle-specific transport events, including transport of the nucleus, mitochondria and chromosomes. A key regulatory step for several of these functions is likely the G2 phase-specific export of CENP-F from the nucleus to the cytosol, where the cytoplasmic dynein transport machinery resides; however, the molecular mechanism of this process is elusive. Here, we have identified 3 phosphorylation sites within the bipartite classical nuclear localization signal (cNLS) of CENP-F. These sites are specific for cyclin-dependent kinase 1 (Cdk1), which is active in G2 phase. Phosphomimetic mutations of these residues strongly diminish the interaction of the CENP-F cNLS with its nuclear transport receptor karyopherin α. These mutations also diminish nuclear localization of the CENP-F cNLS in cells. Notably, the cNLS is phosphorylated in the −1 position, which is important to orient the adjacent major motif for binding into
its pocket on karyopherin α. We propose that localization of CENP-F is regulated by a cNLS, and a nuclear export pathway, resulting in nuclear localization during most of interphase. In G2 phase, the cNLS is weakened by phosphorylation through Cdk1, likely resulting in nuclear export of CENP-F via the still active nuclear export pathway. Once CENP-F resides in the cytosol, it can engage in pathways that are important for cell cycle progression, kinetochore assembly and the faithful segregation of chromosomes into daughter cells.


P1133
Board Number: B136
The contribution of electrostatic interactions to the processivity of inner-arm dynein c.
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Dynein c, one of the axonemal inner-arm dyneins of Chlamydomonas flagella, shows a high processivity although it functions as a single-headed molecule in vitro and has a low duty ratio. In this study, we evaluated the effects of electrostatic interactions on the processivity of dynein c using a high ionic strength solution and deletion of the negative-charge rich region of tubulin. Continuous movement assays revealed that the contribution of electrostatic interactions between dynein c and microtubules to maintain gliding movements was small. The number of dynein c molecules required to maintain microtubule movement was estimated to be one for intact microtubules. Under high ionic strength conditions, that number increased to two, but was not changed when the negative-charge rich region of tubulin was deleted. On the other hand, electrostatic interactions between dynein c and microtubules significantly contributed to the capture of diffusing microtubules and to the start of their translocation. Landing Rate assays revealed that the number of dynein c molecules required to start microtubule translocation was two. That number was increased to five under high ionic strength conditions and by deleting the negative-charge rich region from tubulin. These results suggest that dynein c has at least two different microtubule binding states: one is at the moment of capturing the microtubule before the beginning of movement and the other is during the translocation of the microtubule. Electrostatic interactions significantly contribute to the process of microtubule capture before movement, but are less essential to microtubule movement.

P1134
Board Number: B137
The 3.5Å cryoEM structure of a fast dynein/dynein complex.
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Dynein and its cofactor dynactin form a highly processive microtubule motor in the presence of a coiled coil adaptor, such as BICD2. Different adaptors link dynein/dynactin to distinct cargos. Here we use electron microscopy (EM) and single molecule studies to show that adaptors can recruit a second dynein to each dynactin. Whereas BICD2 is biased towards recruiting a single dynein, the adaptors Hook3 and BICDR-1 predominantly recruit two. Shifting the equilibrium towards a double-dynein complex increases both force and speed by almost two-fold. A 3.5 Å cryo-EM reconstruction of the dynein tail/dynactin/BICDR-1 complex explains how the second dynein is recruited. Our work provides a
structural basis for how diverse adaptors recruit different numbers of dyneins and suggests the mechanism by which adaptors can tune the properties of the dynein/dynactin machine.

**P1135**  
**Board Number: B138**  
**A Conserved Interaction of the Light Intermediate Chain with Dynein-Dynactin Effectors.**  
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Cytoplasmic dynein 1 (dynein) is the major minor-end–directed microtubule-based motor in eukaryotic cells. Dynein cargo selectivity is achieved through interactions with cargo-specific effector proteins that mediate the formation of dynein-dynactin-effector (DDE) ternary complexes. While these effectors are generally unrelated, they share the ability to interact with both dynein and dynactin and to activate dynein processivity. How this is achieved is poorly understood. Here, we describe the identification and characterization of a region of the dynein Light Intermediate Chain 1 (LIC1) that mediates the interaction with unrelated effector proteins. Binding studies with purified proteins and using isothermal titration calorimetry allowed us to map the interaction to a conserved helix within LIC1 and to N-terminal fragments of BICD2, Hook1 and Hook3. A crystal structure of the LIC1 helix bound to an N-terminal fragment of Hook3 reveals a major conformational change of the extended C-terminal helix of the conserved Hook domain to form a hydrophobic cleft where the LIC1 helix binds. Mutating the interacting interface of the LIC1 helix identified in the crystal structure impaired lysosomal positioning in cells. Addition of a peptide corresponding to the LIC1 helix competitively inhibits processive, dynein-driven motility in single-molecule assays using total internal reflection fluorescence (TIRF) microscopy of cell extracts. Together, the results demonstrate the existence of a conserved mechanism of interaction between the dynein LIC1 and dynein-dynactin effectors.

**P1136**  
**Board Number: B139**  
**Dynein Light Intermediate Chains participate differently in fundamental aspects of neocortex development.**  
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Cytoplasmic dynein is the major retrograde microtubule motor and participates in several aspects of neocortex development. These include Radial Glial Progenitor (RGP) cell cycle-dependent interkinetic nuclear migration (INM) and division, the multipolar-to-bipolar transition of newborn neurons, axon extension, and neuronal migration to the cortical plate (CP). The dynein light intermediate chains (LICs) are cargo-binding subunits, but the relative roles of the two LIC genes, LIC1 and LIC2, are not well understood. To test whether they play overlapping or distinct roles in vivo we used in utero electroporation in E16 rat embryos injected intracerebrally with LIC1 and/or LIC2 shRNAs. We analyzed the brain tissue subsequently by fixed and live imaging. LIC1 RNAi led to a marked inhibition of apical INM and a decrease in mitotic index in RGPs. In contrast, LIC2 RNAi had little-to-no effect at this stage. BICD2 expression, which can rescue defects in other apical INM genes and is a known LIC interactor, failed to rescue LIC1 knockdown, suggesting a role for the LIC1-BICD2 interaction in INM. LIC1 RNAi also
blocked further steps of brain development, with an accumulation of cells at the incompletely differentiated multipolar stage and a decrease in the number of cells in CP, their final destination. Expression of truncated LIC1, lacking the cargo binding domain, also caused a decreased number of cells in this region, highlighting the importance of cargo binding function of LIC1 for the progression of the newborn neurons to the CP. In contrast, there was little apparent effect of LIC2 depletion in the number of bipolar neurons in the CP, though the number of neurons reaching the upper regions was reduced. Co-expression of RNAi-insensitive LIC1 or LIC2 each rescued the LIC1 knockdown phenotype, though incompletely. Despite the greater severity of the LIC1 vs. LIC2 knockdown effect, the ratio of LIC1:LIC2 protein levels was unaltered in during the late embryonic period (E16-E20), and even in the adult rat. Nonetheless, pull-down experiments showed a greater affinity of BICD2 for LIC1. Over-all, we find that LIC1 and LIC2 are associated with distinct phenotypic effects during brain development, with a particularly important role for LIC1 in apical nuclear migration in RGP cells and for the steps of neuronal differentiation and migration. In view of the constant ratio of LIC1 to LIC2 during brain development, these results may reflect the stronger interaction of LIC1 with at least one dynein cargo-binding protein. (Supp. by FCT MDPPhD Scholarship PD/BD/113766/2015 to JCG, and NIH HD40182 to RBV.)

P1137
Board Number: B140
Model for RILP mediated Autophagosome Assembly and Transport.
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Autophagy is a highly conserved cellular process used to degrade damaged and misfolded proteins and organelles, to recycle amino acids and to destroy pathogens. Defects in neuronal autophagy lead to toxic protein aggregates, resulting in degeneration and neuronal death, a hallmark of Alzheimer’s, Parkinson’s and Huntington’s Disease. Autophagy is regulated by mTOR kinase, which when inactivated under conditions of cellular stress, leads to formation of autophagosomes (APs), which are retrogradely transported towards the lysosomal compartment by cytoplasmic dynein.

We have identified a novel role for the dynein adaptor RILP (Rab interacting lysosomal protein) in neuronal autophagy. RILP recruits dynein to Rab7-GTP-positive late endosomes (LEs) in nonneuronal cells, but its neuronal function has not been explored. With high resolution live cell imaging, we have found that RILP is associated with and required for retrograde transport of autophagosomes (APs) in neuronal and nonneuronal cells (ASCB 2016). We found that, in addition to a Rab7-binding site, RILP contains three LC3 Interacting Regions (LIRs) and can bind independently to either nascent and mature APs or LEs in neurons. We found, furthermore, that expression of LIR-mutant RILP results in substantially lower numbers of APs, suggesting a novel role for RILP in AP formation as well as transport. To test for a role in AP formation, we have further examined RILP localization to DFCP1-positive AP nucleation sites and ATG5-positive isolation membranes. We find that RILP and its N-terminal motor binding domain bind to ATG5-positive structures in neurons. Further immunofluorescence analysis suggests mutually exclusive ATG5 vs. dynein colocalization with RILP, suggesting competition for RILP binding. This arrangement might serve to ensure that incomplete isolation membranes remain immotile, and that only more mature structures can be transported.

Finally, we now find that small molecule-mediated inhibition of mTOR results in upregulation of RILP mRNA levels in neurons as judged by qRT-PCR, and this increase correlates with induction of autophagy. These results suggest that RILP plays an important role in mTOR-sensitive neuronal autophagosome assembly and transport.
Based on these results, we propose a model for RILP mediated neuronal AP assembly and transport: mTOR inhibition in neurons may upregulate RILP, which in turn stimulates AP assembly by associating with growing isolation membranes. RILP may further transport newly formed APs through their fusion with LEs and lysosomes. This mechanism may be involved in mTOR-sensitive autophagy in neurons to respond to diverse forms of cellular stress.

Supp. by NIH GM102347.

P1138
Board Number: B141
Membrane-associated septins promote dynein-driven transport by scaffolding dynein light and intermediate chain interactions.
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Septins are GTPases that assemble into non-polar hetero-oligomers and self-organize into higher order polymers such as filaments, rings and gauzes. Septins associate with cell membranes and the cytoskeleton, functioning as molecular scaffolds for protein-protein interactions or as diffusion barriers for the spatial compartmentalization of membrane proteins. Here we show that membrane-associated septins promote dynein dependent transport by recruiting cytoplasmic dynein through direct interactions with the dynein light chain Tctex-1 and dynein intermediate chain (DIC). Using the ActA protein of *Listeria monocytogenes* and the TagRFP fluorescent protein, we targeted various septins to mitochondria and we found that SEPT9-tethered mitochondria accumulate at the perinuclear region of COS7 cells. This accumulation was dependent on dynein, because overexpression of dynamitin p50 disperses mitochondria throughout the cell. Staining for the dynein light chain Tctex-1 and intermediate chain DIC showed that they accumulate on SEPT9-tethered mitochondria. *In vitro* GST pull downs using recombinant proteins show that SEPT9 interacts directly with Tctex-1. We identify two Tctex-1 binding motifs on SEPT9 matching the consensus sequence [R/K]-[R/K]-X-[R/K] found in various Tctex-1 targets. Tctex-1 binding null mutants of SEPT9 fail to cause perinuclear clustering of mitochondria or accumulate Tctex-1. Interestingly, Tctex-1, DIC and SEPT9 form a tripartite complex *in vitro* and the presence of SEPT9 increases the DIC-Tctex-1 interaction. Taken together, our findings suggest that SEPT9 promotes dynein-mediated transport by scaffolding the interactions of the Tctex-1 cargo adaptor with the dynein motor complex. On-going studies aim at determining if through this interaction, septins affect the positioning and trafficking of membrane organelles such as the Golgi complex and endolysosomes.

P1139
Board Number: B142
Dynein force production and its regulation.
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The molecular motor dynein is responsible for most microtubule-driven cargo traveling towards the microtubule minus-ends, near the cell nucleus. Though biophysically, mechanisms of many dynein function are well characterized, its regulation in the cell is less understood. Recently, our lab reported that dynein-driven lipid droplet cargos are able to adapt their force production to improve transport when facing opposition to motion, a dynein function that was previously unknown. While we have
identified two important factors involved in this function, NudEL and Lis1, the work presented here will discuss other proteins involved in control of this adaptation pathway.

P1140

Board Number: B143

Molecular basis for dynein dysfunction in a spectrum of motor neuron diseases.
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Proper intracellular transport along axons is crucial for maintaining the health of motor neurons. In these cells, dynein is the primary method by which cargos from distal ends of axons are transported to the soma. As such, dysfunctions in the dynein motor due to genetic mutations are the primary underlying cause in a spectrum of neurodegenerative disorders. This spectrum of dyneinopathies encompasses a variety of muscular dystrophy and neurological diseases. These diseases are typified by missense mutations to the dynein heavy chain with dominant negative phenotypes, cellular degeneration, and are typically childhood onset. However, studying dynein in mammalian tissues remains challenging and limited data is available about the effect of these mutations on the motor. Using the power of budding yeast dynein as a high-throughput platform to study how missense mutations influence motor behavior, we may investigate the molecular basis for dysfunction in mutant dynein isoforms and attempt to reverse the dysfunction.

P1141

Board Number: B144

The Drosophila MAST Kinase Drop out controls Dynein-mediated transport and is required for phosphorylation of cytoplasmic Dynein.
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A family of four Microtubule-associated Ser/Thr (MAST) protein kinases have been implicated in several human diseases including breast cancer, neurodegenerative diseases and inflammatory bowel disease, but the cellular biological function of this kinase family is poorly understood. We identified a single Drosophila MAST kinase homolog encoded by the drop out (dop) gene. Mutations in dop affect several processes depending on Dynein-dependent transport in early embryos including transport of polarity proteins, Golgi-derived vesicles, lipid droplets and rab11-dependent endosomal transport. By creating the first null mutants of Dop, we present evidence that Dop is a key upstream regulator of membrane growth during cell formation in early embryos. We used a SILAC based proteomic approach in embryos to identify substrates of Dop and found a 4.8-fold reduction in phosphorylation of the conserved Ser401 residue of Dynein-light-intermediate chain (Dlic) in dop mutants. We demonstrate that a phospho-mutant form mimicking Ser401 phosphorylation is able to suppress the phenotypes of dop mutant embryos. These results indicate that Dop is a regulator of Dynein-dependent transport during key events in the initiation of plasma membrane growth and may shed light on the mechanisms of MAST kinases in human diseases.
P1142

Board Number: B145

The actin capping protein is involved in dynein function but non-essential for Arp1 filament assembly.

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Cytoplasmic dynein, a minus-end-directed microtubule motor, requires the dynactin complex for a variety of in vivo functions. The backbone of the vertebrate dynactin complex is the Arp1 (actin-related protein 1) mini-filament whose barbed end binds to the heterodimeric actin capping protein (Schafer et al., 1994 J Cell Biol). However, it has been unclear whether the actin capping protein is a dynactin component in lower eukaryotic organisms, especially because there is no evidence that it binds to the budding yeast dynactin complex (Moore et al., 2008 Traffic). Here our biochemical data show that the actin capping protein is a component of the dynactin complex in the filamentous fungus Aspergillus nidulans. Moreover, deletion of the gene encoding capping protein alpha (capA) results in a partial defect in both nuclear distribution and early endosome transport, two dynein-mediated processes. A recently published study using RNAi to knockdown capping protein beta in mammalian cells revealed a critical role of the capping protein in dynein-mediated spindle orientation (di Pietro et al., 2017 Curr Biol). While our conclusion on the involvement of capping protein in dynein function is consistent with this study, capping protein in fungi seems less critical for dynein function than that in mammalian cells. In the ΔcapA mutant, the defect in nuclear distribution is obviously less severe than that exhibited by a dynein heavy chain mutant. The defect in early endosome transport exhibited by the ΔcapA mutant only becomes obvious when an abnormally bigger early endosome is formed via endosomal fusion, but the defect is still less severe than that exhibited by the deletion mutant of p25, an Arp1 pointed end protein (Zhang et al., 2011 JCB, Schroer 2004 Annu Rev Cell Dev Biol). Moreover, although capping protein blocks the elongation or shortening of conventional actin filaments (Cooper and Sept, 2008 Int. Rev. Cell Mol Biol), our biochemical analysis suggests that its loss does not affect the dynactin Arp1 filament in an obvious way. Recently, cryo-EM structural studies found that one dynactin complex binds two dimeric dyneins and one of them binds at the barbed end of the Arp1 filament close to the capping protein (Urnavicius et al., 2017 bioRxiv; Grotjahn et al., 2017 bioRxiv). Based on all these results, we suggest that the capping protein may enhance dynein function not by affecting significantly the assembly of the Arp1 filament but by modulating dynein binding or positioning on dynactin and consequently its activity.

P1143

Board Number: B146

NEEP21 Family Member Calcyon Regulates Biogenesis and Axonal Transport of Late Endosome/Lysosome Related Organelles.

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The findings reported here identify a possible novel mechanism regulating axonal transport and biogenesis of late endosomes and lysosome related organelles (LE/LROs) based on the coordinated coupling of microtubule motor and vesicular coat proteins by the neuron-enriched, transmembrane protein Calcyon (Caly). The C terminus of Caly pulled down proteins involved in microtubule transport (DIC, KIF5A, p150Glued and Lis1) and organelle biogenesis (AP-1 and AP-3) from mouse brain...
homogenates. Removing 10 amino acids from the juxtamembrane domain of Caly abolished motor protein binding, but not AP-3 interaction. A Caly mutant that does not bind AP-3 (ATEA) was unable to pull down motor proteins from brain homogenates, suggesting that adaptor protein and motor protein binding are coupled in vivo. While shRNA mediated knockdown of Caly reduced the motility of LE/LROs labeled by LysoTracker in dorsal root ganglion neuron axons, upregulation of Caly stimulated movement of LE/LROs. Caly upregulation also increased the motility of organelles labeled by the AP-3 cargo GFP-PI4KI\(\alpha\), but not GFP-Rab5 labeled early endosome/sorting endosomes. Whereas Caly upregulation produced equivalent increases in anterograde and retrograde speeds and run lengths, knockdown resulted in longer anterograde but shorter retrograde motile events. Further, the raw number of LE/LROs, as well as the percent of LE/LROs co-localizing with dynein intermediate chain also correlated with Caly upregulation and knockdown. These results suggest that Caly also impacts organelle biogenesis and motor protein recruitment. In contrast to wild type Caly, expression of the ATEA mutant failed to alter either LE/LRO flux or levels of associated dynein, but abolished effects on organelle biogenesis. Taken together, these data support the hypothesis that Caly is a multifunctional scaffolding protein that promotes AP-3 dependent organelle biogenesis and transport in axons.

**P1144**

**Board Number: B147**

She1 affects dynein by interactions with the microtubule and the dynein microtubule-binding domain.

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Cytoplasmic dynein is an enormous \(\sim 1.2\) MDa molecular motor that transports myriad cargoes toward the minus ends of microtubule tracks. Rather than existing as bare tracks, microtubules are bound by numerous microtubule-associated proteins (MAPs) that have the capacity to affect various microtubule-based cellular functions, including motor-mediated transport. One such MAP is She1, a potent effector of dynein motility that plays a role in polarizing dynein-mediated spindle movements in budding yeast. Here we characterize the molecular basis by which She1 affects dynein motility, providing the first such insight into which a MAP can directly modulate motor motility. We find that She1 reduces the rate by which dynein hydrolyzes ATP, likely due to the enhanced microtubule-binding affinity and consequent reduced stepping frequency imparted on dynein by She1. Microtubule binding by She1 is required for it to affect dynein motility. Moreover, we find that She1 and dynein directly interact and that this interaction is sensitive to dynein’s nucleotide-bound state. We narrowed down the She1 binding region to the microtubule-binding domain (MTBD) of dynein. Accordingly, a dynein motor with a mutated MTBD displays reduced sensitivity to She1 in vitro and in vivo. Taken together, our data support a model in which simultaneous interactions between the microtubule and the dynein MTBD provides She1 with the ability to reduce dynein velocity and prolong its microtubule encounters.
Microtubules Nucleation and Organization 1

P1145
Board Number: B149

Upregulation of the beta-tubulin isoform tubb6 (beta 6, class V) in regenerating skeletal muscles of the mdx mouse model of Duchenne muscular dystrophy: too much of a good thing?


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Many essential cellular functions depend on microtubules. It is therefore not surprising that microtubule defects are found in many diseases. The cause of the defects and their link with the pathology are not easy to pinpoint. Our work focuses on the muscle microtubules of the mdx mouse model of Duchenne muscular dystrophy (DMD). Microtubules in mdx are abnormal and lack the regular grid organization typical of fast rodent muscle fibers. It has been proposed that these altered microtubules contribute to the dystrophic pathology. We now have evidence that an aberrantly high level of the beta-tubulin tubb6 (beta 6, class V) may be involved in the mdx microtubule defects. Tubb6, a ubiquitous minor beta-tubulin isoform, is essential for cell division but disturbs microtubules when overexpressed. We have previously reported that overexpressing a GFP-tagged tubb6 but not tubb5 (beta V, class I tubulin), in the WT flexor digitorum brevis (FDB) muscle causes microtubule changes resembling those of mdx. In contrast, knocking down tubb6 (but not tubb5) from the mdx FDB normalizes microtubules. We also find that the long-term overexpression of GFP-tubb6 in WT FDB causes morphological changes suggesting regeneration of damaged fibers. Why is there a large increase in tubb6 mRNA and protein in mdx and (at least of tubb6 mRNA) in DMD muscles? We have observed that tubb6 is also upregulated during differentiation of the C2 mouse muscle cell line, while tubb5 is decreased. In undifferentiated C2 myoblasts, tubb6 is mostly present in mitotic spindles. In C2 myotubes, which need microtubules for elongation, all microtubules show high tubb6 immunofluorescence. WT adult muscle fibers however show a low level of tubb6 by immunostaining or immunoblotting. It is therefore plausible that tubb6 is elevated in mdx because regenerating (i.e. differentiating) muscle fibers need it for elongation. This idea is supported by our recent observation that tubb6-rich myotubes cluster in IgG-positive areas of inflammation and regeneration in mdx muscle sections. No tubb6 accumulation can be detected in matching WT sections. From these results emerges a model: in WT mice, tubb6 is elevated in elongating myotubes but decreases when myotubes mature into fibers; in mdx, tubb6 increases during the initial wave of muscle regeneration (at 3-4 weeks of age) but fails to subsequently come down because inflammation or other stimuli for regeneration persist. A permanently high tubb6 level in mature fibers could cause microtubule defects and further muscle damage. Thus, mdx microtubule defects may result from a normal reaction to muscle damage gone awry. It will be important to further investigate this model and identify the stimuli causing tubb6 upregulation.
P1146

Board Number: B150

Effect of TPX2 extreme C-terminal domain on microtubule dynamics.
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Organization and dynamics of the metaphase spindle critically depend on the microtubule-nucleating activity of TPX2. It has been shown that TPX2 C-terminal half is sufficient to promote microtubule nucleation in vitro1. This half contains two evolutionarily conserved domains: (1) the importin-binding domain located close to the center of TPX2 is essential for the microtubule-nucleating activity1 and (2) the extreme C-terminal domain binds kinesins and controls their activity2,3. The role of the latter domain in regulation of microtubule dynamics remains poorly understood. Here we address this knowledge gap using a small protein, TPX2-CT, from Brachypodium distachyon with a high degree of homology to the extreme C-terminal domain of TPX2 but lacking the N-terminal region and the importin-binding domain. We demonstrate that in vitro at high concentrations TPX-CT can: (1) induces formation of microtubule asters; (2) inhibit catastrophe; (3) restrict microtubule elongation; (4) nucleate microtubules; and (5) bundle microtubules. At lower concentrations TPX2-CT exhibits weak microtubule polymerase and bundling activity. In vivo, the TPX2-CT suppresses microtubule catastrophe resulting in the formation of arrays. The geometry of these arrays can undergo global re-orientation even though microtubule dynamic instability is dramatically reduced. Our modeling experiments demonstrate that inhibition of catastrophe is sufficient to alter the microtubule organization through progressive microtubule elongation and simulates the in vivo reorganization. Inhibition of catastrophe by TPX-CT requires highly evolutionarily conserved residues within a putative coiled-coil domain. These residues confer higher affinity of TPX2-CT to microtubules and increase oligomerization of TPX2-CT. We conclude that the suppression of catastrophe by TPX2-CT in vivo depends on its oligomerization capacity. Our findings advance understanding of TPX2-dependent microtubule dynamics and demonstrate a novel mechanism responsible for generation of acentrosomal microtubule arrays.


P1147

Board Number: B151

Investigating the role of lateral interactions in microtubule dynamics.
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Microtubules are an essential component of the cytoskeleton, and cells regulate their functions by controlling microtubule dynamics. How the dynamics of microtubules are intrinsically regulated remains unknown. Several observations, including drugs that stabilize microtubules and electron micrographs of disassembling microtubules, suggest that lateral interactions are the weak point of a microtubule. Based
on these observations, we hypothesize that lateral interactions are the key site for regulating microtubule dynamics. We focus on the M-loop of β-tubulin and its interaction with H1-S2 and H2-S3 of the neighboring β-tubulin subunit predicting that more flexible loops promote microtubule polymerization while strengthening and structuring these interactions will stabilize microtubules. At low temperatures, yeast microtubules depolymerize but Antarctic animal species have dynamic microtubules. The lateral loops of β-tubulin from several Antarctic animal species are hot spots for sequence differences. We make mutations in the yeast β-tubulin gene, TUB2, mimicking the lateral loops of Antarctic species or treat with microtubule stabilizing drugs and determine the affect on microtubule dynamics. Studying TUB2 mutations located in the lateral loops, we hope to define the role the lateral interactions play in regulating microtubule dynamics.

**P1148**

**Board Number: B152**

Phosphatases participate in the control of microtubule organization through CDK regulation. J. Magescas¹, J.L. Feldman¹;

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The cytoskeleton is a critical regulator of internal cell organization. Among it, microtubules are organized from specific structures called microtubule organizing centers (MTOCs). The vast majority of research has historically focused on the centrosome, however, in non-cycling cells MTOC function can be reassigned to non-centrosomal structures such as the plasma membrane. However, how the cell inactivates MTOC function at the centrosome and maintains it inactive while other sites assume the MTOC function is unknown Activation of MTOC activity at the centrosome is catalyzed by cell cycle regulated kinases (e.g. CDK-1, PLK-1, and Aurora-A/AIR-1), and so one hypothesis is that inactivation of MTOC activity at the centrosome involves phosphatases that could directly or indirectly regulate the function of those kinases. We are using C. elegans as a model to characterize MTOC inactivation at the centrosome, particularly how it is maintained inactive, and test the implication of PPPs in this process. We are using the C. elegans adult germ line as an in vivo model to study the maintenance of centrosome inactivation. The hermaphrodite germline is distributed over two gonad arms, each composed of a thousand germ cells. In the majority of these cells, MTOC function has been reassigned to the plasma membrane and the centrosomes are maintained in an inactive state. For example, the microtubule nucleating complex γ-TuRC associates with the membrane and the centrosome lacks PCM and associated microtubules. We tested a role for phosphatases in the maintenance of centrosome inactivation using pharmacological inhibitors. Inhibition of PPPs in the gonad resulted in a loss of γ-TuRC from the membranes and in the reactivation of the centrosome as an MTOC. Indeed, microtubules and Aurora-A/AIR-1 relocalized to the centrosome following phosphatase inhibition. Using a combination of different inhibitors, we conclude that a PP1 phosphatase is required to maintain the membrane MTOC state and centrosome inactivation in the germline. Additionally, inhibition of PPPs and CDK resulted in only a small reduction of γ-TuRC at the membrane and the centrosome remained inactivate, suggesting that PPPs control CDK activity in the gonad and thus centrosome MTOC activity. Based on these observations we propose that a single or a combination of PPPs control the organization of the microtubule in differentiated germ cells. This happens partially through the control of CDKs activity although not entirely. We are further characterizing this process by identifying the PPPs involved as well as their targets.
P1149
Board Number: B153
Microtubule glycylation regulates cortical microtubule assembly and ciliary array organization.
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Motile ciliary arrays generate directed fluid flow that is essential for respiratory airway mucus clearance, cerebral spinal fluid movement and cell motility. To optimize directed fluid flow, motile cilia within the array are organized and polarized. This is achieved by cortically anchoring the basal bodies that nucleate and organize motile cilia. Basal body associated cortical microtubules are a conserved population of microtubules that position basal bodies within the ciliary array. Using the ciliate model organism, Tetrahymena thermophila, we studied the timing and dynamics of basal body associated microtubule assembly during new basal body assembly, maturation and incorporation into the ciliary array. Tetrahymena possess two major classes of basal body associated microtubules, post-ciliary microtubules and transverse microtubules. Post-ciliary microtubules assemble immediately following new basal body assembly whereas transverse microtubules slowly assemble and elongate. We propose that microtubule elongation promotes basal body attachment to the cell cortex. Both basal body associated microtubule structures are prominently marked with the microtubule post translational modification, glycylation. When glycylation is disrupted in Tetrahymena, transverse microtubules promiscuously elongate during early basal body maturation and fail to reach their complete lengths. Consistent with the importance of basal body associated microtubules in basal body positioning, loss of microtubule glycylation causes basal body disorganization. These data reveal the importance of basal body associated cortical microtubules, and their glycylation, in organizing ciliary arrays.

P1150
Board Number: B154
Fodrin as a regulator of microtubule nucleation.
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Fodrin is a non-erythroid homologue of spectrin, underlying the plasma membrane found in most eukaryotic cells. The functions delegated to this protein include structural integrity of the cell upon interaction with other cytoskeletal components, signal transduction and apoptotic pathways. Reports published from our lab detail the presence of fodrin as a component of the γ-TuRC (gamma tubulin ring complex) derived from neuronal tissue and neuronal lineage cells. The major objective of this study is to understand the functional contribution of fodrin in γ-TuRC mediated mechanisms in the cells. γ-TuRC is a complex association of proteins, utilised in the cells as a major tool for microtubule formation and organisation. Centrosome, being the major hub of microtubule nucleation in mammalian cells, is rich in γ-TuRCs.
Towards understanding the aforementioned objective, we employed both in vitro and in vivo approaches. We chose U251 MG, a glioblastoma cell line as our working system. Fodrin was downregulated by targeted shRNA treatment and centrosomal microtubule nucleation was judged by analysing the astral microtubule intensity around the centrosomes in these cells. It was found that the fodrin downregulated cells show significant reduction in astral microtubule intensity in comparison to the control. We also found that fodrin downregulated cells showed delayed microtubule nucleation as compared to control cells upon microtubule regrowth after cold depolymerisation. Concurrent studies
from our lab show reduced gamma tubulin presence at the centrosome of fodrin downregulated cells. This implies that fodrin might contribute towards efficient recruitment of gamma tubulin, and in fodrin downregulated cells this could result in impaired centrosome functions. For understanding the significance of the direct association of fodrin with γ-TuRC, in vitro assays were designed. Microtubule polymerization was monitored turbidometrically using purified neuronal γ-TuRC. The functional efficiency of this complex was compared with a typical, nucleation competent γ-TuRC devoid of fodrin, purified from HEK 293 cells. The nucleation time analysis revealed a significant reduction in the nucleation potential of fodrin containing γ-TuRC as opposed to fodrin negative γ-TuRC. The overall polymerization was also considerably reduced. Moreover incubation of purified fodrin with canonical γ-TuRC from HEK 293 cells reduced its nucleation potential. Electron microscopic analysis also showed a reduction in the number of microtubules formed by neuronal γ-TuRC when compared to HEK 293 derived γ-TuRC. This shows that even though fodrin is incapable of inducing microtubule nucleation, its association makes gamma-tubulin less competent in affecting nucleation in neuronal cells.

P1151
Board Number: B155
Cytoplasmic Ran regulates acentrosomal microtubule nucleation in neurons.
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The proper formation of microtubules (MT) is essential for neuronal developmental processes, such as differentiation, polarization, and migration. The generation of MTs in post-mitotic neurons highly depends on nucleation by acentrosomal MT-organizing centers (MTOCs). While Golgi outposts have been shown to promote acentrosomal MT formation in dendrites, the acentrosomal MTOCs in axon remain elusive. We have previously shown that TPX2 is an important component of the acentrosomal MTOC in neurons. TPX2 distributes along the neurite shaft and binds to the MTs. In addition, TPX2 depletion in neurons impairs MT formation at the tip and the base of the neurite. Because it has been shown that the small GTPase Ran regulates TPX2 activity and MT nucleation around the chromosomes during mitosis, we set out to examine whether Ran also regulates MT nucleation in neurons. Here we show that the active GTP-bound Ran concentrates in regions similar to TPX2 (i.e. at the tip of the neurite and in the soma). Overexpressing the TPX2 inhibiting protein importin-alpha results in the decrease of acentrosomal MT formation in neurons. Additionally, acentrosomal MT formation decreases when importazole, a Ran signaling interrupting molecule, is applied. Furthermore, both of the neurite length and the microtubule intensity increase when a constitutively active Ran mutant (RanQ69L) is overexpressed in neurons. These data suggest that cytoplasmic Ran regulates TPX2 activity and promotes MT nucleation in neurons. Finally, we will provide data using the optogenetic tool to support this hypothesis.
P1152
Board Number: B156
Regulation of mitotic spindle assembly factor, NuMA, by Importin-β.
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ABSTRACT Ran-GTPase orchestrates mitotic spindle assembly by modulation of the interaction between Importin-α/-β and spindle assembly factors (SAFs). The inhibition of SAFs carried out by importins needs to be done without much sequestration from abundant nuclear localization signal (NLS)-containing proteins. However, the molecular mechanisms that determine NLS-binding selectivity and that inhibit the activity of Importin-β-regulated SAFs (e.g. Nuclear Mitotic Apparatus protein (NuMA)) remain undefined. Here, we present a crystal structure of the Importin-α•NuMA C-terminus complex showing a novel-binding pattern that accounts for selective NLS recognition. We demonstrate that, in the presence of Importin-α, Importin-β inhibits the microtubule-binding function of NuMA. Further, we have identified an uncharacterized high-affinity microtubule-binding region that lies carboxyl-terminal to the NLS, which is sterically masked by Importin-β on being bound by Importin-α. Our study provides mechanistic evidence of how Importin-α/-β regulates the NuMA functioning required for assembly of higher-order microtubule structures, further illuminating how Ran-governed transport factors regulate diverse SAFs and accommodate various cell demands.

P1153
Board Number: B157
XMAP215 functions synergistically with gamma-TuRC to promote microtubule nucleation.
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How microtubules are generated in the cell is a major open question in understanding how the microtubule cytoskeleton is assembled. For several decades, gamma-TuRC has been accepted as the universal microtubule nucleator in the cell. Although there is evidence gamma-TuRC might not be the sole nucleator, identification of other factors has proven difficult. We have developed an assay that resolves individual microtubule nucleation events in Xenopus egg extract. This allows the direct and simultaneous measurement of microtubule nucleation kinetics and microtubule plus-end polymerization in a physiological environment. Using this assay, we have been able to identify factors that are required for microtubule nucleation. Here, we report that XMAP215, the well-characterized microtubule polymerase of the cell, is essential for microtubule nucleation in meiotic Xenopus egg extracts and promotes microtubule nucleation in a concentration-dependent manner. XMAP215 functions synergistically along with gamma-TuRC to promote microtubule nucleation in physiological conditions as well as in vitro.
P1154

Board Number: B158
The role of TPX2 in branching microtubule nucleation.
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The mitotic spindle is a bipolar array of microtubules, which orchestrates the accurate segregation of chromosomes during cell division. Microtubules in the spindle are nucleated by the gamma-tubulin ring complex (γ-TuRC). How γ-TuRC gets activated at the right time and location remains elusive. Recently, it was uncovered that microtubules nucleate from pre-existing microtubules within the mitotic spindle. This process, termed branching microtubule nucleation, is stimulated by the protein TPX2. However, the exact mechanism of TPX2 in this pathway remains unknown. We established the domain organization of Xenopus laevis TPX2 and define the minimal TPX2 version still capable of stimulating branching microtubule nucleation, which we find is unrelated to TPX2’s ability to nucleate microtubules in vitro. Several domains of TPX2 contribute to its microtubule binding and bundling activities. However, the property necessary for TPX2 to induce branching microtubule nucleation is contained within newly identified γ-TuRC nucleation activator motifs. Separation-of-function mutations leave the binding of TPX2 to γ-TuRC intact, while branching microtubule nucleation is abolished, suggesting that TPX2 may activate γ-TuRC to promote microtubule nucleation.

P1155

Board Number: B159
The alpha-tubulin acetyltransferase αTAT1 enriches the cellular population of stable microtubules by selectively destabilizing dynamic microtubules.
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Microtubules are filamentous tubes that are required for organizing the internal structure of cells, maintaining cell shape, and facilitating cell division. In cells, some microtubules remain highly dynamic and undergo multiple cycles of growth and collapse, while others are stabilized and do not exhibit stochastic length changes. Microtubules are also subject to several post-translational modifications. The post-translational modification acetylation has been widely reported to mark stable microtubules, such as those that are particularly abundant in some cellular structures such as neuronal axons. However, the enzyme responsible for α-tubulin lysine 40 acetylation, α-tubulin acetyltransferase 1 (αTAT1), has been reported to increase microtubule dynamics in cells, instead of stabilizing them. How an enzyme that increases microtubule dynamics could also be associated with generating a mark associated with stable microtubules in cells remains a puzzle. In this work, we used in vitro reconstitution experiments and found that αTAT1 is able to acetylate both stable and dynamic microtubules at similar rates without a strong preference for either group. However, αTAT1 itself strongly destabilizes dynamic microtubules while not significantly increasing the basal depolymerization rate of stabilized microtubules. Thus, αTAT1 may enrich the cellular population of stable microtubules by selectively destabilizing dynamic microtubules. We tested this model in cells by analyzing specialized nerve endings called boutons at the Drosophila neuromuscular junction. Boutons have core stable microtubules and peripheral dynamic microtubules, which are associated with the formation of new boutons. We found that the loss of αTAT1 led to an increase in the overall number of boutons as well as the formation of ectopic satellite boutons.
suggestive of increased dynamic microtubules. Although bouton number increased, Futsch, a marker of stable microtubules, was unaffected by the loss of αTAT1. Our results are consistent with the model that αTAT1 acts to shift the balance between stable and dynamic microtubules in cells.

P1156
Board Number: B160
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Different cell states and cell types are characterised by distinct microtubule cytoskeleton architectures which contribute to cell function. Although most of the involved molecular activities have been identified, the design principles for cytoskeleton self-organization are still poorly understood. Biomimetic systems with a limited set of purified motors and stable microtubules have provided some important mechanistic insight into the properties of minimal active filament networks that roughly fall into two classes: (1) contracting networks that often lead to the formation of asters; or (2) percolating networks consisting of extensible microtubule bundles. However, the rules determining the topological outcome of self-organization are still poorly understood. Here we examine the morphogenetic potential for microtubule organization of mitotic human motor proteins kinesin-5 and kinesin-14, two motors with well-established roles in intracellular microtubule cytoskeleton organization during cell division. Using an assay with dynamic microtubules displaying distinctly asymmetric growth properties, we show that different motors with opposing directionality can both form a range of morphologically different structures, including contracting networks or asters with focussed poles, or extensible microtubule networks, albeit at different microtubule nucleation efficiencies, growth properties, or motor and microtubule densities. Morphogenetic phase space is characterised by a broad transition zone between the two extreme network topologies. Numerical computer simulations of active network formation using Cytosim can effectively recapitulate the experimentally observed regimes, allowing us to reveal simple rules explaining the choice of network topology by the system based on biochemical and kinetic characteristics of the motors and the asymmetrically growing microtubules.

Microtubule Cytoskeleton: Techniques

P1157
Board Number: B161
Using smFRET to Understand the Structural Changes that Underlie Tau’s Behavior on the Microtubule Surface.
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The microtubule associated protein Tau is involved in multiple aspects of axonal transport in neurons, regulating microtubule dynamics, kinesin motility, and cargo delivery. Misregulation of Tau function results in a number neurodegenerative diseases, including Alzheimer’s, collectively known as “Tauopathies”. Recent in vitro studies have shown that Tau binds microtubules in an equilibrium
between static and diffusive states. Furthermore, Tau’s dynamic binding equilibrium differs with Tau isoform and correlates with the isoform’s ability to directly inhibit kinesin-1 motility. However, the mechanisms that control Tau’s static-dynamic state equilibrium and define Tau’s different functions have yet to be determined. We recently demonstrated that phosphorylation at tyrosine 18, the last residue in Tau’s phosphatase activating domain, shifts Tau towards the diffusive state on the microtubule surface, both in vitro and ex vivo. These results suggest that long-range structural changes in Tau are responsible for shifts between static and diffusive binding on the microtubule surface, given the large distance between the N-terminal position of Y18 and the C-terminal microtubule-binding region of Tau. However, the structure of Tau on the microtubule surface is not known. To better define the structural states of Tau that underlie its functional behavior, we have developed a novel single molecule fluorescence resonance energy transfer (smFRET) system to image conformational changes of Tau interacting dynamically with the microtubule surface. Using total internal reflection fluorescence (TIRF) microscopy and alternating laser excitation (ALEX), we have observed N- and C-termini dual labeled Tau bound to both paclitaxel and Guanosine-5’-[(α,β)-methylene]triphosphate (GMPCPP) stabilized microtubules in the static and dynamic states. Our results suggest N-C termini interactions occur while Tau is bound statically to the microtubule surface but are lost while in the diffusive state. This work is the first direct evidence of dynamic structural change affecting Tau’s behavior and therefore it’s function on the microtubule surface.

P1158
Board Number: B162
Illuminating intra-cellular transport regulation by post-translational modifications of tubulin with super resolution microscopy.
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The Tubulin-Code hypothesis proposes that Post Translational Modifications (PTMs) of tubulin can mediate unique interactions with motor proteins and has been assessed by in-vitro studies. Here, we investigate the regulation of motor driven cargo transport by Acetylation and Detyrosination in living cells using correlative single particle tracking (SPT) and super resolution microscopy (STORM). By mapping the vesicular trajectories on to the distinct microtubule populations, we elucidate the differential cargo dynamics on post-translationally modified microtubules with high spatiotemporal resolution. Our results show that about 30% of microtubules in epithelial cells are both acetylated and detyrosinated. Stationary lysosomes are preferentially anchored on this microtubule sub-population. Further, motile lysosomes on the detyrosinated-acetylated MTs have shorter run-lengths and higher pause frequencies with respect to lysosome on unmodified MTs. The differences in lysosome motility was due to the detyrosination and not the acetylation state of this MT sub-population. We observed similar results for autophagosomes, suggesting that the impact on motility is likely a general phenomenon affecting diverse sets of organelles and vesicles. Our results suggest that the PTMs are recognized by microtubule motors differentially and modified motility could be cue for organelle interactions such as lysosome-autophagosome fusion.
P1159  
Board Number: B163  
Examining mechanisms regulating microtubule organization in dividing cells using lattice light sheet microscopy.  
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Accurate chromosome segregation must be maintained over multiple cell division cycles to stably propagate genetic information. At anaphase onset, microtubules with opposite polarity interdigitate to form the spindle midzone, a specialized array that functions to keep chromosomes apart and position the cell division plane. The proper assembly of this structure depends on PRC1 (protein regulator of cytokinesis 1), a nonmotor microtubule-associated protein that selectively crosslinks antiparallel microtubules, and Kinesin-4, a plus-end directed motor protein. Prior studies have shown that these proteins form a stable complex in vitro and are thought to “measure” features of microtubule arrays. For example, PRC1/Kinesin-4 complex in vitro can both autonomously regulate the length of antiparallel microtubule overlap in dynamic filament networks and also can form micron-scale “end tags” of different lengths on stable microtubules depending on the size of the filament. However, we do not know how the length-dependent regulation of individual filaments or overlap regions between filaments contributes to spindle midzone assembly in human cells. Here, we use lattice light sheet microscopy to image whole cell volumes of dividing cells at rates varying from 1.8 to 4.4 seconds per volume. We quantitatively investigate the association of fluorescently-labeled PRC1 on microtubule bundles during the metaphase to anaphase transition in hTERT-RPE1 cells. In metaphase, approximately 40 PRC1 bundles are established that span the cell equator and measure 4.6 +/- 1.2 microns in length. Immediately after anaphase onset, PRC1 bundles shrink five-fold to 0.9 +/- 0.3 microns in between the separating chromosomes. We are designing PRC1 mutant constructs that have reduced binding to microtubules which we hypothesize may alter the number of bundles established in metaphase or the length of PRC1 bundles in metaphase and anaphase.

P1160  
Board Number: B164  
An optogenetic approach to control microtubule acetylation in living cells.  
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Dynamics of microtubules are regulated by post-translational modifications, the extent and variety of which depend on the cell type and environmental conditions. Acetylation of specific microtubules regulates cargo selection and trafficking to specific regions of the cell edge. Cell stress causes hyper-acetylation of microtubules, and increased microtubule acetylation correlates with metastasis, indicating that microtubule post-translational modification is important in cancer cell biology. To study the role of microtubule acetylation in live cells, we designed an analog of alpha tubulin acetyl transferase1 (αTAT1) that can be controlled with light. Unlike other tubulin modification enzymes, αTAT1 is highly specific for tubulin in its polymerized form. αTAT1 binds and transfers an acetyl moiety from acetyl coA to lysine 40 on alpha tubulin. A fragment of αTAT1 was sandwiched between the photo-responsive LOV2 domain from Avena Sativa phototropin and a peptide that we had engineered to bind
selectively to the dark state of LOV2 (Wang et al. Nature Methods 13 755-758, 2016). Only in the dark, the LOV2 and Zdk bound to each other, occluding the microtubule binding interface. Peptide linkers at the attachment sites of LOV2 and Zdk were optimized for the most effective caging. Photoactivatable αTAT1 (PA αTAT) could be fully activated in less than a second, and the half-life for return to the off state could be adjusted between 1.7 and 496 seconds by mutating residues around the LOV2 flavin. We observed extensive acetylation of microtubules in living cells upon irradiation of cells expressing PA αTAT1. Use of the analog to elucidate the role of microtubule acetylation in directed motility will be described.

P1161
Board Number: B165
Illuminating the role of Microtubule-Actin Crosslinking via Optogenetics.
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The dynamic cytoskeletal network is composed of microtubules (MTs), actin and intermediate filaments (IFs). How the physical coupling of the cytoskeletal filaments dictates downstream cellular morphological changes remains poorly understood. We developed a novel optogenetic tool (based on iLID light induced dimerization (Guntas et al., PNAS 2015)), SxIP-iLID, to facilitate the reversible recruitment of factors to MT plus ends in an End Binding (EB) protein-dependent manner using blue light. SxIP-iLID constitutively tracks MT plus ends and when activated with blue light (405-500 nm) an SspB-tagged protein is recruited to SxIP-iLID. This system is highly reversible: post activation, SxIP-iLID returns to the dark-state and releases SspB-tagged proteins from MT plus ends. We show that SxIP-iLID tracks MT plus ends and recruits tgRFP-SspB upon blue light activation without altering MT comet velocities. We used this system to investigate the effects of temporally cross-linking MT plus ends and F-actin in Drosophila S2 cells to gain insight into spectraplakin function and mechanism. Spectraplakins are the primary class of proteins that cross-link and integrate the different cytoskeletal filaments, aiding in the dynamic remodeling of the cytoskeleton. The spectraplakin family plays a fundamental role in cellular processes including polarity, morphogenesis, migration and intracellular trafficking. We show that blue light-mediated MT-F-actin cross-linking decreases MT growth velocities and generates a MT exclusion zone in the lamella. We are now positioned to test photo-induced MT-F-actin crosslinking in vivo. Currently, we are introducing this modular system into Drosophila to probe how MT-F-actin cross-linking regulates the establishment of cell polarity and cell migration. SxIP-iLID facilitates the general recruitment of specific factors to MT plus ends with temporal control enabling researchers to systematically regulate MT plus end dynamics and probe MT plus end function in many biological processes.
P1162
Board Number: B166
Controlling cytoskeletal organization and cellular dynamics by localized optical modulation of microtubule dynamics.
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Throughout the cell cycle and during differentiation, the highly dynamic microtubule (MT) cytoskeleton self-organizes to form polarized intracellular tracks that enable directional transport, facilitate chromosome segregation and help to establish cell polarity. MT remodeling through stochastic switching between phases of growth and shortening is critical for all MT functions in cells, but it is unclear how MT dynamics are spatially and temporally coordinated. Amongst the regulators of MT growth dynamics and interactions are a heterogeneous family of >30 plus end tracking proteins (+TIPs) that associate with growing MT ends. End Binding proteins (EBs) associate autonomously with MT ends and are considered core +TIPs, as they recruit most other +TIPs to the MT end. Interestingly, while all growing MT plus ends are decorated with EBs, MT functions vary in different parts of the cell, suggesting that the activity of the +TIP complex is spatially controlled. Because no tools existed to manipulate subpopulations of MTs with high spatiotemporal resolution, we sought to develop a novel optogenetics approach to spatially control the activity of the EB recruited +TIP complex and investigate the functions of different MT populations during cell migration and mitosis. We developed a photo-inactivated (pi) EB1 molecule by utilizing a phototropin LOV2 domain-based protein interaction pair that rapidly dissociates upon blue light exposure. The resulting pi-EB1 replaced endogenous EB function in EB1/EB3 double CRISPR/Cas9 knockout cells and was rapidly inactivated by blue light in a highly reversible manner. Inactivation of pi-EB1 efficiently disrupted the +TIP network at growing MT ends, induced depolymerization of a population of cell edge associated MTs and reduced persistent growth of MTs in the cell body, without affecting the recruitment of the MT polymerase ch-TOG. Patterned illumination allowed inactivation of pi-EB1 with micrometer precision, and enabled spatial control of MT polymerization in migrating and dividing cells. While the MT network is polarized toward the leading edge of migrating cells, it has remained unclear if this is a cause or consequence of directional cell movement. Our results show that local pi-EB1 inactivation results in an immediate aversive turning response, indicating that EB1-mediated +TIP interactions are essential to maintain migration direction. pi-EB1 also allowed us to investigate how subsets of dynamic MTs contribute to various aspects of mitotic spindle organization and positioning, and found that the EB recruited +TIP complex is required for spindle size homeostasis.

P1163
Board Number: B167
A two-step mechanism for inactivation of MTOC function at the centrosome.
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During mitosis, the centrosome acts as a microtubule organizing center (MTOC), creating a radial array of microtubules to separate chromosomes between daughter cells. Microtubules are organized by complexes within the centrosome’s pericentriolar material (PCM), which is steadily recruited to the centrosome, culminating in a peak in MTOC activity in metaphase. After metaphase, PCM is removed from the centrosome thereby attenuating MTOC function. This inactivation of MTOC function at the
centrosome is likely required for the timely exit from mitosis and for subsequent cell cycle events such as centriole duplication and the reassignment of MTOC function to other structures. While a large body of work has revealed the phosphorylation-dependent steps required for MTOC assembly at the centrosome, little is known about the mechanisms reversing this process at the end of mitosis. We are using C. elegans as a model to characterize MTOC inactivation at the centrosome. In C. elegans, the PCM is comprised of two main proteins, SPD-2/CEP192 and SPD-5, which localize the microtubule nucleating complex γ-TuRC to the centrosome. Pairwise analysis of SPD-2, SPD-5, and γ-TuRC components revealed that the PCM proteins decrease at different rates: SPD-2 decreases at a faster rate than SPD-5 and γ-TuRC, leaving a “cage” of SPD-5/γ-TuRC around the centrosome. Following the gradual removal of SPD-2, the SPD-5/γ-TuRC cage fragments into smaller “packets” that are still associated with microtubules. The packets move towards the cell cortex and first lose their association with γ-TuRC and microtubules, leaving only SPD-5 foci in the cytoplasm that eventually disappear. These data strongly suggest that PCM disassembly is a two-step process, beginning with the initial removal of SPD-2 from the centrosome, followed by the fragmentation of the remaining PCM. We used pharmacological and genetic manipulations to determine the mechanisms underlying either step in disassembly. Using inhibitors of serine/threonine phosphatases, we observed a stabilization of the PCM during anaphase, implicating this phosphatase family in the process of disassembly. Because of the cortical directionality of the packets and their association with microtubules, we hypothesized that packet formation relies on cortical microtubule pulling forces. Using RNAi to perturb these cortical forces we observed a loss of the packets and PCM disassembly was instead accomplished through a much slower, steady removal of components. Based on these observations we propose that PCM disassembly is initiated in anaphase through a phosphatase-dependent removal of SPD-2. SPD-2 removal weakens the PCM that is then torn apart by cortical pulling forces, ultimately inactivating MTOC function at the centrosome.

P1164
Board Number: B168
Stimulation of microtubule-based transport by nucleation of microtubules on membrane organelles.
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Microtubule (MT)-based transport can be regulated through changes in organization of MT transport tracks, but the mechanisms that regulate these changes are poorly understood. In Xenopus melanophores, aggregation of pigment granules in the cell center involves their capture by the tips of MTs growing toward the cell periphery, and granule aggregation signals facilitate capture by increasing the number of growing MT tips. This increase could be explained by stimulation of MT nucleation either on the centrosome or on the aggregate of pigment granules that gradually forms in the cell center. To discriminate between these possibilities, we prevented aggregation of pigment granules and compared nucleation activity of the centrosome between the signaling states. Pigment granule aggregation was prevented by injecting melanophores with antibodies specific for a major granule protein, tyrosinase-related protein 1. The injected antibodies crosslinked pigment granules with each other and created granule clusters too large to be effectively transported to the cell center. Quantification of EGFP-EB1-labeled MT plus-ends that emerged from the centrosome in the same cells treated to aggregate or disperse pigment granules indicated that granule aggregation signals did not increase nucleation activity
of the centrosome. However, inspection of time-lapse sequences of fluorescence images of cells indicated MTs often originated within pigment granule clusters produced by the injected antibodies, which suggested that pigment granules nucleated MTs. To directly test MT nucleation activity of pigment granules, we isolated them from melanophores and incubated in the presence of chromatographically purified fluorescently labeled brain tubulin. Fluorescence microscopy revealed short MTs that were often connected to pigment granules, and showed that the amount of assembled MTs was significantly increased if granules were isolated from cells exposed to granule aggregation than dispersion stimuli. This increase in MT amount correlated with recruitment of gamma-tubulin, a major component of MT nucleation templates, to pigment granules, and was suppressed by the gamma-tubulin inhibitor gatastatin or function-blocking gamma-tubulin antibodies. We conclude that pigment granule aggregation signals stimulate recruitment of gamma-tubulin to pigment granules thus increasing their MT nucleation activity. We suggest that generation of new MT transport tracks by nucleation of MTs on leading pigment granules accumulating in the cell center provides a positive feedback loop that enhances capture of trailing pigment granules by MT tips at the cell periphery.

**P1165**
**Board Number: B169**
**Measuring and modeling polymer gradients argues that spindle microtubules regulate their own nucleation.**
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Spindle microtubules are nucleated by accessory proteins whose activity is spatially regulated. It was shown that many spindle assembly factors bind microtubules. Here, we investigate whether binding microtubules changes the activity of nucleators. To study this issue, we use novel FLIM-FRET measurements to map the concentration of microtubules and monomeric tubulin in and around the spindle. We find that oligomeric tubulin is constrained to the spindle, with no detectable gradient around it. This argues that microtubule nucleation is restricted to the spindle. Using mathematical modelling we demonstrate that this is indicative of a feedback from microtubule binding to nucleator activity. Our results strongly suggest that nucleators binding to microtubules stimulates their activity.

**P1166**
**Board Number: B170**
**Regulation of centrosomal proteins by Zika virus-encoded proteins.**
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Over the past two years, the world has become increasingly concerned with the spread of Zika virus (ZIKV) throughout Latin America and now parts of the United States due to its devastating neurological effects in developing fetuses. According to the World Health Organization, congenital microcephaly via Zika virus infection will affect about 2500 babies this year in Brazil alone (WHO 2016). Although much research has been focused on drug targeting to stop infection of ZIKV, not much is known about the mechanism behind fetal microcephaly secondary to maternal ZIKV infection. Inherited genetic diseases that cause fetal microcephaly most often affect centrosome protein-encoding genes (Buchwalter et al, eLS 2016). Recent studies in our lab have turned to centrosomal activity in SNB19 glioblastoma cells to decipher this phenomenon (unpublished data), concluding that ZIKV activates the microtubule-
organizing center (MTOC) of the centrosome. However, not enough is known about the individual proteins in the ZIKV genome or their effects on SNB19 cells individually. In one study, we transfected SNB19 cells with each of the twelve individual proteins encoded by the ZIKV genome to observe their effects independently with the goal of identifying which protein(s) are sufficient to activate the MTOC activity of the centrosome. We found that some specific proteins in the ZIKV genome have observed changes in the phenotype of SNB19 glioblastoma cells. In this study, we will be investigating further into the specific mechanism of activation of the MTOC and other phenotypic changes attributed to those genomic proteins.

P1167
Board Number: B171
Rescuing Microtubules with Human CLASP2.
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Cytoplasmic Linker-Associated Proteins (CLASPs) belong to a conserved family of microtubule-associated proteins that regulate microtubule dynamics in various cellular contexts including cell migration, axonal growth and cell division. In cells, CLASPs localize to microtubule tips and stabilize dynamically growing microtubules, however, the molecular mechanisms underlying human CLASP activity are not understood. Here, we use in vitro reconstitution with purified protein components and Total Internal Reflection Fluorescence (TIRF) microscopy to investigate the molecular mechanisms employed by human CLASP2 to regulate microtubule dynamics. We demonstrate that CLASP2 suppresses microtubule catastrophe and promotes rescue but does not affect the rates of microtubule growth or shrinkage. Strikingly, when CLASP2 is combined with EB1, a known binding partner of CLASP2, microtubules become hyper-stabilized and display an increased frequency of rescue compared to microtubules grown with CLASP2 alone. We show that the synergy between CLASP2 and EB1 is dependent on a direct interaction, since a truncated EB1 protein that cannot bind CLASP2 does not enhance CLASP activity. Further, we find that EB1 targets CLASP2 to microtubule tips and increases the dwell time of CLASP2. In summary, our data show that human CLASP2 is a microtubule anti-catastrophe factor and rescue factor whose activity is enhanced by EB1-mediated enrichment at growing microtubule tips.

P1168
Board Number: B172
Developing a non-invasive intravitral imaging strategy for analysis of pre-synaptic microtubule dynamics at the Drosophila neuromuscular junction.
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Microtubules (MTs) play critical roles in the development of synapses, but their regulation and precise functions are only beginning to be understood at the molecular level. In particular, little is understood about how the dynamic growth and shrinkage of MTs contribute to pre-synaptic growth and morphogenesis. MT dynamics have been analyzed in Drosophila tissue culture and sensory dendrites. However, due to the less favorable signal/noise ratio and higher background inherent to many in vivo and ex vivo tissue preparations, studies of pre-synaptic MT dynamics at the fly neuromuscular junction (NMJ) have been comparatively limited. To address this, we have optimized a novel software platform,
Aivia (formerly SVCell), for automated and unbiased in vivo 3D particle detection and tracking that effectively addresses the challenges of the Drosophila NMJ. We performed non-invasive intravital imaging of EB1-GFP labeled MTs both in sensory dendrites and at the NMJ. We quantified multiple MT dynamics parameters in both cell types, finding striking differences in MT behaviors between sensory dendrites and at the NMJ. We furthermore analyzed sensory dendrite MT dynamics using multiple drivers of EB1-GFP expression and identified significant differences in distinct driver backgrounds. Thus, we demonstrate a novel strategy for intravital imaging and multiparametric analysis of pre-synaptic MT dynamics at the NMJ of intact animals, which we have used to identify distinct MT behaviors in different cell types and driver backgrounds. Our future studies will combine our imaging and software platform with traditional Drosophila genetics to dissect signaling networks and cytoskeletal effectors that orchestrate MT dynamics in the development of the synapse.

P1169
Board Number: B173
Microtubule assembly and disassembly dynamics (MADDY) model: exploring dynamic remodeling of microtubule tip by using tubulin polymerization-depolymerization in silico.
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Microtubules (MT), the primary components of the chromosome segregation machinery, can switch stochastically from growth to shortening, but the mechanisms of these processes at the molecular level are poorly understood. We developed a bead-per-monomer coarse-grained model of the αβ-tubulin heterodimers: an MT lattice is stabilized by the longitudinal and lateral interactions between the tubulin subunits; the protofilaments have a bending rigidity and the GTP or GDP-bound state-dependent equilibrium bending angles. The model is parametrized against the experimental rates of MT assembly and disassembly and biomechanics of MT polymers. We carried out Molecular Dynamics simulations implemented on a GPU of entire MT polymers in the experimentally relevant second timescale, in order to explore the mechanisms of MT growth. A growing MT tip reaching 50-nm in length forms thin sheets curving outward. These gradually straighten with an increasing number of protofilaments as the lateral interactions gradually incorporate them into an MT cylinder. The growth is accompanied by formation of erroneous attachments and/or accumulation of “lattice defects” lacking tubulin dimers. These defects tend to self-correct over time, but a critical number of erroneous attachments might result in a transient catastrophe, after which a normal growth resumes. These findings offer a quantitative platform to link the molecular tubulin characteristics with the physiological behavior of MT on biological scales of length and time. The model can be used to probe the onset of dynamic instability and to explore the mechanism of catastrophe and rescue of MT polymers.
Assembly and Disassembly of Cilia/Flagella I

P1170

Board Number: B175
IDA3 associates with IFT in growing cilia to selectively mediate transport and assembly of axonemal I1 dynein.

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Many ciliary substructures such as dynein arms assemble in the cell body before IFT-mediated transport in the ciliary compartment and docking to the axoneme. How these axonemal cargoes interact with IFT remains largely unknown. Here, we analyze the transport of the conserved inner dynein arm I1/f, a 1.5 MDa dynein complex that preassembles in the cytoplasm and is transported by IFT (Viswanadha et al., 2014). The Chlamydomonas ida3 mutant (Kamiya et al., 1991) preassembles I1 dynein in the cytoplasm but I1 dynein fails to enter the cilium for assembly on the axoneme, resulting in a slow-swimming phenotype. Cryo-ET confirms that I1 dynein is the sole structural defect in ida3 axonemes. Using whole genome resequencing, we identified a premature stop codon (W22X) in the ida3 mutant and loss of a 115 kDa coiled-coil protein with an intrinsically disordered region in the C-terminal half. This mutation co-segregates with the loss of I1 assembly on the axoneme. Several independent intragenic revertants restore I1 dynein assembly and subsequent motility defect. Expression of HA or NeonGreen (NG)-tagged IDA3 also rescues the motility and I1 dynein assembly phenotypes in ida3. Immuno-blotting demonstrates that IDA3 is restricted to the cell body in cells with full-length cilia, but IDA3 enters the ciliary matrix in growing cilia. Live-cell TIRF microscopy of cells transformed with IDA3::NG confirms the selective entry of IDA3::NG during ciliary assembly and indicates IDA3 entry is regulated in a cilium-autonomous manner (Craft et al., 2016). IDA3::NG moves by anterograde IFT into elongating cilia and largely exits the cilia by diffusion. Immunoprecipitation of IDA3::HA from the matrix fraction of regenerating cilia reveals an association of IDA3 with the I1 dynein intermediate chain IC140. GFP-tagged IC140 also moves by anterograde IFT. We propose that IDA3 interacts with IC140 and acts as an adapter selectively linking the I1 dynein complex to IFT to ensure transport into and within the growing cilium.

P1171

Board Number: B176

The CEP19-RABL2 GTPase complex binds IFT-B to initiate intraflagellar transport at the ciliary base.

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Highly conserved intraflagellar transport (IFT) protein complexes direct both the assembly of primary cilia and the trafficking of signaling molecules. Despite its initial purification from isolated...
Chlamydomonas flagella, immunofluorescence indicates the predominant localization of IFT components and motor proteins is at the ciliary base. Given that IFT trains periodically enter into the cilia from this large pool of IFT particles located at the base, it has been long hypothesized that regulatory components accumulate at the ciliary base to initiate anterograde IFT. To date, no critical regulator for the initiation of IFT has been identified. Here, by tandem affinity purification and mass spectrometry (AP-MS) of the human obesity gene CEP19, we discover two interlinked protein complexes that organize and trigger the entry of IFT-B into the cilia: the FOP-CEP350 and the CEP19 complex with the highly conserved RABL2B GTPase. CEP19 is localized specifically to the distal end of the mother centriole, and patients carrying the nonsense mutation of this gene (R82X) causes morbid obesity, male infertility, and intellectual disability. We discover that CEP19 specifically binds to and is tethered at the base of cilia by the FOP-CEP350 complex. We further find that CEP19 makes a stoichiometric complex with the highly conserved RABL2B GTPase. In vitro assay using fluorescent MANT guanine nucleotides revealed that RABL2 is an example of the class of GEF-independent GTPases, which bind to GTP via its intrinsic nucleotide exchange. Interestingly, CEP19 captures GTP-bound RABL2B specifically, thus allowing only activated RABL2 to accumulate at the ciliary base. Furthermore, AP-MS of GTP-locked, but not of wild-type and GDP-locked, RABL2 revealed that all 17 known components of the IFT-B complex were the top and almost exclusive hits in the purification. The overall very high efficiency of copurification of RABL2 GTP with IFT-B suggests that IFT-B complex is a single effector of RABL2B.

What is the role of RABL2B in the regulation of IFT-B complex? CEP19-RABL2B complex did not affect stability, complex formation, or pre-recruitment of IFT-B, but was critical for triggering the entry of IFT into the cilium. IFT particles visualized by structured illumination microscopy were reduced in CEP19/RABL2 knockout cells. The frequency of IFT-B determined by live-cell imaging of GFP-IFT80 was strongly decreased in CEP19/RABL2 knockout cells, while the velocity of IFT was unchanged, suggesting that CEP19/RABL2 trigger the ciliary entry of intraflagellar transport. The CEP19-RABL2B-IFT pathway thus provides a new molecular mechanism directing ciliary trafficking and important for the formation of the cilium.

P1172
Board Number: B177
Diffusion as a ruler: Modeling kinesin diffusion as a length sensor for intraflagellar transport.
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An important question in cell biology is whether cells are able to measure size, either whole cell size or organelle size. Perhaps cells have an internal chemical representation of size that can be used to precisely regulate growth, or perhaps size is just an accident that emerges due to constraint of nutrients. The eukaryotic flagellum is an ideal model for studying size sensing and control because its linear geometry makes it essentially one-dimensional, greatly simplifying mathematical modeling. The assembly of flagella is regulated by intraflagellar transport (IFT), in which kinesin motors carry cargo adaptors for flagellar proteins along the flagellum and then deposit them at the tip, lengthening the flagellum. The rate at which IFT motors are recruited to begin transport into the flagellum is anticorrelated with the flagellar length, implying some kind of communication between the base and the tip and possibly indicating that cells contain some mechanism for measuring flagellar length. Although it is possible to imagine many complex scenarios in which additional signaling molecules sense length and carry feedback signals to the cell body to control IFT, might the already-known components of the IFT system be sufficient to allow length dependence of IFT? Here, we investigate a model in which the
anterograde kinesin motors unbind after cargo delivery, diffuse back to the base, and are subsequently reused to power entry of new IFT trains into the flagellum. By modeling such a system at three different levels of abstraction we are able to show that the diffusion time of the motors can in principle be sufficient to serve as a proxy for length measurement. In all three implementations, we found that the diffusion model can not only achieve a stable steady-state length without the addition of any other signaling molecules or pathways, but also is able to produce the anticorrelation between length and IFT recruitment rate that has been observed in quantitative imaging studies.

P1173

Board Number: B178

Axonemal Lumen Dominates Cytosolic Protein Diffusion inside the Primary Cilium.

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Transport of membrane and cytosolic proteins in primary cilia is thought to depend on intraflagellar transport (IFT) and diffusion. However, the relative contribution and spatial routes of each transport mechanism are largely unknown. Although challenging to decipher, the details of these routes are essential for our understanding of protein transport in primary cilia, a critically affected process in many genetic diseases. By using high-speed super-resolution microscopy, we have mapped the 3D transport routes for various cytosolic proteins in the 250-nm-wide shaft of live primary cilia with an unprecedented spatiotemporal resolution of 2 ms and < 16 nm. Our data reveal two spatially distinguishable transport routes for cytosolic proteins: an IFT-dependent path along the axoneme, and a passive-diffusion route in the axonemal lumen that escaped previous studies. While all cytosolic proteins tested primarily utilize the IFT path in the anterograde direction, differences are observed in the retrograde direction where IFT20 only utilizes IFT, and approximately half of KIF17 and one third of α-tubulin utilizes diffusion besides IFT.

P1174

Board Number: B179

Ran-mediated ciliary entry of the heterotrimeric kinesin-2 motor complex.

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In eukaryotic cells, cilia and flagella are microtubule based protrusive organelles. Their structure and function are remarkably conserved throughout the tree of life. Precise control of protein trafficking into cilia is essential for its structure and function. Two kinesin-2 motor complexes, heterotrimeric KIF3A-KIF3B-KAP3 and homodimeric KIF17, exist in vertebrate cells and regulate the intraflagellar transport (IFT) mechanism responsible for anterograde (base to tip) trafficking in cilia. Previous work demonstrated that RanGTP and importin-β2 regulate KIF17 trafficking to the cilia. But its role in generalized trafficking of other ciliary motors is unknown. The unicellular green alga Chlamydomonas reinhardtii is an excellent model system to identify the conserved mechanisms of ciliary protein entry due to the ease of quantifying flagellar protein/assembly dynamics and biochemically isolating flagella in this system. Here we show that in Chlamydomonas, flagellar entry of KAP (ortholog of human KAP3) is regulated by the small GTPase Ran1. Immunofluorescent staining indicates Flag-Ran1 is mainly localized
to the nucleus and flagella. Chemical disruption of Ran1-importin β-mediated import blocks KAP3 transport to flagella. Moreover, inhibition of Ran1-importin β-mediated import, but not CRM1-mediated export, can shorten flagellar length. Finally, in mammalian cells, we have mapped a potential nuclear localization signal to the C-terminus of KAP3, which may be required for ciliary/flagellar targeting as well. Our results suggest Ran1-mediated protein transport regulates entry of the heterotrimeric KIF3A-KIF3B-KAP3 complex into Chlamydomonas flagella, which may provide new insights into conserved molecular mechanisms regulating ciliary protein gating.

P1175
Board Number: B180
Local regulation of IFT train assembly and injection at eight distinct flagellar pores in the multiciliate, Giardia lamblia.
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Many protists have duplicated organelles that vary in cellular function, such as the oral and locomotory cilia in ciliated protozoa. For instance, Giardia lamblia is a multiciliated parasitic diplomonad protist with four distinct flagellar pairs. Each pair has a unique interphase length and unique associated ancillary structures, suggesting that Giardia has different ways of building, sensing and/or regulating the length and composition of the different flagella in the same cell. Giardia also has extensive non-membrane bound regions of each axoneme in the cytoplasm. In this way, Giardia is an ideal model to evaluate how cells can precisely control the production and distribution of a common pool of precursor subunits to build duplicated cellular structures. To evaluate how Giardia may differentially assemble and regulate more than one of the same kind of flagellum, we tracked fluorescently tagged intraflagellar transport (IFT) particles required for assembly and maintenance of the length of flagella. IFT particles localize along the entire flagellar axoneme with distinct accumulations at the flagellar pore, the site that marks the transition from the cytoplasm to the membrane bound axoneme. IFT particles are dynamic, but not actively transported along the cytoplasmic regions of the flagellar axoneme, while distinct IFT trains traverse the membrane bound regions of the flagellar pairs. We suggest that the membrane bound axonemal regions are assembled and maintained by IFT, while cytoplasmic regions are assembled via an IFT independent mechanism. We also used quantitative live cell imaging to compare IFT dynamics between flagellar pairs in the same cell. While IFT train speed is consistent between all flagellar pairs, longer flagella have more frequent injections of larger IFT trains than shorter flagella. The size and number of IFT trains also scale linearly with the equilibrium length of the flagellum. Perturbation of flagellar length using the microtubule stabilizing drug Taxol promotes the remodeling of IFT trains to scale to the new equilibrium flagellar length. This works suggests that Giardia differentially regulates IFT train assembly and injection at each of the eight flagellar pores to generate four flagellar pairs with unique equilibrium lengths. We are also exploring how the composition of IFT trains is remodeled during injection from cytoplasmic to membrane bound regions of flagella and identifying the components that regulate this remodeling.
P1176
Board Number: B181
Novel IFT-A gene, Thm2, interacts with its paralog, Thm1, in ciliary protein transport and in adipogenesis.
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Primary cilia are antenna-like sensory organelles that project from the apical surface of most vertebrate cells. Primary cilia are built and maintained by intraflagellar transport (IFT) protein complexes, IFT-B and IFT-A, which mediate bidirectional trafficking of proteins along the ciliary axoneme. Here we characterize the ciliary phenotype of IFT-A mutants, Thm1 (also known as Ttc21b) and its paralog, Thm2. Thm1 and Thm2 are orthologous to Chlamydomonas FAP60, which encodes IFT139. Mouse embryonic fibroblasts (MEF) from E12-E14 Thm1-null, Thm2-null and Thm1; Thm2 double-knockout (dko) mice were generated. Using immunofluorescence, Thm1+/− MEF showed shortened primary cilia with more intense staining of IFT-B components (IFT52, IFT81) and of anterograde motor subunit, KIF3A, at bulbous distal tips. BBSome subunits (BBS2, BBS5) also stained more prominently in Thm1-null cilia, indicating that loss of Thm1 results in defective retrograde transport of these IFT-B and BBS proteins. In contrast, less ciliary staining of IFT-A component, IFT140, was observed in Thm1+/− MEF, suggesting a partial disruption in ciliary entry. Yet in instances when IFT140 was present in Thm1+/− cilia, staining was more intense at bulbous distal tips, indicating a retrograde defect. Thm2+/− cilia showed normal length and staining of IFT and BBS proteins, suggesting Thm2 alone is dispensable for ciliogenesis. However, Thm1; Thm2 dko MEF showed even greater accumulation of IFT81 at ciliary bulbous distal tips than Thm1+/− MEF, suggesting that Thm2 works with Thm1 to regulate retrograde transport of IFT81. Ciliary localization of membrane protein, INPP5E, was also examined. Ciliary INPP5E was reduced in Thm1+/− MEF and further reduced in Thm1; Thm2 dko MEF, indicating that Thm1 is required for INPP5E ciliary import and that Thm2 enhances this process. Finally, differentiation of MEF into adipocytes was assessed. Oil Red O-staining of lipid droplets used as a read-out of adipogenesis was increased in Thm1+/− cells and further increased in Thm1; Thm2 dko cells. In summary, our data show that Thm1 mediates retrograde transport of IFT and BBS proteins and ciliary entry of IFT140 and INPP5E, and that Thm2 optimizes Thm1-mediated IFT81 retrograde transport and INPP5E ciliary import. Further, our data imply that Thm2 augments a repressive role of Thm1 in adipogenesis.

P1177
Board Number: B182
A new model for regulation of flagellum length in Trypanosoma brucei.
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Trypanosoma brucei is a flagellated protozoan parasite responsible for sleeping sickness in Sub-Saharan Africa. During its complex life cycle, it undergoes drastic morphological changes accompanied by extensive variations in flagellum length. They can undergo an asymmetric division that produces two daughter cells, one with a flagellum ten times shorter than the other one. Here, we have investigated the mechanisms controlling flagellum length. We show that the total amount of IFT proteins is directly related to flagellum length, with a fixed concentration of IFT proteins per length unit at all stages of flagellum elongation. To determine whether this relationship is causal or not, we have knockdowned the IFT kinesins to decrease the frequency of IFT. In these conditions, IFT was rate-limiting and the
trypanosomes assembled a shorter flagellum. However, its length still increased after cell division until it became fixed before the cell produced a new flagellum. This indicates that the timing of cell division and flagellum maturation are critical. This was confirmed by blocking cell division, leading to an increase in flagellum elongation, but never beyond the length of the parental one. We propose a new model where flagellum length is regulated by the total amount of IFT, the timing of cell division and the apparently irreversible maturation of the organelle. Finally, we are investigating these parameters during the parasite cycle when trypanosomes develop in the tsetse fly, suggesting the existence of a fourth parameter.

P1178
Board Number: B183
The role of IFT concentration at the base of the flagellum.
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Flagella are important organelles required for motility and cellular signalling, found in a brought range of eukaryotes. Flagellum formation is dependent on Intraflagellar Transport (IFT) in order to transport building blocks to the site of assembly at the flagellum tip. IFT proteins are concentrated in an IFT pool found at the base of the flagellum, from where multiprotein complexes (IFT trains) travel bidirectional within the organelle. Trypanosoma brucei is a protozoan parasite responsible for sleeping sickness in Sub-Saharan Africa that relies on flagella and represents an exciting model to study IFT. High-resolution microscopy carried out on live cells revealed that the pool of IFT proteins forms a donut-like structure, of approximately 300nm diameter, that colocalizes with the transition fibers that anchor the basal body to the flagellum membrane. Due to this colocalization, we identified the retinitis pigmentosa TbRP2 protein as a potential candidate in IFT pool maintenance, compartmentalization or control. RP2 is a tubulin cofactor C domain-containing protein that localizes at the transitional fibers in trypanosomes and has been described to be essential for cilia formation in various ciliated organisms. Here we report that TbRP2 forms a similar donut-like structure covering the IFT pool. The TbRP2 signal overlaps with the entire IFT pool and encloses its proximal end. RNAi knock-down of TbRP2 shows a strong effect on IFT distribution. Loss of TbRP2 affects IFT trafficking, consequently leading to cells close to an IFT knock-down phenotype, with construction of shorter flagella, reduced IFT frequency and IFT material accumulating in the flagellum. We propose that TbRP2 is required for correct IFT. It could contribute to the concentration of IFT proteins at the pool or to the assembly of IFT trains. Currently we are using High-resolution live microscopy in order to investigate the dynamics of the IFT pool and the role of TbRP2 in this process.

P1179
Board Number: B184
PKD2-mediated modulation of transition zone protein regulate the nucleation of apical actin network in vertebrate multi ciliated cells.
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Cilia are hair-like structures that play important roles during development and tissue maintenance. Although most cells express non-motile primary cilia, a subset of epithelial cells is decorated with multiple motile cilia to generate a directional fluid or particle flow. Due to its accessibility and genetic...
tractability, the epidermis of the Xenopus embryo has become a model system to characterize the molecular programs that control ciliogenesis in multi-ciliated cells (MCCs). Cilia are microtubule-based organelles; however, their assembly relies on an intact cortical actin network. The most apical layer of this network contains highly branched actin bundles that surround the axoneme-nucleating basal bodies in a mesh-like fashion. The subapical layer contains short actin filaments that connect neighboring basal bodies to establish synchronized beating patterns. We have previously shown that ciliary transition zone (TZ)-associated molecules (such as NPHP4) recruit formins (such as Daam1) to organize the subapical actin network, suggesting that the subapical actin network helps to shape the TZ. Here, we show that Xenopus pkd2 (trpp2), a calcium permeable TRP channel, is required for normal transition zone and subapical actin network organization. Knockdown of pkd2 causes a ciliogenesis defect that was in contrast to the knockdown of nphp family members not due to altered basal body migration to the apical cell surface. The cilia of pkd2 morphants exhibited an extended TZ structure and a concurrent loss of the subapical actin network. Further analysis showed that the TRPP2-interacting adaptor and actin-capping protein CD2AP acts down-stream of Pkd2. Analysis of the subcellular localization using GFP-fusion of CD2AP demonstrated its association with the TZ. Upon the knockdown of pkd2, CD2AP preferentially accumulated at the tight junction accompanied by an increased nucleation of actin network at the tight junction. Based on these observations, we propose a model where Pkd2 interacts with CD2AP at the transition zone to control the nucleation of actin structures within the subapical actin network.

P1180
Board Number: B185
Actin redundancy in Chlamydomonas reinhardtii is necessary for flagellar protein trafficking.
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Flagella of the unicellular green alga Chlamydomonas reinhardtii are nearly identical to cilia of vertebrate cells and provide an excellent model to study ciliogenesis. These biflagellated cells have two actin genes: one which encodes conventional actin (IDA5) and the other encodes a divergent novel actin-like protein (NAP1). Previously, we described a role for actin in the regulation of flagella-building intraflagellar transport machinery and now probe how actin redundancy contributes to this process due to the availability of a nap1 mutant Chlamydomonas strain. Treatment with Latrunculin B, a potent actin polymerization inhibitor, on the nap1 mutant background effectively eliminates all functional filamentous actins in the cell. We find that actins are an absolute requirement for flagellar growth when the preexisting pool of flagellar precursors is depleted. Nap1 mutants treated with Latrunculin B also showed reduced protein synthesis during regeneration, though this cannot account for the complete lack of flagellar growth when the preexisting pool of flagellar precursors is depleted. Finally, cells without functional actins exhibit slowed incorporation of the existing pool of flagellar precursors during flagellar regeneration as well as loss of the flagellar composition-regulating protein, NPHP4 at the transition zone. Both of these results suggest actins are required for proper flagellar gating. These experiments demonstrate that each stage of flagellar biogenesis requires redundant actin function to varying degrees, with trafficking most significantly impaired upon total actin loss. By leveraging genetic tools and powerful flagellar assays in Chlamydomonas, our data now support a model in which cytoplasmic trafficking of newly synthesized proteins destined for cilia/flagella requires functional actins in addition to microtubules.
Formation of Microvilli and Cilia in the Zebrafish Pronephros Requires an Actin-binding Bioactive Peptide Amidating Enzyme.

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The assembly of membranous extensions such as microvilli and cilia in polarized cells is a tightly regulated, yet poorly understood, process. Peptidylglycine α-amidating monoxygenase (PAM), a well conserved membrane enzyme essential for the synthesis of amidated bioactive peptides, was recently identified in motile and non-motile (primary) cilia. Knockdown and knockout approaches revealed an essential role for PAM in ciliogenesis in Chlamydomonas, Schmidtea and mouse. In mammalian cells, changes in PAM levels alter both secretion and organization of the actin cytoskeleton. Here we further explore the PAM-actin relationship using Pam-null mutant zebrafish (generated using CRISPR/Cas9 genome editing), mammalian tracheal epithelial cells and Chlamydomonas. Lack of Pam in zebrafish recapitulates the ultimately lethal edematous phenotype observed in Pam⁺/− mice and reveals additional defects. The pam⁺/− zebrafish embryos display cystic kidneys, hydrocephalus, small eyes and massive edema, but do not exhibit either laterality defects or altered otolith biomineralization, both of which depend on motile ciliary function during early development. However, there is an initial striking loss of microvilli and subsequently impaired ciliogenesis in the pronephros which also becomes highly dilated. Furthermore, we observed undocked basal bodies and cytosolic axonemes lacking a ciliary membrane in the apical cytoplasm of mutant pronephric epithelial cells; a similar phenotype was seen previously in the ventral epithelium of PAM-deficient planaria. So why can the pam⁺/− embryos assemble cilia at early time points but not later in development? Although pam⁺/− zebrafish have no Pam enzymatic activity at 4 days post-fertilization or beyond, pam mRNA is maternally loaded into early zygotes and thus amidated products may be generated at very early developmental stages. Furthermore, it is possible that maternally-derived amidated peptides are stored in the yolk and thus available for several days until depleted. In multiciliated mouse tracheal epithelial cells, we find that vesicular PAM staining colocalizes with apical actin, below the microvilli. In PAM-deficient Chlamydomonas, the actin cytoskeleton is dramatically reorganized and expression of an actin paralogue is upregulated. Biochemical assays reveal that the cytosolic PAM C-terminal domain interacts directly with filamentous actin (k_d = 600 ± 150 nM), but does not alter the rate of actin polymerization or disassembly. Our results point to a critical role for PAM in organizing the actin cytoskeleton during development which could in turn have an impact on both microvilli formation and ciliogenesis. Supported by DK032949, DK044464 and GM051293 (NIH).
P1182
Board Number: B187
The role of FBF1 in Drosophila Ciliogenesis.
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Drosophila melanogaster possesses diverse ciliary types and assembly modes (IFT-dependent or IFT-independent), is an excellent model organism for cilia research. Here, we investigated the role of Drosophila transition fiber protein FBF1. We found that FBF1 is exclusively expressed in ciliated cells (both sensory neurons and male germ cells) in Drosophila. As expected, FBF1 is associated with the basal body transition fiber region in Drosophila, localizes distal to the centriole but below the transition zone. To understand the function of FBF1, we created FBF1 null mutants by using CRISPR-Cas9 system. Consistent with our previous reports in C.elegans, deletion of FBF1 does not affect the normal localization of transition zone protein MKS1 and MKS6, but results in IFT trafficking defects in Drosophila. Consequently, FBF1 mutant flies have short sensory cilia and show severely uncoordinated phenotype. our results indicate that Drosophila FBF1 is essential for IFT-dependent sensory cilia formation through an evolutionary conserved function in IFT trafficking.

P1183
Board Number: B188
Protein dynamics of intraflagellar transport complex recruitment to basal bodies in multiciliated cells.
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Proper cilia formation and function require the bi-directional trafficking of protein cargo along the axoneme microtubules in a process called intraflagellar transport (IFT). This process is powered by kinesin and dynein motors, and the conserved 22-member IFT complex acts as an adaptor between motors and cargo proteins. To function in ciliogenesis, the IFT proteins must accumulate at the basal body. However, the mechanisms by which these 22 proteins are brought together and accumulated at the basal body are currently unclear. Using live imaging in vertebrate multiciliated cells, we are studying the localization, complex interaction, and protein dynamics of IFT complex formation and basal body recruitment. Using Fluorescence Recovery After Photobleaching (FRAP), we investigated the kinetics of IFT protein recruitment to the basal body in multiciliated cells. We find that IFT proteins quickly recover, but the total recovery differs between IFT-A and IFT-B proteins. Complementing both the localization and dynamics experiments, we will be employing quantitative modeling to better understand the mechanism by which IFT is accumulated in multiciliated cells.
**P1184**

**Board Number: B189**

IFT140 is required for anterograde IFT of several subgroups of flagellar membrane proteins in *Chlamydomonas*.

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IFT140 is a “core” subunit of the IFT-A complex which, together with the IFT-B complex and the BBSome, is a component of the IFT trains that transport cargos and transduce signals in flagella. Mutations in human IFT140 result in ciliopathies such as Mainzer-Saldino Syndrome and Jeune-Asphyxiating Thoracic Dystrophy.

We analyzed a novel *Chlamydomonas* mutant (ift140-1) which produces no detectable IFT140 by western blotting. *ift140-1* cells lack flagella and show decreased levels of peripheral and core IFT-A components (IFT139 and IFT122, respectively). The levels of IFT-B proteins and their localization were unaffected by IFT140 loss. Basal body and transition zone ultrastructure also were unaffected in *ift140-1* cells. IFT140 contains predicted N-terminal WD-repeat domains and C-terminal TPR-like domains. To investigate the functions of these domains, we generated a construct that encodes GFP-tagged IFT140 lacking the N-terminal WD domains and used it to transform the *ift140-1* mutant. Transformed cells, referred to as IFT140ΔWD-G-20C, expressed subnormal levels of the truncated IFT140, but still recovered the ability to form half-length flagella that were motile and had normal axonemal ultrastructure. Live-cell TIRF microscopy of IFT140ΔWD-G-20C cells revealed that anterograde and retrograde IFT velocity were decreased by ~5% and ~40%, respectively, relative to control cells. The substantial decrease in retrograde IFT velocity suggests that the IFT140 WD-repeat domains may function in the interaction of the IFT particle with dynein 1b, the retrograde IFT motor, or in the activation of dynein 1b at the flagellar tip. Anterograde and retrograde IFT frequency also were significantly decreased, a phenotype that was more pronounced in longer flagella. We also saw more static IFT particles in ‘long’ IFT140ΔWD-G-20C flagella, which may account for the decreased IFT frequency in these flagella. MS/MS analysis of the IFT140ΔWD-G-20C flagellar membrane + matrix fraction highlighted roles for IFT140 in both anterograde and retrograde IFT. Importantly, loss of the IFT140 WD-repeat domains resulted in complete loss of a small group of proteins from the fraction and a substantial decrease in the level of GTPases and membrane-associated proteins, including myristoylated and geranylgeranylated proteins. Our work provides the first large scale analysis of changes to the flagellar proteome resulting from mutation of an IFT-A component and indicates a critical role for IFT-A in the anterograde trafficking of a wide variety of signaling and membrane-associated proteins. Defective trafficking of these proteins may be responsible for the multitude of phenotypes associated with *IFT140* mutation in humans.

**P1185**

**Board Number: B190**

Cryo-EM structure of anterograde intraflagellar transport trains.

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Cilia are ubiquitous microtubule based eukaryotic organelles that have fundamental motility, signalling and sensing function. For its assembly and maintenance, the cilium requires bidirectional intraflagellar
transport (IFT) of building blocks to and from its assembly site at the tip. Transport proceeds on microtubule tracks, driven by oppositely directed motors: the anterograde kinesin-2 and the retrograde dynein-1b. Both motors bind to IFT protein complexes organised into large structures called IFT trains, which function as adaptors for ciliary cargos. Retrograde dynein motors are loaded as cargos onto anterograde trains and are transported to the tip where they initiate and power retrograde transport. Using cryo-electron tomography and subtomogram averaging we reveal the tripartite architecture of the IFT machinery in situ in cilia of Chlamydomonas. Our reconstructions reveal the relative positions of the IFT protein complexes and possible interactions with cargos.

Centrosome Assembly and Functions 1

P1186
Board Number: B192
The molecular architecture of the yeast spindle pole body core determined by Bayesian integrative modeling.
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Microtubule organizing centers (MTOCs) form, anchor and stabilize the polarized network of microtubules in a cell. The central MTOC is the centrosome that duplicates during the cell cycle and during mitosis assembles a bipolar spindle to capture and segregate sister chromatids. Yet, despite their importance in cell biology, the physical structure of MTOCs is poorly understood. Here we determine the molecular architecture of the core of the yeast spindle pole body (SPB) by Bayesian integrative structure modeling based on in vivo FRET, small-angle X-ray scattering (SAXS), X-ray crystallography, electron microscopy and two-hybrid analysis. The model is validated by several methods that include a genetic analysis of the conserved PACT domain that recruits Spc110, a protein related to pericentrin, to the SPB. The model suggests that calmodulin can act as a protein cross-linker and Spc29 is an extended, flexible protein. The model led to the identification of a single, essential heptad in the coiled coil of Spc110 and a minimal PACT domain. It also led to a proposed pathway for the integration of Spc110 into the SPB.

P1187
Board Number: B193
Coupling of Polo kinase activation to nuclear localization by a bifunctional NLS is required during mitotic entry.
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Introduction: Successful cell division requires the spatiotemporal coordination of multiple events by master regulator enzymes. Defects in its regulation can lead to cancer. Discovered in Drosophila, the
Polo kinase (called Plk1 in humans) has emerged as a conserved master regulator of multiple processes during cell division including mitotic entry, centrosomes maturation, kinetochore function, spindle assembly and cytokinesis. Polo is composed of an N-terminal Ser/Thr kinase domain (KD) and a C-terminal Polo-Box domain (PBD), which mediates protein interactions with substrates and localization to different subcellular structures. In addition, the PBD and KD can interact and inhibit each other, and this reciprocal inhibition is relieved when Polo is phosphorylated at its activation loop. How Polo is regulated for its multiple functions during cell division is not completely understood.

Results: Here, we have elucidated the molecular mechanism for the spatiotemporal regulation of Polo kinase during mitotic entry. We discovered that phosphorylation of Polo at its activation loop site triggers its nuclear import during prophase. This import depends on a Nuclear Localization Signal (NLS) in the kinase domain that is masked by the PBD when Polo is inactive, and that becomes exposed when the activation loop is phosphorylated. In addition, by mediating the interaction of the kinase domain with the PBD, the NLS is required for reciprocal inhibition of both domains of Polo. Disruptions in this mechanism have profound consequences. Preventing the nuclear localization of Polo leads to a failure of Polo to trigger nuclear exclusion of the CDK1-activating phosphatase Cdc25 during prophase, and induces multiple mitotic defects. Using transgenic flies expressing different mutant forms of Polo, we show that coordination of Polo activity and localization is essential in the developing animal.

Conclusion: Our study uncovers an elegant mechanism coupling Polo activation and nuclear localization in mitotic entry and demonstrates the importance of this mechanism in vivo. It brings new understanding of the spatiotemporal mechanisms linking major regulatory enzymes during mitotic entry, namely Polo, Cdc25 and Cdk1. The dual role of an NLS motif in regulating both localization and activity, and its allosteric regulation by phosphorylation, are novel concepts that could apply to other proteins.

P1188
Board Number: B194
Upstream open reading frames control Plk4 translation and centriole biogenesis.
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Centrioles are microtubule-based structures that contribute to the formation of the centrosome, the major microtubule-organizing center of the cell. It is critical that centriole duplication happens precisely once per cell cycle to ensure genomic integrity following cell division. Centriole duplication is regulated by the conserved kinase Polo-like kinase 4 (PLK4) and overexpression of PLK4 promotes centriole overduplication and spontaneous tumorigenesis in mice. Previous work from our lab and others have shown that PLK4 regulates its own stability through self-phosphorylation to promote its degradation by the proteasome. Here, we show that in addition to this post-translational control, PLK4 expression is also subject to tight translational control due to the presence of upstream open reading frames (uORFs) in the 5′ untranslated region (5′UTR) of the PLK4 mRNA. Human PLK4 mRNA contains two uORFs in the 5′UTR. The 5′ proximal uORF1 is a positive-acting element that suppresses translation reinitiation at downstream uORF2, while uORF2 is an inhibitory element that blocks PLK4 expression. Elimination of both uORFs increases PLK4 protein expression and leads to centrosome amplification. Importantly, the presence of uORFs in the 5′UTR of PLK4 mRNA is conserved amongst vertebrates, suggesting that the mechanism of translation reinitiation involving uORFs may be a conserved feature controlling PLK4 expression. Together, our findings uncover a new layer of regulation that acts in concert with post-translational control, to reduce the abundance of PLK4 and limit centrosome duplication to once per cell cycle.
P1189
Board Number: B195
Albatross/FBF1 integrates centrosome dynamics.
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The centrosome is tiny but functionally important organelle that alters their fashions as: centriole duplication, spindle formation and primary cilia formation. Each event is regulated by known key enzymatic reactions, but how these events are comprehensively conjugated is still unknown. Recent reports indicated that primary cilia formation is ascribed to distal appendage proteins including FBF1, the synonym of our-identifying Albatross, but contribution of Albatross to other centrosomal function is not determined. Here we show novel function of Albatross at the opposite, proximal side of centrosomes. By our preceding advantages of specific antibodies, full-length Albatross genes and refined knockdown experiments with add-back, we found that this proximal localization is responsible for centriolar duplication through the recruitment of SAS6, a cartwheel protein of centrioles. Moreover, Albatross contributes to centrosome separation by recruiting mitotic kinase Plk1 on its S348 phosphorylation with possible Cdk1-priming, which was postulated from SSP consensus sequence on Albatross. Finally, this mitotic phosphorylation-dependent binding and its function was confirmed by immunoprecipitation against Plk1-PBD, mitotic Phos-tag assay and rescue experiments with the phospho-defective mutant of Albatross. Taken together, we conclude that Albatross is a novel platform integrating spatially and mechanistically independent centrosomal functions, including primary cilia formation, centriole duplication and centrosome separation.

P1190
Board Number: B196
t3421, a mutation in a novel protein required for centrosome matrix assembly in the one-cell stage C. elegans embryo.
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Centrosomes, the primary microtubule organizing centres (MTOC) of animal cells, consist of a pair of centrioles surrounded by pericentriolar-material (PCM). As in many metazoans, in the nematode C. elegans, spindle bipolarity of the zygote is established by means of sperm derived centrioles, which mature to a centrosome by recruiting maternally contributed PCM components. Spindle-defective protein-5 (SPD-5/CDK5RAP2/CNN), the major PCM scaffold protein in C. elegans, forms a PCM core at non-mitotic centrosomes. SPD-5 through its ability to self-organize, assembles a PCM matrix at mitotic entry, onto which other PCM proteins are loaded. It has been shown that PCM matrix expansion is regulated through the phosphorylation of SPD-5 by Polo Kinase-1 (PLK-1/Plk-1/polo) (Woodruff, J. B. et al. Science, 348, 808-812, 2015). However, how the non-mitotic SPD-5 core is recruited to the centrosome and its contribution the PCM matrix expansion at mitotic entry remains unknown.

Among a set of temperature-sensitive embryonic lethal mutations, we identified the mutant allele t3421, which causes defective spindle assembly in early embryos. Live cell imaging of embryos at a restrictive temperature, revealed the formation of monopolar spindles during the first cell cycle, followed by an abnormal cytokinesis, as well as tri- or tetrapolar divisions in the second cell cycle. Our current work suggests that paternally contributed centrioles are defective in the process of PCM matrix expansion.
assembly during the first mitotic divisions. In particular SPD-5 is absent from centrosome core prior to mitotic entry. As a consequence SPD-5 recruitment during mitosis and the dynamic expansion of the mitotic PCM is severely compromised. We mapped t3421 to a gene coding for an unknown coiled-coil domain protein. Despite its role in the maintenance of the non-mitotic PCM core and centrosome matrix recruitment, the unknown protein predominantly localizes to centrioles. We also present evidence that t3421 strongly enhances a mild PLK-1 loss of function phenotype.

We propose that the novel protein is part of the centrosome core, which acts as a scaffold facilitating SPD-5 and PLK-1 interaction, to allow robust PCM matrix expansion at mitotic entry.

**P1191**

**Board Number: B197**

**CEP135 Isoforms Regulate Centrosome Number in Breast Cancer Cells.**

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Centrosomes are microtubule-organizing centers comprised of a pair of centrioles surrounded by the pericentriolar material (PCM) that nucleates and organizes cytoplasmic microtubules. Normal cells typically possess one or two centrosomes. In contrast, centrosomes can be numerically amplified in breast cancer cells by an event called centrosome amplification. Centrosome amplification is detrimental as it promotes hallmarks of cancer including genome instability and cellular invasion. Likewise, we find that centrosome amplification in breast cancer cells increases microtubule density and nucleation capacity. Centroseome numbers are controlled by centriole duplication and homeostasis occurs in normal cells by preventing centriole overduplication that leads to centrosome amplification. However, it is not known how centriole duplication is misregulated to promote centrosome amplification in cancer cells. CEP135 is a conserved centriolar protein important for centriole duplication. We identified a short isoform of CEP135 (CEP135<sub>mini</sub>). The full length CEP135 (CEP135<sub>full</sub>) isoform promotes centriole duplication whereas the CEP135<sub>mini</sub> isoform represses it. The transcript and protein levels of CEP135<sub>full</sub> relative to CEP135<sub>mini</sub> (CEP135<sub>full:mini</sub> ratio) are elevated in breast cancer cells. Furthermore, decreasing the CEP135<sub>full:mini</sub> ratio is sufficient to reduce centrosome amplification in breast cancer cells. Thus, the regulation of these two isoforms may play an important role in controlling centriole duplication and centrosome amplification. This led us to ask how the isoforms are generated and if this mechanism can be utilized to alter the CEP135<sub>full:mini</sub> transcript ratio. We found that the CEP135<sub>mini</sub> isoform is produced by alternative polyadenylation. It utilizes a predicted, non-consensus poly A signal (AATATA) for its transcription termination, while the CEP135<sub>full</sub> isoform utilizes a consensus poly A signal for its transcription termination. Polyadenylation signal usage is often misregulated in cancer cells suggesting that the altered usage of the CEP135 poly (A) signals promote increased CEP135<sub>full:mini</sub> transcript ratio in breast cancer cells with amplified centrosomes. We propose that the altered usage of CEP135 and other centrosome component poly (A) signals regulates centrosome amplification and its microtubule organization capacity in breast cancer cells.
P1192
Board Number: B198
C. elegans FZR-1, a Co-Activator of Anaphase Promoting Complex, Acts as Genetic Suppressor of zyg-1 in Regulating Centrosome Assembly.
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The centrosome is the primary microtubule-organizing center in animal cells, and is vital for the maintenance of genomic integrity. For the fidelity of cell division, the centrosome must duplicate only once per cell cycle, and duplicated centrosomes establish bipolar spindles for accurate chromosome segregation. Aberrant centrosomes are often associated with human disorders including cancers and microcephaly, where extra centrosomes lead to chromosome missegregation and abnormal cell proliferation. Levels of centrosome proteins are shown to be critical for proper centrosome number and function. Overexpressing key centrosome factors leads to centrosome amplification, while depleting these factors results in a failure of centrosome duplication. In this regard, protein turnover by proteolysis provides a vital mechanism for proper levels of centrosome factors. In this study, we investigate how the anaphase promoting complex/cyclosome (APC/C) and a cofactor FZR-1 (a homolog of human Cdh1) regulates centrosome assembly in C. elegans embryos. The APC/C complex is an E3 ubiquitin ligase that orchestrates the sequential degradation of cell cycle regulators during mitosis and early interphase. FZR-1/Cdh1 modulates the APC/C at late mitosis and events in G1 during the time when centrosomes duplicate by recognizing destruction motifs (D- and KEN-box). Through genetic screening, we identified fze-1 as a suppressor of zyg-1, encoding a conserved kinase ZYG-1/Plk4 that is essential for centrosome duplication. The fze-1 missense mutations restore centrosome duplication and embryonic viability of zyg-1(it25ts) mutants. As the APC/C complex is also shown to suppress the phenotype of zyg-1 mutants, FZR-1 is likely to regulate centrosome assembly as a part of the APC/C complex. Our quantitative analyses revealed that both centrosomal and cellular SAS-5 levels are significantly increased in zyg-1; fze-1 double mutants, compared to zyg-1 mutants, suggesting that SAS-5 can be a putative substrate targeted by APC/CFZR-1-mediated proteolysis. As ZYG-1 is required for the localization of SAS-5 to centrosomes, hyper-accumulation of SAS-5 might compensate for the loss-of-function of zyg-1. Furthermore, our IP experiment indicates a physical interaction between SAS-5 and FZR-1 in C. elegans embryos. Finally, we show that FZR-1 is enriched at nuclei and associated with spindles and centrosomes. Together, our results show that FZR-1 negatively regulates centrosome assembly in close association with ZYG-1 in C. elegans embryos. FZR-1 depletion results in elevated levels of centrosome factors, thereby compensating impaired ZYG-1 function in centrosome duplication, functioning as part of the APC/C complex to control protein stability in a cell cycle dependent manner.

P1193
Board Number: B199
Centrosome number homeostatic mechanisms.
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The centrosome is the major microtubule organizing center in animal cells. It consists of two centrioles with associated pericentriolar material and appendage structures, and functions as the basal body for the formation of the primary cilium. Centrosomes duplicate once per cell cycle and segregate on the mitotic spindle. Normally, cells have one centrosome (two centrioles) in interphase, and two centrosomes (four centrioles) at mitosis. In contrast, cancer cells often have extra centrosomes, a
phenotype that is correlated with genome instability, aggressiveness, poor prognosis, and recurrence of some tumors. It has been shown that when cells experimentally-depleted of centrioles are allowed to re-form centrioles, they initially make a greater-than-normal number by de novo formation, followed by a return to the normal number over relatively few cell cycles, suggesting that a mechanism to maintain centrosome number homeostasis exists. We consider three possible homeostatic mechanisms: 1) limited duplication of centrioles in S phase, 2) asymmetric segregation of centrioles at mitosis, and 3) centriole elimination. To test these, we induced centriole amplification in RPE1 cells expressing centrin-GFP and tetracycline-inducible Plk4, a positive regulator of centriole duplication. We confirmed that these cells amplify centrioles, and gradually return to the normal number of centrosomes. Labeling cells in S phase with an antibody against SASS6, a marker of new daughter centrioles, showed that duplication is not limited to a subset of centrioles, with SASS6 co-localizing with each centrin focus. Further evidence for lack of a duplication limit was the presence of centriole doublets at mitosis, as expected if each centriole was able to duplicate. Proliferation of cells with amplified centrioles was reduced compared to the appropriate controls, suggesting either increased cell cycle length or cell death specifically associated with amplification. Live imaging revealed no significant difference in time in mitosis between control and centrosome-amplified cells. Instead, we found a significant delay in the length of interphase. Lineage tracking and live imaging experiments are in progress to determine centrosomes fate and cell cycle dynamics. In conclusion, our research has the potential to uncover the mechanisms responsible for centrosome number homeostasis, an important defense against cancer progression.

P1194
Board Number: B200
Correlative STORM/EM analysis of the centriole distal appendage architecture.
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The centriole plays a vital role in a variety of cellular processes including microtubule organization, chromosome alignment, and the formation of motile and primary cilia. The process of ciliation relies on specialized centriolar structures known as distal appendages, which localize to the distal end of the oldest centriole. When visualized by electron microscopy, distal appendages appear as “120 nm long electron dense structures emanating from the centriole microtubule walls, consisting of a of small “stem” and an electron dense “head”. If observed from the proximal centriole end, distal appendages display a clockwise twist of “66 degrees with respect to the vector connecting centriole outer microtubules. Distal appendages contain several proteins: Odf2, CCDC41/Cep83, CCDC123/Cep89, SCLT-1, FBF-1, and Cep164. Previous studies have demonstrated the recruitment hierarchy for these proteins showing that Odf2 and CCDC41 accumulate first, recruiting CCDC123 and SCLT-1. SCLT-1 is then needed for the recruitment of FBF-1 and Cep164. Due to the small size of appendages, conventional light microscopy does not provide the resolution to reveal the precise localization of distal appendage proteins (DAPs) on appendages. Here we combine the strengths of two powerful imaging techniques-Stochastic Optical Reconstruction Microscopy (STORM) and electron microscopy (EM)- to unravel the localization and dynamics of several DAPs in RPE-1, Hela, and mIMCD3 cells. STORM analysis revealed that each DAP has a unique nine-fold symmetrical pattern, which appears conserved across cell lines. Correlative STORM/electron microscopy analysis revealed the position of STORM signals with respect to the centriole wall and the characteristic distal appendage electron densities (stem and head). Cep164 signal localizes outermost and encircles the appendage head in a hook-like pattern. Cep164 units touch laterally homogenizing the space between the two electron dense EM signals. FBF-1 has a lobular appearance and co-localizes with the electron-dense appendage head. SCLT-1 and FBF-1 are localized at

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the same distance from the centriole wall. CCDC41 appears as a discrete signal closest to the centriole. Its signal overlaps with Odf2, which is localized around centriole wall in a more uniform pattern. Finally, our analysis reveals the dynamic nature of DAPs during cell cycle. From G1 till late G2 the appendage structure is maintained. From prophase till metaphase the outermost proteins Cep164 and FBF-1 are mostly lost, while the inmost proteins CCDC41 and SCLT-1 remain associated with the centrioles. Our study provides the most detailed analysis of distal appendage architecture to date.

P1195
Board Number: B201
mRNA is a dynamic component of the pericentriolar material in Drosophila early embryos.
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Aberrant centrosome function underlies pathologies with profound significance for human health, including growth deficiency syndromes and cancer. Centrosomes function as microtubule organizing centers, build the mitotic spindle, form the basal body to template cilia in ciliated cells, and serve as platforms for signaling cascades including cell cycle signaling. The centrosome consists of a pair of centrioles surrounded by a protein matrix termed pericentriolar material (PCM). The composition and quantity of PCM determines the microtubule nucleating activity of the centrosome and changes rapidly in cycling cells. The mechanisms responsible for rapid changes to centrosome composition and structure are incompletely understood. Intriguingly, a screen for localized mRNA in Drosophila early embryos identified multiple mRNA transcripts enriched near the spindle poles. Mutations to several of these genes disrupt centrosomes and/or the mitotic spindle, suggesting that mRNA enrichment at the centrosome may be an unexplored mechanism to modulate centrosome function. To test this hypothesis, we combined single molecule fluorescence in situ hybridization (smFISH) with quantitative imaging in Drosophila early embryos to measure the centrosome enrichment of several model mRNAs throughout the cell cycle. Here, we report that centrosome-related mRNAs, including Centrocortin (Cen) and Pericentrin-like protein (Plp), are enriched at centrosomes labeled by the pericentriolar material marker, Centrosomin-GFP. Our data suggests that Cen mRNA moves from a diffuse centrosomal enrichment to clustered peri-centrosomal structures during the syncytial divisions of the early embryo. We conclude that centrosomal mRNAs are dynamic during development and enriched at bona fide centrosomes. We are currently investigating the functional significance of centrosome-localized mRNA, which will inform our understanding of the regulation of this crucial organelle.

P1196
Board Number: B202
Centrocortin regulates actin-related processes in the early Drosophila embryo including axial nuclear expansion in a Rho1-dependent manner.
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In the early Drosophila embryo, many important changes occur during development, including the dynamic actin cytoskeleton rearrangements that control axial nuclear expansion. This process involves movement of the nuclei along the anterior-posterior axis so that they become evenly spaced during the early syncytial mitotic cycles. The mechanism behind this movement is poorly understood, but requires nonmuscle myosin II and the subsequent phosphorylation of its regulatory light chains (RLC). Rho kinase, which is activated by the GTP form of Rho1, regulates RLC phosphorylation and is also required for axial
expansion. Centrocortin (Cen), which is associated with the centrosome and the actin cleavage furrows in the early embryo, is necessary for accurate cleavage furrow formation. Cen mutants display defective cleavage furrows due to disruption in the actin cytoskeleton surrounding the early embryo nuclei. This disorganization of actin leads to inappropriate centroskeleton interactions between multiple nuclei as evident by linked microtubule spindles. In this study, we aimed to establish a link between the Rho1 pathway, Centrocortin, and axial expansion in order to elucidate the system behind axial expansion. We found that Cen interacts physically with multiple actin regulators such as Filamin, Formin, and other actin-associated proteins through mass spectrometry analysis. A hemizygous mutant of Cen combined with a heterozygous mutant of Rho1 results in severe axial expansion failure, indicating a cooperative role for both proteins. The hatch rate of hemizygous Cen mutants is slightly, but significantly, lower than wildtype, while the hatch rate of the double Cen Rho1 mutants is drastically lower indicating a strong interactive role for both of these proteins during early embryogenesis. These data indicate that Cen is in a complex with other actin regulators in the Rho1 pathway in order to regulate the actin dynamics of the early Drosophila embryo.

P1197
Board Number: B203
Characterization of centrosome amplification in the unique cell types of the placenta.
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The centrosome is the microtubule-organizing center of animal cells, and is composed of a pair of centrioles and pericentriolar material. Centrioles are required for cillum formation and mitosis, and their number is tightly controlled. As with chromosomes, centrioles are duplicated once and only once per cell cycle, and are segregated equally in mitosis. In most mitotically cycling cells, these processes are coupled, such that chromosome and centriole number are equivalent. However, the two processes are uncoupled in some differentiated cell types, resulting in amplification of either centrioles or the genome without canonical cell cycle progression. Amplification is often critical to the function of these differentiated cell types; for example, the amplification of centrioles in multiciliated cells is required for the subsequent formation of the hundreds of motile cilia that define that cell type. We are interested in addressing how DNA replication and centriole duplication are uncoupled from the cell cycle, and from each other, in terminally differentiated cells.

We have chosen to focus on trophoblast giant cells (TGCs), a terminally differentiated cell type in the placenta. TGCs undergo modified cell cycles through a process known as endoreduplication, in which the genome is amplified many-fold without cell division. TGCs are important in placental development, specifically for implantation of the embryo and placental remodeling during pregnancy, and some subtypes of TGCs are highly invasive. TGC invasion defects contribute to disease states such as preeclampsia and accreta in which TGCs fail to migrate to the proper location in the placenta. Prior to this work, little was known about the centrosome cycle in TGCs. We show that centriole number in TGCs in vitro increases as nuclear area increases, throughout differentiation. The amplified centrioles in TGCs have known markers of centrosomal maturation, including distal and subdistal appendages, centriolar cap, and pericentriolar material. The TGC centrioles have the potential to act as functional microtubule-organizing centers, but do not form a primary cillum in culture. We are testing whether centriole and genome copy number increase in tandem during differentiation, or whether they are independently amplified. Interestingly, inhibition of either process with specific drugs suggests that centriole duplication and DNA replication can be uncoupled in TGCs. Lastly, we are investigating whether amplified centrosomes in TGCs are present in the developing placenta in vivo. We hypothesize...
that amplified centrosomes in TGCs might contribute to their highly invasive phenotype, based on previous work showing that increased invasiveness is associated with centrosome amplification in cultured cancer cells.

Kinetochore Assembly and Functions 1

P1198
Board Number: B204
Investigating the contribution of phospho-Histone marks to Aurora B kinase activity at kinetochores.

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Dynamic, regulated interactions between kinetochores and spindle microtubules result in amphitelic attachment of sister chromatids, which facilitate proper chromosome bi-orientation prior to cell division. Essential for the regulation of kinetochore-microtubule attachments is the centromere-localized chromosome passenger complex (CPC), which is composed of Survivin, Borealin, INCENP, and Aurora B kinase. In early mitosis, Aurora B kinase phosphorylates kinetochore substrates to reduce the stability of kinetochore-microtubule interactions, thereby preventing premature stabilization of any incorrect connections that form. Centromere recruitment of Aurora B kinase is proposed to depend on two histone modifications: Haspin kinase phosphorylation of histone H3 at Thr3 (H3-pT3) and Bub1 kinase phosphorylation of histone H2A at Thr120 (H2A-pT120). A major premise of the current model describing centromeric CPC recruitment is that phosphorylated H3 and H2A spatially overlap, and this region of overlap directs CPC recruitment to inner centromeres. Contrary to the proposed model, however, immunostaining experiments reveal that H3-pT3 localizes to the inner centromere, while H2A-pT120 distinctly localizes to kinetochores in a “paired dot” pattern. Thus, it is unclear if the “overlapping phospho-mark” model accurately describes centromeric CPC recruitment. In addition, Aurora B kinase and other members of the CPC, including Borealin and INCENP, have been localized distinctly to kinetochores in addition to the inner centromere. How Aurora B kinase is recruited specifically to kinetochores, and whether H2A-pT120 and/or H3-pT3 are required, remains unknown. Here, we set out to determine the contribution of H2A-pT120 and H3-pT3 to the recruitment of Aurora B kinase and additionally, to the activity of Aurora B towards kinetochore substrates. We find that each individual histone phospho-mark is sufficient to recruit the CPC, and localization of CPC components does not, in fact, require a combination of the two histone modifications. Furthermore, such overlap is not required for Aurora B kinase activity towards kinetochore substrates. However, we find that Bub1 kinase activity is essential for kinetochore-associated Aurora B activity independent of its contribution to pH2A-mediated recruitment of Sgo1. In sum, our results suggest that distinct molecular pathways are responsible for the recruitment and activity of Aurora B at centromeres and kinetochores.
P1199
Board Number: B205
Aurora B association with nucleosomes, not transcription, regulates its centromere localization and proper SAC response in human cells.
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Despite the prevailing dogma that transcription in higher eukaryotes is repressed during mitosis, recent studies provided evidence that some level of transcription might still take place. In line with this hypothesis, the master mitotic regulator Cyclin B1 has been suggested to be actively transcribed during mitosis and to be required for a robust spindle assembly checkpoint (SAC), a signaling mechanism that controls the fidelity of mitosis by detecting unattached kinetochores. Furthermore, in vitro studies using Xenopus oocyte extracts have suggested that the localization and activation of Aurora B (required for proper SAC response and error correction) is dependent on centromeric mitotic transcription. However, whether active transcription is required for spindle assembly checkpoint response and Aurora B localization/activity in animal somatic cells remains unknown. Here we use deep sequencing and live cell imaging of human cells in culture to investigate the role of transcription during a prolonged mitosis. Our initial analysis using a well-known transcription inhibitor (Actinomycin D) that intercalates DNA revealed that Aurora B was indeed de-localized from the centromeres. This loss affected mitotic timing and cell fate after nocodazole treatment. However, inhibition of transcription independently of DNA intercalation did not significantly alter cell fate, mitotic duration nor Aurora B localization. Moreover, we found other DNA intercalating agents phenocopied the Actinomycin D effect. In vitro electrophoretic mobility shift assay using reconstituted nucleosome core particles (NCPs) from recombinant histones showed that DNA intercalating agents reduced the interaction of CPC core complex (that include the Aurora B) with nucleosomes. Finally, RNA-seq analysis of cells treated with Actinomycin D failed to reveal de novo transcription of mitotic genes during a prolonged mitosis. Overall our data suggest that centromere structure, rather than transcription, regulates Aurora B localization and SAC response in human cells.

P1200
Board Number: B206
Dual Roles of the Chromosomal Passenger Complex at Centromeres.
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The Chromosomal Passenger Complex (CPC) localizes to centromeres in early mitosis to activate its subunit Aurora B kinase. Aurora B is required for outer kinetochore assembly as well as correction of erroneous kinetochore-microtubule attachments. However, it is unclear if centromeric CPC localization contributes to these functions beyond the activation of Aurora B. Here, we show that an activated CPC that cannot localize to centromeres supports functional assembly of the outer kinetochore, but is unable to correct errors in kinetochore-microtubule attachment in Xenopus egg extracts. We find that CPC has two distinct roles at centromeres: one to properly phosphorylate the Aurora B substrate Ndc80 to regulate attachment, and a second, kinase-independent role in the proper loading of CENP-C and the CENP-H-I-K-M complex to the inner kinetochore. Although a fully assembled inner kinetochore is not required for outer kinetochore assembly, we find it is essential to recruit tension indicators, such as
BubR1 and 3F3/2, to erroneous attachments. Thus, centromeric CPC is necessary for tension-dependent removal of erroneous attachments, and for the kinetochore composition required to detect tension loss.

P1201
Board Number: B207
Optogenetic manipulation of individual kinetochores shows that Aurora B kinase promotes microtubule depolymerization rather than detachment.
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It is well established that Aurora B kinase promotes chromosome bi-orientation in cell division by regulating kinetochore-microtubule (MT) interactions. The mechanism by which Aurora B performs its function is still not clear. Isolated kinetochore proteins and kinetochore particles with phosphomimetic mutations at known Aurora B target sites show reduced affinity to MTs and increased detachment from MTs under force, supporting a model where Aurora B triggers detachment directly. In contrast, kinetochore MT shortening rather than detachment was observed after Aurora B activation by inhibitor washout in vivo, suggesting that Aurora B modulates MT dynamics by triggering depolymerization. To distinguish between two models, we developed an optogenetic approach using a photocaged chemical inducer of protein dimerization to recruit Aurora B to individual kinetochores at metaphase. MT depolymerization at the targeted kinetochore would lead to poleward movement, whereas detachment would lead to anti-poleward movement. We found that the targeted kinetochore moved poleward, inducing the sister kinetochore to move anti-poleward. Our results provide direct evidence for a model in which phosphorylation of Aurora B substrates at kinetochores in vivo promotes depolymerization of kinetochore MTs. Our experimental approach also highlights the potential of manipulating kinetochores with light.

P1202
Board Number: B208
Measuring NDC80 binding reveals the molecular basis of tension-dependent kinetochore attachments.
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During cell division, chromosome motion is driven by coupling the dynamic ends of microtubules to the kinetochore. Erroneous kinetochore-microtubule attachments are corrected to prevent chromosome mis-segregation. The mechanism of error correction remains poorly understood, but is believed to be based on a tension-dependent interaction between microtubules and the kinetochore. The NDC80 complex is the predominant coupler of the kinetochore to microtubules, and is thus directly implicated in tension sensing for error correction. The lack of techniques to quantify the attachment of the NDC80 complex to microtubules in vivo has been a major obstacle to investigate this possibility. Here, we present a method, based on fluorescence lifetime imaging microscopy and Förster resonance energy transfer (FLIM-FRET), to quantitatively measure the fraction of NDC80 complexes bound to microtubules at individual kinetochores in living human tissue culture cells. We found that the fraction of NDC80 bound is positively correlated with the distance between sister kinetochores, a proxy for centromere tension. This positive correlation was significant enough to account for the change in NDC80 binding in the course of error correction. Moreover, we found that Aurora B displacement from centromere
eliminates the tension dependency of NDC80 binding. These results argue that error correction results from the tension-dependent modulation of NDC80 binding affinity by centromere-localized Aurora B kinase.

P1203
Board Number: B209
Coordination between discrete MAD1 domains is required for efficient mitotic checkpoint signaling.
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As a sensitive signaling system, the mitotic checkpoint ensures faithful chromosome segregation by delaying anaphase onset when even a single kinetochore is unattached. The key signal amplification reaction for the checkpoint is the conformational conversion of open MAD2 (O-MAD2) into closed MAD2 (C-MAD2). The reaction was suggested to be catalyzed by an unusual catalyst, a MAD1:C-MAD2 tetramer, but how the catalysis is executed and regulated remains elusive. Here we report that in addition to the well-characterized middle region (MIM), both amino- and carboxyl-terminal domains (NTD and CTD) of MAD1 also contribute to the mitotic checkpoint. In contrast to MIM that stably associates with C-MAD2, MAD1-NTD and CTD surprisingly bind to both O-MAD2 and C-MAD2 in vitro, suggesting their interactions with both substrates and products of the O-C conversion. MAD1-NTD also interacts with CTD. MPS1 kinase interacts with and phosphorylates both NTD and CTD. The phosphorylation reduces the NTD:CTD interaction and their interactions with MPS1. Mutating CTD phosphorylation sites including Thr716 also compromises MAD2 binding and the checkpoint responses. Our results have uncovered previously unknown interactions of MAD1-NTD and CTD with MAD2 conformers and their regulation by MPS1 kinase, providing novel insights into the mitotic checkpoint signaling.

P1204
Board Number: B210
Plk1 anchors the inner kinetochore against tension.
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In early mitosis, Polo-like kinase 1 (Plk1) is recruited to the kinetochore, where it stabilizes microtubule attachments at the outer kinetochore for proper chromosome segregation during anaphase. Its other kinetochore roles are less clear. We previously reported that bulk Plk1 localizes to the inner kinetochore, near chromatin, and functions at this location to ensure high-fidelity chromosome segregation. Here, we evaluate this phenotype in detail to discover the functional defect, which is readily achieved through partial inhibition of Plk1 in RPE1 cells. Partial inhibition preserves most essential functions but causes chromosomes to mis-segregate on bipolar spindles in anaphase. Strikingly, we find that lagging chromosomes, but not segregated chromosomes, lack kinetochore proteins, including Hec1, Knl1, CENP-T, CENP-C, and CENP-A. However, CENP-B is relatively preserved. Importantly, these effects are reproduced with alternate methods of Plk1 inhibition and with both tagged and antibody-detected endogenous CENP-A, confirming a bona fide effect of Plk1 inhibition on CENP-A retention at the inner centromere. The disrupted kinetochores are observed prior to anaphase even though Plk1 activity is not required to initially recruit these kinetochore components. Live cell imaging reveals asymmetrical protein loss from sister kinetochores after midline chromosome

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alignment. The sisters then migrate towards the pole faced by the retained kinetochore. The disrupted kinetochores are silent to the mitotic checkpoint, as demonstrated by concomitant absence of the checkpoint proteins BubR1 and Mad1 at the kinetochore, explaining how cells can proceed into anaphase with misaligned chromosomes. Although Plk1 can indirectly mediate Aurora B localization to the centromere, inhibition of Aurora B fails to phenocopy the defect, suggesting that Plk1-dependent signaling through this kinase does not mediate the effect. These findings support a model where Plk1 is required to tightly anchor the kinetochore to chromatin against tension. Loss of this tight anchoring leads to stochastic disruption of some but not all kinetochores in metaphase; presumably those that are weakly anchored and/or have the most tension. This metaphase defect leads to poleward migration of the sister pair towards the retained kinetochore, but this pair is insufficient to trigger the mitotic checkpoint, enforcing chromosome mis-segregation. Additionally, lagging anaphase chromosomes are commonly generated when a kinetochore is disrupted just before anaphase onset. We conclude that a key inner-kinetochore function of Plk1 is to stabilize the mitotic kinetochore at its root.

**P1205**

**Board Number: B211**

PP1-87B antagonizes Polo kinase and C(3)G, a transverse element of synaptonemal complex, in maintaining sister chromatid co-orientation in metaphase I in *Drosophila* oocytes.

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In meiosis I, a unique mechanism called co-orientation ensures that sister chromatids co-orient and segregate to the same pole at metaphase I. In budding yeast, co-orientation is generated by a complex called Monopolin, while in fission yeast and mice, co-orientation has been shown to be mediated by specialized kinetochore proteins, Moa1 and MEK1N, functioning with meiosis specific cohesin, Rec-8, to regulate sister chromatid co-orientation. Even though this meiosis specific co-orientation is needed for chromosome segregation, knowledge of the mechanism remains limited. Here we show that *Drosophila* protein phosphatase PP1-87B is required for sister chromatid co-orientation. In PP1-87B depleted oocytes, sister centromeres separate, implying defective co-orientation. In fact, this sister centromere separation in PP1-87B depleted oocytes, is suppressed by co-depletion of Polo kinase or a synaptonemal complex (SC) protein C(3)G, indicating that antagonistic mechanisms are involved in co-orientation. We have also found that maintenance of co-orientation in PP1-87B depleted oocytes is microtubule-dependent, unlike the co-orientation defect in the absence of kinetochore protein SPC105R, which is due to Separase-dependent loss of cohesion. Taken together we propose a mechanism where PP1-87B establishes proper sister chromatid co-orientation in meiosis I, by inhibiting Polo kinase and possibly structural chromatin components like the SC. We further hypothesize that maintenance of this co-orientation requires careful regulation of microtubule dynamics. This is the first report showing a functional role of the centromeric SC after prophase. However, whether C(3)G and Polo Kinase interact directly or function through different pathway to antagonize PP1-87B in maintaining co-orientation after metaphase I remains to be tested.
P1206
Board Number: B212
Fin1-PP1 promotes the translocation of the Chromosomal Passenger Complex in early anaphase.
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The chromosomal passenger complex (CPC) is translocated from chromosomes to the spindle during anaphase, and Aurora B/Ipl1 kinase is one subunit of this conserved complex. Previous studies from yeast and mammalian cells indicate that CPC is essential for accurate chromosome segregation, because the CPC promotes bipolar chromosome attachment and spindle elongation. However, the molecular mechanism for CPC translocation during early anaphase and the function of this translocation remain poorly defined. Protein phosphatase 1 (PP1) antagonizes Aurora B/Ipl1, and Fin1 is a regulatory subunit of PP1 in budding yeast S. cerevisiae. Our recent data indicate that kinetochore association Fin1-PP1 is cell cycle regulated and this association promotes the silencing of the spindle assembly checkpoint, which monitors chromosome attachment and prevents anaphase onset. Here, we report that yeast mutant cells lacking Fin1 show compromised CPC spindle translocation during anaphase. Moreover, we found that premature CPC translocation leads to chromosome mis-segregation in the presence of syntelic attachment. Our data indicate that Fin1-PP1 promotes CPC translocation, and the premature CPC translocation causes chromosome mis-segregation.

P1207
Board Number: B213
Interdependent centromeric and microtubule bound pools of the CPC enable kinetochore phosphorylation and resolution of merotelic attachments.
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The attachments of kinetochores to microtubules are tightly regulated to prevent merotelic kinetochore attachments, which generate anaphase lagging chromosomes, micronuclei and genomic instability. The chromosome passenger complex (CPC) prevents and repairs merotelic attachments by phosphorylating the Ndc80 complex, which connects spindle microtubules to kinetochores. It is unclear how inner-centromeric CPC can phosphorylate Ndc80 in the outer kinetochore, which can be 500nm away. Two models have been suggested. First that the CPC is anchored at inner centromeres but then stretches to phosphorylate kinetochores or second, that the CPC directly binds kinetochore localized Sgo1. We provide evidence for a third model using assays that eliminated both of these mechanisms. Specifically, the centromeric pool activates Aurora B kinase within non-centromeric CPC (from the cytoplasm), which can then diffuse from centromeres to phosphorylate kinetochores. We identified a novel microtubule binding activity in the CPC on the Borealin subunit, which became a critical tool to test this model. Consistent with our model of outer kinetochore phosphorylation by diffusible non-centromeric CPC, we show that microtubule-binding site on the Borealin subunit of the CPC is required for efficient phosphorylation of the outer kinetochores, resolution of merotelic attachments and for maintenance of taxol dependent spindle assembly checkpoint arrest. We suggest that microtubule structures near kinetochores direct the diffusion of soluble activated CPC towards kinetochore substrates to control the amount of kinetochore phosphorylation.
P1208
Board Number: B214
SkA3 phosphorylated by CDK1 binds Ndc80 and recruits Ska to kinetochores to promote mitotic progression.
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The spindle and kinetochore-associated (Ska) protein complex is required for accurate chromosome segregation during mitosis and consists of two copies each of Ska1, Ska2, and Ska3 proteins. The Ska complex contains multiple microtubule-binding elements and promotes kinetochore-microtubule attachment. The Ska1 C-terminal domain (CTD) recruits protein phosphatase 1 (PP1) to kinetochores to promote timely anaphase onset. The Ska complex regulates, and is regulated by, Aurora B. Aurora B phosphorylates both Ska1 and Ska3 to inhibit the kinetochore localization of the Ska complex. Despite its multitude of functions at kinetochores, how the Ska complex itself is recruited to kinetochores is unclear. It is unknown whether any mitotic kinases positively regulate the localization of the Ska complex to kinetochores. Here, we show that Cdk1 phosphorylates Ska3 to promote its direct binding to the Ndc80 complex (Ndc80C), a core outer kinetochore component. We also show that this phosphorylation occurs specifically during mitosis and is required for the kinetochore localization of the Ska complex. Ska3 mutants deficient in Cdk1 phosphorylation are defective in kinetochore localization but retain microtubule localization. These mutants support chromosome alignment but delay anaphase onset. We propose that Ska3 phosphorylated by Cdk1 in mitosis binds to Ndc80C and recruits the Ska complex to kinetochores where Ska1 can bind both PP1 and microtubules to promote anaphase onset.

P1209
Board Number: B215
MPS1 N-terminal domains interact to regulate kinetochores levels and normal mitotic progression.
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The spindle assembly checkpoint (SAC) is a mitotic cellular surveillance system that protects faithful chromosome segregation by safeguarding the transition to anaphase. Only when they are all attached to the mitotic spindle can cells start to segregate their chromosomes, ensuring in this way that the two daughter cells that arise contain complete and identical copies of the genome. MPS1 (Monopolar Spindle 1), the most upstream component of the pathway, is a kinase that gets recruited to the kinetochore of unattached chromosomes, a multi-complex scaffold that is assembled to facilitate the interaction with the mitotic spindle. Once localized to kinetochores, MPS1 phosphorylates several target substrates and activates the signalling cascade which results in halting mitotic progression. Domains in the N terminus of MPS1, the NTE (N Terminal Extension) and TPR (Tetratricopeptide Repeat) domains, are important for MPS1 recruitment and binding to kinetochores, though the exact contribution of each remains unclear. MPS1 displays a high turnover rate on kinetochores, a property that is crucial for SAC inactivation in metaphase as stable microtubule-kinetochore attachments displace MPS1. In this study, we examine the interplay between MPS1 N-terminal domains and how this contributes to the regulation of its localization and interaction with the kinetochore. We show by NMR that the NTE and TPR domains are able to directly interact with each other. A mutant of MPS1 containing an extra copy of the NTE
domain was designed to bypass the regulatory effect of the interaction with the TPR domain. Expression of this mutant leads to an increased accumulation of MPS1 on kinetochores which persists even in the presence of microtubule attachments. This results in SAC signalling in metaphase and problems in inactivating the SAC. Further experiments are being done to determine whether MPS1 turnover on kinetochores is affected in this mutant. We conclude that the NTE-TPR interaction we detected is involved in regulating MPS1 levels on kinetochores. Perturbing this interaction potentially affects dynamic localization of the SAC kinase, without which cells cannot inactivate the SAC and normal mitotic progression is abrogated.

P1210
Board Number: B216
Spatiotemporal regulation of spindle assembly checkpoint kinase MPS1.
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Error-free chromosome segregation depends on detection and correction of erroneous chromosome-spindle microtubule attachments, and the ability to delay cell division until all chromosomes have established correct attachments. MPS1 kinase is essential for establishing and maintaining these processes during mitosis. Weakened MPS1 signalling causes genome instability and promotes tumour progression. Despite the central importance of MPS1 for guarding genome stability, little is known about temporal dynamics and mechanisms of its activation and inactivation at relevant locations in the cell. We have developed a highly specific FRET-based biosensor that allows the monitoring of MPS1 activity in living cells. We show that in mitosis MPS1 activity reaches beyond the kinetochore by placing the biosensor on chromatin. Using this tool we measure the dynamics of MPS1 activity in cells progressing through mitosis unperturbed. We measured rapid MPS1 activation before cells undergo mitotic entry and in prometaphase MPS1 activity gradually decreased. Microtubule-attachments to kinetochores repress MPS1 activity and in metaphase no MPS1 activity was measured using the chromatin-targeted biosensor. Before mitotic entry we measured MPS1 activity throughout the nucleoplasm using a diffusible nuclear localised biosensor. In prometaphase we measured cytoplasmic activity of MPS1 revealing MPS1 activity is not restricted to chromatin and kinetochores during mitosis. Finally, we show that on metaphase kinetochores, residual MPS1 activity is present. Currently we are investigating the dynamics of MPS1 activity at kinetochores in response to microtubule attachments and regulators of MPS1 activity. Furthermore, we will investigate differential activity of MPS1 in healthy and tumour cells and tissues.

P1211
Board Number: B217
Monitoring Aurora B kinase activity in response to changes in kinetochore-microtubule attachment stability during mitosis.
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Faithful chromosome segregation is mediated by the bi-oriented attachment of replicated chromosomes to spindle microtubules through kinetochores. Proper regulation of these kinetochore-microtubule (k-MT) attachments is essential for mitotic fidelity. Microtubules dynamically attach to and detach from
kinetochores and the stability of these attachments must be precisely regulated within a narrow range. k-MT attachments must be stable enough to support chromosome movement and satisfy the spindle assembly checkpoint (SAC), but sufficiently unstable to allow for the correction of erroneous attachments. k–MT attachment stability is determined by various microtubule binding proteins and cell cycle regulators that act to either stabilize or destabilize k-MTs. A growing body of evidence suggests that a core control network of mitotic kinases is responsible for integrating inputs from multiple sources and adjusting the set point for k–MT attachment stability during each stage of mitosis. A central component of this network is Aurora B kinase. Here, we test whether Aurora B is responsive to changes in k–MT attachment stability throughout mitosis. Local Aurora B kinase activity is quantitatively measured at the kinetochore in living cells using a Mis12-targeted fluorescence resonance energy transfer (FRET)–based biosensor. Aurora B activity is highest on unattached kinetochores and on unaligned prometaphase kinetochores with decreased inter-kinetochore distance (IKD). As cells transition from prometaphase to metaphase, there is a significant decrease in Aurora B activity, corresponding with a 35% increase in k-MT half-life. Using the microtubule stabilizing drug taxol, we were able to monitor Aurora B kinase activity under conditions of perturbed microtubule dynamics. Low dose (5nM) taxol treatment did not significantly alter Aurora B activity at metaphase kinetochores, despite a 21% increase in k-MT half-life and a 0.2 ± 0.1 μm decrease in IKD. These data suggest that Aurora B activity changes during the prometaphase-metaphase transition, but the kinase is not responsive to further stabilization of k-MT attachments or decreased IKD in metaphase. We also tested whether Aurora B responds to increased activity of a downstream target, MCAK. We previously demonstrated that UMK57 activates MCAK activity and decreases k-MT half-life during metaphase by 30%. Increasing MCAK activity using UMK57 treatment significantly increased Aurora B activity in both prometaphase and metaphase cells without changing IKD. These data suggest that Aurora B activity is responsive to changes in MCAK activity. Future studies will be aimed at determining whether this change in attachment stability acts as a “sensing mechanism” for the modulation of Aurora B activity.

P1212
Board Number: B218
Glycogen Synthase Kinase 3 maintains mitotic arrest by regulating mitotic checkpoint complex levels.
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The cell cycle is regulated by checkpoints which ensure genomic integrity and proper cell division. We are investigating the role of a multifunctional ser/thr kinase, glycogen synthase kinase 3 (GSK3), in regulation of the mitotic checkpoint. Our data show that in multiple cancer cell lines spindle toxin induced mitotic arrest is relieved by GSK3 inhibitors SB 415286 (SB), RO-81220 (RO) and lithium chloride. GSK3 knockout mammalian cells and GSK3 knockout mouse embryo fibroblasts show reduced mitotic index compared to wild type cells in the presence of taxol. Mitotic arrest is dependent on the mitotic checkpoint complex (MCC), composed of Mad2, BubR1, Cdc20 and Bub1. Our data show that GSK3 targets the MCC to regulate mitotic arrest. Co-treatment of GSK3 inhibitors with spindle toxins showed decreased levels of Mad2, BubR1 and Bub1 localization at the kinetochores compared to spindle toxin only. MCC assembly also decreased with SB when treated with taxol, compared to taxol only. The data implies that GSK3 plays a role in maintaining the mitotic checkpoint. Additionally, time-lapse imaging of cells co-treated with taxol and SB show a role for GSK3 in mitotic checkpoint strength regulation. Overexpression of GSK3 in mammalian and MEFs cells show an increase in mitotic index in the absence of any spindle toxins. The Wnt-signaling pathway and the PI3K/Akt signaling pathway are upstream
regulators of GSK3, negatively regulating GSK3 activity. Our data shows that inhibiting Wnt-signaling and PI3K/Akt-signaling in the presence of taxol, induces a longer mitotic arrest compared to taxol alone. This suggests that GSK3 is regulated upstream by the Wnt- and the PI3K/Akt signaling arcs to control the strength of the mitotic checkpoint. Our observations indicate a novel regulator of the checkpoint and novel insight in connecting growth-signaling pathways with mitosis.

P1213
Board Number: B219
The role of kinetochore-mediated regulation of Protein Phosphatase 1 activity in the correction of syntelic attachments in budding yeast.
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Bipolar attachment of two sister kinetochores on a chromosome to microtubules emanating from opposite spindle poles ensures accurate chromosome segregation during cell division. Sister kinetochores that are syntelically attached, i.e. attached to microtubules emanating from the same spindle pole, detach using the Aurora B kinase. Aurora B (Ipl1 in budding yeast) is thought to detect and destabilize syntelic attachments by sensing the absence of centromeric tension arising from the mutually opposing forces produced by sister kinetochores with bipolar attachment. However, the mechanistic basis of such a tension-sensitive correction mechanism is unknown. Here we provide evidence suggesting that budding yeast kinetochores regulate Protein Phosphatase 1 (Glc7 in yeast) activity to selectively stabilize bipolar attachments. We discovered that a conserved patch of four basic residues in close vicinity of the Glc7 recruitment motif in the kinetochore protein Spc105/KNL1 is essential for Glc7 activity in the kinetochore. Substitution of these basic residues with non-polar Alanine residues reduced Glc7 activity, as evidenced by a significantly higher recruitment of the Bub3-Bub1 complex by metaphase kinetochores. Strikingly, this substitution also significantly improved the correction of syntelic attachments: it partially suppressed the growth-defect due to temperature-sensitive mutations in Ipl1 or due to the mutation of the Ipl1 phosphorylation sites in microtubule-binding kinetochore proteins (ndc80-6A and dam1-3A). Importantly, the improvement in the correction of syntelic attachments was not due to the upregulation of the Bub1-Shugoshin pathway. Mutation of the basic residues suppressed growth-defects due to the deletion of either Shugoshin or the kinase domain of Bub1, which licenses Shugoshin recruitment. To understand the functional significance of the basic residues in Spc105, we reasoned that yeast kinetochores may modulate Glc7 activity by neutralizing the basic residues using phosphorylation. Consistent with this reasoning, phospho-mimetic mutation of putative Cdk1 and Ipl1 phosphorylation sites located downstream of the basic patch also marginally improved the correction of syntelic attachments. These and other data suggest an alternative mechanism for the correction of syntelic attachments in budding yeast: the kinetochore up-regulates Glc7 activity to stabilize proper attachments against a constant, tension-independent Ipl1 kinase activity.
P1214
Board Number: B220
Identification and characterisation of spindle checkpoint silencing components in *Schizosaccharomyces pombe*.
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During cell division, the spindle-assembly checkpoint (SAC) is an important mechanism which ensures proper segregation of chromosomes into daughter cells by delaying anaphase onset until all chromosomes are correctly attached to the mitotic spindle via their kinetochores. This reduces the risk of aneuploidy, which is associated with severe consequences such as birth defects and cancer. Once all kinetochores have been properly attached the SAC is rapidly silenced, allowing the cell to progress through anaphase.

Several SAC silencing factors have been identified to date but the mechanisms by which silencing occurs remain unclear. This project aims to identify factors involved in SAC silencing and to characterise their functions. Genetic screening is being carried out in fission yeast (*Schizosaccharomyces pombe*) to identify silencing defective mutants. In designing this screen we aimed to improve upon previous screens by avoiding false positives due to mutations that cause prolonged mitotic arrest for reasons unrelated to checkpoint silencing defects, e.g. disruption of kinetochore function. An ectopic synthetic checkpoint mechanism (SynCheck) developed as part of previous work in the lab is being used to spatially separate checkpoint activation from the kinetochore (Yuan *et al.*, 2016). This involves co-tethering KNL1*scp7* and Mps1 kinase, providing a platform for the recruitment and activation of Bub proteins, which promotes Mad1-Bub1 complex assembly and SAC-mediated metaphase arrest.

Our screen has produced a list of candidate silencing factors including Eaf6, Sol1, and Reg1, a known regulator of the confirmed silencing factor Protein Phosphatase 1, PP1^Dis2^. Assays to confirm and characterise the checkpoint silencing roles of these factors are underway. In addition, we are analysing the actions of PP1^Dis2^ in silencing SynCheck arrests. Several spc7 alleles, defective in the recruitment of PP1^Dis2^, are being tested for their ability to generate and silence SynCheck arrests. This work includes pull-downs of PP1^Dis2^ to check what factors are associated with it in different mutant backgrounds. These studies will greatly enhance our understanding of the molecular mechanism(s) of spindle checkpoint silencing.


P1215
Board Number: B221
Factors Required for Centromere Formation.
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Centromeres are critical chromatin structures that mediate chromosomal segregation during mitosis. In most eukaryotes the centromere is a single chromosomal locus that attaches to microtubules to ensure delivery of one copy of each chromosome to each daughter cell during cell division. Centromere identity does not rely on specific DNA sequences, but rather, is associated with large arrays of repetitive DNA. Centromere specification is thought to occur via deposition of the centromere-specific histone H3
variant, CENP-A. After DNA replication, “old” centromeric nucleosomes are distributed to the replicated chromatids. The deposition of newly synthesized CENP-A occurs at the centromere in the G1 phase in mammals. While this regulatory mechanism is crucial for proper centromere inheritance and function, the mechanism mediating precise CENP-A deposition remains poorly understood. We have demonstrated that CENP-A deposition at the centromere requires ubiquitylation on lysine 124 mediated by CUL4A-RBX1-COPS8 E3 ligase. Introduction of K124R mutation of CENP-A abrogates centromeric localization of CENP-A, while addition of a mono-ubiquitin at the C-terminus of CENP-A K124R restores its centromeric localization (Dev Cell, 2015). We have shown that CENP-A K124 ubiquitylation is epigenetically inherited through dimerization between rounds of cell division. We found that overexpression of mono-ubiquitylated CENP-A, but not wild-type CENP-A, creates ectopic functional centromeres (neocentromeres) at non-centromeric regions, which induces substantial chromosome instability (Cell Reports, 2016). Our working hypothesis is that ubiquitylation of CENP-A is a key requirement for building up protein complexes of the kinetochore. Our preliminary IP-MS with Flag-tagged mono-ubiquitin-fused CENP-A identified dozens of proteins (including MCM2, MCM2, SMC2 and SMC4) that specifically bind to ubiquitylated CENP-A. We will determine the function of isolated factors in formation of the centromere.

**Spindle Assembly 1**

**P1216**

**Board Number: B222**

**CG10126, a calcium-binding microtubule-associated protein, is a target of EGFR signaling and promotes mitosis during Drosophila development.**

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The Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase that regulates signaling pathways critical for development and proliferation in Drosophila. EGFR activity is also highly upregulated in a number of human cancers, making identification of its downstream targets important for understanding both basic cellular processes and disease states. We have identified CG10126, a Drosophila ortholog of the human proteins Calcyphosine (CAPS) and Calcyphosine-like (CAPSL), as a transcriptional target of EGFR signaling. Calcyphosine encodes a calcium-binding protein upregulated in several human cancers, but little is known of its function. Here we examine the role of its Drosophila ortholog, CG10126, during development.

To examine CG10126’s function in flies, we expressed RNA interference constructs at various points in embryonic and larval development. Reducing the level of CG10126 in eye imaginal discs produced adults with small eyes, and while reducing its level ubiquitously in embryos was generally lethal, rare escapers were dramatically reduced in size. To further examine the role of CG10126, we used mass spectrometry to identify the binding partners of CG10126 in the presence or absence of calcium. Specifically in the presence of calcium, CG10126 bound α- and β-tubulin and several spindle assembly proteins, suggesting that it may influence formation of the mitotic spindle. Consistent with this, embryos with reduced CG10126 show a diminished number of mitotic nuclei and abnormal spindle structures. Also in keeping with this, epitope-tagged forms of CG10126 colocalize along the microtubule spindle during mitosis, and in vitro assays suggest that G10126 enhances the rate of microtubule polymerization specifically in the presence of calcium.

Calcium is indispensable for cell cycle regulation (Parry, H., McDougall, A. & Whitaker, M. Biochem Soc Trans 34, 385-388, (2006); Roderick, H. L. & Cook, S. J. Nat Rev Cancer 8, 361-375, (2008)), but Ca\(^{2+}\) also
destabilizes microtubules (Lee and Timasheff, 1977). Our results suggest that CG10126 may promote progression of the cell cycle by enabling spindle assembly even in the presence of the increased calcium levels present during mitosis.

P1217
Board Number: B223
Microtubule-associated tumor suppressor ATIP3 controls Kif2A and aurora kinases to maintain mitotic spindle length.
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Maintaining the integrity of the mitotic spindle is essential to ensure proper chromosome segregation and normal cell division. Any defect in spindle length or symmetry leads to aneuploidy, which is a hallmark of cancer. ATIP3 is a Microtubule-Associated-Protein encoded by candidate tumor suppressor MTUS1 gene whose expression is markedly down-regulated in aggressive breast tumors of the triple-negative subtype. ATIP3 presents potent anti-growth and anti-metastatic effects, making it an interesting therapeutic target for novel anti-cancer strategies (1-3).
Over the past few years our group has been focusing on the molecular aspects of ATIP3. We have shown that in interphase, ATIP3 localizes at the centrosome and along the microtubule (MT) lattice (1). ATIP3 is a potent MT stabilizer whose depletion increases MT dynamic instability at the plus ends through direct interaction with End-Binding protein EB1, a major marker/regulator of MT dynamics (4, 5). During mitosis, ATIP3 co-localizes with the mitotic spindle and spindle poles. Time-lapse videomicroscopy analyses have shown that ATIP3 delays mitosis by increasing the time spent in metaphase. However, the role of ATIP3 in cell division remains unknown.
Our recent results indicate that ATIP3 depletion induces several mitotic abnormalities, including multipolar and asymmetric spindle as well as reduced spindle length. We provide evidence that ATIP3 effects on the mitotic spindle involve the depolymerizing kinesin Kif2A and the mitotic kinase Aurora A (AurKA). We show that ATIP3 maintains spindle length in metaphase by decreasing Kif2A recruitment at the poles. Of interest, ATIP3 interacts with Kif2A in an AurKA-dependent manner. Whether a novel ATIP3-Kif2A-AurKA axis is involved in spindle integrity is under investigation. Together these results may shed light on novel mechanisms regulating mitotic spindle integrity and may deepen our understanding of how loss of ATIP3 may promote aneuploidy, with major consequences in breast cancer.
1. Rodrigues-Ferreira et al., PlosOne 4(10), e7239 (2009)

P1218
Board Number: B224
Preparing frozen Xenopus egg extracts for the study of spindle assembly mechanisms.
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Cell-free extracts prepared from Xenopus laevis eggs are a powerful experimental tool to study various aspects of cell cycle events, such as DNA replication, nuclear organization, and spindle assembly.
However, the utility of this system is often hampered by its low storage stability and high variation in quality. Although frozen stocks may circumvent these problems and are used for researches, they typically lack spindle assembly activity. Here, we discuss a simple two-step method for preparing frozen egg extracts that retain a nearly-endogenous spindle assembly activity. Freshly prepared, cytostatic factor-arrested extracts were processed for the frozen preparation by using a centrifugal filter-based dehydration and slow sample cooling. With the choice of optimal preparation parameters, including the centrifugation period, speed, and filter mesh size, the freeze-stocked extracts cycled through interphase back into metaphase, assembling bipolar spindles in a size, shape, and number that are comparable to those of the freshly prepared extracts. This further allowed us to perform a microneedle-based micromanipulation experiment for tens of spindles in single extract preparations and compare the results among different preparation batches. Our data suggest the existence of a scaling mechanism between the spindle size and stiffness. The extracts are also capable of assembling microtubule asters with Taxol. We anticipate that this method would not only help reveal mechanisms that are masked by sample variation, but also make Xenopus extracts more accessible to researchers.

P1219
Board Number: B225
UBAP2L/PQN-59 is a novel Plk1 regulator, required for chromosome segregation in human cells and C. elegans embryo.
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Humans display a diploid karyotype of 44 autosomes and 2 sexual chromosomes. In proliferating cells, this considerable amount of DNA is replicated in 92 fragments, called chromatids. During mitosis, each chromatid must be represented once in each daughter. How a single cell achieves such a level of accuracy in chromosome segregation is a question that fascinated scientists for decades. Plk1 (Polo Like Kinase 1) is a conserved serine/threonine kinase, which regulates several aspects of mitosis. One of these aspect is the chromosome alignment and segregation. In early mitosis Plk1 accumulates at kinetochores, promoting stable kinetochore-microtubules attachment. Then, Plk1 is removed from kinetochores through ubiquitination. Reduced levels of PLK1 allow chromosome separation and anaphase onset. However, the exact mechanism through which Plk1 functions is not fully understood. Here, we provide evidences that both the uncharacterized protein PQN-59 and its human orthologue UBAP2L regulate PLK1 in C. elegans embryo and human cells. UBAP2L/ PQN-59 depletion causes defects in chromosome alignment and, in human cells, precocious removal of Plk1 from kinetochores and lower levels at centrosomes. In both C. elegans and human cells UBAP2L/ PQN-59 knock-down results in the formation of Plk1 cytoplasmic aggregates providing a possible explanation for the phenotypes observed. We propose UBAP2L/ PQN-59 as a novel regulator of Plk1, counteracting Plk1 aggregation during mitosis.

P1220
Board Number: B226
Investigating the functional role of the GAPVD1-CK1δ/ε interaction.
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Human casein kinase 1 delta (CK1δ) and epsilon (CK1ε) are members of a conserved family of ubiquitously expressed serine/threonine kinases that regulate multiple processes including Wnt
signaling, vesicular trafficking, circadian rhythm, and ribosome maturation. Inhibition of CK1ε using two small molecule inhibitors (SR1277 and SR3029)1 results in a 6-fold increase of mitotic cells exhibiting multipolar spindles, as well as 7-fold and 50-fold increases of mitotic cells with misaligned and unaligned chromosomes, and a significant increase in the percentage of multipolar spindle poles containing no centrioles. These results suggest CK1ε function to promote chromosome alignment, spindle polarity and pericentriolar-material cohesion in mitosis. We sought to investigate the molecular mechanism by which CK1ε may modulate chromosome alignment by determining their interacting partners and substrates. As observed in fixed cells with indirect immunofluorescent microscopy, CK1ε and CK1α endogenously tagged with a fluorescent, multifunctional affinity purification (MAP2) tag localize to centrosomes, the nucleus and throughout the cytoplasm. MAP-tagged CK1ε and CK1α from asynchronous and mitotic cell populations was used as bait to purify and identify associated proteins by mass spectrometry. GTPase-activating protein and VPS9 domain-containing protein 1 (GAPVD1) was consistently the most abundant interacting protein in the purifications of both enzymes. By conventional co-immunoprecipitation, we found that GAPVD1 and CK1ε associate throughout the cell cycle and biochemical experiments showed that GAPVD1 interacts directly with the kinase domain of CK1ε. GAPVD1 contains a GAP domain at the N-terminus and GEF domain at the C-terminus. We found that GAPVD1 is a substrate of CK1ε in vitro and in vivo, with all of the phosphorylation sites located between the GAP and GEF domains. Our results suggest that GAPVD1 is a key interacting partner and substrate of CK1ε and may potentially relate to CK1ε’s function in mitosis.

P1221
Board Number: B227
Controlling candidate physical inputs to the spindle assembly checkpoint.
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To ensure accurate chromosome segregation, the spindle assembly checkpoint (SAC) prevents anaphase until chromosomes correctly attach to the spindle. The SAC detects some aspect of plus-end microtubule attachment to the kinetochore, and generation of tension is not sufficient to satisfy the SAC without it. However, we do not know whether tension is necessary to satisfy the SAC, and this is in part due to the difficulty of controlling tension in otherwise native end-on attachment scenarios. Here, we use laser ablation to change tension in real time, and we currently monitor SAC satisfaction. By detaching a kinetochore-fiber from the spindle for timescales relevant to SAC responses, we show that the SAC is insensitive to loss of tension when attachments are otherwise not detectably changed. What about end-on attachment the SAC detects and how it integrates this information remain unclear, and to address these questions we are developing assays to control end-on attachment features and test signal integration mechanisms. Together, we hope that the ability to control physical parameters of attachments will help push our understanding of what cues the kinetochore integrates to control cell cycle progression.
P1222

Board Number: B228

Tension-dependent anaphase A in the C. elegans embryo.

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Accurate chromosome segregation is essential for maintaining genome integrity across meiotic and mitotic divisions. During this process, a microtubule-based spindle is organized and is linked to chromosomes through the kinetochores. Kinetochore microtubules generate the forces necessary for chromosome alignment in metaphase and for sister chromatid segregation during anaphase. In most cell types, anaphase consists of two distinct processes, referred to as “anaphase A” and “anaphase B”. Anaphase A is the movement of sister chromatids toward the spindle poles due to the shortening of kinetochore microtubules. Anaphase B corresponds to the separations of the two spindle poles from one another. During the first division of the Caenorhabditis elegans embryo, sister chromatid segregation is mainly driven by anaphase B, which primarily depends on cortically anchored dynein that pulls on astral microtubule minus-ends. This in turn generates pulling forces on the two opposite spindle poles. However, genetically reducing dynein-mediated cortical pulling forces significantly decreases spindle pole elongation during anaphase, but does not prevent chromosome segregation. This suggests that alternative mechanisms of force production involved in chromosome segregation must exist in the C. elegans embryo.

By analyzing chromosome segregation in the C. elegans one-celled zygote, we found that in parallel to the anaphase B-driven spindle elongation, the pole to chromosome distance reproducibly decreases during the first 40 seconds following anaphase onset. Therefore, a brief anaphase A step participates in chromosome segregation in the C. elegans embryo. We found that reducing the cortical pulling forces through RNAi-mediated depletion of the dynein cortical anchor proteins, led to a prolonged anaphase A. Furthermore, mechanically or genetically disrupting the anaphase central spindle led to a similar increase in the duration of anaphase A. As decreasing cortical pulling forces or disrupting the central spindle both ultimately lead to a reduced tension within the spindle and lead to prolonged anaphase A, we propose that anaphase A is regulated by tension in C. elegans embryos. Functional analysis demonstrated that the kinesin-13 KLP-7 (ortholog of MCAK) and the kinetochore SKA complex are required for anaphase A in the C. elegans embryo. Overall, our results suggest that a link exists between tension exerted on the spindle and the regulation of kinetochore-microtubule dynamics during anaphase A in C. elegans. We are currently analyzing the molecular details of the tension-dependent anaphase A.

P1223

Board Number: B229

Measuring force responses in the mitotic spindle.

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The mitotic spindle is a self-assembled microtubule structure that associates with chromosomes and mediates the separation of genetic material during cell division. Force plays an important role during mitosis, from physically moving, aligning, and separating chromosomes to acting on regulatory signaling.
pathways. To understand the role of forces in mitosis, it is important to not only understand the molecular underpinnings, but also to make direct measurements of forces throughout the cell cycle. Using magnetic tweezers, forces on the order of 100s of piconewtons can be exerted on intracellular particles to measure force responses during the course of mitosis. To increase precision, we are building on previous work that used wire grids to create tunable, localized magnetic fields by individually controlling the current through each wire. The goal is to probe dynamics of mechanical properties, force generation mechanisms, and force-responsive signaling pathways using the magnetic grid to applied targeted and direct forces.

P1224
Board Number: B230
Cell cycle progression and mitotic spindle assembly following light-induced release of proteins from photodegradable hydrogels.
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Targeted manipulation of molecular processes in closed biological systems is often experimentally advantageous. This is typically achieved by using specific modalities of light microscopy in combination with photolabile proteins that have been engineered to change conformation in response to specific wavelengths. Here we describe the application of a novel photodegradable hydrogel technology that was developed to spatially and temporally control the release of unmodified recombinant proteins. Light-induced release of a nondegradable cyclin B from sea urchin (cyclin B Δ90) from hydrogel “posts” into cell-free interphase extracts (derived from X. laevis eggs) was sufficient to induce nuclear envelope breakdown and accumulation of mitosis-specific protein phosphorylation events. In agreement with previous observations, spindle assembly following Xenopus cyclin B1 (dm) addition to bulk interphase extract was indeed perturbed, suggesting that additional components are necessary to recapitulate the process accurately. We found that addition of a minimal protein mixture was sufficient to induce cell cycle progression of interphase extract into a mitosis-like state capable of assembling spindles with near normal morphology and chromosome alignment. This three component cocktail included Xenopus cyclin B1 (dm), a phosphomimetic mutant of Arpp19 (a greatwall kinase substrate and inhibitor of PP2A-B55), and the chromokinesin XKid. Our results demonstrate the utility of the approach and indicate that a surprisingly small number of proteins is required to induce cell cycle progression and transform interphase extract into spindle assembly competent extract.

P1225
Board Number: B231
The middle region of BUBR1 binds to MAD2 and p31comet.
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Chromosome mis-segregation results in aneuploidy, which is common in many solid tumors. The Spindle Assembly Checkpoint (SAC) is a signal transduction pathway that prevents chromosome mis-segregation by monitoring kinetochore-microtubule attachments. Unattached kinetochores activate the SAC and delay metaphase-to-anaphase transition in cells. The effector of the SAC pathway is the Mitotic
Checkpoint Complex (MCC) which is composed of MAD2, BUBR1, BUB3, and CDC20. The MCC is likely assembled at unattached kinetochores and then diffuses into the cytoplasm to bind and inhibit the Anaphase Promoting Complex/Cyclosome (APC/C), hence causing inhibition of the metaphase to anaphase transition. It was previously thought that the N terminus of BUBR1 (1-486 residues) was sufficient for the assembly and function of the MCC; however, recent evidence suggested that the middle region of BUBR1 also binds to CDC20 and contributes to MCC regulation. Interestingly, the middle region of BUBR1 is largely invisible in the near atomic resolution structures of human MCC:APC/C complexes solved last year, indicating flexibility or heterogeneity. Here, we report that the middle region of BUBR1 (487-700 residues) directly interacts with Mad2 and p31\textsuperscript{comet} in vitro. We also show that the N terminus of BUBR1 binds to p31\textsuperscript{comet}. p31\textsuperscript{comet} is known as a mitotic checkpoint silencing protein, but preliminary data suggested it does not simply compete with MAD2 for BUBR1 binding. We hypothesize that the middle region of BUBR1 may contribute to the stability of the MCC and/or its association with the APC/C. We are conducting in vitro pull down assays and live cell imaging with BUBR1 deletion and overexpression constructs to test this hypothesis.

P1226
Board Number: B232
Aurora A activation in mitosis promoted by BuGZ.
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Protein phase separation or coacervatation has emerged as a potential mechanism to regulate biological functions. We have shown that coacervation of a mostly unstructured protein, BuGZ, promotes assembly of spindle and its matrix. BuGZ in the spindle matrix binds and concentrates tubulin to promote microtubule assembly. It remains unclear, however, whether BuGZ could regulate additional proteins to promote spindle assembly. Here we report that BuGZ promotes Aurora A activation in vitro. Depletion of BuGZ in cells reduces the amount of phosphorylated Aurora A on spindle microtubules. BuGZ also enhances MCAK phosphorylation. The two zinc fingers in BuGZ directly bind to the kinase domain of Aurora A, which allows Aurora A to incorporate into the coacervates formed by BuGZ in vitro. Importantly, BuGZ coacervation activity promotes Aurora A phosphorylation in Xenopus egg extracts. These suggest that BuGZ coacervation promotes Aurora A activation in mitosis.

P1227
Board Number: B233
Study on the function of BubR1 in zebrafish model system.
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BubR1 is a spindle assembly checkpoint (SAC) protein that has two distinct roles in mitosis: SAC signaling and chromosome congression. This checkpoint protein is conserved in eukaryotes, yet considerable differences among species exist. Unlike other vertebrates, interestingly, zebrafish has two BubR1 orthologues: BubR1-N and BubR1-C. Two separated orthologues may be able to perform functions in a similar or not similar manner to those found in mammals. Until now, functions of zebrafish BubR1s in
mitosis have been uncovered. Here we show that depletion of BubR1-N results in shortened mitotic timing and increase of lagging chromosomes. Moreover, we found that BubR1-N has acetylation sites that may be involved in regulating SAC signaling, like its orthologue in mammals. Our results indicate that BubR1-N has the roles in SAC signaling. Future studies involve investigating knock-out phenotypes of two different forms of zebrafish BubR1s. This will lead us to understand the evolution of BubR1 and its function in vertebrates.

P1228
Board Number: B234
Induction of outer radial glia by the random spindle orientation causes severe microcephaly in the Aspm mutant mice.
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ASPM/MCPH5 is one of the major responsible genes for microcephaly in human, and plays an important role in the spindle formation during neural progenitor mitoses. However, it is known that in mouse model system, mutations in the Aspm gene lead to only a modest reduction in brain size, implying that the spindle abnormalities caused by the Aspm mutations may have different impacts on the brain development in rodents and primates. One of the prominent features of the cortical development in gyrencephalic mammals is the emergence of non-apical neural progenitors, outer radial glia (oRG), residing in the outer subventricular zone (OSVZ) at the later stage of the development. To mimic this situation in mice, we used LGN mutant mice, in which the random spindle orientations frequently transform apically dividing radial glia into ectopic oRG-like cells without affecting neural differentiation or cell survival. In the Aspm mutant mice, we found that neural progenitors exhibited p53-dependent apoptosis in a low frequency. Surprisingly, the rate of apoptosis in the Aspm mutant was dramatically increased by inducing oRG-like cells by the LGN mutation. The Aspm LGN double mutant showed a severe reduction in brain size with a decrease in the number of neurons. We propose that the developmental scheme with the OSVZ and oRG is susceptible to spindle malformations in neural progenitors. These findings will help us understand the mechanism underlying a serious microcephaly in the gyrencephalic mammals due to the spindle abnormalities.

P1229
Board Number: B235
Characterization of a novel myosin light chain in mitosis.
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Cell division is a highly regulated process that ensures the proper distribution of genetic material to daughter cells. The mitotic microtubule spindle is a structure that is critical for chromosome congression and chromosome segregation, which is important for the fidelity of cell division. We recently performed a screen for mitotic microtubule interacting proteins and identified a Novel Myosin Light Chain (NMLC) as a putative mitotic microtubule binding protein. Here I present data on the characterization of NMLC and its role in cell division. Immunofluorescence microscopy experiments indicated that NMLC localizes to the spindle poles in a cell cycle dependent manner, only during mitosis. Biochemical purifications and mass spectrometry analyses further indicated that NMLC associates with known spindle pole proteins. Interestingly, depletion of NMLC by siRNA treatment led to defects in chromosome congression during
early mitosis and to aneuploidy in post-mitotic cells. Based on these preliminary data, we hypothesize that NMLC is a novel myosin light chain that functions in spindle pole homeostasis, which is important for proper chromosome congression and segregation during cell division. Future experiments will be focused on testing this hypothesis.

P1230
Board Number: B236
Identification of genetic regions that influence the expression of mitotic checkpoint genes.
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The mitotic checkpoint is a cellular safeguard that prevents the missegregation of chromosomes. Checkpoint failure can lead to abnormal chromosome numbers (aneuploidy), a key characteristic of cancer cells. The accurate control of mitotic checkpoint protein abundances is crucial for the proper functioning of this pathway, and misexpression of checkpoint genes is common in cancer cells. Yet, little is known about factors that control the expression of mitotic checkpoint genes in any organism. We use CRISPR/Cas9-mediated scar-less genome editing in fission yeast to map the regulatory regions of key mitotic checkpoint genes. To identify the minimal region that is necessary and sufficient for proper expression, we transplant the coding sequence of checkpoint genes with differently sized upstream and downstream regions to exogenous loci. To identify the quantitative influence of sub-regions, we delete or exchange these regions at the endogenous locus, and study the influence on mRNA and protein abundance. Our data suggest that, at least for some mitotic checkpoint genes, the regulatory sequences are centered on the transcript, or even the coding sequence. Once our mapping is completed, we aim to determine the relevant features or protein-interaction partners of the crucial regions that we have identified. Given the high conservation of the mitotic checkpoint, we expect that some findings will be applicable to other eukaryotes. This work expands the so far largely protein-centric view of mitotic checkpoint function to the underlying gene expression control, and contributes to a better understanding of non-coding elements in the genome.

P1231
Board Number: B237
Kinesin-12 generates essential force during C. elegans acentrosomal spindle assembly through regulation by TPX2-like protein MESP-1.
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Meiotic divisions in the female gamete, or oocyte, proceed using a microtubule-based spindle that is formed in the absence of centrosomes. Although this unique acentrosomal spindle organization is conserved in oocytes of many species, including humans, the molecular mechanisms underlying force generation in the absence of centrosomes is poorly understood. Our previous work in C. elegans oocytes has shown that upon depletion of either KLP-18/kinesin-12 or MESP-1 (meiotic spindle-1), microtubule minus ends converge into a single monopolar aster and the spindle does not reach bipolarity, suggesting that these proteins produce an essential outward force on microtubules during spindle assembly. In addition, KLP-18 does not localize to spindle microtubules in the absence of MESP-1, indicating that MESP-1 performs a Kinesin-12 targeting role analogous to TPX2 (1). Following on these findings, we are
now investigating the basis of this force generation by testing an interaction between purified KLP-18 and MESP-1 and the resulting activity on microtubules. First, we found that MESP-1 has both microtubule binding and bundling activity, similar to TPX2. Additionally, we expressed and purified segments of the KLP-18 C-terminal stalk domain and performed pulldown experiments, demonstrating that MESP-1 binds to a region near the middle of the KLP-18 stalk. In parallel analysis using these same KLP-18 fragments, we found that while the entire C-terminal stalk was unable to bind and bundle microtubules, a smaller C-terminal fragment and a stalk construct lacking a putative “hinge” region in the center both had microtubule binding and bundling activity. Putting these findings together, we hypothesize that during acentrosomal spindle assembly in C. elegans oocytes, MESP-1 binds to the central region of the KLP-18 stalk, potentially unfolding it and activating the motor’s C-terminal microtubule binding site. Together with the motor domain, this C-terminal domain would then allow KLP-18 to crosslink spindle microtubules and generate the outward force necessary to achieve bipolarity. Altogether, we propose a new mechanism of Kinesin-12 regulation by a TPX2-like adaptor protein and shed light on the interplay between essential motors and their regulatory proteins during acentrosomal cell division. (1) Wolff, et.al. 2016, MBoC

Chromosome Organization

P1232
Board Number: B238
Topoisomerase II inhibitor mediated upregulated Topo IIα SUMOylation and Aurora B kinase regulation.
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Type II Topoisomerase (Topo II) is a unique enzyme that resolves DNA tangles and is essential for accurate chromosome segregation during anaphase. In addition to its role in chromosome segregation, recent evidence indicates that perturbation of the Topo II catalytic cycle in yeast results in a mitotic delay. Supporting that finding, we discovered in Xenopus egg extracts (XEEs) that the α isoform of Topo II is post-transnationally modified with SUMO protein (SUMOylation), which can regulate Aurora B kinase recruitment to mitotic centromeres. Aurora B recruitment at centromeres is regulated via the interaction between SUMOylated Topo IIα and Haspin. Aurora B is an essential kinase component of the chromosome passenger complex (CPC) and is known to be crucial for faithful chromosome distribution to daughter cells during mitosis. SUMOylation of Topo II is conserved among eukaryotes and its role in Aurora B centromeric recruitment is observed in both budding yeast and XEE. We sought to examine whether the mitotic delay observed with a defective catalytic cycle of Topo II is caused by SUMOylation-dependent Aurora B regulation. Supporting that we found the catalytic inhibitor of Topo II (ICRF-193), which has been shown to increase SUMOylation of Topo IIα in somatic cells, causes a mitotic delay in somatic cells. In XEE assays, the addition of ICRF-193 to mitotic XEE increased Topo IIα SUMOylation. Intriguingly, ICRF-193 also promotes Aurora B upregulation at the mitotic centromere and its mislocalization on chromosome arm regions in a SUMOylation dependent manner. These results suggest that ICRF mediated increased Topo IIα SUMOylation and Aurora B upregulation/mislocalization can contribute to mitotic delay observed in somatic cells. Supporting this notion, ICRF-193 induced mitotic delay in somatic cells can be bypassed by an Aurora B inhibitor. With these result, we propose that ICRF-193 mediated mitotic checkpoint activation is initiated by SUMOylation-dependent Aurora B
regulation. Currently, we are investigating potential Aurora B targets that might be responsible for the ICRF-193 mediated mitotic delay.

**P1233**
**Board Number: B239**
**Human centromeres produce non-coding alpha satellite RNAs that are chromosome-specific and required for centromere protein loading.**
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Centromeres serve as the chromatin foundation for the kinetochore and are essential for proper chromosome segregation. In humans, the centromere on each chromosome is defined by a homogenous, repetitive array of alpha satellite DNA. Although alpha satellite DNA was originally thought to be transcriptionally inert, the presence of histone marks at the centromere compatible with active transcription and the known role of non-coding centromeric RNA in other organisms suggest transcription and the resulting transcripts may also be involved in human centromere function. Recent studies confirmed the presence of alpha satellite transcripts, but the exact origin of these non-coding RNAs was unclear, as well as their involvement in the centromere-kinetochore assembly cascade. Using cytological and molecular techniques that allow alpha satellite arrays on different chromosomes to be molecularly distinguished from each other, we have demonstrated that each chromosome produces unique non-coding RNAs that localize in cis to their site of production. Alpha satellite RNAs bind at least two key centromere proteins: CENP-A, the centromere-specific histone variant, and CENP-C, a component of the constitutive centromere-associated network of proteins that bridge the centromere and the kinetochore. Targeted depletion of alpha satellite RNAs from a single chromosome leads to 30% loss of CENP-A due, at least in part, to deficient loading of new CENP-A-containing nucleosomes. CENP-C levels are also reduced by nearly half at the targeted centromere. Importantly, loss of alpha satellite RNA from a single chromosome does not affect the centromeric RNA or protein levels at any other centromere in the cell, highlighting the cis-regulatory nature of these non-coding RNAs, but it does lead to cell cycle arrest prior to mitosis. Collectively, these findings implicate non-coding alpha satellite RNA as an essential player in centromere maintenance and underscore the ability of a single defective centromere to alter cell cycle progression.

**P1234**
**Board Number: B240**
**Negative regulatory network between the three aurora kinases protects mouse gamete euploidy.**
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The Aurora kinases (AURKs) are conserved serine/threonine kinases that regulate chromosome segregation. Mitotic cells require two AURKs (A, B), while mammalian germ cells require a third: (C). The meiosis-specific expression of AURKC has lead to a hypothesis that AURKC functionally replaced AURKB in oocytes. Whether AURKB is required for female meiosis remains unknown. To determine if AURKB is required during female meiosis we utilized an oocyte-specific Cre driver (GDF9) to excise exons 2-6 in the murine Aurkb locus. We compared phenotypes of wild-type mice to that of AURKB or AURKC single knockouts. These studies revealed that AURKB is required for meiosis. In contrast to oocytes lacking
Aurk, oocytes lacking Aurkb were aneuploid. Surprisingly these Aurkb/- oocytes had increased AURKC activity in addition to abnormal AURKC localization on the chromosome arms in metaphase II, suggesting a requirement for AURKB to restrict the activity of AURKC in vivo. To test this hypothesis, we generated mice with one copy of Aurk in the Aurkb/- oocyte background to ask if reducing AURKC levels could rescue the failure phenotype; utilizing oocytes without AURKB and AURKC (double knockout) as controls. Reduction of Aurk in half partially rescued the number of eggs that were aneuploid but did not rescue the Met II mis-localization of AURKC. The aneuploidy defect and mis-localization of AURKC seen in Aurkb/- oocytes could only be rescued by complete removal of Aurk, supporting our hypothesis that AURKB is a negative regulator of AURKC. Surprisingly, double knockout oocytes were euploid and indistinguishable from WT. Here we show that AURKA, the homolog restricted to spindle poles, compensates for the loss of AURKB and AURKC by binding to the Chromosomal Passenger Complex and by phosphorylating AURKB/C substrates. Interestingly, this compensation is dependent on the absence of AURKC as its endogenous expression restricts AURKA to spindle poles. Therefore, these data also imply that AURKC negatively regulates AURKA by preventing its localization to chromosomes in wild-type oocytes. We speculate that AURKB has been retained in oocytes to ensure that AURKC activity is spatially restricted and that this restriction keeps the balance of counteracting AURKA/AURKC activity in check. These studies show, for the first time, the ability for AURKA to functionally complement AURKB/C in vivo. Importantly, these data shed new light on a negative regulatory network among the kinases, which may be critical for generating euploid gametes. This work was supported by grants from the NIH (F31HD089591: A.L.N.; R01GM112801-02: K.S.).

P1235
Board Number: B241
Premature mitotic entry induces replicative helicase unloading, fork collapse, and genome instability.
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DNA replication errors that generate complex chromosomal rearrangements are thought to be important drivers of genome evolution. Although the events that trigger these errors are not well understood, one candidate is premature mitotic entry before the completion of DNA replication. Using a cell-free DNA replication system in Xenopus egg extracts, we found that mitotic cyclin-dependent kinase (CDK) induces fork collapse at stalled DNA replication forks, resulting in complex genomic rearrangements. Upon mitotic CDK addition, the replicative CMG (Cdc45/Mcm2-7/GINS) helicase was ubiquitylated on its Mcm7 subunit and unloaded from stalled forks by the p97 ATPase. Loss of CMG protection resulted in fork breakage, followed by complex end-joining events involving deletions and DNA replication template switching. Together, these experiments define a mechanism by which premature mitotic entry can generate DNA replication-based genomic instability.
P1236

Board Number: B242

Chromosome dynamics simulations reveal the role of condensin and cohesin in building the bottle-brush chromosome architecture.
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Despite their similar molecular structure, cohesin and condensin occupy geometrically distinct regions in mitotic chromosomes across eukaryotes. In budding yeast, this separation is apparent at the pericentric region, the cohesin and condensin-rich region surrounding the centromere. Mitotic chromosomes of higher eukaryotes, and the pericentric region of budding yeast, contain a high density of radial chromatin loops extending from an axial core region, causing the chromosome to resemble a bottle-brush. We constructed novel chromatin dynamics simulations modeling cohesin as a slip ring and condensin as either a static or dynamic loop extruder on a bottle-brush model of the pericentric region. Centromere simulations in which condensin persistently binds to chromatin while cohesin passively slides along chromatin recapitulate the geometric separation observed in live cells. Critically, the physical segregation of condensin and cohesin relies on an extensional force on the chromatin. In the pericentric region of budding yeast, the main extensional force is provided by the mitotic spindle. In the highly condensed chromosomes of higher eukaryotes, and to a lesser extent in the pericentric region of budding yeast, this extensional force is provided the repulsion of the high number or densely packed radial loops extending from a central axis.

The physical segregation of cohesin and condensin within the pericentric region predicts the motion of chromatin within the pericentric region would be deferentially regulated by cohesin and condensin based on the chromatin’s position. We found the dynamics of a 10 kb lacO/LacI-GFP array placed 1.8 kb from CEN15, which co-localized with pericentric cohesin, is primarily confined by cohesin. In contrast, we found the dynamics of a 5.5 kb tetO/TetR-GFP array within a conditionally dicentric plasmid, which co-localizes with condensin, is primarily confined by condensin. We constructed chromatin dynamics simulations of the dicentric plasmid to explore how several molecules of cohesin and condensin could directly alter the plasmid’s dynamics. Simulations containing condensin resembled wild-type experimental dynamics, whereas simulations lacking condensin resembled the plasmid dynamics of cells containing the temperature sensitive condensin mutation, brn1-9. These studies support a model where condensin persistently binds to chromatin while extruding loops, while cohesin slides along chromatin. Extensional force on the chromatin centers condensin within the chromosome’s axis, while cohesin can slip along the chromatid to diffuse to the less crowded radial edges of chromatin loops.

P1237

Board Number: B243

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Candida albicans is an important fungal pathogen of humans that causes superficial to life-threatening systemic infections under immune-compromised conditions. Cell proliferation is critical for survival in the host and infection. However, the networks controlling the cell cycle in C. albicans are poorly understood. To this end, we focused on defining the regulation of the metaphase-to-anaphase

Sunday-150
transition. This is characterized by separation of sister chromosomes through activation of the protease separase. Separase activity is dependent in part on degradation of a separase regulator, securin. Securin is targeted for degradation by the Anaphase Promoting Complex/Cyclosome (APC/C) and its cofactor Cdc20p. However, in C. albicans, only homologues of APC/C cofactors Cdc20p and Cdh1p are characterized, and show conserved as well as novel features. Further, C. albicans lacks a sequence homologue of securin. Collectively this suggests unique features of metaphase-to-anaphase regulation in C. albicans, which may be exploited for new drug target discovery. In order to explore this further, we characterized a homologue of separase, ESP1. Eip1p-depleted cells arrested in metaphase, consistent with Esp1p functioning as a separase. We next explored the hypothesis that C. albicans contains a divergent securin, which may be revealed through identifying the interacting factors of separase, a conserved securin target. Affinity purification of Esp1p from Cdc20p-depleted cells and mass spectrometry revealed Orf19.955p, or Esp1p-Interacting Protein 1 (Eip1p), an uncharacterized protein with no mammalian homologue and specific to Candida species. Co-immunoprecipitation confirmed interactions between Eip1p and Esp1p. Depletion of Eip1p in strains carrying GFP-labelled Histone H2B or b-tubulin resulted in disorganized chromosomes with some fragmentation, and a higher proportion of cells in anaphase. This suggests Eip1p functions in mitosis and chromosome organization. Since eip1/eip1 strains were viable, yet grew poorly, EIP1 is not essential. Eip1p contains putative destruction and KEN boxes, suggesting regulation via degradation like other securins. Consistent with this, Eip1p was reduced when CDC20 was overexpressed, or when cells were blocked in early anaphase via depletion of the polo-like kinase Cdc5p. Finally, Eip1p function was further explored using affinity purification and mass spectrometry. Esp1p, the phosphatase Cdc14p and additional novel proteins were uncovered. Collectively, our results demonstrate that the metaphase-to-anaphase transition in C. albicans involves a novel, Candida-specific protein, Eip1p. The data suggests it may be a functional homologue of a securin, providing important implications for variation in cell cycle circuitry and cell growth.

P1238
Board Number: B244
Condensin complexes promote chromosome movement during mitosis.
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Mitosis is a highly-regulated process that ensures the accurate formation of two genetically identical daughter cells. An important characteristic of mitotic cells is the compaction of genetic material into discrete chromosomes to facilitate their alignment and faithful segregation (Ono et al., 2004). This compaction is produced by the activity of two independent condensin complexes. To better understand the role of condensin complexes in chromosome movement during mitosis, we knocked down the essential condensin subunit CAP-D3 in Condensin II. This led to decreased expression of other condensin subunits in both condensin complexes. Cells depleted of condensin subunits significantly delayed the completion of mitosis and exhibited disrupted chromosome movement. Chromosome oscillations around the metaphase plate were significantly reduced with some chromosomes displaying no perceivable movement. To understand the basis for this change in chromosome motion, we examined the effect of condensin depletion on molecules with known roles in driving chromosome oscillation. We observed no change in the localization or function of the kinesin Kif18A. However, the depletion of CAP-D3 disrupted the polar ejection force generated by the chromokinesins Kid and Kif4A. Neither Kid nor Kif4A localized to mitotic chromosomes in condensin-depleted cells. Thus, the condensin complexes are required for loading of Kid and Kif4A onto mitotic chromosomes. Without these chromokinesins,
Abnormal chromosome karyotypes are a defining characteristic of most cancers. In some malignant tumors, such as esophageal and cervical cancers, tetraploidy is suggested to be an unstable intermediate that triggers aneuploidy, leading to tumorigenesis and tumor progression. Although chromosome stability depends on the proper progression of mitosis, exact knowledge of the mitotic machinery in tetraploid cells has remained elusive. To understand mitosis in tetraploid cells, we examined the response of diploid and tetraploid tumor cells to anti-mitotic drugs. We induced tetraploid tumor cells by treating synchronized HeLa cells with hesperadin, an Aurora B inhibitor that induces mitotic slippage. In both diploid and tetraploid cells, tubulin-binding agents, such as paclitaxel (PTX) and nocodazole (Noc), had similar efficacy and induced apoptosis in prolonged mitosis or the subsequent G1 phase. On the other hand, monastral (Mona), an inhibitor of Eg5 (also known as KIF11), which plays an essential role in centrosome separation during mitosis, induced apoptosis more effectively in diploid cells than in tetraploid cells. Live cell–imaging analysis revealed that, after Mona treatment, approximately 90% of tetraploid cells underwent cell division, whereas only 30% of diploid cells did. Untreated tetraploid cells, which have four centrosomes in mitosis, formed multipolar spindles in approximately half of the cells, but under Mona treatment formed bipolar spindles in most of the cells. Untreated tetraploid cells also tended to generate unequal-sized daughter cells, whereas unequal division was rare in Mona-treated tetraploid cells. Regardless of Mona treatment, tetraploid cells exhibited a similar rate of G1 progenies that showed positive for 53BP1 or γ-H2AX foci after cell division, which indicates that multipolar and bipolar division in tetraploid cells evoked a comparable level of DNA damage response. As a consequence of multipolar division, tetraploid cells generated a sub-tetraploid population with reduced chromosomal content, and after prolonged culture converted to near-diploid cells. On the other hand, Mona-treated tetraploid cells maintained clear tetraploid chromosome contents after 7 days of culture. These data indicate that Eg5 inhibition in tetraploid cells suppressed unequal or multipolar division and caused tetraploid cells to accumulate. Our results suggest that, by controlling the cell division property in tetraploid cells, Eg5 activity dictates the conversion into near-diploid cells or the maintenance of tetraploid cells. Our results provide insight into the mechanism of how tetraploidy leads to aneuploidy and genomic instability.
P1240

Board Number: B246
Rapid degradation and 3D CLEM of condensin uncouple chromatin compaction from chromosome architecture in mitotic cells.
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Condensin is essential for chromosome formation in Xenopus extracts, fission yeast and mouse oocytes. However in somatic cell systems, chromosomes do form in condensin-depleted cells, but are structurally compromised and fail to segregate at anaphase. Here we have studied the role of condensin in vertebrate mitosis by combining auxin-mediated rapid depletion of condensin subunit SMC2 (condensin I and II) with chemical genetics to obtain near-synchronous mitotic entry of chicken DT40 cells. We analysed the outcomes by live-cell and fixed-cell microscopy methods, including 3D correlative light and serial block face scanning electron microscopy. Cultures rapidly depleted of condensin fail to form individualized mitotic chromosomes. The chromatin is compacted normally, but positioned to one side of a bent mitotic spindle. Kinetochores fail to form stable amphitelic attachments to microtubules and cultures exhibit a robust spindle assembly checkpoint-mediated delay in mitosis, eventually undergoing mitotic slippage. Experiments titrating the auxin concentration reveal that differing condensin levels are required for anaphase chromosome segregation, organization of condensin and topoisomerase IIα on the chromatid axes, and formation of a normal chromosome architecture.

P1241

Board Number: B247
The determinants and consequences of cohesion fatigue.
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Cells delayed at metaphase of mitosis undergo cohesion fatigue, an unscheduled and asynchronous separation of chromatids while cells remain in mitosis. All cell types examined exhibit cohesion fatigue, but the rate of fatigue varies between even closely related cell lines. It is unclear what renders these cells to show strong differences in the rates of cohesion fatigue. In addition, the molecular causes of cohesion fatigue are unknown. Herein we investigated the roles of microtubule dynamicity, and the strength and the abundance of Cohesin protein complex in affecting rates of cohesion fatigue. Furthermore, we studied if and how the Cohesin complex was breached during cohesion fatigue. Finally we looked at the consequences of cohesion fatigue in contributing to aneuploidy and genomic instability; we tested the potential of cohesion fatigue to induce chromosome segregation defects and generate micronuclei.

To alter microtubule dynamics we used microtubule stabilizers and destabilizers at low concentrations that only marginally affected normal mitosis. However when combined with treatments that induced metaphase arrest, alteration of microtubule stability led to significant changes in the timing of cohesion fatigue. In HeLa cells, stabilized microtubules accelerated the onset of cohesion fatigue while destabilized microtubules delayed the process. We also studied the effects of compromising the Cohesin complex. Deletion of the Cohesin accessory subunit, stromal antigen 2 (SA2), accelerated cohesion fatigue. Mitotic arrest in Nocodazole before release to metaphase arrest reduced the amount of chromosome-bound Cohesin and accelerated fatigue. In contrast, depletion of Wapl, which increases chromosome-bound Cohesin, significantly delayed cohesion fatigue. Surprisingly, we found no
difference in the amount of core Cohesin proteins bound to chromatids, before and after cohesion fatigue. Using rapamycin-induced tethering, we artificially locked the protein-protein interface (gates) between Cohesin ring components. Locking any of the gates did not affect cohesion fatigue. While massive chromatid separation likely generates inviable daughter cells, shorter delays at metaphase can induce exaggerated separation of kinetochores while arms remain attached. Cells delayed at metaphase transiently to allow kinetochore separation then released to allow anaphase onset exhibited mitosis with chromosome segregation defects and formed micronuclei. Thus metaphase delays with kinetochore separation but without chromatid separation can be a source of chromosome instability.

P1242
Board Number: B248
SUMO-mediated regulation of anaphase progression during *C. elegans* oocyte meiosis.
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*C. elegans* oocytes utilize a unique chromosome segregation mechanism. Instead of end-on kinetochore attachments, microtubules make lateral associations with chromosomes and segregation depends on a complex of proteins containing AIR-2/Aurora B kinase that forms a ring around the center of each homologous chromosome pair. These rings facilitate congression and then are released from chromosomes in anaphase and left at the spindle midzone, where they begin to disassemble. We are investigating the molecular mechanisms driving the assembly and disassembly of this important protein complex, in order to better understand how it contributes to faithful meiotic chromosome segregation. We previously demonstrated that while AIR-2 usually relocates from the rings to the microtubules in early anaphase, AIR-2 relocalization and ring disassembly are both delayed in response to a variety of meiotic errors, revealing a regulatory mechanism that controls the disassembly of the ring complex and aspects of anaphase progression (1). Now, we have gained new insights into this mechanism, through studies of the small ubiquitin-like modifier SUMO. SUMO had been previously shown to be required for ring assembly, and two different ring components (AIR-2 and KLP-19) can be SUMOylated in vitro (2). We therefore hypothesized that SUMO removal from a ring component/components might serve as a trigger for ring disassembly. In support of this, we found that SUMO is ring-associated until mid-anaphase, when the rings usually disassemble. However, under error conditions when rings are stabilized, SUMO and the SUMO conjugating enzymes GEI-17 and UBC-9 stay associated with the persisting rings, correlating SUMOylation and ring stability. To identify factors that facilitate SUMO removal, we depleted each of the four SUMO proteases in *C. elegans* and found that one of these enzymes, ULP-1, is required for both AIR-2 relocalization to the microtubules and for ring disassembly. Moreover, we found that ULP-1 localizes to the ring structures from prometaphase through early anaphase, and that GEI-17/UBC-9 are removed from the rings before ULP-1. These findings raise the possibility that prior to anaphase, the SUMO conjugating enzymes and proteases compete for substrate, enabling removal of GEI-17/UBC-9 in early anaphase to serve as a trigger for ring disassembly by ULP-1. Consistent with this idea, we found that metaphase SUMO fluorescence intensity is increased following ULP-1 depletion, and that ULP-1 can remove SUMO modifications from multiple ring components in vitro. Altogether, we have revealed a role for SUMOylation in regulating ring disassembly and anaphase progression during anaphase of *C. elegans* oocyte meiosis. (1) Davis-Roca, et.al. (2017) JCB (2) Pelisch, et.al. (2017) Mol Cell
P1243
Board Number: B249
A compartmentalized, self-extinguishing signaling network mediates crossover control and faithful chromosome segregation in meiosis.
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During meiosis, homologous chromosomes pair, synapse, and undergo crossover recombination. This process leads to the formation of stable interhomolog connections known as chiasmata, which enable homologs to bi-orient and segregate during the first meiotic division. Defects in meiotic chromosome segregation lead to aneuploidy, the primary cause of spontaneous abortion and congenital birth defects in humans. To ensure faithful meiotic chromosome segregation, meiotic crossovers must be tightly regulated. Each chromosome pair typically undergoes at least one crossover event (crossover assurance) but these exchanges are also strictly limited in number and widely spaced along chromosomes (crossover interference). This has implied the existence of chromosome-wide signals that regulate crossovers, but their molecular basis remains mysterious. Recent work from our group suggests that the synaptonemal complex (SC) is a liquid-like compartment that assembles between homologous chromosome axes through phase separation (Rog et al., 2017). The structural proteins of the SC are highly mobile within assembled complexes, and polycomplexes of SC proteins behave as droplets with liquid-like properties in vivo. Additionally, both SCs and polycomplexes are rapidly and reversibly dissolved by aliphatic alcohols such as hexanediols, indicating that their structural integrity depends on weak hydrophobic interactions. These liquid crystalline properties suggested that the SC might act as a conduit for a crossover interference signal. We now characterize a family of four related RING finger proteins in C. elegans. These proteins are recruited to the synaptonemal complex between paired homologs, where they act as two heterodimeric complexes, likely as E3 ubiquitin ligases. Our genetic and cytological analysis reveals that they act with additional components to create a self-extinguishing circuit that controls crossover designation and maturation. Our finding suggests a signal flows through the liquid crystalline channel between the surface of two homologous chromosomes to mediate crossover control and to ensure faithful meiotic chromosome segregation. In addition, we find that these proteins act upstream of other regulators to direct chromosome remodeling in response to crossover formation, which enables stepwise cohesion loss and chromosome segregation. Work in diverse phyla indicates that related mechanisms mediate crossover control across eukaryotes.

P1244
Board Number: B250
Vive la difference! Evolutionary divergence in meiotic chromosome dynamics among nematodes.
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Meiosis is the specialized cell division process essential for sexual reproduction. Studies of meiosis have expanded our understanding of chromosome architecture and dynamics, cell cycle regulation, and
feedback control. The nematode *Caenorhabditis elegans* is a well-established model for investigating meiosis. Its experimental advantages include a small genome compared to most metazoans, a low number of chromosomes (6 pairs), powerful genetics, and germline organization that facilitates temporal analysis of meiotic progression. Our recent work has elucidated circuitry that acts downstream of homolog synapsis to regulate crossover formation, which appears to be widely conserved across eukaryotes (see presentation by Zhang et al.) However, other aspects of meiotic regulation in *C. elegans* are atypical, including homolog pairing and synapsis in the absence of recombination, and the emergence of specialized chromosome regions – “pairing centers” - that mediate nuclear envelope attachment and microtubule-based chromosome movement.

To better understand how these innovations arose during evolution, we are developing the nematode *Pristionchus pacificus* as a comparative model for the cell biology of meiosis. Like *C. elegans*, *P. pacificus* is a self-fertilizing hermaphroditic species that can be readily cultured in the laboratory. Genome editing with Cas9/CRISPR is fairly robust, allowing reverse genetics and epitope tagging, although large knock-ins have been problematic. While germline organization and meiotic progression appear superficially similar between these two nematodes, we have identified major differences in meiotic mechanisms and their interdependence. Unlike the genomes of *Caenorhabditis*, which have lost the recombinase Dmc1 and its cofactors Mnd1 and Hop2, the *Pristionchus* genome has intact copies of these genes. Disruption of *Ppa_spo-11* or *dmc-1* blocks homolog pairing and synapsis. DMC-1 and RAD-51 localize sequentially to meiotic chromosomes; crossing-over and meiotic progression require DMC-1 but not RAD-51. Perhaps most surprisingly, CO designation occurs much earlier in *P. pacificus*, and synapsis depends on crossover designation by COSA-1 and ZHP-3, analogous to the situation in budding yeast but divergent from what is observed in *C. elegans* or mammals.

Through this comparative approach, we are uncovering core features of the meiotic program that are shared by divergent organisms, as well as illuminating how details of this process are modified during evolution.

**P1245**

**Board Number: B251**

**Sisters keep arms locked through metaphase.**

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Pairing of sister chromatids is critical for faithful transmission of chromosomes to daughter cells. Cohesin complexes deposited during S phase establish cohesion, the force holding these duplicated chromosomes together. As cells enter mitosis most cohesin is removed from condensed sister chromatid pairs via the prophase pathway. The removal of cohesin from chromatids via this non-proteolytic pathway occurs mainly along chromatid arms, with the majority of remaining cohesin concentrated at centromeres. As a result, it is commonly reported that the prophase pathway removes cohesin from chromosome arms thereby causing the loss of cohesion between chromatid arms and giving rise to the classical X-shaped depictions of mitotic chromosomes. But we find that arms of sister chromatids are cohered through metaphase. This sister chromatid arm cohesion is readily apparent in mitotic cells undergoing cohesion fatigue, the force-dependent process whereby sister chromatids are pulled apart in an asynchronous and unscheduled manner within cells delayed in prometaphase or metaphase. During cohesion fatigue loss of cohesion begins at centromeres and proceeds lengthwise along paired chromatid arms from centromeres to telomeres. To unravel this paradox we examined chromosome spreads from variant mitotic cell populations. Paired sister chromatids derived from cells arrested in mitosis by the microtubule depolymerizing agent, nocodazole, often formed the classical X-
shaped structure. In contrast paired chromatids from cells arrested in mitosis by inhibition of Cenp-E, the APC/C, or proteasome exhibited arms that retained tightly parallel orientations, indicating that cohesion was retained. Thus the absence of interactions between the mitotic spindle and sister chromatids appears to promote premature loss of chromatid arm cohesion, while the presence of this interaction sustains arm cohesion. In support of this hypothesis, we found that mitotic cells initially treated with nocodazole, then permitted to reform their mitotic spindles by removing nocodazole but restrained from progression past metaphase by proteasome inhibition, underwent cohesion fatigue more rapidly than mitotic cells that were arrested in mitosis by proteasome inhibition alone. We hypothesize that maintenance of chromatid arm cohesion through metaphase is advantageous and may rescue initial unscheduled loss of centromere cohesion should centromere regions loose cohesion during spontaneous prolonged prometaphase or metaphase. Furthermore maintenance of sister chromatid arm cohesion until the onset of anaphase suggests that regulators controlling the scheduled and synchronous loss of cohesion at anaphase onset are likely targeting both centromere and arm cohesin for destruction.

P1246
Board Number: B252
Actin protects mammalian eggs against chromosome segregation errors.
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Chromosome segregation is driven by a spindle that is made of microtubules but is generally thought to be independent of actin. Here, we report an unexpected actin-dependent mechanism that drives the accurate alignment and segregation of chromosomes in mammalian eggs. Prominent actin filaments permeated the microtubule spindle in eggs of several mammalian species, including humans. Disrupting actin in mouse eggs led to significantly increased numbers of misaligned chromosomes as well as lagging chromosomes during meiosis I and II. We found that actin drives accurate chromosome segregation by promoting the formation of functional kinetochore fibers, the microtubule bundles that align and segregate the chromosomes. Thus, actin is essential to prevent chromosome segregation errors in eggs, which are a leading cause of miscarriages, infertility, and Down syndrome.

P1247
Board Number: B253
Persistent DNA-break potential near telomeres contributes to a chromosome-size bias in break initiation.
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Infertility, spontaneous fetal loss and birth defects in humans result mainly from chromosome segregation errors during meiosis [1]. Faithful meiotic chromosome segregation requires induction of numerous programmed DNA breaks, and repair of these DNA breaks as crossovers to link previously unattached homologous chromosomes [2]. Consequently, proper control of DNA breakage and repair is critical for maintenance of genome integrity [3]. To avoid excessive DNA damage, DNA break-promoting HORMA-domain protein, Hop1, is removed from the meiotic chromosomes by the evolutionarily conserved synaptonemal complex (SC) [4-6]. Previous
research has shown that this negative feedback regulation of Hop1 and DNA breaks by the SC occurs following break initiation and crossover designation and in a chromosome autonomous manner [4-8]. Using ChIP-seq assays we found that while SC efficiently erases telomere-distal Hop1 in *Saccharomyces cerevisiae*, it is not sufficient to remove Hop1 from large telomere-adjacent regions (TARs; ~100 Kb). Several lines of evidence indicate that when all chromosomes have assembled the SC and telomere-distal breaks are suppressed, DNA breaks continue to form in the TARs. Removal of Hop1 from telomere-distal regions and thereby enrichment of Hop1 in the TARs requires activity of the AAA⁺-ATPase Pch2. In addition to telomere-distal regions, Pch2 also restricts Hop1 from centromeres, where crossover recombination maybe deleterious. As TARs comprise a proportionally large fraction of the small chromosomes in the genome, continued DNA breakage in these domains may contribute to the curiously high recombination rate observed on the small chromosomes [8-10] and thereby promote their accurate segregation in meiosis.

References

P1248
Board Number: B254
Intrinsic and extrinsic factors contributing to the stability of CENP-A nucleosomes at centromeres.
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Centromeres are present on each chromosome to orchestrate the accurate transmission of genetic information to daughter cells. Centromeres are epigenetically specified by the presence of nucleosomes containing the histone H3 variant, CENP-A. Nascent CENP-A nucleosome assembly occurs once per cell cycle, following mitotic exit. Once assembled they are exceptionally stable with no measurable turnover, even persisting at the same centromere through disruptive chromatin events like DNA replication (Falk et al., 2015, Science, 348, 699-703). Further, CENP-A nucleosome stability is presumably critical for maintaining centromere identity in slowly dividing cells where typical nucleosome turnover rates would compromise prior to the next round of CENP-A nucleosome assembly the epigenetic centromere mark that they provide. Stability is likely generated through a combination of factors that are either intrinsic (i.e. encoded within the CENP-A protein sequence) or extrinsic (i.e. conferred by direct binding partners). For intrinsic factors, we identified six a.a. residues of the CENP-A protein at its interface with its partner histone, H4, forming hydrophobic stitches that rigidify CENP-A nucleosomes relative to their canonical counterparts containing histone H3 (Sekulic et al., 2010, Nature 467, 347-351). Substitution of all six to the corresponding residues found in histone H3 do not disrupt interactions with the CENP-A
chromatin assembly protein, HJURP, but nonetheless lead to a loss from centromeres (Bassett et al., 2012, Dev. Cell 22, 749-762). We will describe our approach and emerging findings in experiments employing gene editing and CENP-A pulse-labeling to interrogate single a.a. substitutions of the hydrophobic stitch residues to identify those that affect the stability of centromeric chromatin. Indeed, we find that individual substitution of the hydrophobic stitch residues leads to varying reductions in centromere localization, with a subset of them severe. For extrinsic factors, we recently described two forms of stability in asynchronous cell populations conferred within a core centromeric nucleosome complex (CCNC): CENP-C binding across the histone octamer of the CENP-A nucleosome and driving a conformational switch that stabilizes the nucleosome, and CENP-N cross-bridging CENP-A to the nucleosomal DNA (Guo et al., 2017, Nat. Comm. 8, 15775). We are now extending our analysis to synchronized cells to investigate whether or not CENP-C and CENP-N confer CENP-A nucleosome stability consistently through the cell cycle or only at certain key points. A long-term goal of our work is to provide a comprehensive understanding of how epigenetic centromere identity is maintained.

P1249
Board Number: B255
Investigating the Role of CDK-2 in Crossover Recombination in C. elegans.
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Cyclin-dependent kinases (CDKs) and their activating cyclin subunits play central roles in driving cell cycle progression in eukaryotes. The classical view was that CDK2 is essential for cell cycle progression by controlling the G1/S transition. Studies using CDK2 knockout mice, however, have revealed an unexpected role of CDK2 during meiotic prophase, while demonstrating that CDK2 is dispensable for mitotic cell cycle progression and proliferation. Consistent with its meiotic functions, CDK2 has been shown to localize to telomeres as well as crossover sites during mammalian meiosis. However, the role of CDK2 in meiotic recombination remains unknown. Here report that CDK-2, the C. elegans homolog of CDK2, is localized to the crossover sites during meiotic prophase. Using super-resolution microscopy, we show that CDK-2 colocalizes with COSA-1, a cyclin-like protein that plays a conserved role in crossover formation, raising the possibility that CDK-2 might partner with COSA-1 to function as an active kinase. To determine the role of CDK-2 during meiosis, we employed the auxin-inducible degradation system to rapidly deplete CDK-2 from the C. elegans germline. CDK-2-depleted germ cells exhibit normal homolog pairing, synapsis, and meiotic DNA double-strand break formation. However, germ cells progressively lose COSA-1 foci from the distal end following auxin treatment and exhibit an increase in the number of univalent chromosomes in diakinesis, indicating that CDK-2 is required for crossover recombination. We are currently investigating how CDK-2 promotes crossover formation and will present our current findings.

P1250
Board Number: B256
Different Mechanisms of Micronucleus Formation and Impact to Genomic Stability.
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Micronuclei contain whole or fragmented chromosomes excluded from the main nucleus and are often used clinically as a marker of genomic instability. However, it is not fully understood how micronuclei...
contribute to pathogenesis at the cellular level. Furthermore, micronuclei can form through several mechanisms, and it is not clear whether all micronuclei have similar effects on cellular fitness and transformation. For example, micronuclei that form following improper attachments between chromosomes and microtubules of the mitotic spindle during cell division can lead to severe, but localized DNA rearrangements, called chromothripsis, identified as an early event in tumor formation. In contrast, our own studies of cells containing micronuclei from chromosome alignment defects do not show evidence of early events predicted to lead to chromothripsis, such as division with a pre-existing micronucleus or fragmentation of micronucleated chromosomes.

To test whether the impact of micronuclei on genomic stability vary as a function of 1) how they are formed, or 2) whether cells have p53 activity, we induced micronuclei in two different ways and used quantitative fluorescence microscopy to assess a) whether cells that form micronuclei continue to divide, b) the fate of micronuclei after cell division events, and c) the prevalence of micronuclear envelope rupture. Micronucleus formation was induced in human retinal pigmented epithelial (RPE1) cells by either promoting division with improper attachments via siRNA knock down of MAD2 (mitotic arrest deficient 2), an essential spindle checkpoint protein; or promoting division with unaligned chromosomes via knockout of Kif18A, a kinesin protein. Both conditions were performed with and without siRNA knockdown of p53 for comparison.

Our preliminary data show that micronucleated RPE1 cells resulting from unaligned chromosomes undergo cell cycle arrest (rate of division is reduced by 67% compared to non-micronucleated cells). However, the rate of micronucleated divisions doubles with p53 KD, suggesting that an intact DNA damage checkpoint prevents micronucleated cells from dividing. We also observe that micronuclei which form due to unaligned chromosomes (Kif18A knockout) rarely become reincorporated with the primary nucleus following a subsequent round of division. Interestingly, micronuclei that form due to improper attachments are more likely to rupture (Mad2 KD condition; 29% rupture), compared to those that form due to unaligned chromosomes, (Kif18A knockout; 15% rupture), indicating that the fate of micronuclei indeed differs between mechanisms of formation (p < 0.05).

P1251
Board Number: B257
The cytoplasmic DNA sensor cGAS promotes mitotic cell death.
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The cyclic GMP-AMP (cGAMP) synthase cGAS counteracts infections by detecting and binding foreign cytoplasmic DNA. DNA-induced synthesis of cGAMP activates innate immune signalling and apoptosis through the cGAMP receptor STING and the downstream effector IRF3. During interphase the nuclear envelope protects chromosomal self-DNA from cGAS, but the consequences of exposing chromosomes to cGAS following mitotic nuclear envelope disassembly are unknown. Here we demonstrate that cGAS associates with chromosomes during mitosis and binds nucleosomes with even higher affinity than naked DNA in vitro. Nucleosomes nevertheless competitively inhibit the DNA-dependent stimulation of cGAS, and accordingly, chromosomal cGAS does not affect mitotic progression under normal conditions. This suggests that nucleosomes prevent the inappropriate activation of cGAS during mitosis by acting as a signature of self-DNA. During prolonged mitotic arrest, however, cGAS becomes activated to promote cell death, limiting the fraction of cells that can survive and escape mitotic arrest induced by the chemotherapeutic drug taxol. Induction of mitotic cell death involves cGAMP synthesis by cGAS, as well as signal transduction to IRF3 by STING. We thus propose that cGAS plays a previously unappreciated role in guarding against mitotic errors, promoting cell death during prolonged mitotic arrest. Our data also indicate that the cGAS pathway, whose activity differs widely among cell lines, impacts cell fate
determination upon treatment with taxol and other anti-mitotic drugs. Thus, we propose the innate immune system may be harnessed to selectively target cells with mitotic abnormalities.

**Oncogenes**

**P1252**

**Board Number: B259**

Developing a *C. elegans* model to study SF3B1-driven Myelodysplastic Syndromes using CRISPR/Cas9 to introduce a point mutation and RNAi-mediated knockdown of sftb1.

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It is well established that Caenorhabditis elegans is an ideal model system to study the underlying genetic mechanisms of human diseases, particularly developmental diseases. There is a high level of genetic conservation between *C. elegans* and humans. Approximately 65% of human disease genes have orthologs in *C. elegans*. Moreover, these organisms are extremely amenable to genetic manipulation, they are relatively cost effective to maintain in the laboratory, and their developmental processes are well characterized. Myelodysplastic syndromes (MDS) comprise hematologic malignancies that are characterized by dysregulated production of blood cells and poorly formed blood cells. Mutations in SF3B1 were detected in more than 70% of MDS patients with refractory anemia with ring sideroblasts (RARS). SF3B1 is a core component of the U2 small nuclear ribonucleoprotein and it plays a key role in RNA splicing. This study will focus on the most commonly detected SF3B1 mutation in MDS, K700E. First, we performed bioinformatic analyses to determine whether SF3B1 and the K700 amino acid were conserved in *C. elegans*. Sequence homology between sftb1 and SF3B1 is approximately 71%, with sftb1 being 1322 amino acids compared to 1304 amino acids for SF3B1. The lysine residue at position 700 in SF3B1 was also conserved, albeit at position 718 in sftb1. Second, we examined the effect of RNAi-mediated silencing of sftb1 on development, reproduction, growth, and survival. We observed a decrease in overall survival rates following siRNA treatment targeting sftb1 but not control siRNA. This was likely due to impairments in egg laying because we observed altered uterine morphology following RNAi-mediated knockdown of sftb1. Third, we examined the expression patterns of sftb1 in each developmental stage. Finally, we modified a CRISPR/Cas9 vector and a double-stranded DNA repair template to generate offspring carrying sftb1 with the K718E mutation.

**P1253**

**Board Number: B260**

Cross-species oncogenomics approach identifies PTPN11 as an oncogene and potential therapeutic target in melanoma.


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Melanoma is a deadly disease carrying many genetic mutations. A major challenge to the development of effective targeted therapies in melanoma is the identification of true “driver” mutations among numerous “passenger” alterations. Several previous studies support using cross-species comparative oncogenomic approaches for cancer gene discovery. It has been shown that mice and humans share several genetic events in the development of cancer and that these events, conserved across different
species, may point to functionally important alterations targeting “driver” genes. Recently, we analyzed melanoma genomes from a mouse model driven by the loss of PTEN and CDKN2A (INK4A/ARF) by whole exome sequencing. This study identified several conserved cross-species orthologous mutations in Kras, Erbb3, and Ptpn11. In this study, we addressed the functional roles of PTPN11 in melanoma tumorigenesis and tumor maintenance, its effect on RAS/RAF/MEK/ERK signaling pathway, and its activation status in human melanoma.

Melanoma displays frequent activation of the RAS/RAF/MEK/ERK signaling pathway, which is intricately regulated by multiple proteins including PTPN11 (Tyrosine-Protein Phosphatase Non-Receptor Type 11, encoding SHP2). Although implicated as an oncogene in multiple cancer types, the oncogenic role of PTPN11 has not been fully established in melanoma. PTPN11 can be activated by receptor tyrosine kinases (RTKs) and/or by point mutations. Although the mutation rate is low (1~3%), we observed activating phosphorylation on Tyr 542 of PTPN11 in 40% (n=15/38) of melanoma specimens and the majority of human melanoma cell lines (n=14), indicating the potential frequent activation of PTPN11 in human melanoma. PTPN11 knock-down suppressed ERK activation in NRAS mutant (WM1361A, 1366, 1346) and BRAF/NRAS wt (WM3211, MeWo, CHL1) melanoma cells, but not in BRAF mutant (1205Lu, IGR1, 983C) cells. Moreover, we have shown that the expression of active PTPN11 E76K mutant drives soft-agar colony growth in vitro, tumor growth in nude mice, RAS/RAF/MEK/ERK activation, and resistance to MEK inhibition; whereas, knock-down of Ptpn11 reduces colony growth and ERK activation.

We generated a tet-inducible, melanocyte-specific, PTPN11 E76K transgenic mouse model in a Pten and Cdkn2a null background and observed melanoma formation. Implantation of melanoma cells derived from this model showed doxycycline dependent tumor growth in nude mice; additionally, withdrawal of doxycycline and subsequent extinction of PTPN11 E76K caused regression of established tumors, supporting a tumor maintenance role of PTPN11. These data support the oncogenic roles of PTPN11 in melanoma by regulating RAS/RAF/MAPK pathway activation and the value of PTPN11 as a novel and actionable therapeutic target.

P1254
Board Number: B261

On the role of kindlin-3 phosphorylation in cancer cells.
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The contributions of integrins to cellular responses depend upon the dynamic regulation of their activation status, which in turn depends on engagement of binding partners by their cytoplasmic tails. It is well-established that not only talin but also kindlin family members are essential for integrin activation, and both must present for optimal integrin activation. Recent studies in have specifically emphasized the vital role of kindlin-3 (K3) in integrin function in hematopoietic and non-hematopoietic cells, including cancer cells. K3 deficiency in hematopoietic cells leads to episodic bleeding, frequent infections and osteopetrosis, consequences of an inability to activate β1, β2 and β3 integrins. Despite this growing evidence, little is known about the regulation of K3 function. To identify posttranslational modification which might regulate K3 function, we used human platelets and human erythroleukemic (HEL) cells. The stimulation of platelets and HEL cells with agonist resulted in significant increase of K3 phosphorylation, as detected by MS sequencing. T482 or S484 were identified as phosphorylation sites and reside in K3 variable region. When expressed in cells, T482S484/AA K3 decreased soluble ligand binding and cell spreading on immobilized fibrinogen when compared to wild-type K3. Thus, our data emphasizes a role of previously unknown K3 phosphorylation, in integrin activation and provides a basis for functional differences between K3 and its two other paralogs. Our previous work showed that in breast cancer cells K3 is a tumor promoter, which regulates cancer progression by influencing the
crosstalk between β1 integrins and transcription factor Twist to increase VEGF production and to control tumor angiogenesis and metastasis. The importance of K3 in tumor progression led us to examine a role of K3 phosphorylation in breast cancer cells. Stable clones expressing phosphomutants of K3 were generated in MDA-MB-231 breast cancer cells. In vitro analyses determined that K3 phosphorylation is important for breast cancer cell spreading, migration and invasion, activation of β1 integrin and Twist upregulation. While expression of WT K3 increased spreading and invasiveness of MDA-MB-231 by ~40%, the spreading and invasiveness of T482S484/AA K3 expressing-cells was comparable to parental cells or cells expressing EGFP alone. T482S484/AA K3 also decreased HUTS-4 binding (antibody binding to activated β1) when compared to WT K3 cells. Also Twist expression was upregulated in WT K3 cells and it was reduced significantly in T482S484/AA K3 cells. These effects lead to the prediction that phosphorylation of K3 is likely to influence on tumor progression and metastasis in vivo.

P1255

Board Number: B262

NRMT1 mutants naturally occurring in human cancers have altered catalytic activity and cause a decrease in N-terminal trimethylation levels.

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N-terminal methylation of the alpha amino group of proteins has been documented for over 40 years, but the methyltransferases responsible for this modification, N-terminal RCC1 methyltransferases 1 and 2 (NRMT1 and NRMT2), have just been discovered in the last decade. NRMT1 is the only known eukaryotic N-terminal trimethylase, while its homolog NRMT2 is the only known N-terminal monomethylase. N-terminal methylation has been commonly believed to be a general regulator of protein stability, but the identification of NRMT1 and NRMT2 has led to the discovery that it also regulates protein-DNA interactions. Comprehensive determination of the shared NRMT consensus sequence has indicated over 300 potential substrates for the enzymes, including many involved in DNA repair. It has since been shown that specific loss of NRMT1 impairs nucleotide excision repair (NER) and double-strand DNA break repair, as well as, promotes oncogenic phenotypes in breast cancer cells and leads to premature aging phenotypes in mice. These data also suggest that residual monomethylation from NRMT2 cannot functionally compensate for loss of trimethylation. As mutations of NRMT1 naturally occur in human cancer genomes, the objective of this study was to test if these mutations actually alter enzymatic activity. NRMT1 mutants N209I (endometrial cancer) and P211S (lung cancer) displayed decreased trimethylase and increased mono/dimethylase activity as compared to wild type NRMT1. Closer examination demonstrated that the NRMT1 mutants are capable of trimethylation, but they do so at a much slower rate and with a requirement for higher substrate concentration. NRMT1 is a distributive methyltransferase that dissociates after the addition of each methyl group. Both the N209I and P211S mutations are located in the peptide-binding channel and further strengthen the notion that this structural region helps determine affinity for unmodified vs. previously mono- or dimethylated substrates. Expression of the mutants in wild type NRMT1/2 backgrounds showed no change in N-terminal methylation levels or growth rates, demonstrating they are not acting as dominant negatives. Expression of the mutants in cells lacking endogenous NRMT1 resulted in minimal accumulation of N-terminal trimethylation, indicating the impaired biochemical activities of these mutants cannot be overcome in cells with endogenous substrate levels, even after prolonged exposure. It also suggests N209I or P211S homozygosity could mimic NRMT1 knockdown phenotypes and lead to inefficient DNA repair, mutagenesis, and oncogenic transformation. Alternatively, these mutations could serve as
markers for tumors with increased sensitivity to DNA damaging chemotherapeutics or gamma-irradiation.

**P1256**

**Board Number: B263**

**Downregulation of LAT1 (L-type amino acid transporter 1 / SLC7A5) in human cancer.**

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L-Type amino acid transporter 1 (LAT1 / SLC7A5) is highly expressed in cancer cells to support their continuous growth and proliferation and has been suggested as a marker of cancer prognosis in different types of cancer (Semin Cancer Biol 15:254-266, 2005). The present study examined the effect of LAT1/SLC7A5 gene downregulation by RNA interference on the function of the transporter in human cancer cells. Target sites within LAT1 gene (NM_003486) were selected from the respective human mRNA sequences. Nucleotide homology searching was performed against nonredundant and dbEST using BLAST via online connection to NCBI. The commercial siRNA S131011000 that targets the human LAT1 mRNA sequence and negative control siRNA commercial sequences NC-S103650318 and NC-S103650325 (Qiagen) were tested. Cells (human liver adenocarcinoma SK-HEP-1, human fibrosarcoma HT-1080 and human bladder carcinoma T24) were cultured for 48 h (in RPMI and DMEM-hg, respectively). LAT1 mRNA and protein expression, inward transport of $[^{14}\text{C}]$-L-leucine, and effects of siRNA anti-LAT1 upon $[^{14}\text{C}]$-L-leucine uptake were analyzed. Antibodies raised against LAT1 and GAPDH were used, and images were obtained by scanning at both 700 nm and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences). For RT-PCR, total RNA was converted to cDNA and qPCR analysis was made in the StepOnePlus instrument (Applied Biosystems). Primers for LAT1 and for the endogenous control gene GAPDH were used. All cell types expressed LAT1 mRNA and protein. The abundance of LAT1 mRNA differed among the tumor cell lines, being more intense in HT-1080, followed by SK-HEP-1 and T24 cells. The same trend was not verified for protein abundance, with T24 cells expressing more LAT1 protein than the other cell lines. The sodium-independent inward transport of non-saturating concentrations (0.25 μM) of $[^{14}\text{C}]$-L-leucine, the preferred substrate of LAT1, was linear with time for up to 10 min of incubation. In order to determine the kinetics of the transporter, cells were incubated for 1 min with $[^{14}\text{C}]$-L-leucine (0.25 μM) in the absence or in the presence of increasing concentrations of unlabelled substrate L-leucine (1 to 3 mM). The kinetic parameters of $[^{14}\text{C}]$-L-leucine uptake ($K_m$ in mM; $V_{max}$ in nmol/mg protein/min) were determined by non-linear analysis of the specific analysis of inhibition curve for L-leucine and are as follows: SK-HEP-1, $K_m=0.072\pm0.011$, $V_{max}=2.938\pm0.439$; HT-1080, $K_m=0.123\pm0.021$, $V_{max}=5.876\pm0.858$; T24, $K_m=0.120\pm0.013$, $V_{max}=10.346\pm0.996$. Transfection with siRNA against LAT1 gene, but not the negative control siRNAs, reduced the $[^{14}\text{C}]$-L-leucine accumulation in all cancer cell lines. In conclusion, anti-LAT1 siRNA decreased $[^{14}\text{C}]$-L-leucine uptake, possibly by downregulation of LAT1 expression.
P1257
Board Number: B264
Transforming growth factor beta (TGFβ) regulates glutamine metabolism in lung Fibrosis.
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Fibroproliferative diseases are a leading cause of morbidity and mortality featuring localized and systematic tissue/organ fibrosis. Nearly 45% of all deaths in the developed world are caused by chronic inflammatory and fibrogenic disorders. Despite the massive impact of fibroproliferative diseases on human health, there are no efficient therapeutic treatment options that directly target the mechanisms of fibrosis due to their inherently complex and often undefined etiology. Cancer cells reprogram their bioenergetics and biosynthetic requirements by altering metabolic pathways to divert nutrients such as glutamine to satisfy the demand for cellular building blocks to fuel cell growth. Accumulated evidence showed that the reprogramming of tumor metabolism is under the control of various oncogenic signals and growth factors. While TGFβ is considered a pleiotropic cytokine due to its ability to impact numerous diseases and biological processes, the role of nutrient metabolism in profibrotic TGFβ signaling is relatively unexplored or unknown. To that end, our studies have identified a previously uncharacterized relationship between upregulation of glutamine transporters (SLCs) and glutaminase I (GLS1; which converts glutamine to glutamate) with TGFβ. Specifically we have found that (i) glutamine transporters SLC1A5 and SLC1A7 as well as GLS1 are induced by TGFβ in both murine and human lung fibroblasts; (ii) while phosphorylation of SMAD3 occurs independently of SLCs or GLS1, fibroblast migration and expression of TGFβ stimulated profibrotic targets including collagen 1, PAI-1, CTGF, fibronectin and α-SMA requires the action of SLC1A5 and GLS1; (iii) by using pharmacologic and genetic approaches, we have demonstrated that upregulation of SLC1A5, SLC7A5 and GLS1 occurs via the canonical SMAD2/3 pathway as well as non-canonical PI3K/Akt/mTOR pathway. Finally we have found a novel mechanistic insight of the regulatory roles of TGFβ in metabolic dysregulation leading to idiopathic pulmonary fibrosis through histone modifications and autophagy, which will inspire a new frontier in the design and development of antifibrotic therapy.

P1258
Board Number: B265
How does MYC make purines?
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Cancer cells rewire their metabolism to obtain energy and anabolic precursors for proliferation. MYC is thought to be a key driver of cancer cell metabolism with its major function attributed to the transcriptional induction of metabolic enzymes. However, it is completely unknown whether MYC can control cellular metabolism by transcription-independent mechanisms. Our group discovered a cytoplasmic transcriptionally-inactive MYC variant named MYC-nick. MYC-nick is a proteolytic byproduct of MYC that promotes survival of cancer cells under stress conditions (such as hypoxia or nutrient starvation) but does not induce cell proliferation. While MYC-nick does not have the ability to bind DNA, it can interact with acetyltransferases. Our working model is that MYC-nick recruits acetyltransferases to promote acetylation of cytoplasmic proteins, thereby regulating their functions. To determine if MYC-nick has the ability to regulate cell metabolism, we performed metabolomics (in collaboration with Dr. Ralph DeBerardinis) comparing the profiles of fibroblasts expressing MYC and
MYC-nick. Unexpectedly, we found that MYC-nick promotes an increase in purines. Utilizing HPLC-Mass spectrometry (in collaboration with Dr. Noelle Williams), we found that MYC-nick expression induces purines in fibroblasts and in human colorectal cancer cells. Moreover, we also found that purines are elevated in human colon cancer tissues, which express high levels of MYC-nick. The most dramatic result was an increase in the levels of deoxyinosine (a mutagenic purine) in every tested biopsy sample of colon cancer.

We propose that MYC-nick increases the production of purines by shunting glycolytic intermediates into the pentose phosphate pathway to promote de novo purine synthesis. We hypothesize that MYC-nick, by acetylating and regulating the activity of glycolytic enzymes (such as aldolase), promotes synthesis of purines in a transcription-independent manner. Currently, we are testing the role of deoxyinosine and other purines on MYC-nick-induced cell survival and mutagenesis.

P1259
Board Number: B266
Alpha1-antitrypsin-derived C-terminal peptide is a potent oxidative stress inhibitor.
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Acute-phase protein alpha1-antitrypsin (AAT) has been reported as a biomarker in many tumors. A number of tumor cell lines produce the protein. Tumor-associated proteases cleave AAT at the active site producing a bioactive C-terminal peptide C36, which possesses immune suppressing, serine protease-protecting and mitogenic activity (L. Cercek, B. Cercek, 1992, 1993; Zelvyte I. et al., 2003). According to our previously reported data, C36 can also activate metabolism under certain conditions (Maslakova A., 2016). Recently, an exogenous full-length AAT has been established as an oxidative stress protector in a human placental cell line (Feng Y-L et al., 2016, 2017) at the effective concentration of 50uM. We investigated the oxidative stress-protecting activity of AAT-derived C36 peptide in human cell line DU145 under serum starvation condition using cell-permeable 2′,7′-dichlorodihydrofluorescein diacetate. We used a wider C36 peptide concentration range (10pM-50uM) in order to investigate its potential activity at levels that are much lower than physiological levels for the full-length AAT (around 30uM), that might be closer to true peptide concentrations in tumor microenvironment. Indeed, C36 peptide protects cells from oxidation in a dose-dependent manner starting from as low as 100nM (~34% lower overall oxidation level), while 30-50uM gives a more pronounced effect (~49-56% lower overall oxidation level, respectively). The latter inhibitory level is in a good accordance with the previous data on the full-length protein (Feng Y-L et al., 2016), that has been shown to inhibit p38MAPK signaling pathway (Feng Y-L et al., 2017). Our findings indicate that AAT-derived C36 peptide preserves the site crucial for such activity and can potently protect tumor cell from oxidative stress under serum starvation. The study was supported by RFBR project № 16-34-01095 mol_a (“Structural and functional analysis of SERPINA1 gene transcripts and alpha1-antitrypsin protein isoforms in human tumors cultured cell lines”).
P1260
Board Number: B267
Targeting the Notch1 Transcriptional Activation Domain in T-Cell Acute Lymphoblastic Leukemia.
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Notch signaling is an evolutionary conserved pathway which allows for communication between neighboring cells. Notch1, one of 4 mammalian Notch receptors, is required for commitment of thymic progenitors to the T-cell fate and has been implicated in T-Cell Acute Lymphoblastic Leukemia (T-ALL). In fact, over 50% of adult T-ALL patients have tumors in which the Notch1 gene has obtained a hypermorphic mutation, and treating human Notch1 addicted T-ALL cell lines with Notch pathway inhibitor Gamma-Secretase Inhibitors (GSI) in vitro leads to growth arrest and apoptosis. The intracellular fragment of Notch1 (ICN) is composed of several domains, one of which is the Transcriptional Activation Domain (TAD). TAD deleted ICN (ICNΔTAD) interferes with signaling from wild type ICN in mice heterozygous for the mutation, resulting in blunted transcriptional activity. There is also evidence that the TAD is involved in T-ALL progression. Bone marrow transplant (BMT) assays have shown that ICN lacking its C terminal portion, thus lacking the TAD, fail to initiate leukemia. Based on this finding, we will target the Notch1 Transcriptional Activation Domain in an effort to uncover a novel method of modulating Notch1 signaling in human T-ALL.

P1261
Board Number: B268
Investigating the Role of PA28y in DNA Base Excision Repair.
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Increased levels of reactive oxygen species (ROS) are present in almost all cancers. Since increased levels of ROS can cause serious damage to both DNA and proteins, understanding how cancer cells sustain their altered biology could identify ways to selectively target transformed cells. DNA base excision repair (BER) is the primary pathway responsible for the repair of DNA damaged through oxidation. Oxidative damage to DNA generally induces small base lesions, which if left unresolved, can result in transversion and transition mutations. Disruption of the BER pathway is linked to a variety of cancers, including colorectal, gastric, and lung. The proteasome activator, PA28y, has been previously demonstrated to play an important role in the cellular response to oxidative stress through its degradation of oxidatively-damaged proteins. Moreover, expression of PA28y protein is elevated in many cancers and increases in response to treatment with hydrogen peroxide, an ROS. Here, we identify a novel role for PA28y in the repair of oxidative damage in the DNA. Murine embryonic fibroblasts (MEFs) deficient in PA28y have higher baseline levels of oxidative DNA damage. Additionally, PA28y-deficient MEFs exhibited delays in DNA repair after treatment with hydrogen peroxide. Expression of Proliferating Cell Nuclear Antigen (PCNA), a protein important to base excision repair, was compared between the two cell lines since PA28y has been characterized as an indirect activator of PCNA. Baseline PCNA expression was comparable in PA28y-deficient MEFs, however in response to hydrogen peroxide treatment, PA28y-deficient MEFs exhibited no increase in PCNA expression. Conversely, a six-fold increase in PCNA expression was observed in wild type MEFs, suggesting that PA28y is essential for expression of one of the key proteins induced as part of the cell’s DNA damage response. The inability of PA28y-deficient MEFs to induce a key excision repair protein could suggest a possible mechanism for the difference

Sunday-167
in repair kinetics observed between the two cell lines. Our data reveal an expanded role for PA28γ in the cellular response to oxidative damage, playing an important role in the repair of damaged DNA as well as the degradation of damaged proteins.

**P1262**
**Board Number: B269**
The NF45-NF90 complex is required for execution of the mitotic programme and chromosome stability.
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ILF2 and ILF3 are essential genes that are upregulated in several types of neoplasms, yet their exact function remains elusive. These genes encode nuclear factor 45 and 90 (NF45 and NF90), which are RNA-binding proteins involved in mRNA processing and translation. Here we show that NF45 and NF90 form a complex that regulates the expression of many mitotic proteins involved in chromosome stability. Depletion of NF45 or NF90 induces pleiotropic cell-cycle defects, including chromosome mis-segregation and frequent failure of cytokinesis, leading to polyploidy or mitotic catastrophe. Cells depleted in NF45 or NF90 fail to synchronize in mitosis in response to spindle poisons, suggesting defects in mitotic spindle checkpoint. Transcriptome analysis revealed that NF45 and NF90 are necessary for the expression of a cluster of mitotic mRNAs, which also associate with the NF45-NF90 complex. Cancer genomics databases indicate that ILF2 and ILF3 are upregulated in breast cancer, and that their expression tightly correlates with the mitotic cluster. Thus, our data uncover post-transcriptional events regulated by the NF45-NF90 complex that are essential for proper mitotic progression and may be particularly relevant in cancer.

**P1263**
**Board Number: B270**
The potential prognostic marker BaxΔ2 is generated without mutation at a genetic or transcriptional level.
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Proapoptotic BaxΔ2 was originally discovered in colon cancer with high microsatellite instability. BaxΔ2 is a more potent isoform of Bax whose expression was thought to require a mononucleotide deletion (G8 to G7) in the Bax microsatellite region in combination with an alternative splicing event. Interestingly, we have recently found that most BaxΔ2 positive cells were found in healthy tissues and low-grade tumors. After analyzing 50 samples of both normal and tumor tissues for genomic sequencing, we found that the vast majority of the BaxΔ2 positive samples had no microsatellite mutations. One possible explanation was a G7 mutation at the mRNA level, which could happen through transcriptional slippage. To test this, we screened mRNA transcripts from both normal and colon cancer cells. Surprisingly, we found that even though the necessary alternative splicing event is fairly common, point mutations were rarely detected. Our ongoing work involves testing the hypothesis involving Programmed Ribosomal Frameshift, a mechanism by which a ribosome can intentionally skip bases and continue protein expression at a different reading frame (see Manas poster). These results indicate that BaxΔ2 can be generated without mutations at either the genomic or transcriptional levels, and possibly serve as a prognostic marker for cancer therapy.
P1264  
**Board Number: B271**  
**Ribosomal frameshift-mediated expression of BaxΔ2 in human tissues and its correlation with cancer stage.**  
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BaxΔ2 is a novel pro-apoptotic Bax isoform previously identified in patients with microsatellite instability, especially in HNPC (hereditary nonpolyposis colorectal cancer). As many other cancers are prone to microsatellite mutations, we screened BaxΔ2 expression in nearly a thousand TMA samples covering tumors from a variety of organs, normal adjacent tissues, and normal healthy tissues. We found that, unlike ubiquitous distribution of Baxα, BaxΔ2 is mainly scattered in connective and epithelial tissue, and its expression inversely correlates with tumor grade, being very low or negative in high-grade tumors. Interestingly, a predominant amount of BaxΔ2 was found in normal and normal adjacent tissues. It is well established that production of BaxΔ2 requires an alternative splicing event and a guanine deletion in the microsatellite region. However, we found that the great majority of BaxΔ2-positive tissue/cells contain no microsatellite mutation at either genomic or transcript levels. When the correct alternative splicing event happens in the absence of the microsatellite mutation, it leads to an early stop codon unless a frameshift occurs. Therefore, we propose an expression mechanism involving Programed Ribosomal Frameshift (PRF). PRF is a phenomenon by which a ribosome can purposely skip one or more bases, either backwards (⁻) or forward (+), and continue protein expression at a different reading frame. To test this, we generated a double tagged construct at the N- and the C-termini of BaxΔ2 with no mutation. Only if a +1 PRF event happens will both the N- and C- tags be expressed. We found that, though rare, +1 PRF does happen and leads to expression of BaxΔ2 from constructs with no microsatellite mutation. In conclusion, production of BaxΔ2 can occur without genetic mutation and its expression could serve as a prognostic biomarker.

P1265  
**Board Number: B272**  
**Nedd9 influences lung cancer tumorigenesis through regulation of autophagy.**  
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Non-small cell lung cancer (NSCLC) has a low survival rate, with metastasis contributing to the vast majority of deaths. Expression of the NEDD9 (HEF1/Cas-L) protein is elevated and promotes metastasis in a large subset of NSCLC tumors and other malignancies. NEDD9 functions as a scaffold for multiple critical effectors in integrin/FAK/SRC and receptor tyrosine kinase signaling cascades, with overexpression enhancing signaling by these pro-oncogenic proteins. However, we found that a Nedd9 constitutive null genotype (Nedd9⁻⁻) enhanced tumor growth in an inducible 129S/Sv-Krastm3Tyj/Trp53tm1Brn (KP) model in which Kras mutation is induced specifically in lung tissue by inhalation of adenovirus bearing the Cre gene. Pathological examination of tissues indicated Nedd9 null genotype also was associated with higher invasive capacity in vivo, including direct invasion to the heart, as well as elevated proliferation rate, decreased apoptotic activity, and changes in the expression of
proteins such as vimentin, associated with mesenchymal status. These results contradicted previous studies in which depletion of NEDD9 by RNAi reduced the growth and invasion of established lung cell lines and tumors, based on cell culture and xenograft assays. To gain insight into these paradoxical results, we performed Reverse Phase Protein Array (RPPA) analysis to characterize signaling in isolated tumors. This revealed numerous tumor-intrinsic changes associated with a Nedd9-/- genotype, affecting GYS1 and other proteins in the glycosgen synthesis metabolic pathway, and including notable upregulation of BECLN1 and ATG3, indicative of elevated autophagy. Subsequent in vitro analysis coupled with immunohistopathological and metabolic analysis of tumor tissue and tumor-derived cells confirmed and extended these results. Overall, our data support a model in which NEDD9 provides critical support for early stages of NSCLC growth, and progression beyond this early stage in the absence of NEDD9 requires extensive metabolic reprogramming. This reprogramming includes upregulation of the AMPK signaling axis and inhibition of the mTOR pathway, resulting in activation of autophagy responses and changes in oxidative phosphorylation and metabolism. Our data for the first time identifies NEDD9 as a regulator of these processes, and emphasizes distinct requirements for NEDD9 in early tumorigenesis versus in established tumors.

P1266
Board Number: B273
Survivin governs mitochondrial architecture by regulating phosphatidylethanolamine (PE) availability.
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Survivin is an essential protein with established roles in mitosis and the inhibition of cell death. It is overexpressed in all cancers and its abundance correlates with tumour resistance to irradiation (IR). In normal cells, survivin expression is confined to the G2 and M phases; however, in cancer cells it is also present during interphase when it can be found in the nucleus, the cytosol and the mitochondria. PE is a phospholipid with a small head-group that facilitates negative curvature of membranes, thereby increasing their accommodation of proteins; thus it is enriched in specific membranes, such as the mitochondrial cristae and the cytokinetic furrow. Here we report the remarkable discovery that mitochondrial survivin regulates phosphatidylserine decarboxylase (PSD) the enzyme that converts phosphatidylserine (PS) to PE, the exclusive source of PE in the mitochondria. Our data suggest that by controlling PSD thereby governing mitochondrial topology and potentially altering cellular metabolism, as well as sensitivity to IR. This novel molecular insight suggests that many of the apparently disparate roles of this “multitasking” protein may be fundamentally linked to membrane architecture, and offers a completely unexpected perspective on its contribution to cancer and other metabolic disorders.

P1267
Board Number: B274
Increased Rac activity is required for Ha-RasV12-induced multilayer cellular aggregates in Madin-Darby canine kidney cells.
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Ha-RasV12 overexpression induced multilayer cellular aggregates during confluence in MK4 cells (inducible Ras harboring MDCK cells). Because activation of Yes-associated protein (YAP) induced cellular transformation, epithelial-mesenchymal transition (EMT) and loss of contact inhibition, we examined
whether the activation of YAP mediated Ha-RasV12 induced-multilayer cellular aggregates. We found that Induction of Ha-RasV12 triggered YAP nuclear translocation and subsequently YAP-targeted gene expression. However, verteporfin (VP), a YAP/TEAD binding inhibitor, failed to prevent Ha-RasV12-induced multilayer cellular aggregates. Overexpression of Cav1 inhibited Ha-RasV12-induced YAP activation and multicellular cell aggregates, whereas knockdown of Cav1 in MDCK cells only resulted in activation of YAP, but not cellular aggregates and cellular transformation. Activities of Rac and RhoA, both associated with cell extrusion, were increased in Ha-RasV12 overexpressed MK4 cells. EHT1864 (Rac inhibitor) abolished multilayer cellular aggregates in Ha-RasV12-overexpressed MK4 cells, whereas Y27632 (ROCK inhibitor) induced multilayer cellular aggregates in MK4 cells. However, neither EHT1864 nor Y27632 inhibited Ha- RasV12-induced YAP nuclear localization. Taken together, these data indicate that Cav-1 downregulation is required for Ha-Ras V12-induced YAP activation and cellular transformation. However, only Cav1 downregulation and Rac activation, but not YAP activation, are required for Ha-Ras V12-induced multilayer cellular aggregate.

P1268
Board Number: B275
The role of InHibitor of Growth (ING) 2 in Breast Cancer Treatment.
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Breast Cancer is the most frequent form of women's cancer, affecting 1 in 8 during their lifetime with >24,000 new cases in women and >200 diagnosed in men in Canada in 2017. Although curable if detected early, breast cancer still accounts for 13% of all cancer-related deaths in Canada, underscoring the need for additional accurate predictors of early breast cancer development. Our lab discovered the first ING gene (ING1) and found that it was inactivated in breast tumours and breast cancer cell lines. INGs contain a conserved PHD domain and ING PHD domain/H3K4me3 binding targets the ING2-mSin3A-HDAC1/2 complex to the nucleosome to mediate gene repression. We hypothesised that expression of ING2 in breast epithelial cells might serve as a harbinger of cellular transformation, and levels of ING2 may have clinical value. Our studies indicate that normal breast epithelial cells did not express detectable levels of ING2 while all breast cancer cell lines examined expressed significant levels of ING2. Over-expression of ING2 repressed the induction of ING1, but ING1 over-expression did not affect ING2 levels. Consistent with an oncogenic role, Her2+ breast cancer cells showed a considerably higher ING2 levels and a significant correlation between ING2 and Her2/Neu levels. Elevated levels of ING2 also correlated with an increased sensitivity of breast cancer cells to SAHA that is known to target ING2. Furthermore, higher levels of ING2 was noticed in triple negative breast cancer samples relative to normal breast epithelium. The expression of ING2 in breast cancer cells correlated with the association of Myeloid zinc finger 1 with the ING2 promoter. Our data suggest that ING2 out-competes ING1, reducing its tumour suppressive effect, to induce cellular transformation and might serve as a novel biomarker for breast cancer diagnosis.
P1269
Board Number: B276
To Understand the Structural Role of Pyruvate Kinase M2 in Epigenetic Mechanism.
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Cancer cells undergo metabolic reprogramming characterized by high glucose consumption and lactate production. This phenomenon of aberrant metabolism is known as aerobic glycolysis or the Warburg effect. Pyruvate Kinase, the terminal glycolytic enzyme is the master regulator of glycolytic flux and plays a pivotal role in regulating the cellular metabolism. Pyruvate Kinase M2 (PKM2) is an oncofetal isoform generated as a result of alternative splicing of the PKM mRNA transcript. It exhibits a low basal activity and thus diverts the glycolytic intermediates in the anabolic pathways for sustained cell proliferation conferring growth advantage to the cancer cells. Recently the non-glycolytic or nuclear function of PKM2 has come into prime focus. PKM2 exacerbates cancer progression by exerting its role as a key nuclear protein kinase phosphorylating plethora of nuclear proteins. On oncogenic/mitogenic stimulation dimeric PKM2 undergoes nuclear translocation wherein it binds to the promoter regions of oncoproteins and initiates an array of epigenetic modifications. Studies have reported that the nuclear PKM2 modulate the gene expression by phosphorylating Histone H3 of the nucleosome complex that ensues a cascade of epigenetic changes. However the structural details and the mechanistic regulation of this interaction is still elusive. In our study we have tried to gain a structural insight of the PKM2 interaction with the nucleosome and Histone H3. Fluorimetry experiments and Surface Plasma Resonance (SPR) results reveal a direct interaction of PKM2 with the Histone H3. Electrophoretic mobility shift assay using non—denaturing polyacrylamide gel suggest that PKM2 interacts with the reconstituted FAM-labelled nucleosome. Thus, our results establish that PKM2 directly binds to the nucleosome. We aim to decipher the architecture of the PKM2-nucleosome complex using X-ray crystallography and cryo-electron microscopy. Structural intricacies of the PKM2 with nuclear proteins or nucleosome may help us to understand the mechanism of PKM2 mediated epigenetic changes. Furthermore this study will indirectly help us decipher the PKM2 driven oncogenicity as well as possible targets or pathways of cancer in context with the cancer cell metabolism.

P1270
Board Number: B277
LIN9 is a mitotic vulnerability in triple-negative breast cancer that is targetable with BET inhibitors.
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LIN9 is a member of the MuvB transcriptional regulatory complex and is involved in several critical cellular processes, including embryonic development, cell cycle progression, and mitosis. It is also a component of the metastasis-predicting Mammaprint gene signature in breast cancer. We discovered...
P1271
Board Number: B278
Wnt/β-catenin signaling, genomic instability and DNA break formation in hematopoietic cells: Role of Topoisomerase IIα.
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Wnt/β-catenin signaling has an essential function in hematopoiesis and deregulation of its activity is a key factor in the development of leukemia. Here we studied Wnt/β-catenin signaling on genomic instability and DNA strand break formation and examined β-catenin interaction with topoisomerase IIα (Topo IIα), as this enzyme plays an important role in cleavage and re-ligation of double-stranded DNA during replication, transcription and DNA damage repair. Using single cell electrophoresis (Comet assays) and γ-H2AX immunofluorescence staining, we observed a significant dose-dependent increase in DNA breaks in KG-1 human hematopoietic cells treated with CHIR (98014), a specific Wnt/β-catenin signaling pharmacological activator. Treatment of cells with the drug induced the co-localization between β-catenin with γ-H2AX foci and also between β-catenin with Topo IIα. Interestingly, activation of the signaling cascade enhanced γ-H2AX co-localization with Topo IIα protein. We finally confirmed the interaction of β-catenin with Topo IIα by co-immunoprecipitation experiments. Our results suggest that sustained transcriptional stress induced by the signaling cascade is responsible for genomic instability and DNA strand break formation in hematopoietic cells and as such may participate during malignant transformation to leukemia.
Funding: Fondecyt 3150612 to M.F.V. and 1140353 to G.D.V.
**P1272**

**Board Number: B279**

**Characterization of the Interferon Regulatory Factor 4 Pathway in Melanoma Cells.**

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Melanoma skin cancers have poor prognosis at the metastatic stage despite recent advances in targeted therapies and immunotherapy. Therefore, identification of novel pathways and therapy targets is highly desirable. Like most cancers, abnormal gene regulatory and epigenetic mechanisms are also linked to melanomas.

Interferon regulatory factor 4 (IRF4) is a transcriptional regulator with crucial roles in the development and functioning of immune cells, and is implicated in malignant transformation. We have previously demonstrated the critical role of IRF4 in mature B-cell-derived cancer cells, and unraveled its mechanisms of action. These and related other studies, therefore, highlighted IRF4 pathways as therapy targets in cancer. Beyond immune cells, genetic association studies linked variation at the IRF4 locus with pigmentation phenotypes and skin cancers. Recent work also identified the role of IRF4 in the pigmentation process of normal melanocytes. However, despite the observed genetic associations and expression, the role of IRF4 in melanoma remains largely unexplored.

Therefore, we set out to characterize the functions of IRF4 in melanoma cells using cell and molecular biological, and genome-wide approaches. In order to identify the genome-wide targets of IRF4 in melanoma cell lines, we have performed chromatin immunopreipitation sequencing (ChIP-seq) and transcriptomic (RNA-seq) assays, and analyzed them integratively. Complemented with The Cancer Genome Atlas (TCGA) data analyses, our data point to a role of IRF4 in epigenetic regulation of melanoma cells, among other cancer- and development-related pathways. In parallel studies, we have taken a candidate approach to identify the upstream regulators of IRF4 expression in melanoma cells. Here, our results uncover a known melanoma master transcription factor and a major signaling pathway with therapeutic targeting options as upstream regulators of IRF4 expression. Furthermore, our preliminary cell biological studies implicate IRF4 as a critical factor in melanoma cell proliferation and survival.

Taken together, our work on IRF4 is mechanistically complementing at the cellular and molecular level the published genetic studies on melanoma patient samples, delineating this novel pathway in melanoma cells, and therefore will potentially be pointing to new therapy strategies in melanoma.

**P1273**

**Board Number: B280**

**HPV16 E6 and E7 Oncoproteins are Negative Regulators of Invadopodia Activity but Promote Migration in Head and Neck Squamous Cell Carcinoma.**

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Human papilloma virus (HPV)-positive head and neck squamous cell carcinomas (HNSCC) are frequently diagnosed at low tumor stages but with positive nodal metastatic disease in contrast to HPV-negative tumors of the same region. The mechanisms that regulate tumor progression of HPV-positive head and
neck cancers are poorly understood. The ability to degrade the extracellular matrix (ECM) is thought to be necessary for the spread of tumor cells. Invadopodia are actin-rich cellular protrusions that facilitate extracellular matrix (ECM) degradation and have been implicated in tumor cell invasion and metastasis in HPV-negative HNSCCs. Thus, we sought to compare the invasive phenotypes of HPV-negative and positive head and neck cancer cell lines in vitro. Using a panel of HPV-negative and HPV-positive cell lines, we found that in general, the HPV-negative cells exhibited more invadopodia activity. Selecting one representative cell line from each group, we found specifically that both invadopodia numbers and ECM degradation are significantly increased in HPV-negative SCC-25 cells compared to HPV-positive SCC-47 cells. To determine if this difference was dependent on HPV status, the HPV-specific oncoproteins E6 and E7 were overexpressed (OE) and knocked down (KD) with lentiviral particles in SCC-25 and SCC-47 cells, respectively. A reversal of results was noted with a significant decrease in invadopodia formation and degradation area in SCC-25 OE cells and vice versa in SCC-47 KD cells. Given these differences in invasiveness, transwell migration assays were used to assess migration patterns as a potential etiology for early metastasis. Increased migration was observed in the SCC-47 cells compared to the SCC-25 cells; however, SCC-47 KD cells migrated less than SCC-47 control cells. Thus, despite the clinical findings that HPV-positive HNSCCs metastasize early, our data suggest that tumor progression occurs through an alternative mechanism that is not dependent on invadopodia-mediated degradation of ECM but instead favors increased cell migration.

P1274
Board Number: B281
Dual role of mitochondria in tumor initiation and progression.
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Oncocytomas are benign tumors of epithelial origin. To determine origin of tumor formation we sequenced benign oncocytomas, which revealed two subtypes and pathogenic mitochondrial mutations in electron transport chain (ETC) as a common factor in the two subtypes (Joshi et al., 2015). Therefore, we aimed to determine if pathogenic mitochondrial ETC mutations are 1) the driver mutations in these tumors; 2) responsible for limiting tumorigenesis. Metabolomics of human renal oncocytomas showed modest increase in levels of (S)-2-hydroxyglutarate (2HG), an oncometabolite implicated in epigenetics regulation. Oncocytomas showed higher GSSG/GSH ratio when compared to normal kidney tissue, suggesting oxidative stress in these tumors. Oncocytoma primary cells were observed to be highly glycolytic. They used glutamine to generate αKG and citrate via reductive carboxylation, and produced 2-HG at elevated levels. Epigenome sequencing of the patient samples is underway to identify epigenetic changes, which might be responsible for the tumor initiation. To check the effect of ETC impairment in tumorigenesis, ETC was genetically impaired by CRISPR knock out of complex II subunit, SDHB in \textit{Kras}\textsuperscript{G12D}, \textit{p53}\textsuperscript{7/} driven mouse lung cancer cell line or chemically inhibited by using complex I inhibitor in \textit{Kras}\textsuperscript{G12D}, \textit{p53}\textsuperscript{7/} driven lung tumor mouse model. The deletion of SDHB significantly reduced the growth of cancer cells both in vitro and in an allograft mouse model by induction of ROS mediated DNA damage and G2-M growth arrest. The growth of the SDHB knock out cancer cells was partially rescued by the treatment with ROS scavenger, N-acetyl cysteine. The chemical inhibition of complex I
increased the mice survival rate by reducing the lung tumor burden and producing fibrotic, slow growing tumors. Our studies suggest that ETC pathogenic mutations may initiate tumors by oxidative stress and epigenetic regulations but ETC functions are preserved in aggressive tumors for growth.

**Tumor Invasion and Metastasis 1**

**P1275**  
**Board Number: B282**  
Tissue explant imaging reveals spatially coordinated migration patterns in the tumor core.  
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Invasion of cancer cells into the stroma is a critical step in the malignant progression of carcinoma and can lead to metastasis. Cancer cell invasion is associated with an increased migratory capacity of cells leading to their dissemination to distant tissues. Until now, cancer cell migration has been described at the so-called “invasive front”, the region where cancer cells reach stromal tissue. The core of tumors has been considered as a relatively immobile tissue. However, due to the lack of technology required to image the tumor core in real time, this has never been directly addressed. In order to investigate cancer cell dynamics in a native tumor core, we use a mouse model which gives rise to spontaneous aggressive intestinal carcinoma. We explore cell migration phenotypes in tumor explants using ex vivo long-term 3D imaging by two-photon microscopy. We find that cancer cells in the tumor core are not stationary, as previously thought, but are rather remarkably dynamic and migrate with a range of cell speeds and persistence. Moreover, we observe a correlation between migration direction and distance between cells, where cells close together have a correlated direction of migration and move in local "currents". Such collective behaviors of neighboring cells give rise to large-scale tissue dynamics, such as collective streaming and vortex-like migration features. Although cells exhibit stop-and-start migration patterns with intermittent pauses, we do not observe any substantial cell pausing during division. This suggests that dividing cells in cancer tissues can still flow with their neighbors during division. This study represents the first investigation of the live dynamics of cells in the tumor core of spontaneously developing tumors. Our work will help understand collective behaviors of cells in the tumor core and sheds light on cancer migration as well as other dynamics systems, such as developing organisms.

**P1276**  
**Board Number: B283**  
A novel window for high resolution imaging of the lung reveals mechanisms of metastatic breast cancer progression.  
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It has become clear that the tumor microenvironment dominates the metastatic phenotype of tumor cells. In addition, the cell biology mechanism behind tumor cell dissemination from the primary tumor is becoming well understood thanks to high resolution multiphoton intravital imaging. Imaging shows that therapeutic agents, including cytotoxic chemotherapy and receptor tyrosine kinase inhibitors, have the ability to alter this microenvironment to either promote or reduce metastatic dissemination from the
primary tumor. Unknown however, is if the mechanism of tumor cell dissemination from the primary tumor is conserved in metastatic tumors and if the above treatments impact the metastatic site differently from the primary tumor, essential information for the design of long term treatment strategies. Each of the major metastatic sites (bone, lung, and liver) are inaccessible internal organs limiting analysis to standard assays (FACS, histopathology, etc.) which destroy the tissue and thus only give single time point, snap-shot analyses without vital information about the tumor microenvironment. Only intravital imaging through implantable imaging windows gives the ability to return to the tissue multiple times over course of disease progression and treatment. The lung, the central metastatic site in breast cancer patients, is the most difficult organ to study using intravital microscopy due to its perpetual motion. Recent advances in intravital microscopy have enabled visualization of the live intact lung, but are limited in time over which they can be utilized (hours) and require major, invasive surgeries. Both of these limitations reduce their usefulness to the study of single cell events in the lung and are susceptible to introducing artifacts. We have addressed these limitations by developing an implantable, permanent, lung imaging window which allows high-resolution multiphoton imaging of the intact, breathing (without ventilation), murine lung over days to weeks of repeated imaging. This window does not use vacuum to immobilize the lung tissue, thereby avoiding artifacts associated with vacuum lung windows. Using our new window, we have documented over weeks, and for the first time, tumor cell arrival, the cell motility steps used during extravasation, tumor cell survival, dormancy, and progression to micro-metastases, all at subcellular resolution. Further, we have observed, for the first time, the presence and intravasation activity of the Tumor MicroEnvironment of Metastasis (TMEM) in metastatic lung lesions directly demonstrating use of the same mechanism of tumor cell dissemination from metastatic lung tumors as observed in the primary tumor opening new strategies for the treatment of metastatic tumors to prevent metastatic progression and death.

P1277
Board Number: B284
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Metastasis is the main cause of mortality and morbidity in cancer patients, because it spreads beyond the site of origin, leading to damage in distant organs and tissues. Key steps of the metastatic cascade involve intravasation and extravasation, where single tumor cells from the primary tumor intravasate into the bloodstream and extravasate into the stroma of secondary organs to form metastatic colonies. Previously, we have shown that inhibiting cancer cell extravasation is a realistic target for halting cancer metastasis when invadopodia function is inhibited. We have also recently determined that extravasating cancer cells release extracellular vesicles (EVs) in vivo. Using intravital imaging, we determine that cancer cell EV release leads to significant cell volume loss and a reduction in metastatic potential. Cell membrane blebbing is the main cellular mechanism of EV generation and release. Since cell death pathways such as apoptosis and necroptosis also induce membrane blebbing and release EVs, we hypothesized that modulating these cell death pathways leads to cell volume reduction and inhibition of cell extravasation.

Various metastatic cancer cell lines were cultured and injected into the chorioallantoic membrane (CAM) of chicken embryos for imaging and quantifying circulating EVs. Staurosporine (STS) and dimethyl fumarate (DMF) were used to induce cancer cell apoptosis and necroptosis respectively. We performed
intravital imaging of cancer cell EV release, extravasation, and metastatic colony formation by high-resolution confocal microscopy. To quantitate EVs, we used nanoscale flow cytometry to analyze blood samples from the CAMs and conditioned media. We observed an increase in circulating EVs and reduced extravasation and metastatic colony formation rates when membrane blebbing was modulated. Although pro-apoptotic cancer cells exhibited an increase in cancer cell EV release that resulted in reduced extravasation rates, extravasating cancer cells did not exhibit caspase-3 activity during EV release, indicating that apoptosis is not involved in EV generation. Pro-necrotic cancer cells also exhibited an increase in cancer EV release. This was accompanied by cell volume reduction and a decrease in cancer cell extravasation and metastatic colony formation rates. Moreover, inhibition of necroptosis significantly reduced EV release, improving extravasation and metastatic colony formation rates.

Overall, our findings suggest that a reduction in cell volume by EV release facilitates extravasation but excessive release is counterproductive. Additionally, our findings on the inhibition of necroptosis and the pro-necrotic process implicate that necroptosis is a more critical regulator of cancer metastasis.

P1278

Board Number: B285

Distinct ECM proteins of breast cancer metastatic niches in multiple organs.

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Metastasis remains the cause of the vast majority of cancer-related deaths, and one of the most insidious aspects of metastatic cancer is the incredible adaptability of cells from a single primary tumor to survive in multiple, vastly different secondary sites. Nevertheless, how cells from the same primary tumor are able to adapt to all these environments is not fully understood. At each secondary site of metastasis, tumor cells create a metastatic niche, a microenvironment conducive to their survival and proliferation. A critical component of each niche is the extracellular matrix (ECM), which provides structural support, migration control, and growth and survival signals. However, a comprehensive comparison of the ECM components of metastatic niches at various secondary tumor sites has not yet been conducted. We isolated metastases from the bone marrow, brain, liver and lung, which were all derived from a common population of MDA-MB-231 breast cancer cells. We then enriched these tumor samples for ECM proteins and used quantitative mass spectrometry to analyze their ECM composition. Across all samples, 247 ECM and ECM-associated proteins were identified, of which 44 were exclusively produced by the human tumor cells and 142 were made solely by the murine stroma. Strikingly, both the tumor-derived and the induced stroma-derived ECM and ECM-associated proteins differ at each site; that is, the niches created are distinct. By abundance, most of the proteins produced only by tumor cells were secreted factors, while the proteins made only by stromal cells were mostly ECM glycoproteins. The set of proteins produced by both the tumor and stromal cells consisted mainly of collagens. Overall, greater than 90% of the total ECM protein abundance within metastases originated from stromal cells. Using these data, protein abundance was compared across all metastatic sites in order to determine which ECM proteins were most significantly elevated in each particular tissue relative to the others. By comparing all metastatic to all normal samples, proteins that were broadly elevated across all metastatic tissues were also identified. Following this analysis, ongoing work is focused on a set of ECM proteins produced by tumor cells specifically in metastases to the brain, testing whether the metastasis of MDA-MB-231 cells to the brain is dependent on the expression of these proteins by injecting knockdown cells into circulation and observing their resulting metastatic growth and tropism. This investigation will
provide insight into the fundamental biology of metastatic niches, as well as provide potential markers of metastatic breast cancer at various sites for imaging and therapy.

**P1279**
**Board Number: B286**
Liver metastasis is facilitated by the adherence of circulating tumor cells to vascular fibronectin deposits.
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The interaction between circulating tumor cells (CTC) and endothelial cells during extravasation is a critical process during metastatic colonization, but its mechanisms remain poorly characterized. Here we report that the luminal side of liver blood vessels contains fibronectin deposits that are enriched in mice bearing primary tumors and are also present in vessels from human livers affected with metastases. Cancer cells attached to endothelial fibronectin deposits via talin1, a major component of focal adhesions. Talin1 depletion impaired cancer cell adhesion to the endothelium and transendothelial migration, resulting in reduced liver metastasis formation in vivo. Talin1 expression levels in patient CTC’s correlated with prognosis and therapy response. Together, our findings uncover a new mechanism for liver metastasis formation involving an active contribution of hepatic vascular fibronectin and talin1 in cancer cells.

**P1280**
**Board Number: B287**
3D collagen fiber architecture regulates cell migration phenotypes by modulating MMP activity.
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Cancer cells interact closely with the extracellular matrix (ECM), and defined collagen ECM architectures have been correlated with metastatic potential in vivo. However, the role of local ECM structure on the regulation of metastatic motility phenotypes is not completely understood. In previous studies, we identified a confining collagen architecture characterized by short, thin fibers and small pores that triggers a conserved metastatic migration phenotype and transcriptional response independently of matrix stiffness and density. To further explore the role of this confining architecture on the development of an invasive motility phenotype, we sought to characterize the coordination of the four key biophysical processes used by cells to migrate through 3D environments: cell adhesion, actomyosin cytoskeleton polymerization and contraction, and protease mediated degradation of the surrounding ECM. These processes were quantified in HT-1080 fibrosarcoma cells using: i) fluorescence recovery after photobleaching (FRAP) to study actin polymerization dynamics in cellular protrusions labeled with LifeAct, ii) traction force microscopy (TFM) of matrix-embedded beads to study cell adhesion and contractility, and iii) fluorescent dye quenched (DQ) collagen degradation to study MMP activity. We observed that cells embedded in matrices with small pores and short fibers, produce smaller and shorter
lived protrusions as compared to cells embedded in a matrix with longer and thicker fibers. Actin FRAP studies revealed that this difference in protrusion lifetime was not caused by differences in actin polymerization rates. TFM and protrusion-ECM displacement correlation showed that in confining architectures, cells fail to properly bind and pull on the surrounding matrix. Rather, they displayed a chaotic probing phenotype, that suggests early breaking or degradation of the small fibers composing the matrix. Interestingly, DQ collagen degradation assays showed that cells in the confining architecture degrade the surrounding collagen significantly more than cells in the porous architecture. Preliminary results suggest that this increase in collagen degradation is accompanied by increased collagen internalization. Moreover, gene expression analysis using RNA sequencing showed that cells in the confining architectures upregulate uPARAP (MRC2), a mannose receptor protein implicated in the internalization of degraded collagen fragments. Taken together, our results suggest that the short fibers associated with confining 3D collagen architectures promote the upregulation of a collagen degradation and internalization pathway that could trigger downstream intracellular signaling cascades.

P1281
Board Number: B288
Role of Laminin Matrikines in Phenotypic Switching of Motile Cancer Cells.
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Dissemination to distant organs and formation of metastatic outgrowth are the leading causes of cancer-related death. The metastatic process heavily relies on cancer cell motility and much effort has been made to decipher the physiologic mechanisms governing cell movement. Recently, increasing evidence has shown that different cues present in the tumor microenvironment may modify cell behavior. Homotypic interactions between cancer cells themselves, but also heterotypic interactions between cancer cells and stromal cells have been described in the context of cell fate. However, the dynamic role of the extracellular matrix on cancer cell decision-making came into the spotlight only recently. Studies show that the extracellular matrix continuously exchanges both mechanical and chemical signals with cancer cells. For instance, cancer cells pull on the extracellular matrix and use it as a structural support for migration. In addition, cancer cells remodel the extracellular matrix via degradation in order to invade into the healthy tissues, and intravasate into the blood vessels or nerves. Our previous work demonstrated that motile cancer cells which are entering perivascular niche within the primary tumor, switch their phenotype from migration to extracellular matrix degradation by invadopodia. Such degradation subsequently leads to intravasation and metastasis. In contrast, invadopodia inhibition prevents future metastasis.

Our working hypothesis is that the matrikines created by invadopodia have the capability of regulating the level of further degradation by acting as a phenotype switching triggers. Our preliminary data shows that soluble beta 1 laminin fragments, released by degradation of perivascular basement membrane, can inhibit further degradation by invadopodia. We are interested in mapping matrikines that may modulate cancer cell plasticity in each step of the metastatic cascade. Further, we believe that understanding their effects on the cancer cell behavior will be a powerful tool to develop new therapeutic treatments and prevent early metastasis.
P1282
Board Number: B289
Collective epithelial intravasation in breast cancer metastasis.
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A key goal in cancer research is to understand the underlying mechanisms of the metastatic process, by which cancer cells spread from a primary tumor to other sites and form secondary tumors. Indeed, metastasis is responsible for around 90% of cancer patient mortality. Despite its clinical importance, metastatic dissemination is an inefficient process. In experimental animal models, less than 0.02% of the single tumor cells leaving the primary tumor ultimately develop into metastatic lesions. Our lab has found that cells from primary tumors sometimes escape as clusters and that cell clusters are more efficient in forming lung metastases than single cancer cells.

We now focus on a key step of the metastatic process: intravasation. This process is facilitated by molecular changes that promote the ability of cancer cells to cross endothelial cell barriers that form the walls of microvessels. Previous studies have shown that tumor cell clusters are able to travel through pulmonary circulation in vivo. However, the mechanism by which tumor clusters gain access to the systemic circulation is still unknown. Our lack of understanding of this mechanism is due to the difficulties in establishing tumor models in which tumor-vessel interactions can be visualized and characterized. To overcome this barrier, we use a microfluidic approach with a physiologically and morphologically realistic vasculature along with 3D organotypic culture of mammary gland tumor organoids embedded in collagen-I gels. This approach allows us real-time and quantitative assessment of tumor-vessel interactions under in vivo like conditions. Preliminary evidence shows tumor organoids pushing through the tumor-vessel boundary resulting in the disruption of the vessel structure and function. We observed in real-time an intravasation event that preceded the disruption of a vessel. Our current work focuses on imaging optically cleared primary tumors to correlate the tumor-vessel structures observed in vivo with those observed in vitro. We anticipate that deeper understanding of the mechanisms of tumor cell cluster intravasation will provide new insights for targeted anti-metastatic treatments.

P1283
Board Number: B290
Cell-density dependent migration in pancreatic ductal adenocarcinoma.
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Pancreatic cancer is the 4th most common cause of cancer-related death in the United States with only 9% of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) living to the 5-year mark. Despite this, relatively few improvements have been made to the treatment of PDAC. Gemcitabine, a drug approved in 1996, is now being used in combination with nab-paclitaxel, a therapy that is one of
two standard first-line treatment options despite only having increased overall survival by two months. My objective is to dramatically improve overall survival and ultimately impact the current 5-year survival statistic by providing a novel combination therapy designed to treat both the solid tumor and inhibit the migration of cancer cells in patients with PDAC. Our lab has recently proven that cells display a migratory phenotype which only occurs when a high number of tumorigenic, metastatic cells are in a localized environment, such as would be found near a growing tumor. The cause behind this was determined to be paracrine signaling via a precise ratio of secreted interleukin 6 (IL-6) and interleukin 8 (IL-8). Cells exposed to these cytokines display enhanced invasion in comparison to cells that are not in an interleukin-rich environment or cells whose IL-6 and IL-8 receptors have been blocked. While this was shown in breast cancer, preliminary findings lead us to believe that this is also true in pancreatic cancer models. An in vitro 3D migration assay conducted with various metastatic PDAC cell lines seeded in collagen I shows that there is an increase in the mean square distance (MSD) of cells embedded in a high-cell-density environment when compared to low density. This migratory phenotype can be prevented with treatment of a combination therapy of Tocilizumab and Reparixin. Based on these results, we designed an in vivo experiment utilizing a patient-derived xenograft mouse model. The mice were randomly assigned to one of four treatment arms; saline (control), Gemcitabine, Tocilizumab + Reparixin, or Gemcitabine + Tocilizumab + Reparixin. We anticipate the results from the tumor volume and the analysis of the excised lung, liver and lymph nodes will show that the combination of Gemcitabine, Tocilizumab, and Reparixin has a synergistic effect on preventing tumor growth, as well as providing the best protection against metastases. In conclusion, because metastases are generally considered to be the cause of death in most PDAC patients, utilizing this novel combination therapy which targets both the growth and invasion of cancer cells has the potential to revolutionize treatment efficacy for PDAC patients.

**P1284**

**Board Number: B291**

**NHE1 overexpression disrupts organization of epithelial cell monolayers and accelerates collective cell migration.**

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Metastasis is the main cause of cancer patient mortality and substantially complicates treatment strategies. The Na⁺/H⁺ Exchanger 1 (NHE1) is upregulated in many cancers and plays a major role in cancer cell motility and thus metastasis. NHE1-mediated acid extrusion levered the increased intracellular acid production in cancer cells and contributes to the acidification of cancer tissue. Moreover, NHE1 recruitment to the front of migrating single cells promotes cell movement, in part by regulating intracellular and extracellular pH (pHᵢ, pHₓ). A number of studies have shown that collective cell migration is common in epithelial cancers, but how NHE1 affects organization and morphology of migrating epithelial cell collectives has not been investigated. To test this, NHE1-GFP was overexpressed in normal epithelial MDCK cells. NHE1 overexpression increased the steady state pHᵢ of MDCK cells from 7.17 to 7.41 and the H⁺ transport capacity after an acid load by 49%. During collective cell migration, NHE1-GFP localized in the leading edge of leader cells as well as migrating cells further back in the cell sheets. Overexpression of NHE1 alone significantly increased collective cell migration by 17%, and this effect was potentiated by stimulation with EGF and low serum concentrations. Within the migrating sheets, NHE1 overexpressing
cells were less organized, formed more migration fingers, and the cell spacing was increased. Combined NHE1 overexpression and EGF stimulation further decreased organization of cell monolayers and migration fingers. This difference was not reflected in detectably altered pH of migrating cells, underlining the importance of intracellular signaling for NHE1 in migration. In summary, we found that overexpression of NHE1 alone is sufficient to accelerate collective epithelial cell migration and disturb the organization of mammalian epithelial cells in migrating sheets. Live tracking of migrating MDCK cells overexpressing NHE1 can identify whether NHE1 can interfere with cell-cell coordination and regulation of directionality during migration.

P1285
Board Number: B292
Loss of MTSS1 results in increased metastatic potential in pancreatic cancer.
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Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival rate of 7%. This dismal outlook is largely due to the inability to diagnose the disease before metastasis occurs. 53% of patients afflicted with pancreatic cancer are diagnosed at the metastatic stage. Thus, there is a critical need to better understand what causes early tumor cell dissemination and metastatic progression in this disease. One of the hallmarks of early stages of PDAC is inflammation. It is well known that patients with chronic pancreatitis have a much higher chance to develop PDAC. While many studies have focused on elucidating the mechanisms by which this inflammation drives PDAC progression, few have focused on the role inflammation plays in metastasis. Using a novel inflammation-driven mouse model of PDAC, we were able to uncover a subset of genes that were not only regulated by increased inflammation, but also correlated with poor PDAC patient prognosis. Here, we functionally test the role of one of the genes found in this subset that is involved in metastatic progression, namely, metastasis suppressor protein 1 (MTSS1). We show that loss of MTSS1 leads to increased invasion and migration in PDAC cell lines. Moreover, PDAC cells treated with cancer-associated fibroblast-conditioned media also have increased metastatic potential, which is augmented by loss of MTSS1. Additionally, overexpression of MTSS1 in PDAC cell lines leads to a loss of migratory potential in vitro and an increase in overall survival in vivo. Furthermore, we present a novel regulatory mechanism for the stabilization of MTSS1 via the tumor suppressor protein, phosphatase and tensin homolog (PTEN). Our data show that PTEN loss in PDAC cells results in both increased metastatic potential in vitro, and in a decrease in MTSS1 expression. Furthermore, we show that ectopic MTSS1 expression rescues this effect. Additionally, we demonstrate that PTEN forms a complex with MTSS1 in order to stabilize it from proteosomal degradation. Finally, we show that the inflammatory tumor microenvironment, which makes up over 90% of PDAC tumor bulk, is capable of downregulating PTEN expression, potentially uncovering a novel extrinsic, upstream mechanism of MTSS1 regulation. Collectively, these data offer new insight into not only the role and regulation of MTSS1 in suppressing tumor cell invasion and migration, but also a different glimpse as to what molecular mechanisms could be leading to early cell dissemination in PDAC.
P1287

Board Number: B294
CCL18 from tumor-associated macrophages promotes breast cancer metastasis via ACAP4-ARF6 signaling cascade.
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Tumor-associated macrophages influence cancer progression and metastasis. Our study showed that cytokine CCL18 derived from breast tumor-associated macrophages promotes breast cancer metastasis via PITPNM3 (Chen et al., 2011. Cancer Cell). However, the signaling cascade underlying CCL18-elicted breast cancer metastasis remains elusive. Here we show that CCL18 stimulates acetylation of ACAP4 which liberates ACAP4 from plasma membrane and release into the cytosol. Surprisingly, this acetylation tunes ACAP4 effector binding for elevation of ARF6 GTPase activity. Importantly, acetylation of ACAP4 promotes the invasion and metastasis of breast cancer xenografts, whereas suppressing PCAF abrogates these CCL18-elicted, ACAP4-mediated effects. These findings indicate that ACAP4 acetylation plays a critical role in CCL18-elicted breast cancer metastasis. Currently, we are studying CCL18-elicted cellular dynamics using a combination of human breast cancer organoids with spectral imaging.

P1288

Board Number: B295
Cellular localization of ER chaperones may predict cancer patient prognosis.
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Background: Anterior Gradient 2 (AGR2) is a member of the Protein Disulfide Isomerase (PDI) family which regulate protein homeostasis and the unfolded protein response (UPR) pathway. AGR2 has also been characterized as a proto-oncogene and a potential biomarker when secreted. We investigated the interplay between AGR2’s activity as an endoplasmic reticulum (ER) chaperone versus its role as a secreted protein in the context of cancer progression and response to therapy. Materials and Methods: In order to characterize the role of the ER resident versus secreted protein, we performed a comprehensive protein-protein interaction screen (BioID and AP-MS/MS) on distinct cellular compartments. Ingenuity Pathway Analysis (IPA) of the interaction screen was used to identify novel signaling axis. These findings were confirmed in vitro, in vivo, and with clinical cohorts from The Cancer Genome Atlas (TCGA). The prognostic significance of AGR2 expression and cellular localization on response to chemotherapy was assessed on metastatic prostate cancer tissue and circulating tumor cells (CTCs) Results: We identified over 800 proteins across three different cancer cell lines (FDR <0.01). Enrichment of pathways associated with the ER versus secreted AGR2 interactome highlighted differences in several cancer related pathways (angiogenesis, PI3/AKT, HIPPO). A combination of in vivo and in vitro assays confirmed that localization of AGR2 can either promote or inhibit pathway activity (e.g. Hippo pathway). For example, cancer cell spheroid formation was accelerated by dosing with recombinant AGR2 while overexpression of ER AGR2 inhibited this phenotype. The distinction between ER versus secreted AGR2 was most pronounced in various in vivo models that collectively confirmed the metastasis promoting phenotype of secreted AGR2. Importantly, TCGA and CTC expression data suggest
that increased ER AGR2 sensitizes cancer cells to chemotherapy while secreted AGR2 promoted resistance.

Conclusion: Taken together, our results identified opposing biological phenotypes predicated on AGR2 localization. The PDI activity of AGR2 seems to be most pronounced in the ER. Our findings also raise an interesting possibility for screening of secreted AGR2 as a means to guide therapy options.

P1289
Board Number: B296
Myoepithelial cells are a dynamic barrier to epithelial dissemination.
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The normal mammary gland forms a branching ductal network, arranged in a bilayer: an inner luminal epithelial and an outer myoepithelial layer of cells. Most breast cancers arise in luminal cells and such luminal-derived tumors can generate life-threatening metastases. The first step of breast metastasis is tumor invasion past the myoepithelial layer; however, the role of myoepithelium during tumor progression and metastasis remains poorly understood. Hence, it is important to understand if myoepithelial cells play a functional role during cell invasion. To investigate behaviors at the single-cell level and in real-time, we used 3D culture of mouse mammary epithelial clusters, termed organoids. We have previously demonstrated that constitutive expression of the prometastatic transcription factor Twist1 in the murine mammary gland leads to cell dissemination. In the current study, we characterized the dissemination potential of specific mammary epithelial cell populations. Using time-lapse confocal microscopy, we observed that in the ubiquitous-Twist1 organoids, myoepithelial cells could actively limit the escape of luminal epithelial cells. To further study this potential role of myoepithelium in regulating cell dissemination, we generated mouse models in which myoepithelial or luminal cell-specific promoters drove Twist1 expression, while the remaining cells were Twist1-negative. In organoids with myoepithelial-specific Twist1 expression, there was cell-autonomous myoepithelial cell dissemination. Remarkably, in the organoids with luminal-specific Twist1 expression, we observed a major reduction in dissemination when compared to control organoids expressing Twist1 in both luminal and myoepithelial cell types (5 fold decrease in dissemination). To test the functional role of myoepithelial cells in controlling luminal cell dissemination, we combined Twist1+ luminal cells with varying numbers of normal Twist1- myoepithelial cells. The results showed that an increasing number of normal myoepithelial cells added to Twist1+ luminal cells acted to decrease dissemination. To elucidate the molecular mechanisms for this myoepithelial barrier function, we used lentivirally-delivered shRNA in luminal-specific Twist1+ organoids to knockdown expression of myoepithelial-specific contractility genes. Our results reveal that knockdown of α-smooth muscle actin in the luminal-Twist1 model markedly enhanced dissemination (3 fold increase). Collectively, our data showed that the normal myoepithelium acts as an active suppressor of luminal cell dissemination, and myoepithelial contraction is required for this function.
P1290
Board Number: B297
Emerin regulation of nuclear structure in cancer cell invasion.
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Breast cancer accounts for approximately 20-25% of cancer diagnoses each year. Metastasis is the spread of cancer from the site of the original tumor to another site in the body and responsible for most of the deaths. Metastasis requires cancer cells to enter and exit the blood vessels through incredibly small gaps; without this, metastatic tumors cannot form. The cell’s nucleus is much larger than these gaps and thus must be ‘squished’ to 10% of its original diameter for the cell to squeeze through these small gaps. The nucleus is very rigid in normal cells and resists the compressive forces required to contort the nuclei to fit through these gaps. However, metastatic cancer cell nuclei are more compliant and compress more easily. The molecular changes underlying this increased compliance remain poorly understood. Our studies focused on emerin, an integral membrane protein of the nuclear envelope, because of its well established roles in nuclear structure and function. The nuclear area of emerin-null myoblasts was measured to directly test if emerin expression was important for nuclear size. 400-600 wildtype myoblasts or emerin-null myoblasts from three biological replicates were used for this analysis. Nuclear area was measured using the NII Plugin in ImageJ software (NIH). The NII Plugin measures aspect, area, radius ratio and roundness of selected nuclei. The area of wildtype nuclei was 44% larger than emerin-null nuclei. Wildtype emerin was expressed in emerin-null myogenic progenitors to test if increased emerin expression rescued nuclear size and structure. Emerin expression increased nuclear size by 49% in emerin-null cells, comparable to the differences seen between wildtype and emerin-null cells. Previous studies showed the MDA-231 invasive breast cancer cells had decreased nuclear size and abnormal nuclear structure. Emerin protein expression was measured in invasive breast cancer cells (MDA-231, Hs578t and MDA-157), control breast fibroblasts (MCF10A) and primary fibroblasts (normal). Emerin protein levels were decreased 1.4- to 1.8-fold in the invasive breast cancer cell lines, compared to control cells. There was no statistical difference between emerin levels in MCF10A cells and primary fibroblasts (p=0.47; Figure 5). Thus decreased nuclear size in breast cancer cell lines correlated with decreased emerin expression. Our future studies will continue to test the hypothesis that emerin plays a critical role in regulating nuclear size and stiffness and that disruption of emerin expression contributes to the increased invasiveness of breast cancer cells by reducing nuclear size and stiffness. We anticipate our studies will yield a more complete understanding of cell invasion and migration and uncover novel therapeutic targets for metastasis.

P1291
Board Number: B298
Leader cells are defined by DNA hypermethylation and aberrant gene expression during collective lung cancer invasion.
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Collective cancer cell invasion, wherein packs of cancer cells invade together, is present in patient samples of most solid tumor types and contributes to cancer metastasis. Within these collective invasion
packs, highly-invasive leader cells pioneer migration and invasion while highly-proliferative follower cells travel behind them. Our lab previously developed an image-guided genomics technique to isolate and culture individual leader and follower cells, termed Spatiotemporal Genomic and Cellular Analysis (SaGA). Since epigenetic mechanisms, such as DNA methylation, regulate phenotypic plasticity and cell differentiation in many cellular contexts, we hypothesize that leader and follower cell phenotypes can emerge through epigenetic reprogramming of lung cancer cells. We performed DNA methylation profiling using a DNA methylation array wherein leader cells showed significant global DNA hypermethylation compared to both follower cells and the H1299 lung adenocarcinoma parental population. Furthermore, integrating DNA methylation analysis with RNAseq analysis identified gene expression patterns unique to leader cells that correlate with these changes in DNA methylation. Differentially methylated CpG islands overlapping promoters correlated with significant gene silencing or overexpression of 57 genes in leader cells, including multiple putative tumor suppressors and oncogenes. In addition, leader cells showed differential methylation at FANTOM5 enhancers correlated with many gene ontology pathways critical for collective cancer invasion, including VEGF signaling, which we have previously shown to be critical for collective pack formation. In addition, inhibition of DNA methylation using 5-aza-2'-deoxycytidine (DAC) significantly abrogated collective invasion of 3-D spheroids of H1299 parental cells and even more significantly in spheroids of purified leader cells. DAC treatment also rescued expression of genes in leader cells that had little to no gene expression and high promoter DNA methylation compared to parental and follower cells. Our data suggest a mechanism wherein global DNA hypermethylation can drive the leader cell phenotype and wherein DNA methylation regulates gene expression critical for leader cell behavior. Subsequent validation of differentially methylated target genes may determine functionally-relevant genes driving leader cell-dependent collective cancer invasion.

P1292
Board Number: B299
Repair factor loss and genome variation in cancer cell invasion.
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Migration through micron-size constrictions has been seen to rupture the nucleus, release nuclear-localized GFP, and cause localized accumulations of ectopic 53BP1 – a DNA repair protein. Here, constricted migration of two human cancer cell types and primary mesenchymal stem cells (MSC) increases DNA breaks throughout the nucleoplasm as assessed by endogenous damage markers and by electrophoretic ‘comet’ measurements. Migration also causes multiple DNA repair proteins to segregate away from DNA, with cytoplasmic mis-localization sustained for many hours as is relevant to DNA repair times. Partial knockdown of repair factors that are known to also regulate chromosome copy numbers is seen to increase DNA breaks in U2OS osteosarcoma cells, with nucleoplasmic patterns similar to migration, and knockdown likewise increases aberrant levels of DNA without affecting migration. Migration-induced nuclear damage is nonetheless reversible for wild-type and sub-cloned U2OS lines, whereas DNA arrays and sequencing reveal lasting genomic differences. Gains and losses of hundreds of megabases in many chromosomes are typical of the changes and heterogeneity in bone cancer. Phenotypic differences that arise from constricted migration of U2OS cells are further illustrated by a clone with a highly elongated and stable MSC-like shape that depends on microtubule assembly.
downstream of the transcription factor GATA4. Such changes seem consistent with reversion to a more stem-like state upstream of cancerous osteoblastic cells. Migration-induced genomic instability can thus associate with heritable changes. Next, we tested such migration-induced genomic aberrations in vivo, by using a metastatic mouse model followed by genomic comparisons between the cells derived from primary and metastatic sites.

**P1293**

**Board Number: B300**

**Metastasis by tumor epithelial clusters requires E-cadherin expression.**

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**Introduction**: Metastasis is the major driver for cancer related deaths. The genetic/ epigenetic loss of E-cadherin (E-cad) is linked to increased invasion of cancer cell lines and therefore, has been inferred to promote metastasis. Consistent with this concept, invasive lobular breast cancer cells lack E-cad expression and commonly metastasize. However, contrary to this concept, more than 80% of breast cancers are invasive ductal and these tumors retain their E-cad expression in both the primary tumor and distant metastases. We recently demonstrated that cancer cell clusters expressing E-cad contribute to >95% of metastases arising from these tumors. The current study aims to dissect the direct functional contribution of E-cad towards metastasis.

**Results**: To address this question, we used MMTV-PyMT mice, a frequently used metastatic model of invasive ductal carcinoma. We bred these mice to also include additional transgenes that allow for a Cre-inducible, GFP reported deletion of E-cad. Primary tumor organoids isolated from these mice were infected with Adeno-Cre recombinase to delete E-cad. We observe that loss of E-cad expression results in increased invasion and dissemination in 3D organotypic culture. Disseminated cells typically lack E-cad, but retain expression of other epithelial markers such as cytokeratins. To test the requirement of E-cad expression for metastasis, we transplanted E-cad deficient tumor organoids into immunocompromised host mice. Tumors in these mice grow slower but have a more invasive front compared to tumors with intact E-cad expression. Interestingly, however, cancer cells that have lost E-cad do not contribute to any observable metastases. From *in-vivo* tail vein assays, we concluded that E-cad null cancer cells are defective at seeding distant organs. To then test if E-cad expression promotes survival of cancer cells, we performed colony formation assays using flow sorted cancer cells. E-cad null cancer cells show a 7-fold decrease in colony forming potential. We are currently investigating molecular signaling alterations downstream of E-cad deletion that confer a decreased metastatic potential.

**Conclusions**: Consistent with previous reports, loss of E-cad increases tumor invasion and dissemination. However, our results reveal that E-cad loss in cancer cells can inhibit metastatic colonization. Interestingly, we observe an uncoupling of the relative efficiency of local invasion/ dissemination and distant metastatic outcome. **We propose an alternate route of collective epithelial metastasis in which cancer cells need to maintain gene expression consistent with their epithelial origin to efficiently metastasize.**
P1294

Board Number: B301

ZEB1 becomes a transcriptional activator upon interacting with YAP.
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Early dissemination, metastasis and therapy resistance are central hallmarks of aggressive cancer types and the leading cause of cancer-associated deaths. Dissemination of tumour cells is enabled by an aberrant activation of the epithelial to mesenchymal transition (EMT) program. The EMT-inducing transcription factor ZEB1 is a crucial stimulator of these processes, particularly by coupling the activation of cellular motility with stemness and survival properties. The potent effects of ZEB1 cannot be solely explained by its well-established role as a transcriptional repressor of epithelial genes. We analysed in which context ZEB1 can act as a transcriptional activator and discovered a functional link between ZEB1 and YAP, the key downstream effector of the Hippo pathway. We found ZEB1 to be an activator of a set of classical YAP target genes in breast cancer cell lines, and that ZEB1 and YAP synergistically co-regulate this common ZEB1/YAP gene set. We could further demonstrate that ZEB1 and YAP directly interact. Intriguingly ZEB1 was not found to interact with TAZ, the YAP paralogue, indicating functional differences between YAP and TAZ. We are currently modelling the ZEB1/YAP interface in silico, in order to further dissect the binding determinants and determine how this interaction is regulated upstream. The ZEB1/YAP common target gene set is a predictor of poor survival, therapy resistance and metastasis formation in breast cancer, proving clinical relevance of our findings. The identification of this ZEB1/YAP crosstalk directly links two cancer promoting pathways and provides insight into how ZEB1 switches to a transcriptional activator in order to drive tumour progression beyond EMT.

P1295

Board Number: B302

Constricted migration suppresses cell cycle progression.
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As a cancer cell invades adjacent tissue, penetrates a basement membrane barrier, or squeezes into the smallest blood capillaries, its nucleus can be highly constricted. Such nuclear stress has recently been shown to enhance DNA damage and genome variation. However, little is known about the interaction between constriction-induced damage and cell cycle/DNA replication, even though the latter is increasingly being appreciated as a major source of cancerous mutations. Here, U2OS osteosarcoma cells, U251 glioblastoma cells, and A549 lung carcinoma cells were seeded at three different densities (low, medium, high) on 2D plastic and were also migrated through rigid 3 μm and 8 μm pores. In each case, cell cycle analysis was performed using total DNA content combined with EdU incorporation, as measured by fluorescence microscopy. For all three cell types, G2 was found to be suppressed most in 2D by high density, indicating possible contact inhibition. In 3D, G2 was suppressed most among cells.
that migrated through 3 μm pores, even though this was the lowest density condition. These findings suggest that constricted migration inhibits cell cycle progression, perhaps due to checkpoint activation by constriction-induced DNA damage.

**Cancer Therapy: Chemotherapy and Drug Resistance**

**P1296**

**Board Number: B303**

Leptomycin B sensitizes ovarian and endometrial cancer cells to TRAIL and cisplatin induced apoptosis through synergistic modulation of crucial apoptosis regulators.

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The vast majority of malignant tumors found in human can be fought through the use of chemotherapeutic drugs. However, chemoresistance is a major hurdle in the treatment of cancer, especially in the case of ovarian cancer (OC) as well as in endometrial cancer (EC). Both of these cancers display high mortality rates due to the establishment of chemoresistance. It is thus of utmost importance that we develop novel approaches to overcome this problem and restore chemosensitivity in cancer cells. Nuclear transport has been shown to be crucial in cell fate dynamics. CRM1, also known as XPO1, is a nuclear export receptor that recognizes proteins bearing NES domains. Substantial CRM1 enrichment is indicative of highly invasive and resistant tumors and is generally suggestive of reduced patient survival. Our hypothesis is that CRM1-driven nuclear exclusion of Par-4, p53 and FoxO1 could be an acquired resistance mechanism in cancer cells and interference with this mechanism could be an interesting therapeutical approach.

To explore this hypothesis, we first treated various OC and EC cell types with Leptomycin B (LMB), cisplatin and TRAIL, either singly or in combination, in order to observe whether Par-4, p53 and FoxO1 would accumulate to the nucleus; immunofluorescence revealed a drastic enrichment of p53 and FoxO1 in the nuclei of cells following LMB treatments as well as combination of LMB and TRAIL. We then treated multiple OC and EC cell lines with the aforementioned drugs to determine whether the tumor suppressors nuclear enrichment induced by CRM-1 inhibition could sensitize tumor cells to apoptosis induction; to this end, we assessed caspase-8 cleavage, as well as MCL-1, XIAP, cFLIP, caspase-3 cleavage and TRAIL decoy receptors expression. We then confirmed the results pertaining to cell death by flow cytometry (Annexin V/PI). The obtained results suggested that the combination of LMB and TRAIL largely increased cell death and apoptosis when compared to their respective single use; this effect is enabled through the synergistic downregulation of apoptosis inhibitors with the concomitant upregulation of apoptotic inducers. Namely, we observed the downregulation of DcR2, XIAP, MCL1 and c-FLIP as well as the upregulation of DR4, DR5, cleaved PARP, cleaved caspase 3 and caspase 8.

Taken together, these results suggest that CRM1 controls the export of various tumor suppressors which contributes to the acquired chemoresistance seen in both OC and EC. The use of CRM1 inhibitors in combination to chemotherapeutic drug regimen could be an attractive strategy to reduce chemoresistance in the context of gynecological cancer therapy.
P1297
Board Number: B304
Imaging effect of cellular heterogeneity on anti-cancer drug responses in breast cancer.
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Cell-to-cell variations are ubiquitous in any cell population and the evolution and ramification of this variability is less well understood. In cancers, the heterogeneity of cancer cells introduces significant challenges in designing effective treatment strategies. In this work, we study the cellular heterogeneity of breast cancer cells MDA-MB-231 cells in their response to drugs. We found that single-cell progenies derived from parental MDA-MB-231 cells exhibiting distinct progeny-to-progeny morphologies and gene expression patterns. These progenies show distinct growth inhibition response to several common anticancer drugs suggesting that treating tumor with chemotherapy can induce the changes of cancer subpopulation. To directly observe the change of subpopulation in progeny mixture system in response to drug, we fluorescently labeled three functionally distinct progenies by different colors and established high throughput microscopy analysis system to measure the population composition of the progenies under a broad spectrum of treatments and dosages. Our results showed that progenies subpopulation can significantly change under different treatments and dosage such as Taxol for more than 50%. Since these progenies exhibit different in vivo function, our results suggested that different chemotherapies can change tumor behaviors as result of changing cell subpopulations compositions. Together, our study highlights that evaluating cancer subpopulations responses to anticancer treatments is critical in identification of optimal personalized cancer treatments.

P1298
Board Number: B305
Investigating the role of CD79B in primary CNS lymphoma response to ibrutinib.
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Primary central nervous system lymphoma (PCNSL) is an aggressive tumor, of which almost all are diffuse large B cell lymphoma (DLBCL) by immunophenotype. Targeted cancer gene resequencing (586 genes) in 177 PCNSL patient biopsies identified recurrent somatic mutations in several genes that mediate B cell receptor (BCR) signaling. The most highly mutated genes were myeloid differentiation primary response gene 88 (MYD88) and cluster of differentiation 79B (CD79B), with frequencies of 58% and 41%, respectively. Since Bruton Tyrosine Kinase (BTK) is a central node in the BCR pathway, we
examined the activity of the BTK inhibitor in patients with relapsed or refractory primary CNS lymphoma. We observed a response rate of 77% (10/13 patients), including five complete responses. None of the complete responders, but the majority of tumors with partial ibrutinib response, harbored a CD79B mutation. RNA expression profiling and gene set enrichment analysis of human PCNSL biopsies showed an enrichment of mammalian target of rapamycin (mTOR)-related gene sets in CD79B mutant tumors. In a novel PCNSL cell line derived from a newly diagnosed PCNSL patient carrying a CD79B hotspot mutation, ibrutinib synergized with both PI3K and mTOR inhibitors to induce cell death. In summary, our results suggest a role of mutant CD79B in mitigating ibrutinib response in PCNSL, at least in part through PI3K/mTOR pathway. Drug combinations targeting both the PI3K/mTOR and BCR pathways may be more efficacious than single agent therapies for PCNSL patients harboring the CD79B hotspot mutation.

P1299
Board Number: B306
Development of a Cocktail Therapy against Human Malignant Melanoma by Combining Autophagy Inhibitors and Vemurafenib.
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Metastatic melanoma, a malignancy originating from pigment-producing melanocytes, is the most aggressive form of skin cancer. Most patients with metastasized melanoma harbor the activating mutation gene, BRAF V600E. Vemurafenib (PLX4032), a BRAF inhibitor (BRAFi), develops drug resistance in the patients. Autophagy is a self-salvaging mechanism for cells to deal with different stresses and promotes cancer cell survival and growth. However, the role autophagy contributes to resistance to anti-tumor drugs is not well studied. In this research, we determine the effects of autophagy inhibitors, as well as a combination of autophagy inhibitors and Vemurafenib, on cell viability and cell migration. Autophagy inhibitors significantly reduced cell viability and inhibited cell migration of human malignant melanoma cells and displayed an additive cytotoxicity with PLX4032 in both Vemurafenib sensitive and resistant melanoma cells. Moreover, autophagy inhibitors alone, or combined with Vemurafenib, induced apoptosis in both Vemurafenib sensitive and resistant melanoma cells. We further examined the signaling pathways that autophagy inhibitors may employ to exert cytotoxic effect and overcome BRAFi resistance. Autophagy inhibitors alone activated Erk and worked synergistically with BRAFi to inhibit autophagy by reducing the expression of Atg12. In conclusion, our results suggest that a combination of autophagy inhibitors and BRAFi would be a potential therapeutic strategy to treat melanoma and to overcome the drug resistance in the melanoma cells.

P1300
Board Number: B307
The prognostic value of RAS pathway biomarkers in late-stage breast cancer.
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Introduction: Metastatic breast cancer exhibits diverse and rapidly evolving intra- and inter-tumor heterogeneity. Patients with similar clinical presentations often display distinct tumor responses to standard of care (SOC) therapies. Genome landscape studies indicate that EGFR/HER2/RAS pathway activation is highly prevalent in malignant breast cancers. The identification of therapy-responsive and prognostic biomarkers is paramount important to stratify patients and guide therapies in clinical oncology and personalized medicine.

Methods: In this study, we analyzed matched pairs of tumor specimens collected from 182 patients who received neoadjuvant systemic therapies (NST). Statistical analyses were conducted to determine whether EGFR/HER2/RAS pathway biomarkers and clinicopathological predictors, alone and in combination, are prognostic in breast cancer.

Findings: SIAH and EGFR outperform ER, PR, HER2 and Ki67 as two logical, sensitive and prognostic biomarkers in metastatic breast cancer. We found that increased SIAH and EGFR expression correlated with advanced pathological stage and aggressive molecular subtypes. Both SIAH expression post-NST and NST-induced changes in EGFR expression in invasive mammary tumors are associated with tumor regression and increased survival, whereas ER, PR, and HER2 were not. These results suggest that SIAH and EGFR are two prognostic biomarkers in breast cancer with lymph node metastases.

Interpretations: The discovery of incorporating tumor heterogeneity-independent and growth-sensitive RAS pathway biomarkers, SIAH and EGFR, whose altered expression can be used to estimate therapeutic efficacy, detect emergence of resistant clones, forecast tumor regression, differentiate among partial responders, and predict patient survival in the neoadjuvant setting, has a clear clinical implication in personalizing breast cancer therapy.

P1301
Board Number: B308
Analysis of the Nature of Paclitaxel Resistance in APC Knockdown Breast Cancer Cells. B.J. Berkeley1,2,3,4, A.H. Arnason2,3, J.R. Prosperi1,2,3;
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Adenomatous Polyposis Coli (APC) is a multi-domain tumor suppressor protein that binds to proteins including β-catenin, axin, and microtubules (MTs). APC is lost in many epithelial cancers and up to 70% of sporadic breast cancers, with a tendency towards triple negative breast cancers (TNBCs). In a mouse breast cancer model of APC loss, MMTV-PyMT;ApcMin/+; our laboratory previously demonstrated that APC loss resulted in metaplastic-like tumors, a subtype of TNBCs that often develops resistance to chemotherapy. Using the human breast cancer cell line, MDA-MB-157, we created APC knockdown cells (APCKD) using lentiviral mediated shRNA knockdown of APC, which demonstrated resistance to the Taxane family chemotherapeutic agent, Paclitaxel. This APCKD model has an increased proportion of tumor initiating cells (TICs), a subpopulation of highly tumorigenic cells found in most cancers that are resistant to traditional chemotherapeutic agents. Given that Taxanes and APC both alter MT dynamics, we sought to understand the molecular mechanisms of APC-mediated resistance. Based on RNA-seq data, we hypothesized that genes involved in the G2/M transition or those co-identified in PTX treated APCKD cells and a TIC signature list would be responsible for PTX resistance. We used three approaches to examine the molecular architecture and cell cycle phase pattern of Paclitaxel resistant APCKD cells: Western blots, RT-qPCR and flow cytometry. Four genes were identified as being selectively regulated by PTX in the APCKD cells and overlapping with the TIC signature (PLEKHG2, GPR37, MLF1, and IGSF3). RT-
qPCR confirmed the RNA-seq data, and future studies will investigate the effect of manipulating expression of these genes. Western blot analysis of the checkpoint proteins CDK1 (Thr14, Thr161, Tyr15) and Cyclin B1 showed no significant difference in expression between the parent MDA-MB-157 and APCKD cells after PTX treatment. Finally, cell cycle analysis demonstrated that PTX treatment of APCKD cells resulting in G2/M arrest similar to the parent cells. Combined, this suggests that the PTX resistance of APCKD cells is not mediated by the G2/M checkpoint but by an alternative mechanism. We propose that APCKD cells evade apoptosis induced by cell cycle arrest in G2/M by utilizing mutant proteins, such as kinases or phosphatases, which interact with checkpoint proteins directly. As the functionality of checkpoint proteins hinges on their phosphorylation profile, an examination of this in APCKD cells and parent cells may explore this as a potential PTX resistance mechanism. By understanding the molecular nature of PTX resistance in APCKD cells, a prospective therapeutic target may be identified to work towards a targeted treatment for some TNBC types.

P1302
Board Number: B309
Mapping mechanisms of drug sensitivity and resistance: genetics and environment.
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Gemcitabine is a powerful chemotherapeutic that targets DNA replication, killing proliferating cells. Generally, it is a last-line of defense in high-fatality cancers and is one of relatively few options used to treat pancreatic cancer. The window of opportunity is short because gemcitabine treatment tends to develop resistance. However, it is largely unknown which patients are candidates for successful gemcitabine therapy, and which will not respond. This decreases the window of opportunity to treat the tumor, and exposes patients to gemcitabine’s side effects without its tumor-cell killing benefit. We use defined mutations in fission yeast (Schizosaccharomyces pombe) to profile gemcitabine sensitivity and its relationship with environmental stress. Our transgenic yeast pipeline aims to categorize loss-of-function mutations that promote sensitivity or resistance in individual cells. We report that loss of the replication checkpoint is an important predictor of gemcitabine sensitivity, but that dose-sensitivity varies even between mutants of the same class. This correlates with enhanced resistance during later drug exposures. Yet, not all mutants that survive gemcitabine are resistant, suggesting that population-wide heterogeneity is a critical consideration when defining outcome. We have found that transient environmental stress has a profound effect upon checkpoint mutant survival, defining the conditions of cell death in different drug classes. In this way, we define conditions that allow checkpoint mutants to survive gemcitabine, and the changes in cells that survive drug. Long-term, genetic profiling for chemotherapy drugs may help to suggest which cells are more likely to respond, and whether targeted synthetic lethality strategies may enhance patient- and cancer-specific treatment.
P1303

Board Number: B310

Single cell profiling of phospho-protein levels in chronic lymphocytic leukemia.

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Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy which covers 40% of all leukemia cases in the Western world. The disease shows high biological and clinical heterogeneity, and it has become a priority to understand the underlying abnormalities in order to provide tailored treatment. Here, cell signaling aberrations which may serve as biological indicators for suitable therapy were characterized. Phospho-specific flow cytometry was applied to map basal and induced phosphorylation levels of 20 phospho-epitopes on proteins relevant to B-cell signaling in B cells from 22 CLL patients and 25 normal controls. CLL cells displayed similar or lower basal phosphorylation levels than normal B cells, with the exception of STAT3 (pY705) which was elevated. Importantly, STAT3 inhibitors normalized the STAT3 (pY705) level and reduced cell viability in CLL cells. After BCR stimulation, CLL cells showed significantly impaired phosphorylation levels for several of the analyzed phospho-proteins. However, the level of Akt (pS473) was more effectively increased in IgHV unmutated CLL (UM-CLL) patient samples and was significantly higher than in M-CLL cells. Notably, the PI3Kdelta inhibitor idelalisib potently reduced Akt (pS473). Moreover, the signaling impacts of the cytostatic drugs fludarabine, doxorubicin and vincristine were also investigated, but only minor effects were observed. In conclusion, signaling aberrations could be identified by phosphoflow cytometry and were normalized by small molecule drugs. This approach can thus be applied to identify relevant drug targets as well as drug effects in the individual patient.

P1304

Board Number: B311

Pyrrolidine dithiocarbamate reverses Bcl-xL-mediated apoptotic resistance to doxorubicin by inducing paraptosis.

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Elevated Bcl-xL expression in cancer cells contributes to doxorubicin resistance, leading to failure in chemotherapy. In addition, the clinical use of high-dose doxorubicin in cancer therapy has been limited by issues with cardiotoxicity and hepatotoxicity. Here, we show that co-treatment with pyrrolidine dithiocarbamate (PDTC) attenuates doxorubicin-induced apoptosis in Chang-L liver cells and human hepatocytes, but overcomes doxorubicin resistance in Bcl-xL-overexpressing Chang-L cells and several
hepatocellular carcinoma (HCC) cell lines with high Bcl-xL expression. Additionally, combined treatment with PDTC and doxorubicin markedly retarded tumor growth in a Huh-7 HCC cell xenograft tumor model, compared to either mono-treatment. These results suggest that PDTC/doxorubicin co-treatment may provide a safe and effective therapeutic strategy against malignant hepatoma cells with Bcl-xL-mediated apoptotic defects. We also found that induction of paraptosis, a cell death mode that is accompanied by dilation of the endoplasmic reticulum and mitochondria, is involved in this anti-cancer effect of PDTC/doxorubicin. The intracellular glutathione levels were reduced in Bcl-xL-overexpressing Chang-L cells treated with PDTC/doxorubicin, and PDTC/doxorubicin-induced paraptosis was effectively blocked by pretreatment with thiol-antioxidants, but not by non-thiol antioxidants. Collectively, our results suggest that disruption of thiol homeostasis may critically contribute to PDTC/doxorubicin-induced paraptosis in Bcl-xL-overexpressing cells.

P1305
Board Number: B312
Targeting Ribosome Assembly Factors Selectively Protects p53 Positive Cells rom Chemotherapeutic Agents.
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Many chemotherapeutic agents act in a nondiscriminatory fashion, targeting both cancerous and noncancerous cells in S phase and M phase. One approach to reduce the toxic side effects in normal tissue is to exploit the differences in p53 functionality between cancerous and noncancerous cells. For example, activating p53 signaling by nongenotoxic means can transiently arrest noncancerous p53 positive cells in G1 phase and protect them from the cytotoxic effects of chemotherapeutic drugs. However, since most cancerous cells have faulty p53 signaling, they will proceed to cycle, and continue to be affected by the drug. In this study we asked if this G1-phase arrest and cytoprotection can be achieved by targeting ribosome biogenesis. Through the expression of a dominant negative mutant ribosome assembly factor Bop1, we were able to transiently inhibit rRNA maturation. Using this genetic model, we have shown that inhibition of rRNA maturation protects 3T3 cells from chemotherapeutic agents camptothecin and methotrexate. This cytoprotection is associated with a transient arrest of cells in G1 phase, and is p53 dependent. We have also shown that the depletion of ribosomal protein Rps19 via shRNA arrests cells in G1 phase and protects them from camptothecin. However, this G1 arrest and cytoprotection was not achieved in shRNA-mediated depletion of several other tested ribosomal proteins, indicating distinct cellular responses to different targets in ribosome biogenesis. Using a mixed population of isogenic p53 positive and p53 negative cell lines, we have further shown that camptothecin in combination with the inhibition of ribosome biogenesis selectively kills p53 negative cells. We propose that the inhibition of select post-transcriptional ribosome assembly steps can enhance the efficiency of existing chemotherapeutic treatments.
P1306
Board Number: B313
Endoplasmic reticulum-mitochondria contact sites as a signaling platform of multidrug cancer resistance.
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Most high-risk neuroblastoma patients succumb to lethal therapy resistant disease, selected for during the course of intensive multimodality treatment. Therapy resistance is largely attributed to insensitivity to drug-induced apoptosis, however the exact mechanisms remain unknown. Apart from integrating death signals, mitochondria (mito) interact with the endoplasmic reticulum (ER) at close contact sites known as mitochondria associated membranes (MAM) of the ER to regulate calcium and lipid transfer and apoptotic sensitivity, a process often derailed in therapy resistant cancers. ER-mito contact sites are enriched in tethering and mitochondrial remodeling proteins including MFN2, PACS2 and DRP1. Pathologic deregulation of these contact sites has been implicated in the genesis of neurodegenerative and metabolic disorders. Here, we show that disruption of bona fide ER-mito proteins in therapy sensitive neuroblastomas induces apoptotic insensitivity and a shift towards a resistant phenotype. Isolated mitochondria from matched isogenic tumor pairs obtained from the same patient at diagnosis (DX; therapy sensitive) and relapse (REL; therapy resistant) were assessed for their apoptotic response downstream of therapeutic stressors tBid and Bim by measuring cytochrome C release by ELISA (BH3 profiling). Electron microscopy (EM) image analyses of ER-mito contact sites revealed that REL tumors contain up to 70% fewer ER-mito interactions than their matched DX tumors, as confirmed by IB for organelle-specific proteins. shRNA gene silencing of MFN2 or PACS2 in DX cells revealed a resistance-like phenotype as confirmed by BH3 profiling and chemotherapeutic drug response in vitro. A 60% decrease in MFN2 protein in DX cells partially phenocopied the resistance profile of isogenic REL cells. The degree of apoptotic resistance correlated with the extent of protein knockdown for MFN2. Treatment of shMFN2 and shPACS2 DX cells with ABT-737, a BH3 mimetic, or carboplatin, increased their IC50s multiple-fold compared to control cells, paralleling their blunted mitochondrial apoptotic response. In DX cells, chemical disruption of ER-mito communication by targeting DRP-1 inhibition with mitochondrial division inhibitor 1 (Mdivi-1) lead to decreased cytochrome C release. Likewise, blockage of the opening of the mitochondrial permeability transition pore by treatment of DX cells with cyclosporine A also resulted in lower cytochrome C release and apoptotic attenuation. Our data implicate ER-mito contact sites as positive regulators of apoptosis, whose disruption is associated with apoptotic attenuation and therapy resistance. We present a potential mechanism for broad therapy resistance arising under therapeutic stress that selects for reduced communication of ER with mitochondria.

P1307
Board Number: B314
Susceptibility of Cancer Cells to Sodium Phenyl Butyrate is Associated with DJ-1 Expression and Downstream Signaling.
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Sunday-197
Cancer resistance to aggressive therapeutic regimens is one of the major setbacks in cancer treatment and recovery. It is thus critical to identify the molecular players and pathways that promote tumor resistance and recurrence. Sodium-4-phenyl butyrate (PBA) is a commonly used histone deacetylase inhibitor (HDAC). HDAC inhibitors are a new class of antineoplastic agents currently being tested in clinical trials. In this study, we sought to evaluate the anticancer effects of PBA in various cancer cell lines and determine the underlying mechanisms of action. Our results show that PBA inhibits cell migration and tumor progression both in vitro and in vivo. We also demonstrate that the expression levels of the oncogene DJ-1 increase after treatment with BPA indicating a protective pro-tumor role for DJ-1. Knock down of DJ-1 with siRNA decreased cell motility by inhibiting the tumor suppressor PTEN and activation of the PI3K pathway further confirming the neoplastic role of DJ-1. We therefore conclude that PBA is a potential therapeutic agent for controlling cancer metastasis and that DJ-1 protein expression levels can be used as an indicator to predict resistance to PBA therapy.

P1308
Board Number: B315
Highly malignant gallbladder G-415 cancer cells express a p53 point mutation and are sensitive to D-propranolol-induced EGFR internalization.

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Endocytic trafficking regulates EGFR function and can be targeted to counteract cancer malignancy. A recent approach resulting in harmful effects upon tumoral cells has been to induce EGFR endocytosis and inaccessibility to ligand stimulation through the inhibition of phosphatidic acid (PA) hydrolysis with D-propranolol (Shaughnessy-FEBS J-2014). This pathway involves PA-mediated activation of type 4 phosphodiesterases (PDE4), which reduces cAMP levels and PKA activity. As in other conditions that decrease basal PKA activity, the EGFR becomes accumulated in recycling endosomes due to both increased internalization and reduced recycling. Indeed, this pathway has to be tested in cancerous cells bearing different malignant contexts. Here we first studied gallbladder G-415 cancer cells that are highly aggressive and resistant to the chemotherapy drug gemcitabine. In G-415 cells, D-propranolol induced EGFR endocytosis and sorting to recycling endosomes through the PA/PDE4/PKA pathway, accompanied by inhibition of cell proliferation, migration and invasion. Interestingly, sequence analysis revealed a p53 point mutation (R282W), one of the five most abundant p53 missense mutations in human cancers, not previously described in G-415 cells. Other p53 mutants (R175H or R273H) have been shown to enhance recycling of β1-integrins and EGFR providing high migration/invasion capabilities. Therefore, we compared the effects of D-propranolol in H1299-p53 null cells with transfected H1299 cells expressing either p53 R175H or p53 R273H. In all these cells, D-propranolol induced EGFR accumulation in recycling endosomes, indicating inhibition of EGFR recycling and thus a counteraction of the p53 mutant influence. Our results suggest that D-Propranolol could be effective against gallbladder cancer cells and other cancerous cells carrying gain-of-function p53 point mutations. (Financed by CONICYT Doctoral Scholarship to JBC, CONICYT Basal Grant PFB12/2007 and Fondecyt #1141127 to AG).
Mitotic slippage is the major outcome for cells treated with microtubule inhibitors.

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The response of cancer (HT1080, A549, U118) and immortalized normal (HaCaT, 3T3) cells to microtubule inhibitors (Nocodazole, Taxol, Vinorelbine) was analysed by time-lapse microscopy. The cells had entered mitosis were tracked for 72 hours under the drug treatment in the dose range from 0.03 nM to 3000 nM, or after preliminary incubation in the drug-containing media for 72h. All types of cells display same dose-dependent fates for each drug used. With increase in dose mitotic cells underwent the following fates: normal division, delayed bi-polar division, abnormal (asymmetric and/or incomplete) division, mitotic slippage, and death of mitotic cell without or after exit into interphase. The exact doses where each fate was dominating depended on cell line and drug. The duration of mitotic arrest of the cells finely divided, regardless the type of division and behavior of daughter cells did not exceed 10 hours.

Above a threshold concentration after which cells were arrested for a long time in mitotic stage several outcomes were observed: abnormal division, mitotic slippage with survival, death after mitotic slippage, or death in mitosis. The duration of mitotic arrest for the last three outcomes was similar (10-30 hours) irrespective to drug type and cell line.

Mitotic slippage, the most commonly observed behavior in the wide range of concentrations (Nocodazole: 100-1000 nM; Taxol: 10-1000 nM; Vinorelbine: 3-1000 nM) of the drugs, occurred always after prolonged mitotic arrest and resulted in formation of multinucleated cells. Similarly multinucleated cells were also formed after abnormal division with incomplete cytokinesis. The cells undergoing mitotic slippage after Nocodazole treatment acquired on average 2-5 medium-to-large sized nuclei; Taxol treatment resulted in the formation of 5-16 micronuclei; and Vinorelbine treatment gave 2-7 small-to-medium nuclei. Occasionally, at high concentrations of all drugs, cells after mitotic slippage formed one large nucleus. The majority of cells after mitotic slippage at moderate concentrations remained alive for >72 h. At highest concentrations tested (1-3 \textmu M) prolonged survival, death after mitotic slippage, and death in mitosis could be observed at reasonable frequency.

To conclude, cultured cells after irreversible mitotic arrest with different microtubule inhibitors mainly escape into interphase, form multinucleated cells and survive for prolonged time. Mitotic cell fate only partially depends on the drug type and drug concentration.

This work was supported by Grant # 0472/GF4 from MES (Republic of Kazakhstan).
P1310
Board Number: B317
Label free proteomics profiling of MCF7 and K562 cancer cells treated with mitomycin C and dicarbamoyl mitomycin C identifies main cellular networks leading to inhibition of tumor cell proliferation.
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Mitomycin C (MC), a frequently used anticancer drug, induces DNA damage via DNA alkylation and inter-strand mediated cross-links (ICLs). Decarbamoyl mitomycin C (DMC), a mitomycin derivative which lacks the carbamate at C10, generates similar ICLs lesions thought to be the main mechanism responsible for MC cytotoxicity, and DMC. A higher toxicity of DMC for human cancer cells with or without a functioning p53 has been investigated by independent research groups. This finding was the basis for the hypothesis that the opposite stereochemistry of the ICLs generated by MC (trans) and DMC (cis) was responsible for an enhanced DMC mediated p53-independent cell-death (Cheng, SY et al., Int J Oncol. 2016 Nov; 49(5)). To better understand the mechanisms underlying DMC vs MC toxicity we employed global and label free quantitative proteomics assays for profiling the changes in the proteomes associated with the cellular signaling networks modulated by the two drugs in MCF7 (p53-proficient), and K562 (p53-deficient) in comparison with control, untreated cells. The proteomics platform employed the nanoLC-ESI MS/MS sequencing of tryptic/Glu-C/Lys-C generated peptides from total cell lysates, as well as a Q-Exactive quadrupole orbitrap mass spectrometer coupled with the label free quantification (LFQ) method for data analysis. The global proteomics analysis retrieved 1380-1400 proteins (FDR <1.0% for proteins and <0.8% for peptides) for each treated and untreated sample. The biochemical and cellular pathways were quantitatively investigated using the ingenuity pathway analysis (IPA; Ingenuity Systems), and the protein ratios extracted from LFQ analyses. The bioinformatics analysis predicted that MC and DMC can significantly increase cell death of tumor MCF7 and K562 cells. This was projected to be most likely accomplished by coordinated inhibition of cell proliferation; activation of apoptosis; and downregulation of proteins involved in the DNA repair machinery. Remarkably, biomarkers of the DNA damage repair machinery showed at least a two-fold down-regulation (p<0.05) in the MC and DMC treated samples compared with untreated control: e.g., proteins from the double strand (ds) break repair nuclease family (MREII); MUTS (MSH2) mismatch repair system; apurinic/apyrimidinic endodeoxynuclease (APEX1); PARP1, PCNA and X-Ray repair cross complementing 1 (XXCR1) family (BER pathways, and ds DNA breaks repair pathways). The label free proteomics approach utilized in this research confirmed that integrative cellular and “omics” assays are valuable tools for providing a deeper understanding of the structure-activity relationship of MC and DMC in p53-proficient and p53-deficient cancer cells.
P1311
Board Number: B318
Acute changes in leukocyte populations following focal irradiation of the intestine.
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Radiotherapy is indicated for the treatment of half of all cancer patients. However, incidental irradiation of the bowel results in dose-limiting side effects in the majority of patients receiving abdominal or pelvic radiation treatment. The major late stage complication in the irradiated bowel is connective tissue fibrosis. In the intestine, this process is likely driven by the immune response in the acute phase following focal irradiation. We recently developed a mouse model which involves surgically implanting a radiopaque marker onto the surface of the intestine in order to replicate highly focal clinical radiotherapy. Mice were then imaged with cone beam computed tomography to locate the marker, and irradiated with 18 Gy of 5x5 mm collimated x-rays onto the marked intestine using the Small Animal Radiation Research Platform (SARRP). Irradiated mice exhibited 100% survival with minimal weight loss after irradiation when compared to mock-irradiated controls. Irradiation significantly impaired crypt regeneration as assessed by a 5-ethylcytosine-2'-deoxyuridine (EdU) cell proliferation assay one day post-irradiation. Furthermore, a TdT dUTP nick end labeling (TUNEL) assay indicated a localized increase in crypt apoptosis in the irradiated area, suggesting the marked intestine was successfully irradiated. Beginning 3.5 days after irradiation, there was significant ablation of intestinal structure with very few proliferating cells in the irradiated area. The intestinal barrier was maintained in the acute phase by markedly hyperplastic crypts on the edges of the irradiated area. Furthermore, a severe innate immune response was observed in the irradiated intestine 3.5, 7, and 14 days post-irradiation as evidenced by increased neutrophil and macrophage infiltration. This inflammatory process induced a systemic leukocytosis which was driven by an increase in neutrophils in the peripheral blood. Two months post-irradiation, fibrosis was observed in the irradiated area by Masson’s trichrome stain. Our results suggest that a major inflammatory reaction in the acute phase precedes fibrosis.

P1312
Board Number: B319
Repurposing Verteporfin for chemotherapeutic treatment of endometrial cancer.
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Endometrial cancer (EMCA) is the most prevalent gynecologic cancer in women in the United States. The majority of women diagnosed with EMCA are surgically cured with a hysterectomy; however, a considerable subset of patients shows persistent or recurrent tumors that are refractory to current chemotherapies. For these women, who are diagnosed with advanced stage disease, unfavorable histologic subtypes, and disease recurrence, survival is poor as there are no adjuvant therapies proven to be effective. Chemotherapy has a critical role for these patients with poor prognosis as well as for patients with increased risk of recurrence or persistent disease after surgery. Platinum-based chemotherapy is the standard first-line chemotherapy with carboplatin plus paclitaxel being the most commonly used regimen. However, response rates to first-line chemotherapy are low, with even lower responses for recurrent diseases. Hence, it is of utmost importance that novel chemotherapeutic agents
are identified to increase or restore chemo-sensitization to platinum-based chemotherapy. Verteporfin (VP, Visudyne™) is a benzoporphyrin derivative used in the treatment of adult macular degeneration. We investigated the therapeutic efficacy of Verteporfin (VP) alone or in combination with Cisplatin or Paclitaxel on Type 1 (HEC-1-B) and Type 2 (ARK-1) EMCA cell lines. Our results show that migration and invasion capabilities of EMCA cells were inhibited by VP treatment. In patient-derived organoids, VP is able to induce caspase-3 mediated apoptosis. Cytotoxicity assays were performed with VP, Cisplatin, Paclitaxel alone or in combination. VP and Cisplatin or VP and Paclitaxel exhibited synergetic effects when inducing cytotoxicity in EMCA cells. In order to determine the efficacy of VP in changing cellular transcriptome, we performed RNASeq of EMCA cells after treatment with VP. Analysis of RNA-seq data with MultiQC and DESeq shows that in both cell lines, 547 common genes were downregulated, whereas 452 genes were upregulated. To test the efficacy of VP under in vivo conditions, we developed an orthotopic mouse model of EMCA using HEC-1-B-GFP cells and administered VP (100 mg/kg body weight of mouse) by IP injection. We observed both responders as well as non-responders to VP treatment. Taken together, our results suggest that VP is a promising chemotherapeutic agent for the treatment of endometrial cancer.

P1313
Board Number: B320
Genomic correlates of imaging response in men receiving intense neoadjuvant androgen deprivation therapy.
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We are performing a correlative genomic and molecular pathology study to an ongoing clinical trial (NCT02430480), in which patients with locally advanced prostate cancer receive 6 months of GnRH agonist plus enzalutamide as intense neoadjuvant androgen deprivation therapy (ADT). Each patient receives multiparametric magnetic resonance imaging (mpMRI) at baseline and prior to radical prostatectomy (RP). The underlying biology and associated molecular features of tumor from patients who respond or fail to respond remain unknown. We are drawing associations between pre-treatment mpMRI, somatic alterations in pre-treatment mpMRI-targeted biopsies, ROIs in post-treatment mpMRI, tumor histology, and the molecular features of residual tumor in RP specimens. Imaging and anatomical landmarks on pre- and post-treatment mpMRI were used to match targeted biopsies to RPs. A series of IHC stains, including AR, GR, PTEN, SYP, ERG, and PIN-4, were performed to guide laser capture microdissection (LCM) and characterization of residual disease. RNA and DNA were simultaneously extracted from LCM biopsy and RP tissues. Paired-end RNA-seq and gene expression analysis, and whole exome sequencing with somatic mutation calling and copy number analysis, were subsequently performed. For the first patient analyzed, we have discovered two adjacent tumor clones, distinguished by distinct somatic copy number alterations and mutations in the diagnostic biopsies. Alignment of these biopsies to pre- and post-treatment mpMRI revealed that one clone corresponded to the imaging responder, while the other aligned with the imaging nonresponder. Pathologic examination of matched RP sections revealed residual tumor for both ROIs, and bioinformatic analysis indicated that both clones persisted after treatment with minimal genomic divergence from the biopsy, suggesting intrinsic resistance. All foci were PTEN intact, AR-V7 negative, and < 1% positive for Ki-67. Common to all tumor foci (pre- and post-treatment) were single-copy losses of chromosome 16q overlapping with ZFHX3, although the breakpoints of the 16q loss were different for both clones. The mpMRI-nonresponding
clone also harbored single copy losses to chromosomes 6q, 8p, and 12p, while the mpMRI-responding clone showed losses to 13q (encompassing BRCA2 and RB1), 15q and 18q, and was positive for microsatellite instability. The major genomic events were shared between biopsy and RP tissue from each clone. These data demonstrate feasibility in comparing mpMRI response, pathologic treatment response, and the underlying genomic features that drive tumor development versus treatment resistance, to ultimately evaluate the clonal evolution of resistant tumor and associate molecular features of primary disease with resistance or response.

P1314

**Board Number: B321**

Models of Ovarian Cancer Cell Resistance to Doxorubicin and Cisplatin.

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Resistence to chemotherapy is a major cause of treatment failure in ovarian cancer. In contrast to the reversal of pre-existing resistance, the prevention of its development requires the use of models in which resistance occurs rapidly and predictably. We have utilized our previous model of cisplatin resistant ovarian cancer to identify potential resistance preventatives. The aim of the current study is to design a model of resistance to the chemotherapy drug, Doxorubicin (Dox). Replicate culture wells of A2780 ovarian cancer cells were pretreated in sub-toxic Dox doses ranging from 2 uM to 8 uM for 24-48 hours. Replicate wells were exposed to PBS to serve as controls. At the end of this pretreatment period, the medium was replaced with either control medium or a higher, acute dose of Dox (10 uM). To test for cross-resistance to cisplatin (CP), additional culture wells were pre-treated with subtoxic doses of CP or Dox prior to treatment with a higher, toxic dose of either drug. To track the response of cells over time, the cells were counted daily, and the means and standard deviations were calculated and compared to the mean starting cell counts. Drug resistance was identified as an increase in cell number following treatment with the high dose. Of the pretreatment doses and intervals tested, two different models resulted in Dox resistance within five days. This was determined by cell counts recorded after 24 hour exposure to high dose drug: there was an increase in pre-treated cell count of 8-22%, as compared to the decrease in control cell cultures ranging from 11-31%. Cross-resistance to both Dox and CP was also observed. Studies are underway to characterize mechanisms involved in Dox resistance and cross-resistance, and to use these models to test potential preventatives.

Cancer Therapy: Natural Products

P1315

**Board Number: B322**

Epigallocatechin-3-gallate has antitumor effects due to induction of differentiation and apoptosis in a model of acute promyelocytic leukemia mice.

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Clinical studies show that there is no consensus regarding therapy for older patients with acute leukemia, who are susceptible to aggressive chemotherapy and ineligible to bone marrow (BM) transplantation. Thus, the search for effective compounds with less adverse effects is required.
Catechins are a major compound that has been standing out over the last years and is distributed in many teas. Epigallocatechin-3-gallate (EGCG), the major polyphenol present in green tea, has been shown to inhibit tumor formation and growth in different animal models for human cancer. In this context, the aim of this study was to investigate the effects of EGCG using acute promyelocytic leukemia (APL) as a model. For the APL model, NOD/SCID received 2 Gy irradiation followed by transplantation with intravenously injection in caudal vein with leukemia cells obtained from hCG-PML-RARα transgenic mice. After 12 days, the establishment of the disease was confirmed by leukocytosis (>30x10^3/µL), anemia (<10g/dL), thrombocytopenia (<500x10^3/µL) and the presence of 1% of blast at the peripheral blood (PB). The mice were randomly selected to receive EGCG (25mg/Kg/day) or vehicle (saline), intraperitoneally. The mice were then sacrificed after 5 days treatment and PB, BM and spleens were collected for hematological and flow cytometry analyses. Hematological analysis revealed that the treatment with EGCG reduced leukocyte number (p=0.0153; n=10), and increased platelets number (p=0.0015; n=10) and hemoglobin level (p=0.019; n=10). A reduced number of undifferentiated cells (blast cells) (p=0.0021; n=9) and an increase of neutrophils (p=0.0194; n=9) were also detected in PB. EGCG treatment significantly increase the APL mice survival (medium survival 13 vs 15 days, p=0.0017). Notably, EGCG also reduced the expression of CD117+ in the BM cells while increasing Gr-1+/Mac-1+ expression possibly related to a myeloid differentiation (p=0.0161; n=4). Apoptosis however, was not detected in the BM cells, despite the fact that EGCG treatment induces apoptosis of total spleen cells (p=0.0197; n=10) measured by Annexin/PI. This apoptotic effect was confirmed by activation of caspase-3 and -9. In addition, a significant increase in reactive oxygen species (ROS) production was detected in CD117+ (p=0.0255; n=10) and Gr-1+ (p=0.0062; n=10) BM cell populations. ROS induces apoptosis through the loss of mitochondrial transmembranopotentials and activation of caspase-3 and -9. Moreover, excess of ROS might also induce differentiation of myeloid leukemic cells to mature cells. Taken together, our results demonstrate that EGCG has anti-leukemic effects on APL model by inducing apoptosis and cell differentiation, and suggest that this could be an interesting compound for leukemia treatment.

P1316
Board Number: B323
Ganoderma lucidum derivative fraction effects in triple-negative breast cancer.
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Breast cancer (BC) is known as the leading cause of cancer among women in the United States and Puerto Rico. Moreover, triple-negative breast cancer (TNBC) is characterized by the absence of estrogen and progesterone receptors as well as lacking the presence of the epidermal growth factor receptor 2 (HER2). Since there are no targeted therapies available, currently, there are very few options of treatments for TNBC. Thus, the development of novel strategies is necessary to combat this deadly disease. The objective of this study is to identify the effects of fractions derived from Ganoderma lucidum extract (GLE) and test their efficacy in TNBC cells. Pulverized GLE (10.0g) was dissolved in 150mL of isopropanol and refluxed for 24h. The resultant extract was filtered, and the solvent evaporated. The crude oil was fractionated via silica gel column chromatography. Based on MS and NMR analysis, 8 fractions were prepared for biological evaluation. Further purification via preparatory HPLC and structure elucidation of active fractions is currently under investigation. Additional GLE (10.0g) was extracted with isopropanol for 24h, and the crude compound was purified by silica gel column chromatography (hexane/ethyl acetate, 1:9) to generate 14 fractions. Further assessment of the
fractions resulted in similar chemical composition so they were combined into 8 unique fractions based on MS, where F3 proved to have the greatest efficacy. TNBC cells (MDA-MB-231 & SUM-149) were then treated with increasing concentrations of F3 ranging from 0 to 0.3 mM for 72h. Our results show that F3 significantly decreased MDA-MB-231 and SUM-149 cell viability. The IC50 for F3 in MDA-MB-231 cells was 12.7 μM compared to 0.3 mg/mL of GLE. The IC50 for F3 in SUM-149 cells was 14.7 μM compared to 0.2 mg/mL of GLE. Thus, our data suggests that the GLE F3 fraction is a potential compound against TNBC. This work was supported by NIH NIGMS #GM111171 (MMM), SGRP 2017-00143 (MMM and GOS), NIMHD #MD007583 (MMM), GM103475 (UPR MMM) and Title-V-PPOHA #P031M105050 and Title-V-Cooperative #P031S130068 from the U.S. Dept. of Education.

P1317
Board Number: B324
The cytotoxic effects of purified *Ganoderma lucidum* compounds on triple negative breast cancer.

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Successful breast cancer therapies involve a systemic approach combining chemotherapy, surgery, and other options including targeted therapies. Triple negative breast cancer (TNBC) patients are diagnosed with a form of cancer that lack hormone (ER & PR) and growth factor (HER2) receptors targeted during therapy. TNBC's aggressive nature and the lack of approved targeted therapies often leave patients distressed about the prognosis of their disease. The objective of this study is to examine the effects of purified compounds extracted from *Ganoderma lucidum* (GLE) in TNBC cells and compare its effects to those observed with similar steroidal clinical therapeutic agents. Pulverized GLE (10.0g) was dissolved in 150mL isopropanol and refluxed for 48h. The resultant extract was filtered, and the solvent evaporated. The crude oil was fractionated via silica gel column chromatography to provide 8 fractions (10%EtOAc-5%MeOH). Based on TLC, MS and NMR analysis, 8 fractions were prepared for biological evaluation and two fractions were further purified based on biological activity. These fractions were purification via preparatory HPLC and structure elucidation provided GA and an isoform LZ, which are currently being elucidated. MDA-MB-231 & SUM149 TNBC cells, and non-cancerous MCF-10A cells were seeded and treated for 72h with increasing concentrations ranging from 0 to 64μM of GA, LZ, or eight clinical candidates (SJ-1010, 1030, 5692, 7272, 7380, 7493, 7420, or 8468) with similar chemical core scaffold were used to evaluate potential biological target(s). Cell viability was determined by fluorescence detection. Finally, we assessed apoptotic activity using Annexin V staining in SUM149 cells treated with GA. Results demonstrate that GA causes a dose-dependent decrease in TNBC viability, with an IC50 of 23.0 and 40.3μM, in 231 and SUM149 cells respectively. LZ did not appear to confer any significant cytotoxic effect on TNBC cells, suggesting that its mode of action is different to that of GA. Treatments with two clinical candidates showed some degree of cytotoxicity, suggesting that stereochemical alterations of GA affect its effectiveness. None of the compounds were cytotoxic to non-cancerous cells at the concentrations tested. GA has potential therapeutic properties against TNBC. Our findings indicate that either synergetic mechanisms are present when cells are treated with the pure extract or we have yet to isolate the compound responsible for the observed activity of the pure extract. This work was supported by NIH GM111171 (MMM), MD007583 (MMM), SGRP 2017-00143 (MMM), MD008149

Sunday-205
Cancer cell metastasis, the process by which cancer cells migrate and form new tumors elsewhere in the body, accounts for approximately 90% of cancer-related deaths. Consequently, finding new ways to hinder cancer cell metastasis is crucial for the effective treatment of late-stage cancer patients. For the last thirty years, the majority of anticancer drugs have originated in some form from natural compounds. These compounds continue to be the main source of disease treatment. Naturally occurring flavonoids have been found to have anticancer effects on various cancer cell lines. However, the effects of flavonoids have not been extensively studied on breast cancer cells. In this project, we tested the effects of acacetin and pinostrobin on MDA-MB-231 invasive breast cancer cells. Using scratch and transwell assays, both acacetin and pinostrobin inhibited MDA-MB-231 cell migration in a dose-dependent manner. However, neither of the drugs had any effect on cell proliferation when tested over a wide range of drug concentrations. In addition, both acacetin and pinostrobin produced approximately a 58% and 40% inhibition on cell adhesion, respectively. Interestingly, while both acacetin and pinostrobin inhibited cell adhesion, there was no measureable difference in focal adhesion formation, compared to the control, when treated with various concentrations of either acacetin or pinostrobin. These results suggest that both acacetin and pinostrobin mediate their effects on motility by preventing breast epithelial cell adhesion. However, additional studies are necessary to better understand how acacetin and pinostrobin alter cell adhesion dynamics to influence MDA-MB-231 cell migration.

Triale negative breast cancer (TNBC) represents approximately 20% of all breast cancer cases worldwide. Prognosis for this subtype of cancer is especially dire due to the absence of Estrogen (ER), Progesterone and Human Epidermal Growth Factor receptors, usually targeted by neoadjuvant therapies like Tamoxifen. *Myrothamnus flabellifolius* (MF), a well-studied South African medicinal herb, has shown significant potency against TNBC with minimal effect on normal cells. In this study, we use miRNA profiling, Ingenuity Pathway Analysis (Qiagen), MTT, immunoblots and immunofluorescence to characterize the targeted cytotoxic mechanism of MF in TNBC. We found deregulation in the expression of several oncogenic and anti-cancer miRNAs in TNBC post MF treatment. Using Ingenuity Pathway Analysis to analyze the miRNA expression profiles of the treated cells, we determined Estrogen Signaling as a pathway significantly affected by MF in TNBC. Immunoblots confirmed increase of the ERa/ERß ratio
in TNBC, a well-studied marker for Tamoxifen-therapy sensitivity in TNBC. MTT cell viability assay and Annexin V staining confirmed increased sensitivity to Tamoxifen post treatment with low doses of MF, with up to 70% cell death at half the IC\textsubscript{50} of Tamoxifen in TNBC. This study establishes a potential non-toxic therapeutic to supplement Estrogen-receptor targeting therapies in TNBC by increasing the ER\textsubscript{a}/ER\textsubscript{ß} ratio. The data also evaluates the ER\textsubscript{a}/ER\textsubscript{ß} ratio as a potential target for combination therapies in TNBC.

P1320
Board Number: B327

\textit{Ganoderma lucidum} in combination with carboplatin inhibits the DNA damage response in triple negative breast cancer cells.

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Management of triple negative breast cancer (TNBC) is challenging because of a lack of targeted therapy, aggressive behavior and poor prognosis. Interest in using platinum agents, such as carboplatin as a line of treatment in these patients emerged from data suggesting frequent DNA repair defects. However, carboplatin resistance may occur as a result of multiple factors including increased activation of DNA repair mechanisms. Studies show that \textit{Ganoderma lucidum} extract (GLE) sensitizes cancer cells to different anti-cancer therapies. Thus, we aim to investigate the effects of GLE in combination with carboplatin and to elucidate its effects in DNA Damage Response (DDR) signaling. Our hypothesis is that GLE sensitizes breast cancer cells to carboplatin therapy decreasing DDR. SUM-149 and MDA-MB-231 triple negative breast cancer cell lines were treated with several concentrations of carboplatin, GLE, or both treatments simultaneously for 72h. Our results demonstrate that when carboplatin and GLE are used in combination, their IC\textsubscript{50}s decreased in both breast cancer cell lines when compared to each treatment alone. Our data revealed that GLE and/or the combination decreases the expression of 53BP1, p-CHK2, p-CHK1, p-ATR, p-ATM and p-BRCA1 in SUM-149 and/or in MDA-MB-231 cells. In addition, the phosphorylation of the pro-apoptotic protein p53 was upregulated by the combination. Immunofluorescence studies on DNA repair proteins 53BP1 and phosphorylated \textit{\gamma}H2AX performed to detect DNA double strand breaks suggest that GLE and their combination impair DDR. Taken together, our results provide evidence of GLE’s potential in chemosensitizing TNBC cells to carboplatin therapy affecting the DDR. These results highlight GLE’s anti-breast cancer therapeutic potential. This work was supported by NIGMS SC3GM111171 (MMM), NIMHD G12MD007583 (MMM), P20GM103475 (UPR-pilot MMM), NIMHD G12MD008149 (MMM), NIMHD G12MD007587 (MMM), NIGMS R25GM110513 (TRF), NIMHD G12MD007579, and Title-V-PPOHA P031M105050 and Title-V-Cooperative P031S130068 from the U.S. Dept of Education, and PRSTR T SGRP 2017-00143 (MMM). The content is solely the responsibility of the authors and does not necessarily represent the official views of the supporting agencies.
P1321
Board Number: B328
THE EFFECT OF RESVERATROL ON CELL VIABILITY IN THE BURKITT'S LYMPHOMA CELL LINE RAMOS.
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Resveratrol is a polyphenolic natural compound produced by a variety of crops. Currently, resveratrol is considerer a multi-target anti-cancer agent with pleitropic activity, including the ability to prevent the proliferation of malignant cells by inhibiting angiogenesis and curtailing invasive and metastatic factors in many cancer models. However, the molecular mechanisms mediating resveratrol specific effects on lymphoma cells remain unknown. To begin tackling this question, we treated the Burkitt’s lymphoma cell line Ramos with resveratrol and assessed cell survival and gene expression. Ours results suggest that resveratrol shows a significant anti-proliferative and pro-apoptotic activity on Ramos cells, inducing the DNA damage response and modulating the expression of several genes that regulate the apoptotic process and their proliferative activity. This work was supported by grants FONDECYT REGULAR 1141067, and DID UACH S-2016-48.

P1322
Board Number: B329
Purification and characterization of natural chemopreventative compounds for the treatment of prostate adenocarcinoma.
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Metastatic prostate adenocarcinoma is the leading cause of morbidity and the second-leading cause of mortality among American men. The development of prostate cancer is propagated by a plethora of genetic and proteomic abnormalities. Yet, the specific biology underpinning the initiation and promotion of tumor development remains to be elucidated. The protein arginine methyltransferase (PRMTs) family of proteins regulates epigenetic and post-translational modifications within protein signaling pathways. Under abnormal conditions, these proteins can function to constitutively drive the growth and/or proliferation of dysregulated cells. It has been reported that protein arginine methyltransferase 5 (PRMT5) is over-expressed and/or upregulated in numerous cancer types, including breast, colorectal, leukemia, lung and melanoma cancers. Previously, we have demonstrated that PRMT5 is upregulated in prostate cancer and correlates with disease progression. More specifically, we have demonstrated that PRMT5 localized to the cytoplasm, functions to drive both growth and proliferation of prostatic tumors. A growing body of literature reports antitumor activity from the Dendrobium genus of orchid plants. We have extracted, purified and begun to characterize natural PRMT5 inhibitory compounds from the orchid plant, Dendrobium denneanum. Additional preliminary studies using crude extracts on LNCaP and PC3 prostate cancer cell models have revealed halted growth through G2 cell cycle arrest. A compound purified from Dendrobium elicited a cell cycle G1-growth arrest, in addition to inhibition of proliferation of both cell models at a low-micromolar concentration. We hypothesize that purified phytochemical compounds obtained from the orchid plant may exhibit an inhibitory effect of PRMT5 activity on the progression of metastatic prostate cancer. In order to accomplish this objective, we will: (i) elucidate the molecular structure of the purified compound, (ii) identify the selectivity to other methyltransferases
and determine the global effects of inhibition on cell cycle regulatory gene targets and (iii) test the purified compound for prostate cancer prevention in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Targeting of PRMT5 for inhibition potentiates novel therapeutic opportunities for better understanding the aberrant molecular regulation of prostate cancer growth and proliferation as well as possible therapeutics for mitigation of the disease.

P1323
Board Number: B330
Rosehip (Rosa canina) Extracts Decrease Human Breast Cancer Cell Migration and Invasion.
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Triple Negative Breast Cancer (TNBC) is an aggressive form of breast cancer, characterized by the characteristic lack of receptors such as human epidermal growth factor receptor-2 (HER-2), the estrogen receptor (ER), and the progesterone receptor (PR) which is normally targeted in adjuvant therapy in effective therapies. Major concern for side effects of commercial drugs and the emergence of drug-resistant cancer cells, has created interest in the use of naturally occurring substances that possess chemo-preventive and chemotherapeutic properties in cancer treatment. Rosehip (Rosa canina) extracts which have traditionally been used as dietary supplements to relieve symptoms associated with diarrhea, gastritis, and rheumatoid arthritis, have also been shown to prevent cancer cell growth and migration in various cancers. Specifically, we have previously demonstrated rosehip extracts can prevent brain tumor cell growth, induce a G0/G1 cell cycle arrest and stall cell migration. This study investigated the efficacy of rosehip extracts in preventing cell migration and invasion in triple negative (HCC1806 and HCC70) breast cancer cells. Treatment with rosehip extracts (1 mg/ml, 250 µg/ml, 25 µg/ml, and 25 ng/ml) caused a decrease in cell migration in each of the breast cancer cell lines tested. Additionally, pretreatment of these cell lines with rosehip extracts decreased the level of AKT, MAPK, and NF-κB phosphorylation, suggesting that these extracts prevent TNBC cell migration by blocking these signaling mechanisms. Anti-invasion effects were also observed after exposure to rosehip extracts (1 mg/mL and 25 µg/ml). The observed decrease in cell invasion correlated with decreases in MMP expression. These data suggest rosehip extracts are capable of decreasing cell invasion by down-regulating MMP expression. Rosehip extracts also had a synergistic effect with Doxorubicin (20µM), a chemotherapeutic agent currently used to treat breast cancer, to deplete triple negative breast cancer cell migration. Taking together these data demonstrate rosehip extracts are capable of decreasing cell migration and invasion in triple negative breast cancer cells by attenuating MAPK, AKT, and NF-κB signaling mechanisms. Additionally, these data demonstrate the enhanced effect of Doxorubicin with rosehip in preventing breast cancer cell migration and invasion. Additionally, these results demonstrate rosehip extracts may serve as an alternative or compliment to current chemotherapeutic treatments for triple negative breast cancer.
P1324

Board Number: B331

Involvement of HIF-1α signaling in leukemia model by polyphenols of green tea.

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Green tea (GT) is chemically characterized by the presence of large amounts of polyphenols, which are known as the most effective substances with the ability to inhibit tumorigenesis. Acute myeloid leukemia is an aggressive hematologic malignancy and there is no sufficient evidence that supports a protective role of GT intake on its development. Our previous findings showed that GT administration to leukemia mice reduced leukocytosis and blasts in the peripheral blood (PB), bone marrow (BM) and spleen (SP), and increased mature cells in the BM. We also observed that GT induced apoptosis; reduced the number of CD34+ and CD117+ neoplastic clones; and increased ROS production on Gr1+ cells but reduced on CD34+ and CD117+ cells from the BM. Studies have shown that reactive oxygen species (ROS) increase the expression of CXCR4 in cancer cells through nuclear translocation of HIF-1. We then studied CXCR4 expression and localization of HIF-1α on CD34+ and CD117+ cells of acute promyelocytic leukemia mice. After 2 Gy irradiation, leukemia cells from hCG-PML-RARα transgenic mice were injected in the tail vein of NOD SCID mice. Hematologic counts were monitored and the following criteria was used for the diagnosis of leukemia: presence of at least 1% of blast in PB associated with leukocytosis above 30 000 cells/L, hemoglobin levels below 10 g/dL, and thrombocytopenia below 500×103 cells/L. Twelve days after transplantation, mice were then submitted to daily oral treatment (gavage) with 250 mg/kg GT or vehicle only (water) for 5 consecutive days and were sacrificed. Our results show that GT decreased the expression of CXCR4 in the surface of CD34+ (197±8 vs untreated 142±20) and CD117+ cells (9028±1367 vs untreated 4196±970), detected by flow cytometry. Reduction of HIF-1α activation was also observed, demonstrated by the lower co-localization of HIF-1α with the nuclei, using ImageStream imaging flow. Literature data have shown that GT polyphenols can inhibit the activation of HIF in cancer cells. Besides, a tumor microenvironment that is rich in ROS may influence CXCR4-mediated expression and functions, encouraging tumor development. Expression of CXCR4 can induce leukemia cell trafficking and homing to the marrow niche, where SDF-1, its ligand, retains leukemia cells in close contact with stromal cells that provide growth and drug resistance signals. Therefore, it is possible that GT could affect ROS levels on neoplastic cells, which may impair HIF-1α activation, ultimately reducing CXCR4 expression and cell survival. The impairment of SDF-1/CXCR4 axis induced by GT could cause loss of adhesion of leukemia cells in the BM, leading to mobilization out of their niche and becoming these cells more susceptible to chemotherapy drugs.

P1325

Board Number: B332

The Antitumorigenic Effects of Natural Compounds, Conessine and Cardamonin, on MDA-MB-231 Breast Epithelial Cells.

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In addition to their potential of reducing tumor growth, natural compounds, such as cardamonin and conessine, may be able to stifle cancer cell migration and invasion in order to hinder tumor cell metastasize. Conessine and cardamonin are natural compounds that have been shown to have
antitumorigenic properties on some forms of cancer cells, but little has been researched on the effects of these compounds on breast epithelial cells. Conessine inhibited MDA-MB-231 cell proliferation in a dose-dependent manner, while increasing concentrations of cardamonin increased cell proliferation. However, MDA-MB-231 cell migration decreased when treated with either conessine or cardamonin using both scratch assays and transwell assays. Furthermore, conessine inhibited cell adhesion at varying concentrations with an approximately 39% reduction in cell adhesion when treated with 40uM conessine. However, cardamonin had no effect on cell adhesion. In addition, there was a 20-29% reduction in focal adhesion area, as assessed using an anti-FAK397 antibody, when treated with either conessine or cardamonin. These results suggest both conessine and cardamonin inhibits MDA-MB-231 breast epithelial cell migration and adhesion. However, additional experiments are necessary to further elucidate the mechanisms by which these natural compounds mediate their effects.

P1326
Board Number: B333
Suppression of breast cancer cell proliferation by combinations of the phytochemicals fisetin, luteolin and hesperetin.
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Epidemiological studies suggest that a diet rich in plant flavonoids may prevent cancer. Fisetin, luteolin, and hesperetin, dietary flavonoids found in a variety of fruits and vegetables, have been shown to exhibit anticancer activity in vitro. In this study, we evaluated the antiproliferative effects of fisetin, luteolin, and hesperetin, individually and in combination, using MCF-7 breast cancer cells. We found that fisetin, luteolin, and hesperetin each inhibited MCF-7 cell proliferation in a dose dependent manner. Furthermore, we found that co-administration of lower doses of fisetin and luteolin or hesperetin and luteolin led to a greater inhibition of cell proliferation than either agent alone at the same dose and indicate an additive effect. These results suggest that combinatorial treatments using fisetin, luteolin, and hesperetin may be an effective chemotherapeutic strategy against breast cancer. Additionally, these findings support the hypothesis that consumption of flavonoids may play an important role in breast cancer chemoprevention.

P1327
Board Number: B334
Ganoderma lucidum extract (GLE) decreases stemness properties via STAT3 regulation in Triple Negative Breast Cancer models.
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Triple-negative breast cancer (TNBC) accounts for approximately 15-20% of all breast cancer (BC) cases worldwide. TNBC is an aggressive breast cancer subtype with poor clinical outcome, increased mortality rate, high risk of early recurrence, and the lack of effective therapies. This aggressive nature may be explained by the presence of breast cancer stem cells (BCSCs), which are involved in tumor initiation, progression, metastasis, recurrence, and therapy resistance. With rising evidence that cancer stem cells (CSCs) are present within the tumor, it is vital to understand the molecular mechanisms that regulate self-renewal, differentiation, and maintenance of stem cell properties. Interestingly, the signal transducer and activator of transcription 3 (STAT3) participates in the development and progression of
BCSCs, but its role in TNBC remains unclear. Moreover, BCSCs are defined by the CD44+/CD24-phenotype, which correlates with a worse prognosis in TNBC patients. Furthermore, the transcription factors Nanog, Sox2, and Oct4A, are essential in maintaining CSCs phenotype, where activation of STAT3 leads to enhanced Oct4 and Sox2 expression. Importantly, it has become a major challenge developing non-toxic targeted therapies to improve the outcome of TNBC patients. In this regard, our laboratory investigates the role of *Ganoderma lucidum* extract (GLE), a medicinal mushroom used in complementary medicine. We hypothesize that STAT3 regulation by GLE will decrease stem cell properties in our TNBC cell line, MDA-MB-231 cells. Our in vitro results demonstrates at 24hr GLE significantly decreases total JAK2 and STAT3 and their phosphorylation, ALDH, and Nanog expression in MDA-MB-231 cells. In addition, at 72hr GLE significantly decreases total and the phosphorylation of JAK2 and STAT3, ALDH, and the three transcription factors (Sox2, Nanog, and Oct4A). Furthermore, our flow cytometry results at 24hr and 72hr show that GLE significantly decreases the CD44+/CD24- population in MDA-MB-231 cells. Additionally, our in vivo studies demonstrate that GLE significantly decreases tumor volume of mice injected with CD44+/CD24- cells, and tumor weight of GLE treated mice was marginally significantly lower (p=0.053) compared to controls. We can conclude that STAT3 regulation by GLE decreases stemness of TNBC. This work was supported by NIGMS SC3GM111171 (MMM), NIMHD G12MD007583 (MMM), P20GM103475 (UPR-pilot MMM), NIMHD G12MD008149 (MMM), NIMHD G12MD007587 (MMM), NIGMS R25GM110513 (TRF), NIMHD G12MD007579, and Title-V-PPOHA P031M105050 and Title-V-Cooperative P0315130068 from the U.S. Dept of Education, and PRSTRT SGRP 2017-00143 (MMM). The content is solely the responsibility of the authors and does not represent the official views of the supporting agencies.

P1328

**Board Number: B335**

**Epigallocatechin gallate-induced magnetic nanoparticle uptake by glioma cells: Mechanism via nitric oxide/cGMP signaling.**

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Magnetic nanoparticles (MNP) can be potentially used as carriers in a drug delivery system with magnetic targeting; MNP interaction with tumor cells may be crucial in achieving therapeutic efficacy. Epigallocatechin gallate (EGCG), a polyphenolic component in green tea, enhances MNP uptake by glioma cells (Lu et al., Nanoscale 6:10297, 2014), but the mechanism remains unclear. Since EGCG has been demonstrated to interact with a 67 kDa laminin receptor (67LR) and activate nitric oxide/cGMP pathway to induce apoptosis, we asked whether EGCG-induced MNP uptake is mediated by nitric oxide/cGMP signaling. Uptake of MNPs coated with carboxymethyl-dextran (200 nm) by LN229 glioma cells was determined by measurement of cell-associated MNPs (MNPcell) using potassium thiocyanate colorimetric method. EGCG at micromolar range increased MNPcell in a concentration-dependent manner, and the effects of EGCG were synergistically enhanced by magnetic field. Inhibition of nitric oxide synthase using N⁵-O⁴-Nitro-L-arginine methyl ester hydrochloride (L-NAME) significantly attenuated EGCG-induced MNPcell increase in a concentration-dependent manner in the absence, but not presence of the magnet. In addition, heparin, a 67LR-binding glycosaminoglycan, significantly attenuated EGCG-induced MNPcell increase in the absence, but not presence of the magnet. Pretreatment with MLuC5, an antibody against 67LR, and NSC47924, a 67LR inhibitor, attenuated EGCG-induced MNPcell increase by up to 35% and 22%, respectively. In addition, Bay 63-2521, an activator of soluble guanylate cyclase, and
zaprinast, a phosphodiesterase inhibitor, significantly increased MNP<sub>cell</sub>. These results indicate that cGMP level in glioma cells is positively associated with levels of MNP<sub>cell</sub>. Although EGCG may also interact with epidermal growth factor receptor (EGFR) and modulate EGFR signaling, C225, an antibody against EGFR, exerted no effect on EGCG-induced MNP<sub>cell</sub> increase. In conclusion, EGCG-induced nitric oxide/cGMP signaling, probably via interaction with 67LR, may mediate the enhancing effects of EGCG on MNP uptake by glioma cells.

P1329
Board Number: B336
The place of Phytobiological Compounds in Colorectal Cancer: An in vitro study.
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Introduction: Colorectal cancer is a leading cause of cancer death worldwide with progressive appearance of resistance towards permitted chemotherapies. Novel complementary treatments using antioxidants were reported to have beneficial effects in its management. Aim: The aim of this study is to assess the effect of a combination of antioxidants (Curcumin, quercetin, cruciferex, green tea extract and resveratrol) on colorectal cancer cells and delineate the possible mechanism of action. Materials and methods: Two CRC cell lines HCT116 (P53/-), and HT29(P53+/+) were used, in addition to a human leukemic monocyte cell line THP1, cultured and treated with increased concentrations of the phytobiological (PB) mixture ranging from 5 to 150 μg/ml for different time periods. Two methods were used to determine the anti-proliferative and cytotoxic activity: The Trypan Blue Exclusion Assay and the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In addition, Annexin V-FITC Apoptosis Assay was used. Results: PB had a dose related cytotoxic effect at 24, 48 and 72h against HCT116, HT29.On the other hand, the effect was not significant on THP1.At 48H, the IC50’s were 50.73±50 μg/ml for HCT116, 46.94 ± 150 μg/ml for HT29 and 72.45±150 μg/ml for THP1. Annexin data is in progress. Conclusion: PB was found to have a promising anti-proliferative effect for both types of cancer cells. Concerning the Annexin V-FITC Apoptosis Assay to identify the underlying mechanism of action of PB against colorectal and leukemic cancer cell lines, the results are still preliminary.

P1330
Board Number: B337
Cytotoxic activity of a new lectin from Rhizoctonia solani in vitro.
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Lectins are specific carbohydrate-binding proteins, which hold great potential for cancer therapy<sup>1</sup>. Micromycetes are promising and insufficiently explored resource of lectins with different structures and activities. A new lectin preparation from mycelium of <i>Rhizoctonia solani</i> was previously isolated and characterized<sup>2</sup>. This lectin is a galactose-specific, Ca/Mg-dependent protein with dimeric structure and molecular weight of about 36 kDa.

In this study, cytotoxic activity of the lectin preparation was evaluated on different human cells, including mammary cancer (MCF-7), prostatic carcinoma (PC-3), cervical carcinoma (HeLa), embryonic kidney (HEK-293) cells as well as human skin fibroblasts (HSF). According to MTT proliferative assay, the
lectin preparation possessed noticeable toxicity towards the cancer cells with its half-maximum inhibitory concentration ($IC_{50}$) varied from 3 to 10 μg/ml, which are comparable to those for some plant lectins. Relatively low activity of the lectin was observed for HEK-293 cells and HSF, indicating preferable cytotoxic effect of the lectin towards cancer cells.

The variation in $IC_{50}$ values for cancer cells versus untransformed cells as well as between cancer cell lines can be attributed to differences in composition of the plasma membrane of mammalian cells, e.g. structure of the carbohydrate component on their surface. The specific membrane-bound carbohydrates in the cancer cells seem to assist their interaction with the lectin and promote lectin-induced cytotoxicity7.

Our results show a potential of the lectin isolated from R. solani in selective killing of cancer cells. The molecular structure and mechanism of cytotoxicity of this lectin preparation will be studied elsewhere.


*This work was performed according to the Russian Government Program of Competitive Growth of the Kazan Federal University.*

**P1331**

**Board Number: B338**

**Title:** Epigallocatechin-3-gallate regulates BRCA1 expression in triple negative breast cancer.


Authors: Fernando J. Garzon, Lisset A. Duran and Lissette Delgado-Cruzata

Breast cancer is a hostile disease that is responsible for tens of thousands of deaths of mostly women every year. Diet is considered an important factor in breast cancer prevention. Some studies suggest that green tea consumption has the potential to reduce breast cancer risk. Specifically catechins, a group of green tea polyphenols, have been shown to have anti-tumor effects. One of the catechins, epigallocatechin-3-gallate (EGCG), has been shown to suppress proliferation, to inhibit migration and invasion, as well as to induce apoptosis in ER+ and ER- breast cancer. Studies suggest epigenetic mechanisms underlie these effects in ER-positive breast cancer cell lines, by modulating DNA methylation through the inhibition of DNA methyltransferase (DNMT1). However less is known about the epigenetic effects of EGCG in ER- and triple negative breast cancer and whether its effects are mediated by changes in miRNA expression levels. The aim of this study is to investigate the effects of EGCG in triple negative MDA-MB-468 breast cancer cells. Specifically how EGCG affects BRCA1 expression levels and BRCA1 targeting microRNA. We treated MDA-MB-468 with 25μM and 50μM of EGCG for 48 hours and use Taqman assays to determine expression levels. We found a statistically significant decrease in BRCA1 gene expression with higher EGCG treatment concentrations (BRCA125μM= 29.96%+6.05% (p=0.009); BRCA150μM=16.20%+2.49% (p=0.006) when compared to untreated). We also found that while not statistically significant the levels miRNA 182, which has been identified as a miRNA that regulates BRCA1, were higher with increasing concentrations of EGCG (miR-18225μM= 108.62%+0.72% (p=0.381); miR-18250μM= 112.71%+0.08% (p=0.302) when compared to
untreated). Our results suggest that BRCA1 expression can be modulated by treatment with EGCG, and that is possible that miRNAs have a partial role in regulating the expression of this gene as a response to treatment.

**P1332**

**Board Number: B339**

THE FLAVONOID QUERCETIN MODULATES NRF2 EXPRESSION AND INDUCES APOPTOSIS IN XENOGRAFT MODELS AND LEUKEMIA CELLS LINES.

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Nuclear factor-erythroid 2 related factor (NRF2) is persistently activated in many human tumors, including acute myelogenous leukemia, in this way inhibition of nrf2 activity may be a promising target in leukemia therapy. In the present study, we evaluated the effect of Qu, a natural polyphenolic flavonoid compound, as a modulator of NRF2. This study was performed in vivo in human xenograft acute myeloid leukemia (AML) models, and in vitro using leukemia cells lines. Qu treatment induced apoptosis and resulted in an increased cell arrest in G1 phase of the cell cycle. Reduced tumor growth, up regulated BCL2L11 and BAX at message levels and downregulated NRF2 at protein and message levels (p<0.05). In our previous work (Alvarez, 2016) we found that Qu treatment up-regulated the expression profile of 63% (n=17) and 24% (n=4) (in vitro and in vivo, respectively) of the 84 miRNAs evaluated. Of these, 42% (n=7) and 100% (n=4) corresponding exclusively to miRNAs that target anti-apoptotic genes and to miRNAs that have been demonstrated to have pro-apoptotic functions. Further, expression levels of miR-1, miR- 206 and miR-133a and b were validated in xenograft model samples, resulting in a significant up-regulation of the expression levels in treated animals compared to controls (p<0.05). The up regulation of miR-1 and mir206 are related to decreased levels of NRF2 as these miRs levels were described as being regulated by NRF2, as a gain of NRF2 function in cancer cells attenuated expression of them. Therefore, Qu acting as an inhibitor of NRF2 might be a promising compound that may have application in AML treatment, however, the mechanism by which Qu decrease NRF2 expression remains to be investigated.

**P1333**

**Board Number: B340**

The Supernatant of RAW 264.7 cells M1 Polarized with Phellinus linteus Prevents the Migration and Invasion in Prostate Cancer Cells by Inhibiting the Epithelial-mesenchymal Transition Pathway.

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Most deaths from prostate cancer are due to treatment-resistant metastasis. *Phellinus linteus* has been used for centuries to prevent and treat several diseases in East Asia. Unlike cancer chemotherapy, radiotherapy and anticancer drugs, natural products that have been used from centuries can safely be given antitumor treatment without serious side effects. Once activated by interferon gamma or lipopolysaccharide (LPS), macrophages can kill cancer cells directly or by secreting the factor that kills the cancer cells indirectly. Epithelial-mesenchymal transition (EMT) is associated with stem cell markers of prostate cancer cells. By acquiring the characteristics of stem cells, resistance to apoptosis is increased, aging is reduced, immune response escapes, and ultimately resistance to treatment is
Achieved. EMT is considered an important step in cancer metastasis. The new components that target these EMT molecules can effectively block the interaction between cancer cells and other tissues. Phellinus linteus hot water extract (PL-HWE) was treated into mouse macrophage RAW264.7 cells to observe NO release that is an indicator of macrophages polarization into M1 type. iNOS, IL-1β, IL-6, TNF-α, COX2 and PSG2 were detected at the mRNA level by real-time PCR to confirm that the RAW 264.7 cells were polarized into M1 type. The supernatant of RAW 264.7, which was identified as the M1 type, was treated into prostate cancer cells at volume ratio of 5%, 10%, and 20% for 48h. As a result, mRNA and protein involved in EMT pathway were determined. Real-time PCR at the mRNA level, western blot at the protein level, and wound healing assay were performed to confirm. Based on this research, it can be a new way of preventing cancer metastasis through immunomodulation.

P1334
Board Number: B341
Anticancer effects of cleistanthin A and its analogue in colorectal cancer cells.
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Colorectal cancer (CRC) is ranked as the third most common cancer and cancer-related mortality worldwide. Although the treatment outcome is very much improved for early stages of disease, however long-term survival rate is relatively poor for patients with an advanced stages due to the development of metastasis. Therefore, searching for anti-metastatic agent for CRC is urgently needed. Cleistanthin A (CA) is phytochemical derived from Phyllanthus taxodiifolius Beille (Euphorbiaceae family). In this study we investigated the anticancer activities as well as their underlying mechanisms of CA and its analogue (MUC535) in CRC cell lines, HCT-116 and SW480. By MTT assay, CA and MUC535 induced cytotoxic effects against HCT-116 and SW480 cell lines in concentration- and time-dependent manner. IC50 values of CA at 72 h in HCT-116 and SW480 cells were 0.63+0.25 micromolar and 1.26+0.29 micromolar, respectively. Moreover, IC50 values of MUC535 at 72 h in HCT-116 and SW480 cells were 0.079+0.019 micromolar and 0.59+0.198 micromolar, respectively. The cytotoxic activities of both compounds were not observed at 24 h. Treatment with CA and MUC535 for 24 h significantly reduced migration and invasion of HCT-116 and SW480 cells in dose-dependent manner. Treatment with 10 micromolar of CA suppressed CRC cell migration and invasion approximately by 60% and 40% for HCT-116; 60% and 70% for SW480 cells, respectively. Moreover, treatment with 10 micromolar of MUC535 suppressed CRC cell migration and invasion approximately by 30% and 35% for HCT-116; 50% and 35% for SW480 cells, respectively. Mechanistically, treatment of CA and MUC535 decreased focal adhesion kinase (FAK) Y397 phosphorylation in both CRC cell lines. Collectively, our results indicate that CA and its analogue (MUC535) suppress CRC cell migration and invasion partly through FAK-related signaling pathway. Therefore, CA and MUC535 have potential to be developed as anti-metastatic agents for CRC.
Colorectal cancer (CRC) is the third most common type of cancer in the world and the second leading cause of death among people (Siegel, Desantis, & Jemal, 2014). Rumex crispus extracts, a source of anticancer compounds, have demonstrated induction of apoptosis in colorectal cancer cells (DLD-1). Bhandari (2015) extracted water soluble compounds from the roots and leaves of Rumex crispus and screened those extracts for compounds that induced apoptosis in DLD-1 cells. A compound referred to as L19 was isolated and has been studied in more detail in this project. In this research, specific genes involved in apoptosis induction as a result of treatment of the DLD-1 cells with L19 are identified.

Accelerated solvent extraction (ASE) followed by high performance liquid chromatography (HPLC) was utilized to obtain larger amounts of L19. GC-Mass spectrometry was used to identify the compound L19. Apoptosis was measured by detecting the levels of caspase 3, 6 and 7 using the APO-one assay (Promega, Corp.) after treating the DLD-1 cells with varying concentrations of L19. Furthermore, the mechanism of apoptosis was determined using qRT-PCR and gene specific primers for caspase 8,10 (extrinsic pathway), 9 (intrinsic), 4, 5 and 12 (ER stress). RT2 Profiler™ PCR Array for human apoptosis genes was used to explore additional effects on genes involved in apoptosis. Microarray analysis using the Human OneArray® Microarray from Phalanx Biotech Group (California, USA) to determine which genes of other key cellular pathways are affected by the compound. A time course at 8, 12, and 24 hours exposure of the cells to L19 followed by qRT-PCR showed that caspases 1, 3, 6, 8, 9, 10, and 12 exhibits up regulation by 12 hours. RT² Profiler™ PCR Array using 12 hour cDNA showed a down regulation for BCL2 which was the inhibitor for intrinsic pathways (Favaloro et al., 2012). CASP 9, 3, Apaf-1, Bax and P53 genes, which are key factors for the intrinsic pathways in apoptosis, are shown to be up regulated. CASP 12 and BH3 proteins which are involved in the ER stress pathways had up regulation as well (Puthalakath, 2007). FAS, FASLG, CASP 10, 8, 7, 6, and 3 which are involved in the extrinsic pathway had the largest up regulation.

Colorectal cancer (CRC) is one of the most common type of cancer worldwide. To improve treatment outcome, new chemotherapeutic drugs are required. Aberrant activation of Wnt/β-catenin signaling pathway has extensively been reported to play an important role in colorectal cancer progression. Previously, the anticancer property of cleistanthin A (CA), a plant-derived compound from Phyllanthus taxodifolius Beille, has been demonstrated. However, the detailed molecular mechanisms have not been investigated. Therefore, we aimed to investigate the anticancer activity of CA on Wnt/β-catenin signaling pathway in human colorectal cancer cell lines, SW480 and HCT-116. We found that CA...
exhibited dose- and time-dependent cytotoxic activity against both CRC cell lines. CA enhanced DNA damage and suppressed the expression of survivin, an anti-apoptotic protein leading to CRC apoptosis cell death. In addition, CA suppressed the canonical Wnt signaling pathway as illustrated by reduction of β-catenin protein expression and β-catenin-mediated transcriptional activity. To further confirm the inhibitory effect on Wnt signaling pathway, the effect of CA on the expression of Wnt target genes was examined by real-time PCR. We found that the expression levels of Wnt target genes including Axin2, cyclin D1 and survivin were significantly reduced. The inhibitory effect on Wnt signaling pathway-mediated by CA was GSK-3β-independent since the transcriptional activity-mediated by GSK-3β insensitive β-catenin was still able to suppress by CA. Taken together, our results demonstrate for the first time that CA induces CRC cell apoptosis partly through induction of DNA damage and inhibition of Wnt/β-catenin signaling pathway. Therefore, CA has the potential for further development as a novel anticancer agent for colorectal cancer.

**P1337**

**Board Number: B344**

Characterization of monoclonal antibodies from mice immunized with phycocyanin as a medicinal substance.


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Background: Spirulina platensis is a blue-green algae. Phycocyanin (PC) is a light-harvesting, pigment-binding protein isolated from S. platensis (N. T. Eriksen, 2008). PCs isolated from blue-green and red algae are classified as crude PC (C-PC) and R-PC, respectively (A. N. Glazer, 1984; C. M. Hilditch, et al., 1991). C-PC has been reported to have various medicinal properties, including antioxidant, inflammatory, and antitumor effects. However, the molecular mechanisms underlying these properties remain poorly understood. Therefore, to reveal these molecular mechanisms, we investigated the function of PC, particularly regarding the anti-tumor effect, and then determined making monoclonal antibodies (mabs) against the PC.

Materials and Methods: C-PC derived from S. platensis was kindly provided by Dainippon Ink Corporation (Ichihara, Chiba, Japan). Phycocyanobilin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other materials were from Wako and Sigma-Aldrich.

Female Balb/c mice were immunized with C-PC and Freund’s complete or incomplete adjuvant multiple times over several months. We obtained several mabs in accordance with previously reported methods (K. Yoshimura et al., 1996). Two established mabs (S5C2 and T1) were characterized with immunocytochemistry (ICC), Western blotting, enzyme-linked immunosorbent assay (ELISA), and mab isotyping. Results and discussion: ICC revealed that S5C2 and T1 recognized intact cyanobacteria, S. platensis, which forms spiral-shaped, multicellular filamentous cyanobacteria. Interestingly, these mabs appear to recognize different constituents in osteosarcoma U2OS cells. Western blotting of C-PC with these mabs also suggested that both mabs bind these constituents, revealing a few bands corresponding to the major components of PC. ELISA indicated that both mabs reacted with the entire PC fraction, whereas those almost did not bind phycocyanobilin. A mouse isotyping antibody kit showed that the isotypes of both mabs are IgM and kappa. In the present study, we indicate the characterization of those two mabs from mice immunized with C-PC and discuss the possible applications.
Curcumin is a natural product extracted from C. longa plant with remarkable anti-cancer activity and low toxicity. However, the extensive preclinical and clinical studies have shown that curcumin exhibit poor bioavailability and fast metabolism. The product of metabolic destruction of curcumine, dehydrozingerone (DZG), is stable under physiological conditions and possesses expressed antitumor properties. Several studies demonstrated promising pharmacological potential of DZG and its synthetic modifications. In this work we explored several bioisosteric analogs of DZG based on pyridoxine (vitamin B6) scaffold. Specifically, we synthesized a series of DZG bioisosteres, in which the aromatic part was replaced by substituted pyridoxine rings, and studied their biological properties. The obtained compounds were screened for cytotoxicity against several tumor cell lines (mammary gland, lung adenocarcinoma, glioblastoma, colorectal carcinoma, and melanoma cells) in comparison with DZG and doxorubicin (DOX). Most of the studied compounds demonstrated high cytotoxic activity against the tumor cells with IC_{50} in the range of 0.5-6.6 µM, which is comparable to cytotoxic activity of DOX and 6-20 times higher than cytotoxicity of DZG. At the same time, the cytotoxicity of the leading compounds against the normal HEK293 cells was lower than that of DOX though higher than that of DZG. As a result, several compounds had high selectivity indexes (SI from 6 to 57) as compared to DZG and DOX. According to flow cytometry data, the leading compounds arrest the cell-cycle in G2/M phase and inhibit further transition of cells to G0/G1 phase. Similar action is typical of DZG and some commercial antineoplastic agents such as paclitaxel. Interestingly, our DZG analogs show only slight influence on the membrane potential of mitochondria and probably cannot serve as apoptosis inducers. By analogy to DZG, they also demonstrate expressed antioxidant properties and reduce the level of reactive oxygen species in tumor cells. Our compounds also significantly suppress the migration activity and invasiveness of tumor cells in vitro. The leading compound also shows excellent safety in in vivo acute toxicity experiment (LD_{50} > 2000 mg/kg of weight, mice, p.o.). The obtained results demonstrate that the new pyridoxine-based bioisosteres of DZG are promising antineoplastic agents which can be recommended for further in vivo studies on human tumor xenograft models.

This work was performed in the framework of the state task № 1.5086.2017/7.8 of the Ministry of Education and Science of the Russian Federation, and was supported by the Program of Competitive Growth of Kazan Federal University, and by the Program of Competitive Growth of I.M. Sechenov First Moscow State Medical University.
Cancer Stem Cells

P1339
Board Number: B346
The WAVE3-YB1 interaction regulates cancer stem cells activity in breast cancer.
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Resistance to therapy is the main cause of tumor recurrence and metastasis, and cancer stem cells (CSCs) play a crucial role in this process, especially in triple-negative breast cancers (TNBCs), for which chemotherapy is the main course of treatment. Unfortunately, no FDA-approved treatment is currently available for this subtype of BC, which explains the high rate of mortality in patients with TNBC tumors. WAVE3, a member of the WASP/WAVE actin-cytoskeleton remodeling family of protein, has been established a major driver of tumor progression and metastasis of several solid tumors, including those originating in the breast. Recent studies found WAVE3 to mediate the process of chemoresistance in TNBCs. The molecular mechanisms whereby WAVE3 regulates chemoresistance in TNBC tumors remains largely unknown, as does the role of WAVE3 in CSC maintenance. Here we show that WAVE3 mediates chemoresistance by promoting CSC self-renewal and transcription of CSC-specific genes. Our data show that WAVE3 is enriched in the CSC-subpopulation of TNBC cell lines. Knockout of WAVE3 via CRISPR/Cas9 significantly depletes CSC-subpopulation and inhibits transcription of CSC transcription factors. Mechanistically, we established a link between WAVE3 and the Y-box binding protein 1 (YB1), a transcription factor and CSC-maintenance gene. Indeed, the interaction of WAVE3 with YB1 is required for the translocation of YB1 to the nucleus of cancer cells, and the subsequent activation of transcription of CSC-specific genes. Collectively, our findings identify a new WAVE3/YB1 signaling axis that regulates the CSC-mediated resistance to therapy and opens a new therapeutic window for the treatment of TNBCs.

P1340
Board Number: B347
SOX2/OCT4 biosensor intravital imaging reveals the invasive breast cancer stem cell phenotype and its association with TMEM in vivo.
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Cancer stem cells play an important role during metastatic progression of breast cancer. However, the phenotypes specific to breast cancer stem cells have not been evaluated at single cell resolution in vivo. Here, we employ high-resolution intravital two-photon microscopy in orthotopic xenograft tumors, formed from human breast cancer cell lines expressing a previously characterized SOX2/OCT4 transcription-based fluorescent stem cell biosensor (SORE6) (Tang et al 2015). Using this high resolution imaging technology we found that SORE6+ stem cells: (a) constitute a minority population of the primary mammary tumors, (b) move approximately ten times slower than non-stem breast cancer cells.
(0.1 vs 1.1 μm/min, respectively), (c) are migratory toward blood vessels and, (d) compared to non-stem cells, they are enriched for ECM degrading invasive cellular protrusions called invadopodia. All these phenotypes are specifically associated with the disseminating population of tumor cells in the primary tumor site (Gligorijevic et al 2014) and, in addition, invadopodia are required for transendothelial migration during intravasation (Roh-Johnson et al 2013). We examined the mechanism of cancer stemness initiation and using live cell imaging found that stemness in cancer cells is induced upon physical contact with a macrophage. Interestingly, previous work has shown Mena\textsuperscript{INV} upregulation in cancer cells upon physical contact with a macrophage and Mena\textsuperscript{INV}—dependent transendothelial migration during intravasation. Intravital imaging in mice confirmed that stem cells have a three-fold higher incidence of direct contact with a macrophage compared to non-stem cells. We also checked the relationship of stem cells with the three cell complex called TMEM, composed of a macrophage, Mena\textsuperscript{Hi} tumor cell and endothelial cell, all in direct contact. TMEM was previously shown to be the doorway for intravasation of tumor cells in primary mammary tumors (Harney et al 2015) and is validated as a prognostic of metastasis in human breast cancer patients (Rohan et al 2014; Robinson et al 2009). Intravital imaging confirmed the presence of cancer stem cells in TMEM. Distance histograms of stem cells relative to TMEM sites showed that stem cells are accumulated close to TMEM. Interestingly, Mena\textsuperscript{INV} expressing cancer cells were similarly seen to accumulate close to TMEM, consistent with the observations that Mena\textsuperscript{INV} expression and stemness are both induced by macrophage contact. We conclude that stemness, as well as Mena\textsuperscript{INV} expression, is induced in breast cancer cells by direct contact of cancer cells with macrophages, and that these stem cells utilize Mena\textsuperscript{INV}—dependent invadopodia to invade into surrounding stroma and reach TMEM sites to intravasate into blood vessels.

P1341
Board Number: B348

Suppression of the growth of cancer stem cells developed from iPSCs by soluble form of human Cripto-1.
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Cripto-1 (CR-1) is a GPI-anchored signaling protein of epidermal growth factor (EGF)-cripto-1-FRL-1-cryptic (CFC) family and plays a significant role in early developmental stages and in the different types of cancer cells, epithelial to mesenchymal transition and tumor angiogenesis. Previously, we developed cancer stem cells (miPS-LLCcm) from mouse IPS cells cultured in the conditioned medium of Lewis lung carcinoma cells for 4 weeks. We found the expression of CR-1 during the conversion. To investigate the biological role of CR-1 in cancer stem cells, we have prepared a C-terminally truncated recombinant human CR-1 protein (rhCR-1) in E. coli, in which GPI (glycosphatidylinositol) anchored part has been removed by insertion stop codon through PCR based site-directed mutagenesis. This soluble form of CR-1 was designed in the plasmid pBO1801 to be expressed under the control of T7 promoter with His-tag. The soluble form of rhCR-1 protein was produced as inclusion bodies in E. coli BL21 (DE3) harboring plasmids pLysS. The protein was denatured and solubilized in the presence of 8M urea and purified by nickel column and CM650M cation-exchange column chromatography. Then the recombinant protein was refolded in a redox buffer and subsequently dialyzed against 1xPBS. The soluble form of rhCR-1 effectively suppressed the growth of miPS-LLCcm cells in a dose dependent manner. Further biological effect of rhCR-1 is under investigation.
P1342
Board Number: B349
Investigating the role of YAP and TAZ in medulloblastoma cancer stem cell formation and asymmetric cell division.
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Medulloblastoma is the most common malignant brain tumor of childhood. It is very aggressive and few effective treatments are available. The presence of cancer stem cells (CSC) correlates with medulloblastoma aggressiveness, and efforts have been done to identify signaling pathways that control CSC maintenance and proliferation. Researchers have described in others brain tumors that, similarly to normal stem cells, CSC are able to undergo asymmetric cell division (ACD). This kind of mitosis ensures the formation of one stem cell and one cell committed to differentiation. In tumors, low rates of ACD might be related to increased proliferation and aggressiveness, since the occurrence of symmetric divisions will increase the pool of CSC. Recently, some elements of Hippo pathway (a pathway related to the control of organ size, tumorigenesis and stem cell phenotype) were related to ACD in flies. In this context, the aim of this project is to investigate the contribution of the effectors of Hippo pathway (the transcription factors YAP and TAZ) to medulloblastoma stem cells formation and their ability to undergo ACD. For that, we generated YAP, TAZ and YAP/TAZ knockout cells from two different medulloblastoma cell lines (DAOY and USPMed13) using CRISPR technology. To evaluate the formation of CSC and to culture these cells for different experiments, tumorspheres were formed in low attachment plates in a defined culture medium. After tumorspheres culture for 7 days, when the expression levels of RNAm and protein of pluripotency genes were increased, spheres were dissociated and cell proliferation and the rates of ACD were analyzed in different culture conditions. The rates of ACD were analyzed based on NuMA and Numb location in mitotic cells using confocal microscopy. The rates of ACD could be modulated according to the culture: in culture medium that stimulates cell differentiation, the rates of ACD were increased when compared to culture conditions that stimulates proliferation. No difference in cell proliferation was observed in YAP knockout cells when culture in monolayers. However, fewer tumorspheres were formed from these cells when compared to controls, and their morphology was altered in both cell lines. Regarding ACD, YAP knockout cells showed decreased rates of ACD when cultured in the medium that stimulates cell differentiation. However, in YAP knockout cells the expression of TAZ was increased, what could overcome the absence of YAP. Now the same experiments are being performed with TAZ and YAP/TAZ knockout cells. Taken together, the results indicate that medulloblastoma CSC are able to undergo ACD, and that YAP and TAZ are important for CSC formation and ACD cell division occurrence in medulloblastoma cells.

P1343
Board Number: B350
The Snail/Let-7 axis induces stemness in ovarian, breast, and pancreatic cancer cells.
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One big challenge in cancer therapies is to find effective treatments to relapses and metastases. They are caused by a high-stemness subpopulation within tumors. The increase of stemness in cancer cells is
associated with the loss of miRNA let-7. The mechanisms of let-7 in cancer cells are not known. However, evidence from reprogramming studies is consistent with a role for the epithelial-mesenchymal transition (EMT) factor Snail in let-7 repression. EMT is a process by which epithelial cells gain migratory properties to become mesenchymal cells. EMT in cancer cells results in metastases and chemoresistance. In other words, after EMT, cancer cells have gained stemness. This leads us to believe, just like in reprogramming, Snail is repressing Let-7 directly in cancer cells. The goal of our study is to investigate the interaction between Snail and let-7 and its role in regulating cancer cells’ differentiation status. Hence, we hypothesize that Snail directly represses let-7, leading to increased stemness in cancer cells. To approach this, we treated breast (MCF-7), pancreatic (PANC-1), and ovarian cancer cells (OVCAR8, OVSAHO) with TGFβ or EGF; both growth factors are known to induce EMT. We also overexpressed Snail by using an estrogen-receptor fusion protein. Using qPCR, we detected the expression levels of mRNAs including EMT and stemness markers as well as miRNA let-7. These results were further confirmed by immunofluorescence. Luciferase assay and Chromatin immunoprecipitation (CHIP) were used to test the repressing action and the direct binding of Snail on let-7. After either EMT induction or Snail overexpression, an increase in pluripotency markers, as well as a decrease in let-7, was observed, indicating the negative association between Snail and let-7. CHIP data shows that overexpression of Snail increases the level of binding at let-7 family members’ promoter regions, demonstrating that let-7 is a direct target of Snail. In conclusion, our study has shown that Snail directly represses let-7 transcription and causes an increase in stemness in cancer cells.

P1344
Board Number: B351
O-GlcNAc transferase regulates breast cancer tumor-initiating cells.
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Tumors are heterogeneous with a sub-population of quiescent stem-like cells that when isolated can regenerate the whole tumor. Chemotherapy targets rapidly dividing cells and may not readily target these tumor-initiating cells (TIC) contributing to resistance and relapse. TICs, like cancer cells, rewire metabolism to meet demands of increased growth and biosynthesis. The hexosamine biosynthetic pathway utilizes glucose to generate UDP-GlcNAc. O-GlcNAc Transferase enzyme (OGT) uses UDP-GlcNAc as a substrate for adding O-GlcNAc moieties to nuclear and cytoplasmic proteins, akin to phosphorylation. Our lab has shown that OGT/O-GlcNAc levels are elevated in multiple cancers and reducing OGT in breast cancer cells blocks growth in-vitro and in-vivo. Since OGT directly modifies proteins associated with pluripotency in embryonic stem cells, we hypothesized that OGT may regulate breast cancer TICs. Here, we show that breast cancer cells enriched in tumor initiating activity in mammosphere cultures contain elevated levels of OGT/O-GlcNAcylation. Inhibition of OGT genetically or pharmacologically reduces mammospheres forming efficiency and reduces the CD44H/CD24L and Nanog-expressing TIC population. Conversely, over-expressing OGT increases mammospheres formation and preliminary evidence shows an increase of tumor initiation in-vivo. Analysis of potential mechanism revealed that OGT overexpression increased mRNA expression of transcription factor c-Myc, protein expression of cancer stem cell markers CD44, Snail and epithelial to mesenchymal transition markers including Vimentin and Fibronectin. These results suggest that OGT plays a key role in regulation of breast cancer tumor initiating cells in-vitro and in-vivo.

Sunday-223
P1345  
**Board Number: B352**  
Expression of ALDH Isoforms in Colon Tumorigenesis.  
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**Background:** Tumorigenesis is driven by stem cell (SC) overpopulation. Aldehyde dehydrogenase (ALDH) is a marker for SCs in several tissues, and there are 19 known isoforms of ALDH that each have specific roles and are cell and tissue-type dependent. Although a handful of ALDH isoforms have been implicated in various cancers, the remaining isoforms still remain to be investigated, especially in colorectal cancer (CRC). **Hypothesis:** There is a unique ALDH isoform signature in normal colonic SCs and that unique expression pattern changes during CRC initiation and progression. By identifying the phenotype of the normal colonic SC and comparing it to the colonic cancer SC, we can identify what role these isoforms play in colon cancer biology. **Methods:** We analyzed matched normal colon epithelium and colorectal tumor tissues to see ALDH isoform mRNA expression and verified protein expression of select isoforms by Western blot. ALDH1A1 protein was knocked down by siRNA, and its effect on cell proliferation was analyzed. **Results:** Select ALDH isoforms were expressed in the normal colon epithelium and aberrantly expressed in the matched tumor tissues. ALDH1A1, ALDH1A3, ALDH1B1, ALDH2, ALDH4A1, and ALDH7A1 were identified as important isoforms. Knockdown of ALDH1A1 protein was validated and revealed that it is important for the regulation of cell proliferation. **Conclusions:** The ALDH1A1 knockdown was successful; however other functional tests of the ALDH1A1 knockdown have yet to be completed, as well as the knockdown of the other ALDH isoforms. **Implications:** Ultimately, the major contribution of targeting colonic CSCs, via ALDH isoform expression, will lead to new, more effective treatments for colorectal cancer to limit CSC growth and inhibit tumor recurrence.

P1346  
**Board Number: B353**  
Hypoxia and cancer stem cell activity are linked during tumor cell dissemination and metastasis in breast tumors.  
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**Hypoxia,** an established hallmark of cancer, is responsible for many pro-metastatic changes that occur within aggressive tumors, including increased angiongenesis, tumor cell invasion, dormancy, and dissemination. Hypoxia may be associated with the induction of cancer stem cells, a rare population thought to be responsible for progression, metastasis, recurrence and drug resistance. Here, we describe the creation of a double-reporter MDA-MB-231 cell line for the visualization of hypoxic and cancer stem cells in real time in vivo. The double-reporter cell line contains both our previously published novel optical reporters; for hypoxia (based upon a genetically encodable fluorescent protein, mCherry, and expressed only under hypoxic conditions) (Wang et al 2016), and for cancer stem cells (SORE6-Dscp-GFP) (Tang et al 2015). Using multiphoton microscopy, hypoxic tumor cells were found to
postgraduate students exhibit a more persistent slow migration phenotype, associated with blood vessels, and had increased invadopodium-associated collagen degradation and intravasation activity, all as compared to normoxic cells in the same tissue location in vivo. In addition, hypoxic tumor cells migrated toward human epithelial growth factor gradients in vivo and were present in the CTC population supporting hypoxia involvement in blood vessel directed streaming migration (Leong et al 2016) and intravasation (Wang et al 2016). Since these are characteristics of dissemination and metastasis competent breast tumor cells (Gligorijevic et al 2014), we determined if hypoxic tumor cells with these phenotypes expressed the cancer stem cell reporter (SORE6-DsCop-GFP). We found that the SORE6+ cancer stem cells exhibited the hypoxic tumor cell phenotypes including slower migration than non-stem breast cancer cells (0.1 vs 1.1 \( \mu m/min \), respectively), migration toward blood vessels, and enrichment for ECM degrading invasive cellular protrusions called invadopodia. Furthermore, in the primary tumor, we observed that stem cells were co-labeled with the hypoxia reporter when in association with blood vessels. Finally, we found, in spontaneous metastases in the lung, that the majority of tumor cells were SORE6+ cancer stem cells during early arrival from the primary tumor. Our results show, for the first time by intravital imaging of live tumor cells in both primary and metastatic breast tumors, that hypoxia and stemness are linked within the same migratory and disseminating population of tumors cells leading to the dissemination of tumor initiating cancer stem cells to the lung.

P1347

Board Number: B354

CD82 expression affects acute myeloid leukemia chemosensitivity.
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Acute myeloid leukemia (AML), the most common acute leukemia, is a myeloid lineage cancer that accumulates in the blood and bone marrow, interfering with the normal production of blood and immune cells. Relapse and resistance to therapeutics are among the most challenging obstacles in the management of AML. Recent studies suggest that disease relapse is predominantly due to a population of leukemia stems cells that seek shelter in the bone marrow, resulting in increased cell survival and resistance to chemotherapeutics. Therefore, the interactions between AML cells and the bone marrow microenvironment are critical for AML disease progression and relapse. Recently, our lab identified the tetraspanin CD82 as critical regulator of AML cell bone marrow adhesion and homing. More specifically, we find that AML cells from primary patient samples with increased CD82 expression display enhanced bone marrow homing in preclinical animal models. In the current study, we test the hypothesis that CD82 overexpression in AML cells promotes chemoresistance. We investigated the role of CD82 expression on AML chemosensitivity using the conventional therapeutic, daunorubicin. Flow cytometry analysis of active caspase 3, 7 expression indicates that overexpression of CD82 promotes chemoresistance in AML cells. Additionally, we find increased resistance in AML cells when the N-linked glycosylation sites on CD82 are disrupted. In contrast, we detect an increase in chemosensitivity when CD82 is knocked down. CD82 overexpressing AML cells also have increased IL-8 gene expression and cytokine production following daunorubicin treatment, indicative of a chemoresistance phenotype. Next, we investigated the mechanism by which CD82 modulates AML chemosensitivity, specifically focusing on the WNT-signaling pathway. Our data suggest that CD82 overexpressing AML cells become more chemosensitive to daunorubicin after treatment with a \( \beta \)-catenin inhibitor. RT-PCR and western blotting analysis show an increase in \( \beta \)-catenin gene and protein expression in CD82 overexpressing AML cells treated with daunorubicin. In combination, these results suggest that differential expression of CD82 modulates chemosensitivity in AML cells. Moreover, our data suggest the possible role of \( \beta \)-
catenin signaling in chemosensitivity downstream of CD82. Future studies will be directed at elucidating CD82-mediated chemoresistance and AML relapse in animal models.

P1348
Board Number: B355
Histone demethylase inhibitor JIB-04 blocks the self-renewal of human colon cancer stem cells. H. Cho1,2, M. Kim1,2, Y. Jang1,2;
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Cancer stem cell (CSC) exists as a minor population of tumor mass, which shares similar properties associated with normal stem cells like pluripotency and self-renewal. Since CSCs possess resistance for drug-, radio therapy, untouched CSCs after conventional therapy lead to develop recurrent disease and induce metastasis by promoting epithelial-mesenchymal transition (EMT). Therefore, understanding the mechanism by which cancer stem cells maintain self-renewal and discovery of novel compound selectively targeting CSCs are important to annihilate cancer. Although JIB-04, a small molecule inhibitor of the jumonji family of histone demethylase, was reported to have selective anticancer effects in lung cancer and prostate cancer cell lines and reduce cell growth and proliferation of drug-resistant cancer cell in brain and lung cancer, effects of JIB-04 on CSCs were not elucidated yet. In this study, we investigated the function of JIB-04 on CSCs and its underlying mechanism. Our data showed that JIB-04 decreased cancer stem cell properties like expression of CD133, CSC surface marker, self-renewal ability and tumor initiating ability in three colorectal cancer cells. Furthermore, we found that JIB-04 down-regulated the expression of Wnt signaling target genes associated with colorectal CSC progression. Collectively, our results suggest that JIB-04 may be a novel therapeutic agent for colorectal cancer.

Gene Regulation and Genome Structure

P1349
Board Number: B357
Gene Annotation of Contig17 Within Dot Chromosome of Drosophila eugracilis. A.M. Herken1, T. Sadikot1;
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With the use of many open-source computational genomic tools for sequence alignment, prediction models, the Drosophila genome browser, and model checker contig17 of Drosophila eugracilis, located within the dot chromosome was studied for gene features both genes and pseudogenes. The goal of the study was to annotate and identify all genes located within this contig sequence. To do this the Drosophila melanogaster sequence was used as a reference for conservation-based analysis. Various gene predictions show there are three potential genes in the contig. These genes are apolpp, actbeta, and sv. Each gene contained a varying number of isoforms. Analysis of this contig showed all of the predicted genes to be present within the contig. The genes are conserved between D. melanogaster and D. eugracilis. A non-canonical donor site, GC instead of GT, was found in one of the introns of many isoforms of sv. This non-canonical donor site is conserved between D. melanogaster and D. eugracilis.
P1350
Board Number: B358
Annotation of contig40 of the Drosophila eugracilis dot chromosome.
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The genome of Drosophila melanogaster was completed in 2000 and it has since become one of the most studied species in biology. D. melanogaster serves as a model organism for studying many developmental and cellular processes common to higher eukaryotes, and is also used as a reference species for computational analysis and annotation. The Genomics Education Partnership (GEP), sponsored by Washington University, Saint Louis, is a collaborative effort designed to incorporate undergraduate students in genomics research involving various Drosophila species. Students are tasked with certain steps in taking raw sequence data to a high-quality finished sequence; the completed data is used to answer questions in genomics. For this project, the DNA sequence of contig40 of Drosophila eugracilis dot chromosome was analyzed and compared to D. melanogaster for the presence of protein coding genes and other genomic features. Sequence analysis and data collection were carried out using several open-source computational genomic tools for sequence alignment, gene-prediction and Drosophila genome browsing. The data files and resources for this project were made available through the GEP. Based on BLAST analysis using the D. melanogaster genome as a reference and other gene prediction models, there appeared to be strong support for the presence of two genes, zinc finger homeodomain 2 (zfh2) and Asator, and weak support for a third gene, discs overgrown (dco). Data analysis revealed that resource information supporting the presence of zfh2 and Asator was consistent, and that the support for dco contained incongruences that indicate its absence in the contig. The annotated model produced in this research project was submitted to the GEP for their data repository.

P1351
Board Number: B359
Annotation of contig 20 of dot chromosome in Drosophila eugracilis by comparison to Drosophila melanogaster genome using bioinformatics techniques.
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The genome of Drosophila melanogaster was completed in 2000 and it has since become one of the most studied species in biology. D. melanogaster serves as a model organism for studying many developmental and cellular processes common to higher eukaryotes, and can also be used as a reference for identifying genes and other genomic elements in other Drosophila species. The goal of this project was to analyze and annotate a contig sequence of chromosome 4 from the D. eugracilis genome. Sequence analysis and data collection was carried out using a number of open-source computational genomic tools for sequence alignment, gene-prediction and Drosophila genome browsing. The data files and resources for this project were available through the Genomics Education Partnership (GEP) sponsored by Washington University, Saint Louis. Data analysis and interpretation required careful screening of the DNA sequence of interest, identification of gene markers and comparison of the predicted gene features to a reference D. melanogaster DNA sequence. Four genes were predicted to be in the contig: PlexA, ATPsynbeta, PlexB, and ATPsynbetaL. It was determined that the PlexA and ATPsynbeta genes existed in the contig as predicted by the D. melanogaster reference model and that the PlexB and ATPsynbetaL are falsely predicted to be in this regions. It was also determined that the toy
gene exists in the contig despite the ortholog not being marked as such by the *D. melanogaster* alignment.

**P1352**

**Board Number: B360**

Annotation and cross-species comparison of *Drosophila* genes.

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*Drosophila melanogaster* has a small fourth chromosome (3.5% of the genome * )*, that has very large heterochromatin domains which are rich in HP1. Our goal for this project is to understand the mechanisms of gene expression in this heterochromatic environment. In the Genomics Education Partnership Project, we are using comparative genomics to identify conserved DNA regulatory sequences, by comparing the *D. melanogaster* Muller F element to those of other fly species that deivered from *D. melanogaster* ~10 million years ago, aiming to find regions of homology surrounding the transcription start sites. I have annotated the genes on a 40,000 base-pair piece from the *Drosophila eugracilis* chromosome four. Sequence comparisons using BLASTX, gene predictors like Gene Scans, and RNA sequence data were used to annotate coding genes in this region. I identified two genes: *myo* (3 isoforms) and *ey* (4 isoforms) and annotated protein coding exons and splice sites for each isoform. The *myo* amino acid sequence is identical for each isoform in *D. eugracilis*, with 70.3% amino acid identity compared to the *D. melanogaster* *myo* protein. The *D. eugracilis ey* isoforms are also similar to the *D. melanogaster ey* protein sequences, with a range from 73% to 78% identity to their corresponding *D. melanogaster* isoform. Putative Transcription Start Sites for each gene were investigated, including a search for known transcription factor binding sites. By pooling results, GEP students are annotating several megabases of DNA, and contributing to an understanding of heterochromatic genes.

**P1353**

**Board Number: B361**

Influence by ecdysone and transcription on developmentally regulated DNA re-replication in Sciar* DNA puffs.

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The polytene chromosome DNA puffs of the fly Sciar* bypass cell cycle regulation during development: (1) mitosis is skipped and the polytene cells alternate between G and S phase, reaching 4096C in larval salivary glands; (2) re-replication occurs at the DNA puff loci in late larval salivary glands. To investigate how these rogue replication origins override the normal controls that ensure that an origin fires no more than once per S phase, we have sequenced the Sciar* genome (using Illumina, PacBio, MinION and BioNano Irys sequencing) and identified 14 DNA puff sequences based on their increased read copy number, with validation by qPCR. The amplification levels range from 32-fold (DNA puff II/9A), to 16-fold (DNA puff II/2B) and 8-fold or less (the remaining DNA puffs). The width of the amplicons was 267-607 kb. FISH allowed correlations between the DNA puff sequences and polytene chromosome map positions.

To investigate the interplay between transcription and re-replication, and whether transcription helps to open the chromatin for re-replication, we have sequenced the transcriptome of Sciar* salivary glands before, during and after DNA puff amplification. Most DNA puff loci do not begin transcription until after re-replication, where the amplified DNA can serve as abundant template for massive transcription...
required to build the larval cocoon at the next stage of development. Intriguingly, we found a microRNA that is present during re-replication and is complementary to one strand of the DNA puff II/9A origin. We are extending this observation to explore its presence at the other DNA puff loci and its possible role in re-replication.

We found by qPCR that re-replication was prematurely induced in the 14 DNA puffs by injection of the steroid hormone ecdysone into young larvae prior to the developmental stage when DNA amplification would normally occur. A match to the ecdysone receptor binding site (EcRE) occurs at DNA puff II/9A adjacent to the ORC binding site where we previously mapped the start site of DNA synthesis. This EcRE has been maintained during evolution and is present in a polymorphic strain of Sciara. Using methodology we have developed for genome editing in Sciara, we are investigating by mutational analysis if the DNA puff II/9A origin EcRE is required for DNA puff amplification. This would suggest that the ecdysone receptor functions as a re-replication factor in addition to its well-studied function as a transcription factor.

**P1354**

**Board Number: B362**

A function of the inverted repeat sequences located in the upstream of the mouse Oct3/4 gene.

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A DNA sequence that reads the same from 5’ to 3’ in either strand is known as an inverted repeat (IR) or a palindrome. A vast many studies have been heretofore performed to understand the biological significance of IRs. However, it is still elusive. We found a cluster of 6 IRs in the ~1.5 ~ ~2 kb upstream of the mouse Oct3/4 gene that is known as a key gene for pluripotency. Here, we suggest that this cluster plays some important role in the expression of the gene. The expression of the Oct3/4 is regulated by the proximal promoter (PP), the proximal enhancer (PE) and the distal enhancer (DE), which is flanked by the IR cluster. In order to know whether this cluster is implicated in the Oct3/4 expression, the CRISPR/Cas9-based genome editing was performed using mouse ES cells, by which this cluster was replaced with mirror repeat sequences that retain the sequences of the repeat units in the respective IRs. In the resulting cells, the level of the Oct3/4 expression was found reduced. Since the IR cluster is located in the downstream vicinity of the DE, the cluster seems to have some important role to make the enhancer function efficiently. In the meeting, we will also show the resulting effect on the phenotype of the ES cells.

**P1355**

**Board Number: B363**

The histone methyltransferases Set1 and Set5 promote subtelomeric gene silencing and telomere maintenance in *Saccharomyces cerevisiae*.


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Telomeres, the nucleoprotein structures found at the ends of linear chromosomes, provide a protective function for the genome. Alteration to the chromatin structure at or adjacent to these protective caps leads to dysfunctional telomeres, which are associated with genomic instability and implicated in cellular aging and many types of cancer. In *Saccharomyces cerevisiae*, the enzymes Set1 and Set5 belong to a family of methyltransferases known to target specific lysine residues on histones. The H3K4
methyltransferase Set1 has been linked to both transcriptional activation at gene promoters and silencing at telomeres. Set5, which modifies H4K5, K8, and K12, has recently been shown to aid Set1 in promoting silencing at telomeres. However, while we have some insight regarding the role for Set1 in silencing, the mechanism is still largely unclear. Additionally, it is not known how Set5 contributes to silencing at telomeres. Here, we investigated the role of Set1 and Set5 in the regulation of gene repression at native telomeres in Saccharomyces cerevisiae. Our results suggest that gene derepression in the absence of Set1 and Set5 is largely through a Sir protein-independent mechanism. Instead, our data suggest a link between the regulation of H4K5ac and K4K8ac and Set1- and Set5-mediated gene regulation. Additionally, we determined that transcriptomes from cells lacking both Set1 and Set5 show a strong correlation with transcriptomes belonging to mutants that display defects in telomere maintenance, revealing a specific role for Set5 and Set1 in pathways required for telomere integrity, rather than just gene silencing. In addition, we have determined that the cytoplasmic exonuclease Xrn1 acts synergistically with Set1 to promote gene repression, suggesting a possible link between Set1 and RNA-dependent repression of telomeric genes. Overall, our data provides support for a Sir-independent mechanism for telomeric gene repression by Set1, together with Set5, and identifies potential alternate mechanisms through which Set1 in particular may promote telomeric silencing.

P1356
Board Number: B364
Temperature effects on TPE, trinucleotide repeat stability, and chromosome loss in Saccharomyces cerevisiae.
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We investigated the influence of temperature on the genetic stability of Saccharomyces cerevisiae through the observation of the telomere position effect, trinucleotide repeat expansion and chromosome loss. Previous research has shown that genes placed adjacent to an S. cerevisiae telomere exhibit reversible transcriptional repression, a phenomenon known as the Telomere Position Effect (TPE). This specific alteration of genetic expression is not observed when the same genes are distanced from the telomeric heterochromatin. Using a cell viability assay, we investigated the ability of an S. cerevisiae telomere to silence the URA3 gene at a modified telomere via TPE at three different temperatures. We found that expression of URA3 was low at 23°C and 30°C relative to expression at 37°C. Our findings are consistent with previous studies which indicated that high temperatures diminish telomere length. Trinucleotide repeats are sequences of DNA composed of a repetitive pattern of three nucleotides. Though trinucleotide repeats exist naturally, expansion of these genomic sequences is known to be a cause of several serious human diseases, such as Huntington's Disease and Fragile X Syndrome. We investigated the relative levels of CTG trinucleotide repeat expansion in the euchromatin of S. cerevisiae grown at 23°C, 30°C, and 37°C. Our data show that temperature affects expansion of CTG trinucleotide repeats in S. cerevisiae. Using an ADE3/8 color assay and a disome strain of S. cerevisiae, we examined the impact of temperature on chromosome loss. Our data indicate a correlation between high temperature and chromosomal instability.
P1357  
**Board Number: B365**  
*Impact of ethanol on markers of heterochromatin maintenance and trinucleotide repeat expansion in* Saccharomyces cerevisiae.  
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Using three separate assays, we have examined ethanol’s impacts on gene silencing via the Telomere Position Effect (TPE), trinucleotide repeat expansion, and chromosomal stability in Saccharomyces cerevisiae. Using a cell viability assay, we examined how ethanol influences the repression of gene expression through TPE. Our results show that S. cerevisiae grown in 5% ethanol undergoes increased silencing of a gene placed proximal to a modified telomere. Expansion of trinucleotide repeats is known to be a cause of several serious human diseases, such as Huntington's Disease and Fragile X Syndrome. We investigated the effect of ethanol on the level of CTG trinucleotide repeat expansion in the euchromatin of S. cerevisiae. Our data indicate that the addition of ethanol to the growth medium increases levels of trinucleotide repeat expansion. Genomic instability is a major cause behind many illnesses, including cancer. Using the ADE3/8 color assay with a disomic strain of S. cerevisiae, we investigated ethanol’s impacts on chromosome loss. Our work shows that ethanol increases chromosomal stability in yeast.

P1358  
**Board Number: B366**  
*A single cell view of MYC's gene regulatory and oncogenic mechanism.*  
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We seek to determine how the MYC oncogenic transcription factor modulates stochastic gene expression in single living cells. Several models have recently emerged that question the prevailing view of MYC as a gene-specific transcription factor and instead envision its oncogenic mechanism as a global amplifier to elevate the existing gene expression program in a cell. We evaluated these competing paradigms of MYC’s function to shed light on its in-vivo oncogenic behavior by using single-molecule RNA fluorescence in-situ hybridization (smFISH) and live cell imaging of transcription. To simulate MYC’s oncogenic activity we overexpressed MYC in the U2-OS human osteosarcoma cell line. This cell line contains an exogenous β-globin reporter gene, transcription of which is visualized in live cells by fluorescently tagging the RNA with the MS2-PP7 stem loop system. Our results show that MYC overexpression doubles the population β-globin transcript levels and increases the duration and magnitude of live transcription events. To our knowledge this is the first living, single cell evidence to suggest that MYC's mechanism of action may be extend the duration and initiation rate of active gene periods.

As a way to directly assess the effect of the MYC protein on gene expression, we have also engineered a photo-inducible version of MYC (Pi-MYC) to achieve spatial and temporal control of the transcription factor within living cells. We report the first characterization of Pi-MYC, which translocates to and from cell nuclei within 8 minutes of light induction/removal, faster than any existing translocation-inducible MYC. This allows us to directly measure the way in which a transcription factor modifies the kinetic steps of transcription in living cells.

Sunday-231
Regulatory and Noncoding RNAs

P1359

Board Number: B367

Role of microRNAs in brain tumors as diagnostic and prognostic markers.
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Fifteen percent of all primary brain tumors (BT) are Glioblastoma Multiforme (GBM). GBM is the most malignant type of BT; having an unknown cause. It is a type of astrocytoma that arises from astrocytes and supportive brain tissue primarily located in the cerebral hemisphere, spinal cord or brain. Classified from grade I-IV histologically by the World Health Organization (WHO) combining the type of tumor with the assignment of a defined malignancy grade; Grade IV is known as GBM. Currently, most cancers diagnosis are determined by histological examination of the affected tissue obtained by biopsy or surgical removal. These are expensive procedures that can result in risk to patients and required consistent evaluation. Thus giving rise to an interest in the field of circulating nucleic acids as potential biomarkers. Blood-based testing has the ability to carry out screening and repeated sampling in patients undergoing therapy for monitoring the disease. Previous studies have suggested the use of microRNAs as prognostic and diagnostic markers. MicroRNAs are small non-coding RNAs that bind to 3′-untranslated (UTR) regions of target messenger RNAs to regulate protein synthesis. It is relevant the need to find a better and more economic diagnostic model. If a correlation of microRNAs expression can be found between the endogenous control group of microRNAs and the experimental group in symptomatic and asymptomatic plasma and saliva samples from GBM patients, then a technique can be developed for diagnostic and prognostic of GBM, finding a better and cheaper technique that can make the disease diagnostic much easier, assigning a prognosis to each patient. The expression of microRNA-27a, microRNA-1225-5p, microRNA-143, microRNA-92b, and microRNA-27b were evaluated in plasma samples by Real-Time PCR (qPCR) using microRNA-16 as endogenous control. Mann-Whitney statistical test showed significance in microRNAs relative expression when comparing symptomatic and asymptomatic plasma samples for miRNA-92b.

P1360

Board Number: B368

Regulation of proinflammatory chemokine CXCL5 by microRNA hsa-miR-605.
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Complex regional pain syndrome (CRPS) is a disease characterized by chronic pain and inflammation. Recent studies on CRPS patients have sought to identify biomarkers that can assist physicians in diagnosis and serve as therapeutic targets. MicroRNAs (miRNAs) are small noncoding RNAs that can negatively regulate gene expression by binding to the 3′ untranslated region (3′UTR) of mRNAs. Our previous studies investigating circulating miRNAs in blood from patients with CRPS found a 22-fold downregulation of hsa-miR-605 in poor responders relative to responders prior to ketamine treatment. Here we investigated the functional significance of miR-605 downregulation. This miRNA is predicted to target CXCL5, a proinflammatory chemokine involved in the recruitment and activation of neutrophils.
We hypothesized that the reduced expression of miR-605 in poor responders could contribute to an increase in CXCL5 expression. We confirmed miR-605 binding to CXCL5 3’UTR and an upregulation of CXCL5 mRNA in poor responders relative to responders in whole blood from CRPS patients. We then investigated whether overexpression of miR-605 can decrease endogenous levels of CXCL5 in multiple cell lines. While there was no significant effect in primary endothelial cells, we found a small but consistent decrease of CXCL5 mRNA in miR-605 transfected neuroblastoma cells. Conversely, downregulation of miR-605 in primary endothelial cells resulted in significant upregulation of CXCL5 mRNA. Efforts are underway to determine if these miR-605 induced changes in CXCL5 mRNA are reflected at the protein level, and function as determined by neutrophil migration in vitro. Thus, investigating target mRNAs of differentially expressed miRNAs can provide important insights on aberrant gene expression that contributes to disease pathology.

P1361
Board Number: B369
Identification of growth-suppressive microRNAs that regulate genes involved in cell cycle progression and apoptosis by miRNA library screening.
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Micro(mi)RNAs are small non-coding regulatory RNA molecules, that have a wide impact on the regulation of gene expression. Considerable evidence demonstrates that miRNAs are involved in a variety of fundamental biological processes including cell proliferation, apoptosis, and differentiation. In cancer, miRNAs can function as oncogenes or tumor suppressors to either promote or protect against cancer, respectively. In this study, a screen was performed using 300 miRNA mimics to identify miRNAs that downregulate A549 tumor cell growth. Among 300 miRNAs, we selected miR-28-5p, -323-5p, -510-5p, -552-3p, and -608 that were the most effective in downregulating cell growth. As revealed by flow cytometry, overexpressing miR-28-5p, -323-5p, and-510-5p induced G1 arrest, whereas that of miR-608 induced cell death in a caspase-dependent manner. Quantitative real-time PCR and western blot analyses demonstrated that B cell lymphoma 2-like 1 (BCL2L1), D-type cyclin 1 (CCND1), CCND3, cytochrome b5 reductase 3 (CYB5R3), phosphoinositide 3-kinase regulatory subunit 2 (PIK3R2), specificity protein 1 (SP1), and phosphorylated Akt were all downregulated in miR-608-transfected cells, while Bcl-2-interacting killer (BIK) was upregulated. Moreover, in an NCI-H460 xenograft model, miR-608 was determined to have a suppressive function on tumor growth.

P1362
Board Number: B370
Nascent companions of Hobbit.
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Hox genes are a group of highly conserved transcription factors that regulate anterior-posterior patterning in developing embryos. In mammals there are four clusters (A through D) that contain a total of 39 Hox genes. These genes have to be tightly regulated such that their spatio-temporal expression profile and consequently regulation of their downstream effectors is properly executed. This occurs in part due to enhancers acting both locally and long-range within and around the Hox clusters. Additional transcriptional complexity arises due to the presence of long non-coding RNAs (IncRNAs) that are
embedded within and around the Hox coding regions. lncRNAs have been shown to be linked to rapid epigenetic changes in chromatin states within the Hox clusters. Therefore, it is possible that regulation of lncRNA transcripts may be have an additional input into regulating adjacent coding regions in the Hox clusters during development and organogenesis. For this project, the HoxB cluster is being studied as a model to better understand the regulatory interplay between coding and noncoding transcripts during neural differentiation of mouse embryonic stem cells (mESCs). Interspersed in the region in and around Hoxb4 and Hoxb5 are 3 retinoid dependent enhancer elements and 3 lncRNAs. To understand the interplay between the enhancers and promoters and the temporal order of expression of transcription we are employing single molecule Flourescent in-situ Hybridization (smFISH) approaches. We labelled the Hoxb4 gene and the lncRNAs (Hobbit, HoxBlinclnc, etc) and then probed the mESCs programmed for neural differentiation by Retinoic Acid (RA) treatment. The probe sets were visualized and then each probe set was quantified for the presence of nascent transcripts in the nucleus and the mature single transcripts in the cytoplasm. Our results show that Hobbit and Hoxb4 are activated similarly during neural differentiation. In contrast, HoxBlinclnc is expressed in uninduced mESCs and appears to decrease with neural differentiation. Through this approach, we identified populations of cells with distinct transcription profiles that provide insight into how transcription of the Hoxb4 gene is dynamically correlated with the expression of the lncRNAs, Hobbit and HoxBlinclnc. This approach is allowing us to dissect the order of the regulatory events in the middle of HoxB cluster and to explore long range effects of enhancers on multiple genes.

P1363
Board Number: B371
Nonstop decay in C. elegans: examining a possible role for small noncoding RNAs.
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In C. elegans, small noncoding RNAs known as 22G RNAs are synthesized by RNA-dependent RNA polymerases and target thousands of protein-coding mRNAs, repetitive sequences, and cryptic transcripts throughout the genome. In some cases, 22G RNA synthesis appears to be triggered as a secondary event downstream of targeting by C. elegans piRNAs (known as 21U RNAs) in a pathway that defends against non-self RNA species. However, the synthesis of many 22G RNAs is unaffected by loss of 21U RNAs, and the triggers for biosynthesis of these 21U-independent 22Gs remain elusive. Using a comparative genomic approach in geographically distributed wild isolates of C. elegans, we have identified a locus (F43E2.6) at which loss of the stop codon is correlated with increased production of 22G RNAs. The F43 mRNA has a short 3’UTR with no further in frame stop codons, raising the intriguing possibility that 22G RNAs could be involved in a nonstop decay pathway in C. elegans. F43 is also targeted by an unusual Dicer product; however this Dicer product does not appear to act as a primary siRNA to trigger F43 22G RNAs. First, the Dicer product is expressed equally in strains with large differences (more than 10 fold) in 22G RNA levels. Second, this product is expressed only during embryogenesis, whereas F43 22G RNAs are expressed most strongly in adults. Third, F43 22G RNAs are genetically independent of the Dicer pathway. We have demonstrated that the 22G RNA expression phenotype at the F43 locus is genetically linked to the locus itself, and are currently generating worms carrying CRISPR-edited F43 loci that bear or lack a stop codon to determine whether the loss of a stop codon is indeed causal for increased 22G RNA production. In addition, we are sequencing small RNAs from additional nonstop and nonsense alleles to ask whether this phenomenon is general, and are using
a genetic approach to ask whether 22G RNAs function in a pathway with the known effectors of nonstop decay *dom-34* and *hbs-1*.

**P1364**
**Board Number: B372**
**The Role of Protein Arginine Methylation in the Repression of tRNA Biogenesis under Stress.**
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Under favorable conditions, robust tRNA biogenesis by RNA polymerase III (Pol III) is responsible for balancing a cell’s protein synthetic capacity with its growth requirement. As such, regulation of transcription by Pol III is closely linked to a cell’s metabolic activity and Pol III transcription needs to be tightly controlled, especially under stress. Previously, data from our high-resolution genome-wide localization analysis revealed an enrichment of the budding yeast *Saccharomyces cerevisiae* type-I protein arginine methyltransferase, Hmt1, at tRNA genes and mutants lacking Hmt1 display aberrant levels of tRNA (Milliman et al., 2012). In our current study, we have identified a role for Hmt1 in facilitating proper repression of Pol III-mediated transcription under stress. We show that association of Hmt1 to the tRNA genes is dependent on Pol III-transcribed gene activity and we have identified a subunit of Pol III as a substrate of Hmt1. Using a non-methylatable mutant of this subunit, we further demonstrate a role for this methylation in promoting optimal repression of tRNA biogenesis under stress. Lastly, we demonstrate the conservation of this modification in the human homolog of the Pol III subunit. Taken together, our data indicate a novel role for protein arginine methylation in the repression of Pol III-mediated tRNA biogenesis during stress and this role is likely to be evolutionarily conserved.

**P1365**
**Board Number: B373**
**MyoD Enhancer RNA in Gene Regulation.**
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Adult stem cells (satellite cells, SCs) in skeletal muscle play significant roles in muscle regeneration and represent important targets for the treatment of muscular dystrophies. Myogenesis, the great ability of SCs to differentiate to skeletal muscle, is carried out by the activity of a series of transcription factors, including MyoD and Myogenin (MyoG), is. The gene expression of the muscle-specific master regulator MYOD1 is modulated by one of its own enhancer, distal regulatory regions (DRR). Enhancer RNAs (eRNAs) are a novel class of noncoding RNAs (ncRNA) expressed from active enhancers in the tissue-specific manner. DRR enhancer element is extensive occupied by its protein MyoD and transcribed into RNA species. This RNA transcripts corresponding to DRR enhancer (DRRRNA) upregulates downstream Myogenin gene. RNA fluorescence in situ hybridization imaged the cellular distribution and dynamic interaction of endogenous DRRRNA and nascent Myogenin RNA. In addition, chromatin isolation by RNA purification reveals that DRRRNA is engaged at the Myogenin locus. RNA pull down coupled to mass spectrometry found that the components of chromatin structure regulator cohesion, SMC3 and NIPBL, are identified as DRRRNA interactors. Similar to DRRRNA, knockdown of either DRRRNA associated–protein SMC3 or NIPBL disrupts myogenesis. Altogether these findings display a functional role of eRNAs in gene regulation by trans-acting. The mechanism by which distal eRNA affects specific gene expression is through organizing chromatin architecture. Insights of eRNA function are expected to advance our
understanding of muscle cell-type-specific transcriptional circuitry and contribute towards a cell-based therapeutic approach for muscle diseases.

**P1366**
**Board Number: B374**
*Repeat E anchors Xist RNA to the inactive X chromosome compartment through CDKN1A-interacting protein (CIZ1).*

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X chromosome inactivation is an epigenetic dosage compensation mechanism in female mammals driven by the nuclear long non-coding RNA, Xist. While recent genomic and proteomic approaches have provided a more global view of Xist’s function, how Xist RNA localizes to the inactive X-chromosome (Xi) and spreads in cis remains unclear. Here, we report that the CDKN1-interacting protein, CIZ1, is critical for localization of Xist RNA to the Xi chromosome territory. Stochastic optical reconstruction microscopy (STORM) shows a tight association of CIZ1 with Xist RNA at a single molecule level. CIZ1 interacts with a specific region within Xist exon 7, namely the highly repetitive repeat E motif. Using genetic analysis, we show that loss of CIZ1 and Repeat E phenocopy each other in female cells. In both cases, Xist RNA delocalizes from the Xi and disperses into the nucleoplasm. Interestingly, the Xi is exquisitely sensitive to CIZ1 levels, as either increasing or decreasing CIZ1 levels results in delocalization. This delocalization is accompanied by a decrease in H3K27me3 on the Xi. These data reveal that CIZ1 plays a major role in ensuring stable association of Xist RNA with the Xi.

**P1367**
**Board Number: B375**
The *R1* retrotransposon in *Drosophila* rDNA is transcribed by RNA Pol I upon heat shock.

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The ribosomal DNA of *Drosophila melanogaster* resides within centromere-proximal nucleolar organizers on both the X and Y chromosomes. Each locus contains approximately 200 tandem rDNA genes that encode 18S, 5.8S, and 28S rRNAs for ribosome biogenesis. Spacer sequences ITS1 and ITS2 located within these rDNA units can be used as markers for pre-ribosomal RNA transcription, but do not contribute to ribosome formation. In arthropods like *Drosophila*, some rDNA units are inserted with R1 or R2 retrotransposons at specific sites within the 28S regions. These units cannot produce functional 28S rRNA and hence do not contribute to the formation of functional ribosomes. It is not yet clear whether R1 has its own promoter, but R2 is co-transcribed with rRNA from the rDNA promoter. Previous data from our lab show that R2 transcription is elevated upon nucleolar stress caused by the *Nopp140* gene deletion as compared to wild-type. To further assess whether R1 and R2 are stress induced retrotransposons, we treated wild type third instar larvae with heat shock. R1 expression was induced upon heat shock as measured by semi-quantitative RT-PCR and qPCR. Expression of R2 and the *copia* retrotransposon, however, did not change upon heat shock. Likewise, transcription of rDNA, as measured by the expression of ITS1 or ITS2, remained unchanged. To determine which RNA polymerase is responsible for R1 expression upon heat shock, we inhibited RNA Pol I transcription in S2 culture cells using a low dose of Actinomycin D or with CX5461. The observed downregulation of R1 expression upon Actinomycin D or CX5461 treatments indicated that R1 is co-transcribed by Pol I along with the rest of
the rDNA. To check for involvement of RNA Pol II, we inhibited Pol II transcription with α-amanitin. Even after inhibition of Pol II, R1 expression was upregulated upon heat shock. This suggests that under heat shock conditions R1 is transcribed by Pol I. Heat shock induced R1 transcription was also assessed in a genome wide PRO-Seq data set submitted to NCBI-GEO. We analyzed R1 and rDNA expression in this data set. Using computational analysis, we compared R1 and rDNA expression patterns upon RNAi mediated depletion of the Heat Shock Factor (HSF) with and without heat shock. Heat shock induced expression of R1 was reduced in the absence of HSF, suggesting HSF plays an active role in R1 induction upon heat shock. Although R1 and R2 elements insert within the rDNA and both are transcribed by Pol I, our observations suggest that they behave differently in their regulated expression. R1 and R2 elements are endogenous markers for generally silent rDNA genes. Any increase in their expression rates would indicate changes in nucleolar chromatin structure.

P1368
Board Number: B376
Optical trapping studies of glmS ribozyme riboswitch folding and catalysis.
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In many Gram-positive bacteria, expression of glucosamine-6-phosphate synthase (GlmS) is regulated by the glmS riboswitch. This riboswitch, a regulatory element found in the 5’ UTR of the glmS mRNA, controls gene expression by an unusual mechanism: the element folds to form a self-cleaving ribozyme. The self-cleavage reaction, in turn, is activated by the GlmS enzymatic product and cell wall precursor, glucosamine-6-phosphate (GlcN6P), which serves as a cofactor for the ribozyme. Self-cleavage of the riboswitch targets the entire glmS mRNA for degradation by intracellular RNases, thereby downregulating GlmS expression. We used optical tweezers to investigate folding and catalysis of the ribozyme core domain, a minimal version that retains GlcN6P-dependent self-cleavage activity. Measurements of the folding dynamics revealed a series of distinct intermediate states. By applying controlled optical loads and scoring the transition probabilities among these states as a function of force, we determined the free energy landscape of ribozyme formation, from the fully unfolded state to the fully folded state. Single-molecule measurements of self-cleavage in optically trapped ribozymes revealed that the fully folded state was catalytically active in a GlcN6P-dependent manner. The self-cleavage rate was also load-dependent, decaying in sigmoidal fashion with increasing force. Combined, results from our measurements of glmS ribozyme core folding and self-cleavage activity shed light on how folding of specific structural features relates to catalytic function.

Post-Transcription Gene Regulation

P1369
Board Number: B377
Multiple mechanisms coordinately drive RNA-binding protein localization to RNA granules.
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Protein activity is intrinsically tied to its location in a cell. Many RNA-binding proteins are active when clustered in non-membrane bound RNA granules. One example of RNA granules is the germ granules of the nematode C. elegans, which are required for reproductive cell development and fertility.
binding protein FBF-2 requires localization to the germ granules in order for function. In addition to
germ granules, germ cells contain a variety of other RNA granules. Yet, the mechanisms that direct FBF-2
partitioning between multiple subtypes of RNA granules are unclear. RNA granule assembly is thought to
rely on multiple molecular interactions including protein binding to RNA and protein-protein
interactions mediated either by small linear peptide motifs (SLiMs) or intrinsically disordered regions
(IDRs). RNA-binding protein FBF-2 has both IDRs and SLiMs. Which of these are important for the in vivo
localization of FBF-2?
Localization and function of FBF-2 depends on its association with a small protein DLC-1 (Wang et al.,
2016). DLC-1/LC8 family members promote assembly of protein complexes by interacting with SLiMs in
their binding partners. We found three DLC-1-interacting SLiMs in FBF-2, located in regions of predicted
disorder (IDRs). To test whether SLiMs are required for FBF-2 localization to RNA granules, we mutated
or deleted all three SLiMs and generated an FBF-2 rm variant that fails to interact with DLC-1. FBF-2 rm
did not localize to germ granules when expressed in the worm. Instead, FBF-2 rm was found in distinct
cytoplasmic clusters. Surprisingly, FBF-2 rm enrichment in clusters was also reduced compared to
enrichment of wild type FBF-2 in germ granules.
Prior studies suggested that SLiM multivalency (multiple SLiMs per protein) is instrumental for protein
clustering. To test whether a single SLiM is sufficient for FBF-2 localization to the germ granules, we
generated a transgene with a single DLC-1-binding SLiM. We found an intermediate localization pattern,
where the mutant protein only partially overlapped germ granules.
These data suggest that SLiMs are important for both effective protein clustering in the cytoplasm and
for specific localization to appropriate RNA granules (germ granules) even in the presence of IDRs and RNA-binding activity. Coordinate activity of multiple RNA granule assembly mechanisms is thus required
for regulation of RNA-binding protein localization in vivo.

P1370
Board Number: B378
Single-Molecule Imaging Reveals Dynamic Biphasic Partition of the RNA-Binding proteins G3BP1
and IMP1 in Stress Granules of Living Neuronal Cells.
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Maintenance of cellular polarity as well as fast response upon extracellular cues requires fast and tightly
regulated expression of proteins, especially in morphologically complex cells like neurons.
Ribonucleoprotein (RNP) granules contribute to the regulation of gene expression via posttranscriptional
coordination of mRNA translation, localization and degradation. These self-assembling structures lack a
membrane and can be considered as dynamic microcompartments.
The Ras GTPase activating protein SH3 domain binding protein 1 (G3BP1) and the Insulin like growth
factor II mRNA binding protein 1 (IMP1) are two paradigmatic RNA-binding proteins (RBPs) that are
present in cellular stress granules (SGs). Previous studies have shown that these two proteins colocalize
in SGs and that overexpression of either of them is sufficient to induce SG formation (Moschner et al.,
2014). According to in vitro experiments, SGs are formed by liquid-liquid phase separation (LLPS) and
remain amorphous, while being held together by surface tension. However, the dynamic organization of
SGs and their components with single-molecule resolution and, hence, their behavior remain unclear.
To follow the distribution and dynamics of G3BP1 and IMP1 in stressed neuronal cells, we performed
fluorescence decay after photoactivation (FDAP) measurements and single-molecule localization
microscopy (SMLM) of fluorescence-tagged proteins. The complementary approaches allow for
comparing the dynamics of protein exchange between SGs and the local dynamics of protein diffusion and interaction within SGs.

Our data show that IMP1 is largely constrained to SGs, while G3BP1 dynamically shuttles in and out of them. Within SGs on the other hand, both proteins exhibit alternating binding and diffusion with very similar characteristics. Quantitative analyses of the diffusion parameters confirm liquid-droplet like properties of SGs but also reveal remarkably immobile binding sites, which are transiently occupied by both RBPs and which we termed “nanocores”. In conjunction with the very different exchange kinetics observed by FDP, our studies demonstrate an unexpected disconnect between SGs partitioning and internal diffusion and interaction dynamics of G3BP1 and IMP1.


P1371
Board Number: B379
Identification of Rbfox associated proteins in striated muscles by BioID.
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Rbfox1 and Rbfox2, sequence-specific RNA binding proteins, regulate alternative splicing of a variety of transcripts essential for cardiac and skeletal muscle development. Our lab has previously reported that both Rbfox1/2 undergo tissue-specific alternative splicing and produce multiple isoforms specific to brain, heart and skeletal muscle. However, the functional role of Rbfox isoforms and the associated protein interaction remains to be elucidated. Here, we used a proximity-labeling proteomics approach called BioID (Proximity-dependent Biotin identification) to identify the protein interaction network of individual Rbfox1/2 isoforms. This technique is particularly useful in identifying weak or transient interactions in living cells that are not detected by Co-immunoprecipitation (Co-IP) or yeast two-hybrid systems.

We started our experiments using the C2C12 mouse myoblast cell line as a model since C2C12 cells can be differentiated into myotubes in culture, similar to primary myoblasts. We first used immunofluorescence staining to compare the subcellular localizations of the BioID-Rbfox1/2 with the native protein. The results demonstrate that the fusion protein’s subcellular localization is not altered in comparison to native Rbfox1/2 isoforms. Additionally, all the BioID-Rbfox1/2 isoforms with the RNA recognition motif exhibited similar splicing activities as native proteins. Next, we tested the biotinylation of endogenous proteins in C2C12 cells expressing BioID-Rbfox1/2 isoforms, either in the presence or absence of exogenous biotin, using Western blots probed with Streptavidin- Alexa Fluor 700 conjugate. The results indicate that in the presence of exogenously added biotin, BioID-Rbfox1/2 isoforms strongly stimulate biotinylation of a wide range of endogenous proteins. These biotinylated proteins reside in the same cellular compartment and colocalize with BioID-Rbfox1/2 isoforms by immunofluorescence. These results indicate that BioID-Rbfox1/2 isoforms can be targeted to proper subcellular locations without affecting their activities and can biotinylate endogenous proteins in a proximity-dependent manner.

Next, we used affinity capture to isolate the biotinylated proteins in undifferentiated and differentiated C2C12 cells expressing individual BioID-Rbfox1/2 isoforms and identified them by mass spectrometry for functional characterization. Notably, BioID-Rbfox2 isoforms yielded more than 100 high confidence protein interactions including known interactors of the Rbfox2. The identified protein interactors are directly related to mRNA metabolic processes and post-transcriptional regulation of gene expression.
RNA modifications have long been known to play important structural and functional roles in noncoding RNAs. More recently, however, technological advances in next generation sequencing and mass spectrometry have revealed the distributions of chemical modifications on mRNA transcripts as well. The most abundant internal mRNA modification, $N^\delta$-methyladenosine (m$^\delta$A), is linked to the regulation of mRNA stability, splicing, localization, and translation. However, the mechanisms by which mRNA modifications influence cellular processes remain largely mysterious. Moreover, numerous other modifications have been found on mRNA about which we have an even more limited understanding. We recently reported that $N^\gamma$-methyladenosine (m$^\gamma$A), previously characterized in tRNA and rRNA, occurs on thousands of transcripts in eukaryotic cells. m$^\gamma$A is chemically distinct from m$^\delta$A, carrying a positive charge that can have dramatic effects on RNA structure and folding. m$^\delta$A is also distinct in its distribution, and is enriched around the first splice site. It is found primarily in more structured regions, is dynamic in response to stress, and correlates positively with protein level. While these characteristics suggest that m$^\delta$A has cell biological functions that differ from m$^\gamma$A, many questions remain unanswered. Current work is focused on identifying the machinery that regulates m$^\delta$A installation and understanding how methylation influences transcript fate.

The eukaryotic-specific Rpl22 (eRpl22) family in Drosophila melanogaster includes duplicated ribosomal protein (RP) genes eRpl22 and eRpl22-like that are differentially expressed: eRpl22 is expressed ubiquitously and eRpl22-like expression is tissue-restricted with highest levels in the male germline. Co-expression of both paralogues in the male germline is suggestive of specialized functions for paralogue-specific ribosomes within maturing sperm. Compelling evidence for ribosome heterogeneity at the level of core RPs affecting mRNA translation has recently been shown (Shi et al., 2017). We have proposed that each paralogue is incorporated into ribosomes in early mitotic stages of spermatogenesis. At later stages, however, the role of eRpl22 may change, as the paralogue undergoes testis-specific SUMOylation and phosphorylation, and is sequestered in the nucleoplasm of meiotic spermatocytes (Kearse et al., 2013). Numerous mRNAs transcribed in primary spermatocytes remain translationally inert until later stages. Activation of previously silenced mRNAs as well as translation of mRNAs transcribed in post-meiotic mid to late elongating spermatids requires ribosome activity at stages of spermiogenesis well after meiosis is completed. Based on this temporal cascade of gene expression throughout spermatogenesis, we have proposed that paralogue-specific ribosomes specify a unique translome required for sperm production, with eRpl22-like ribosomes likely translating most testis-specific mRNAs synthesized. To test this hypothesis, we performed RNAseq on testis eRpl22- and eRpl22-like-specific ribosomes, captured by immunoprecipitation using paralogue-specific antibodies from ribosome profiles (polysome fractions). Our preliminary analysis of >1500 RNAs from ~12,000
ribosome-associated RNAs shows distinct classes of mRNAs translated on paralogue-specific ribosomes and numerous non-coding RNAs. mRNAs specifying constitutively expressed genes involved in DNA maintenance and transcription are commonly enriched on eRpl22 ribosomes. Transcripts known to be expressed in early mitotic stages, such genes involved in regulating mitotic nuclear division, are found on both ribosome types, with a slight bias toward eRpl22 ribosomes. Most meiotic and later stage testis-specific transcripts are enriched on eRpl22-like ribosomes. Overall, our RNAseq data are congruent with the proposal that eRpl22 paralogues translate different classes of testis mRNAs. Ongoing experiments include the use of computational tools to determine if conserved mRNA sequences are required for paralogue-specific translation, and the use of stable S2 cell lines transfected with eRpl22-like to explore translation requirements for testis-specific mRNAs.

P1374
Board Number: B382
Understanding the endogenous regulation of Ataxin-1 in SCA-1.
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Spinocerebellar ataxia type-1 (SCA-1) is an autosomal dominantly inherited, progressive neurodegenerative disease caused by the abnormal expansion of a ‘CAG’ tri-nucleotide repeat within the coding region of the ATXN1 gene. The CAG expansion is translated into a polyglutamine (PolyQ) tract in the N-terminus of the Ataxin-1 protein. Mutant Ataxin-1 expression is necessary for both the onset and progression of this currently untreatable disease. Novel therapeutic strategies such as RNAi that down regulate overall Ataxin-1 protein levels have shown to mitigate SCA-1 associated phenotype in the mouse models of the disease. Thus, understanding the endogenous cellular mechanisms that regulate Ataxin-1 expression, and how this regulation may be disrupted in SCA-1, is key to the development of novel, clinically relevant therapeutics. Here we seek to better define the post-transcriptional regulation of ATXN1 by evaluating the role that the 5'UTR of ATXN1 plays on the expression of Ataxin-1, in the context of both the WT and the CAG-expanded transcript. In order to determine if the 5'UTR ATXN1 can independently regulate Ataxin-1, we have designed highly versatile reporter-based assays. In preliminary studies, we find that the 5'UTR ATXN1 sequence exerts a strong regulation of protein levels (GFP reporter gene). Analysis of steady-state RNA levels by quantitative PCR appear to indicate decrease in RNA levels. Since cellular post-transcriptional gene regulation is mainly exerted by altering mRNA stability and/or translational efficiency, we have designed studies to investigate the role the 5’UTR of ATXN1 plays in regulating ATXN1 mRNA stability and translational efficiency.

P1375
Board Number: B383
Alterations in S-adenosylmethionine synthesis regulate stress granule assembly and composition.
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While regulation of RNA metabolism and metabolism are two of the most ancient functions of the cell, there have been relatively few studies into crosstalk between these two regulatory networks. We have recently completed a visual screen of all 440 metabolic enzymes in the S. cerevisiae GFP strain collection to identify metabolic enzymes that assemble into novel structures. This screen identified 17 metabolic enzymes that are localized to stress granules, which are sites of post-transcriptional gene regulation.
This localization does not appear to be used for substrate channeling since the enzymes that are recruited to the stress granules are not in consecutive steps of a biosynthetic pathway. As a result, we examined the possibility that stress granule composition and assembly might be controlled by specific metabolic states and metabolites.

We have found that while many types of stress cause RNA granules to form, only chronic nutrient stress causes metabolic enzymes to be recruited to stress granules. In order to determine whether the products or intermediates of specific metabolic pathways regulate stress granule assembly, we focused on methionine/S-adenosylmethionine biosynthesis since several of the enzymes in that pathway are recruited to stress granules. Interestingly, mutations in this pathway that decrease levels of S-adenosylmethionine (AdoMet) triggered stress granule assembly. In contrast, mutations that increased AdoMet levels blocked stress granule assembly. Further analysis revealed that this assembly defect is specific to the proteins that act at the 5’ end of transcripts arguing that AdoMet acts at a specific step in stress granule assembly. Interestingly, elevated AdoMet only affects the assembly of stress granules that are formed in response to chronic nutrient stress. Thus, chronic nutrient stress triggers the assembly of stress granules that 1) recruit components of the methionine/S-adenosylmethionine biosynthetic pathway and 2) that are regulated by the products of that pathway. This argues that stress granule assembly is differentially regulated in response to distinct metabolic stresses. Furthermore, the role of this metabolic pathway appears to be evolutionarily conserved since the human S-adenosylmethionine synthetase also forms foci in yeast in response to chronic nutrient stress. The targeting of metabolic pathways to sites of post-transcriptional gene regulation presents a new opportunity for understanding how crosstalk between distinct regulatory networks is controlled.

P1376
Board Number: B384
Zthi2 and ZTHI3: Prospects in Breeding and Genetic Engineering for Thiamine Biosynthesis and Accumulation.
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Tropical Zea mays contain significant levels of thiamine, a critical vitamin required for growth and development in living organisms. Thiamine biosynthesis has been studied in several organisms including Arabidopsis, Zea mays and Escherichia coli. The genes involved in Zea mays are Z-thi1 and Z-thi2, ZTHIC, ZTHI3, Ztpk1 and Ztpk2 which code for thiazole synthase, pyrimidine synthase, pyrimidine-phosphate kinase/thiamine mono-phosphate synthase (TMPS) and thiamine pyrophosphokinase respectively. Therefore, it is necessary to determine interrelatiprorgammes.onships that may exist between these bio-synthetic genes in relation to metabolite contents and enzyme activities in Zea mays varieties for thiamine breeding programme. Forty-one Zea mays inbred lines were used in this study and a combination of biochemical and molecular biology techniques (HPLC and spectrophotometry qRT-PCR, SDS-PAGE, Western blotting and sequencing) were employed. We report a significant positive correlation between Zthi2 expression and seed thiamine accumulation thus making it a “candidate” gene for seed thiamine bio-accumulation in Zea mays. The ZTHI3 gene had the highest expression (2.13 - 4.23) but its corresponding enzyme, TMPS, had the least activity (0.080 - 1.424 pmol. mg⁻¹ protein.min⁻¹). Western blot analysis detected the presence of both active di-meric (95 kDa) and inactive mono-meric (55 kDa) forms of the TMPS protein with a greater accumulation of the 55 kDa inactive monomer suggesting a post translational mechanism of regulating its activity. Our results further suggest that TMPS is a rate limiting enzyme in the thiamine bio-synthetic pathway. Thus, making
the Zthi2 and ZTHI3 genes target genes for thiamine bio-accumulation studies and breeding programmes

**P1377**

**Board Number: B385**

**Translational regulation of DEAD-box helicase Ded1/DDX3 medulloblastoma mutations.**

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Alterations in translation regulation have emerged as major contributors to cancer progression, but in many cases the molecular pathology remains unclear. Multiple concurrent genome-wide studies of the most common pediatric malignant brain tumor, medulloblastoma, identified the DEAD-box helicase named DDX3 as a highly mutated gene within multiple subtypes of this disease. Although the overall survival rate is 70-80%, the current treatment protocol for medulloblastoma causes serious long-term cognitive and endocrine side-effects, thus a better understanding of the molecular mechanisms is needed to design effective targeted therapies. DEAD-box proteins are the largest family of RNA helicases, and they have critical roles in multiple aspects of gene expression and RNA metabolism, orchestrating mRNA fate and cellular function. Proposed functions of DDX3 and its yeast ortholog Ded1 include activation and repression of translation; however, the cellular mechanisms are not fully defined. In order to examine the cellular defects caused by the medulloblastoma-associated mutations (mam) identified in DDX3, we made equivalent, conserved mutations in the yeast gene Ded1. Intriguingly, although ded1-mam cells exhibit growth defects, most do not display substantial defects in general translation. Alternatively, we have observed particular 5’ UTR structures of mRNA to be specifically affected in mutant cells using reporters containing stem loops. This may reflect changes in the ability of the ded1-mams to resolve mRNA secondary structures in the 5’ UTR during translation initiation and suggests that translation of a subset of mRNAs may be differentially affected in mutant cells. Quantitative reverse transcription PCR experiments will potentially verify whether specific genes either known to be dependent on Ded1 or contain a long 5’ UTR have altered translational status. We propose that these defects are essentially hypomorphic for DDX3/Ded1 cellular function, and the molecular mechanism will ultimately contribute to the pathology of medulloblastoma.

**Nuclear Lamina and Laminopathies**

**P1378**

**Board Number: B387**

**Mutant lamins alter genome integrity, proteostasis, and redox homeostasis in muscle disease.**

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Mutations in the human LMNA gene cause a wide spectrum of diseases that include three types of muscular dystrophy: congenital, Limb Girdle Type IB, and Emery-Dreifuss. There are currently no treatments for individuals with these types of muscular dystrophy. The development of treatments requires a thorough understanding of the pathogenic mechanisms caused by mutant lamins. To better understand the molecular basis of the muscle pathology, we generated genetically tractable Drosophila models of lamin-associated muscular dystrophy. Fruit flies with muscle-specific expression of mutant lamins develop muscular dystrophy, characterized by reduced motility and premature death. Depending on the specific mutation, diseased muscles can exhibit abnormally spaced nuclei, atypical chromatin
morphology, nuclear actin rods, increased DNA damage, altered proteostasis, and loss of redox homeostasis. Using human muscle biopsy tissue from individuals with mutations that correspond to the Drosophila models, we observed similar abnormal phenotypes. Therefore, we used Drosophila to genetically test candidate genes for modifier function. Muscle-specific expression of RNAi and over-expression transgenes encoding autophagy and redox regulators and members of the DNA damage response pathway suppressed mutant lamin-induced lethality. Our findings demonstrate that lamins play a vital role in maintaining muscle physiology and genome integrity and offer potential pharmacological targets for therapy.

P1379

**Board Number: B388**

**Myofibril contraction and cross-linking drive nuclear movement to the periphery of skeletal muscle.**

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Nuclear movements are important for multiple cellular functions and are driven by polarized forces generated by motor proteins and cytoskeleton. During skeletal myofiber formation or regeneration, nuclei move from the center to the periphery of the myofiber for proper muscle function. Centrally located nuclei are also found in different muscle disorders. Using theoretical and experimental approaches, we demonstrate that nuclear movement to the periphery of myofibers is mediated by centripetal forces around the nucleus. These forces arise from myofibril contraction and cross-linking that “zip” around the nucleus in combination with tight regulation of nuclear stiffness by lamin A/C. In addition, an Arp2/3 complex containing Arpc5L together with γ-actin is required to organize desmin to cross-link myofibrils for nuclear movement. Our work reveals that centripetal forces exerted by myofibrils squeeze the nucleus to the periphery of myofibers.

P1380

**Board Number: B389**

**Mechano-protection by lamin-A against DNA damage as the developing heart stiffens and strengthens.**

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Across all terminally differentiated adult tissues, the lamins are the main structural proteins of the nucleus that increase in A- to B-type stoichiometry (‘lamin-A:B’) when plotted versus collagen-I and tissue stiffness [Swift et al, 2013]. To understand mechanisms and functional contributions, beating hearts from chick embryos at different stages were characterized with various perturbations, revealing that lamin-A:B again scales with the progressive increase in collagen-I, and that lamin-A levels change
within ~1 hr when tissue is softened or stiffened by perturbing collagen or actomyosin contractility. Upon tissue softening, phosphorylation and solubilization of lamin-A increase to facilitate its degradation by constitutive matrix metalloproteinase 2 (MMP-2) within the nucleus. DNA damage decreases upon inhibition of actomyosin contractility and moreso if lamin-A is kept high by inhibiting its solubilization. Transcriptional control of lamin-A using retinoids likewise influences DNA damage levels. Isolated cardiomyocytes on collagen-coated soft or stiff gels show that lamin-A again increases monotonically versus matrix stiffness with regulation by phosphorylation and by MMP-2, while decoupling from rhythmic beating. Knockdown of lamin-A in iPS-derived cardiomyocytes on stiff matrices increases frequency of nuclear rupture, loss of DNA repair factors from the nucleus, and DNA damage, with rescue by actomyosin inhibition and by soft matrix. Lamin-A thus couples to actomyosin tension and matrix stiffness in order to minimize DNA damage.

**P1381**

**Board Number: B390**

**Exploring genomic reorganization during differentiation of emerin-null and EDMD mutant myogenic progenitors.**

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The human genome is contained within the nucleus. Genomic organization dynamically changes during development to regulate tissue development and tissue-specific functions. The nucleus is surrounded by a membrane composed of two lipid bilayers called the nuclear envelope. Many integral membrane proteins reside in the nuclear envelope. Recently, some of these nuclear envelope proteins were implicated in regulating genomic organization. Our lab focuses on one of these proteins, emerin, to study its function in the nuclear envelope. We are specifically focusing on how emerin regulates genomic organization and gene expression during development and skeletal muscle regeneration. Chromatin at the nuclear lamina at the nuclear envelope is associated with transcriptional repression. We predict association of repressed chromatin with the lamina is mediated through lamin-binding proteins at the nuclear envelope, including emerin. Supporting this hypothesis, we previously showed emerin was required for genomic organization of selected loci in myogenic progenitors. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) using lamin A antibodies will be performed in differentiating emerin-null and EDMD mutant myogenic progenitors to test how chromatin organization is affected by emerin loss or mutation. How genomic organization is altered in EDMD causing mutants will then be examined. ChIP-seq with emerin antibodies will also be done to test if the interaction of emerin with the genome is sequence-specific. Lamin A/C and B1 as well as emerin antibodies have been optimized for the ChIP-seq. This allows us to move on to ChIP-qPCR. We hypothesize altered genomic organization in EDMD causing mutants will cause significant changes in temporal expression of differentiation genes.
P1382  
Board Number: B391  
Expression profiling of differentiating myogenic progenitors lacking emerin or expressing EDMD-causing emerin mutants identifies molecular pathways responsible for their impaired differentiation.  
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Mutations in the gene encoding for emerin (EMD), an ubiquitously expressed inner nuclear membrane protein causes X-linked Emery-Dreifuss muscular dystrophy (EDMD), an inherited disorder causing progressive skeletal muscle wasting, irregular heart rhythms and contractures of major tendons. Impaired muscle regeneration is thought to be involved in the skeletal muscle defects seen in EDMD, but the underlying mechanisms remain poorly understood. To identify molecular pathways implicated in the EDMD mechanism, RNA sequencing was performed on differentiating wildtype and emerin-null myogenic progenitors. Our analysis revealed 340 genes were found to be uniquely misexpressed during the transition from day 0 to day 1 of differentiation in wildtype cells. 1,605 genes were found to be uniquely misexpressed in emerin-null cells during this transition. 1,706 genes were found to be shared among both wildtype and emerin-null cells during this transition. Ingenuity Pathway Analysis (IPA) was done on the transcripts uniquely misexpressed in emerin-null cells at this transition. IPA identified significant inhibition of the STAT3 pathway, TGF-β signaling pathway, VEGF signaling pathway and growth hormone signaling pathway. Functional enrichment analysis using IPA identified the significant inhibition of biological functions associated with the growth of muscle tissue and the myogenesis of skeletal muscle. Most disease-causing emerin mutations are nonsense mutations resulting in complete loss of emerin, there are four disease-causing EMD mutations that express emerin protein at near wildtype levels and localize correctly to the nuclear membrane. We hypothesize studying these emerin mutants will elucidate novel emerin functions important for the EDMD disease mechanism. We are currently generating myogenic progenitors (H2K cells) expressing wildtype emerin and each EDMD-causing emerin mutant in an emerin-null background using lentiviral transductions. We have achieved a selection efficiency of about 85% for emerin-null cells transduced with wildtype emerin and these transduced cells maintain their differentiation potential. Importantly, myotube formation and myoblast fusion were rescued in emerin-null cells transduced with wildtype emerin. RNA sequencing analysis will be performed during consecutive days in differentiation of EDMD-causing emerin mutants and wildtype emerin transduced emerin-null cells to identify important changes in the transcriptional program.

P1383  
Board Number: B392  
Nanoscale Nuclear Envelope Dynamics and Spatial Organization of the Muscular Dystrophy Protein Emerin.  
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Emery-Dreifuss Muscular Dystrophy (EDMD) is a laminopathy caused by mutations of emerin, an inner nuclear envelope protein that participates to nucleo-cytoskeleton mechanotransductions, maintenance of nuclear shape and nucleus stiffening against applied tensions. The mechanosensing functions of
emerin and the reasons why its mutations cause altered nuclear mechanics and muscle disease remain unclear. To address the molecular pathogenesis of EDMD at the nanoscale we used single molecule and three-dimensional superresolution optical microscopy in human cells. At the nuclear envelope, the diffusional mobility and the spatial organization of wild type (WT) emerin and a variety of clinically relevant emerin mutants were quantified by single particle tracking photo-activated localization microscopy (sptPALM) and direct stochastic optical reconstruction microscopy (dSTORM) imaging in rescued emerin-null cells. We identified different subpopulations of WT emerin associated with the endoplasmic reticulum, the outer or the inner nuclear envelope and show that it forms diffraction-limited nanoclusters at the nuclear membrane. We further show that the diffusion of emerin and its nanoscale clustering are directly impacted by mutations that cause EDMD and that a complex interplay between lamin A binding, actin binding and emerin/emerin interactions dictate the dynamics and the nanoscale spatial distribution of emerin at the nuclear envelope. To study how specific mechanical strains at the nuclear membrane influence the normal and pathogenic nanoscale organizations of emerin, we also developed a simple cell micropatterning strategy that provides control of the nucleus architecture and allows steady-state changes in the mechanical landscape of the nuclear envelope while permitting nanometer accuracy single molecule microscopy. Super-resolution imaging of emerin in nuclei subjected to varying mechanically strains reveals that the mechanotransducing functions of emerin are coupled to its clustering state and its nanoscale distributions within the inner nuclear envelope. Together, sptPALM, dSTORM superresolution imaging, emerin mutants and cell micropatterning reveal subtle biophysical properties of nuclear emerin that would otherwise be undetected by traditional diffraction-limited microscopy techniques and help elucidate key molecular mechanisms of EDMD.

P1384

Board Number: B393

Attractive and repulsive nuclear interactions are regulated by distinct genes linked to Emery-Dreifuss Muscular Dystrophy and Centronuclear Myopathy.

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Syncitia, cells with multiple nuclei, are less prevalent than their mononucleated counterparts. However, there are many examples of syncitia in biology. The developing Drosophila blastoderm and skeletal muscle are commonly studied examples as are less frequently studied examples such as placenta and osteoclasts. Yet little is known about how the presence of many nuclei in each of these cells impacts their specific biology. Therefore it is crucial to understand how and when each nucleus interacts with the other nuclei. In skeletal muscle, the rapid movement of myonuclei to the cell center to join already incorporated nuclei suggests attractive interactions. Conversely, there are repulsive interactions between nuclei later in muscle development, which keep nuclei spaced distant from one another. Myonuclei in Drosophila muscles undergo a similar set of dynamic movements during myogenesis. After fusion, nuclei separate into two clusters that move directionally toward opposite poles of the muscle. During directional movement, the nuclei remain in tightly associated clusters. Disruption of two different genes, bocksbeutel (dEmerin) and klarsicht (dNesprin), blocked the initial separation of nuclei into two distinct clusters. This clustering phenotype was similar to nuclear positioning defects caused by the disruption of ensconsin (dMAP7), a MT-associated protein that regulates nuclear movement via microtubules. In ens mutants, nuclei fail to separate into distinct clusters and remain associated together, indicating that the MT machinery responsible for separating nuclei was disrupted. However, live-embryo time-lapse microscopy of bocks depleted embryos demonstrated a nucleus could
occasionally escape the cluster and move directionally, indicating that the force generating machinery and spatial cues were functional. Thus, bocks and klar were necessary to regulate the repulsive interactions between nuclei. Conversely, disruption of Amphiphysin, inhibited the attractive interactions between nuclei. This was evident by the regular dissociation of nuclei from clusters and the presence nuclei in the center of the muscle. Together these data indicate that nuclei do exhibit both attractive and repulsive interactions in skeletal muscle that are regulated by distinct proteins at specific developmental times.

P1385

Board Number: B394

Bocksbeutel regulates nuclear positioning by a klarsicht-dependent mechanism.

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Nuclear movement is conserved throughout eukaryotes. Although some mechanisms that drive nuclear movement in diverse cell types have emerged, it is not clear how conserved these mechanisms are between different cell types. Furthermore, the mechanisms by which nuclear movement impacts cellular function are virtually unexplored. To answer these questions, we have focused on the mechanisms of nuclear movement in skeletal muscle. The high correlation between mispositioned nuclei and muscle disease suggests that nuclear position is a critical determinant of muscle function. Furthermore, the factors that have been identified as important for nuclear position in muscle overlap with the factors that move nuclei in most cell types. In these studies we examined genes linked to a specific disease, Emery-Dreifuss Muscular Dystrophy (EDMD), because the proteins encoded by the genes linked to EDMD localize to the nuclear envelope and regulate nuclear movement in other cell types. Although many of these genes have been implicated in nuclear position, the mechanisms by which they contribute to nuclear position are not known. To identify the mechanisms by which these genes regulate nuclear position in muscle, we have determined the network of genetic interactions between EDMD-linked genes that regulate nuclear position during muscle development in Drosophila. The most striking phenotype was the single-file arrangement of nuclei in a klarsicht (nesprin);bocksbeutel(emerin) double-heterozygote. Remarkably, although bocksbeutel and Otefin, another emerin ortholog in Drosophila, genetically interacted to regulate nuclear position, we detected no interaction between Otefin and klarsicht. Based on the genetic interaction between klarsicht and bocksbeutel, we hypothesized that bocksbeutel may affect klarsicht localization. Indeed, immunofluorescence experiments demonstrated that nuclear levels of klarsicht were reduced in bocksbeutel mutant animals compared to controls. Conversely, klarsicht levels were increased in Otefin mutant animals compared to controls. Measurement of klarsicht transcript levels mirrored these changes indicating that bocksbeutel is important for the expression of klarsicht whereas Otefin is necessary to repress klarsicht expression. Together these data suggest that bocksbeutel (Drosophila emerin) indirectly regulates nuclear position by its effects on klarsicht (Drosophila nesprin). Additionally, bocksbeutel-dependent regulation of klarsicht is opposed by the effects of another Drosophila emerin, Otefin.
P1386
Board Number: B395
Lamin A regulates the activity and dynamics of nucleoli.
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The nuclear lamina is an intermediate filament meshwork that underlies the nuclear envelope, extends through the nuclear volume, and interacts with the genome. Previous analyses have linked lamins to processes including transcription, replication, gene silencing, and cell migration. However, embryonic stem cells lacking all lamins can proliferate and differentiate1. This surprising finding underscores how little we understand about the essential functions of lamins. Mutations to lamins cause a wide spectrum of pathologies, including Hutchinson-Gilford progeria syndrome (HGPS). HGPS is a rare, fatal premature aging disorder caused by a mutation to lamin A that generates a truncated protein termed progerin. Using HGPS as a model system to probe lamin biology, we discovered that A-type lamins have a role in regulating the activity of nucleoli. Nucleoli are membraneless organelles within the nucleus that coalesce around actively transcribing ribosomal DNA (rDNA) loci. Nucleoli produce ribosomal RNA, participate in ribosome biogenesis, and receive inputs from cellular signaling pathways. In HGPS-derived cell cultures and in normal cells induced to ectopically express progerin, nucleoli are enlarged. In HGPS, enlarged nucleoli are also more active and produce more ribosomes, which in turn results in globally elevated protein translation. Progerin expression drives the global depletion of heterochromatin marks by an unknown mechanism2. We find that loss of repressive DNA methylation on rDNA loci allows their over-activation in HGPS, suggesting that lamin A influences the chromatin state of rDNA. Depletion of lamin A from normal cells also allows nucleoli to expand and produce more ribosomal RNA. Intriguingly, depletion of lamin A increases the nuclear dynamics of nucleoli. We speculate that A-type lamin networks within the nucleus limit both nucleolar mobility and transcriptional activity of rDNA loci. Since the expression of A-type lamins increases dramatically as cells exit pluripotency and varies widely across differentiated cell types, it is possible that nucleolar activity also changes in tandem with shifts in lamin A expression. These findings generate new insight into the cellular defects associated with HGPS and define a new function for lamin A in regulating the nucleolus.


P1387
Board Number: B396
Chromatin histone modifications and rigidity affect nuclear morphology independent of lamins.
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Nuclear shape and architecture influence gene localization, mechanotransduction, transcription, and cell function. Abnormal nuclear morphology and protrusions termed “blebs” are diagnostic markers for many human afflictions including heart disease, aging, progeria, and cancer. Nuclear blebs are
associated with both lamin and chromatin alterations. Currently, it is believed that lamins dictate nuclear morphology, but the contributions of altered chromatin compaction remain unclear. We show that modulating chromatin histone modification state dictates nuclear rigidity and is sufficient to both induce and suppress nuclear blebs. Treatment of mammalian cells with histone deacetylase inhibitors to increase euchromatin or histone methyltransferase inhibitors to decrease heterochromatin results in a softer nucleus and nuclear blebbing, without perturbing lamins. Oppositely, treatment with histone demethylase inhibitors increases heterochromatin and chromatin nuclear rigidity, which results in reduced nuclear blebbing in lamin B1 null nuclei. Notably, increased heterochromatin also rescues nuclear morphology in a lamin A mutant model for the accelerated aging disease Hutchinson-Gilford Progeria Syndrome. Thus, chromatin histone modification state is a major determinant of nuclear blebbing and morphology via its contribution to nuclear rigidity.

P1388  
Board Number: B397  
Chromatin state contributes to nuclear mechanics.  
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In mammalian cells, an integrated network of heterochromatin, a polymer mesh of intermediate filaments (lamins), and integral inner nuclear membrane proteins provides a robust nuclear structure that protects the genome. In particular, this network plays a critical role in maintaining nuclear integrity under mechanical strain, yet it is unclear how chromatin, and its condensation state, individually contributes to the overall structure and mechanics of the nucleus. Here we show that deletion of epigenetic modifiers is sufficient to alter nuclear deformability in yeast. We use a novel three-dimensional (3D) image reconstruction software that tracks fluorescently-labeled nuclear envelope fluctuations over time, as well as a novel force spectroscopy assay that employs optical tweezers to directly measure the viscoelastic properties of isolated nuclei. We find that deletion of the heterochromatin reinforcing factor Swi6 (an HP1 orthologue) results in increased nuclear deformability, while loss of the H3K9 demethylase Epe1 (a KDM2B orthologue) that drives heterochromatin spreading results in decreased nuclear deformability. Our results support the hypothesis that changes in epigenetic modifications, in addition to influencing the transcriptome, have a strong influence on nuclear mechanics. As epigenetic modifications of histones are strongly suggested to contribute to certain disease states, understanding how these chromatin changes act in parallel to influence nuclear integrity and genome stability will be essential to elucidating the full effect of epigenetic alterations on disease pathogenesis.

P1390  
Board Number: B399  
Ablation of SUN2-containing LINC complexes drives defects in developmental myofibrillogenesis and cardiac hypertrophy without fibrosis.  
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The cardiomyocyte cytoskeleton, including the sarcomeric contractile apparatus, forms a cohesive network with cellular adhesions at the plasma membrane and nuclear-cytoskeletal linkages (LINC complexes) at the nuclear envelope. Human cardiomyopathies are genetically linked to the LINC
complex and other components of the nuclear lamina, but a concrete understanding of disease etiology in these patients is lacking. Here we show that disruption of SUN2 LINC complexes in mice produces pronounced defects in sarcomere organization, intercalated disc structure, and integrin engagement at the costamere. These changes initiate early in development with profound alterations in sarcomerogenesis that precede loss of nuclear integrity. Surprisingly, SUN2-null mice display enhanced cardiac contractile function, which is associated with altered integrin activity, increased AKT/MAPK signaling, and cardiac hypertrophy. However, these mice do not exhibit increased fibrosis or up-regulation of pathological hypertrophy markers, suggesting that ablation of SUN2 LINC complex drives hypertrophy but not pro-fibrotic signaling. Together these results suggest a new model for the origin of cardiomyopathies in nuclear envelopopathies and laminopathies.

P1391
Board Number: B400
Coordinated increase of nuclear tension and lamin-A with matrix stiffness out-competes Lamin-B Receptor, which favors soft tissue phenotypes.
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Matrix elasticity sensed by a cell or measured by a physical probe reflects both the intrinsic softness of the matrix and also how thick or thin the matrix is. Mesenchymal stem cells (MSCs) help show that cells and their nuclei spread in response to a thickness-corrected matrix stiffness, with increases in nuclear tension and nuclear stiffness resulting from increases in myosin-II and lamin-A,C. Linearity between the widely varying projected area of a cell and its nucleus across many matrices, timescales, and myosin-II activity levels indicates a constant ratio of nucleus-to-cell volume. Nuclear envelope fluctuations are suppressed on stiff matrix relative to soft matrix, consistent with a “four-fold increase in tension on the nucleus and also consistent with estimates from traction force microscopy and calculations based on lamin-A,C’s increase. Transcriptomes of diverse tissues and MSCs further show increases in lamin-A,C anti-correlate with levels of lamin-B receptor (LBR), which contributes to lipid synthesis. Adipogenesis (soft) as well as osteogenesis (stiff) of MSCs confirm anti-correlation of protein, and competition of lamin-A,C and LBR for lamin-B is evident in responses to matrix elasticity, various knockdowns, myosin-II inhibition, and constricted migration. Rigidity-driven contractile stress thus tenses and stiffens the nucleus to oppose soft tissue phenotypes.

P1392
Board Number: B401
Dynein pulling forces on ruptured nuclei counteract lamin-mediated repair mechanisms in vivo.
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Recent work done exclusively in tissue culture cells revealed that the nuclear envelope (NE) undergoes nuclear rupture and repair in interphase leading to transient mixing of nuclear and cytoplasmic components. The duration of transient NE ruptures depends on lamins, however the underlying mechanisms and relevance to in vivo events is not known. Here, we use the C. elegans zygote to show that the NE recovers from lamin- or laser-induced NE ruptures during pronuclear positioning in vivo. By
monitoring the rapid recovery from rupture in the C. elegans zygote, we show that lamin restricts nucleocytoplasmic mixing prior to full recovery of NE rupture sites. Dynein forces that position nuclei increase the severity of, but do not induce, transient NE ruptures. In contrast, increasing dynein forces that facilitate nuclear migration induce irreversible nuclear permeabilization and NE collapse prior to mitotic regulated NE breakdown. Surprisingly, embryonic lethality does not correlate with the high incidence of transient NE ruptures, but rather with stochastic chromosome scattering caused by premature NE collapse, suggesting that embryos can tolerate transient losses of NE compartmentalization. In addition to presenting the first mechanistic analysis of transient NE ruptures in vivo, this work delineates the dynamics of NE rupture and recovery and establishes lamin’s role in promoting NE recovery from rupture by opposing dynein forces that position nuclei.

P1393
Board Number: B402
Dual roles for nuclear envelope constituents in the cytoplasm.
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The nuclear envelope (NE) is a highly specialized extension of the endoplasmic reticulum whose roles include organization of peripheral heterochromatin, separation of the nuclear and cytoplasmic compartments, and nuclear positioning via mechanical linkages with the cytoskeleton. It is therefore perhaps no surprise that mutations in NE constituents are associated with several rare human diseases. Identifying NE constituents and their function in health and disease is key to uncovering the underlying mechanisms of NE-associated diseases. We have utilized the BioID method to identify a lamin-associated transmembrane kinase called VRK2A. We have shown that VRK2A phosphorylates and subtly alters the nuclear mobility of barrier to autointegration factor (BAF), a small dynamic DNA-binding protein that localizes to the nuclear envelope and nucleoplasm with roles in cell division and NE assembly. BAF also exists in the cytosol, where it binds DNA, such as DNA introduced during viral or bacterial infection. We observed that VRK2A co-localizes with BAF at induced cytosolic DNA foci, and depletion of VRK2 significantly increases the number of these cytosolic BAF-DNA foci. Ongoing studies seek to determine the mechanisms by which BAF and VRK2A function to regulate cytosolic DNA and investigate the relevance of this pathway to diseases of the NE.

P1394
Board Number: B403
LAMIN B1 TETHERS TO CHROMATIN AND ORGANIZES ITS HIGH-ORDER STRUCTURE in Mammalian Cells.
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Lamin B1 constitutes a key component of nuclear lamina and is implicated in nuclear skeletal, gene regulation, differentiation, and senescence. However, whether and how lamin B1 organizes the three-dimensional (3D) structure of chromatin remain elusive. Here, we applied a combination of chromosome conformation capture (Hi-C), super-resolution imaging, and single chromosomal loci tracking to characterize the chromosome architecture in wild type and lamin B1 depleted human breast tumor cells. Globally, the Hi-C interaction matrices of lamin B1 depleted cells indicate a relative loss of long-range and gain of short-range interactions within chromosomes. At the chromosome territory (CT)
level, trans-interaction between chromosomes increases significantly. At the compartment level, although the overall organization of the chromatin into topologically associated domains (TADs) is conserved, ~10% of genomic regions switches between active (A) and repressive (B) compartments. Through direct measurements of single genomic loci dynamics in living cells, we find that lamin B1 can confine genomic loci dynamics in nuclear periphery. The Ig-like domain of lamin B1 has been known to mediate the interaction between Lamin B1 and chromatin. Disruption of the lamin B1-chromatin interaction by loss of the Ig-like domain resulted in increased chromatin mobility. These results suggest that lamin B1 tethers to chromatin and organizes its 3D structure at the compartment level. We propose that like yeast, mammalian cells also use tethering as a mechanism to organize and compartment their genome to adapt complex intra-nuclear processes.

P1395
Board Number: B404
The role of the BAF/VRK1 signaling axis on the DNA damage response in NGPS.
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The nuclear envelope is a dynamic structure involved in the regulation of nuclear function and architecture. Adjacent to the inner nuclear membrane (INM) is the nuclear lamina. The lamina is primarily composed of lamins, and provides structural scaffolding and an interface with chromatin. The small dimeric BAF protein is another important component of the lamina. BAF binds to chromatin (both DNA and histones) and to LEM domain-containing proteins of the INM, such as Lap2 and emerin, acting as a bridge that “snaps” DNA to the nuclear lamina. A subset of diseases called laminopathies or envelopopathies result from mutations in several components of the nuclear lamina or envelope. Hutchinson-Gilford Progeria Syndrome (HGPS) and Emery-Dreifuss Muscular Dystrophy (EDMD) are diseases that arise from a lamin A and an emerin mutation respectively. These syndromes highlight the importance of the nuclear envelope’s structural integrity in normal cell function. Nestor-Guillermo Progeria Syndrome (NGPS), a recently discovered novel progerioid syndrome, results from the homozygous inheritance of a mutation that causes an amino acid substitution in BAF from Ala-12 to Thr (A12T). The mechanism of disease pathology is still unclear and the role of the BAF A12T mutation in the disease phenotype has yet to be characterized. NGPS is an envelopopathy in which cells display aberrant nuclear architecture. This phenotype is also seen in cells which are deficient in VRK1, a nuclear kinase that phosphorylates BAF. It has previously been described that BAF A12T mesenchymal stem cells display a defect in their ability to maintain stemness and to differentiate into their known lineages in vitro. We aim to understand whether BAF and VRK1 signaling pathways are affected by the A12T mutation and whether disruption of these pathways in mesenchymal stem cells contributes to the differentiation defect. The literature indicates that VRK1 depletion results in a dampened DNA damage response, and that BAF interacts with DNA damage response proteins. Therefore, we propose that the DNA damage response pathway may contribute to the NGPS phenotypes. Future investigations will address whether involvement of histone modifications and global changes at the epigenetic level drive the disease.
P1396
Board Number: B405
Subcellular localization of dystrophin-associated proteins is altered in Hutchinson-Gilford progeria syndrome cells.
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The dystrophin-associated protein complex (DAPC) provides a link between the extracellular matrix and the cytoskeleton, regulating various cellular processes, including adhesion, cell signaling and cytoskeleton stability. Interestingly, several DAPC components, namely dystrophin Dp71, β-dystroglycan (β-DG) and α-dystrobrevin (α-DB) were found interacting with nuclear lamins (lamin A/C and lamin B1) in the nucleus of different cell lines, which implies that these proteins are involved in nuclear envelope-associated functions. As a first step to elucidate the participation of Dp71, β-DG and α-DB in nuclear structure/function, in this study we analyzed the expression and subcellular localization of these proteins in Hutchinson-Gilford progeria syndrome (HGPS) cells, a well-studied laminopathy characterized by premature aging. Protein localization was analyzed by indirect immunofluorescence and confocal microscopy, while protein levels were assessed by western blotting assays, using primary antibodies against each of these proteins. We found that Dp71, β-DG and α-DB distribute in both the cytoplasm and the nucleus of wild type fibroblasts, while HGPS fibroblasts exhibit decreased nuclear localization for the three proteins. Altered localization of Dp71, β-DG and α-DB in HGPS cells might be related to progerin-induced nuclear morphology abnormalities; however, further experiments are required to define a role for Dp71, β-DG and α-DB in the maintenance of nuclear morphology and their relationship with progerin.

P1397
Board Number: B406
Lamin A/C mutant myonuclei experience nuclear envelope rupture and DNA damage that is reduced upon microtubule stabilization.
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Lamin A/C is an inner nuclear membrane protein that confers the stiffness of the nucleus. Mutations in lamin A/C cause a broad spectrum of human diseases that include various muscular dystrophies and dilated cardiomyopathy. The cause of these tissue-specific diseases is unclear; however, one potential explanation could be that lamin mutations alter the stability of nuclei, which is particularly deleterious in mechanically active tissue. To this end, we found that lamin A/C mutations alter nuclear stability in primary mouse myoblasts, which persists during differentiation into myofibers, resulting in highly elongated myonuclei and severe nuclear damage. Using both endogenous proteins and live-cell reporters, we found that lamin A/C-mutant myonuclei experience transient nuclear ruptures that progressively increase in number during myofiber maturation. This loss of compartmentalizing was associated with an increase in DNA damage, measured by γH2AX and 53BP1 accumulation, and an overall decrease in myofiber viability. This led us to hypothesize that nuclear envelope ruptures are induced by mechanical forces present in differentiated myofibers, resulting in widespread DNA damage and myofiber pathology. Since microtubules are required for nuclear movement, we tested whether microtubules could be involved in inducing myonuclear rupture. We used pharmacological approaches
to either destabilize (nocodazole) or stabilize (paxlitaxel) the microtubule network in lamin A/C-mutant myofibers. Interestingly, the destabilization of microtubules increased the amount of nuclear damage and rupture events, while stabilizing microtubules dramatically reduced nuclear damage and rupture events, which also corresponded to an increase and decrease in DNA damage, respectively. Lastly, to investigate whether the DNA damage in lamin A/C-mutant myotubes may be underlying their decreased viability, we inhibited the DNA damage repair pathways using ATM or DNA-PK inhibitors. Inhibiting the activity of DNA-PK, a kinase required for non-homologous end joining DNA repair, improved the health of lamin A/C-mutant myofibers. Taken together, our findings suggest that lamin A/C mutations associated with muscle disease mechanically weaken the nucleus, and result in severe nuclear damage coupled with increased DNA damage in muscle fibers that could contribute to the muscle-specific phenotypes seen in many laminopathies. Notably, muscle fibers from aged animals and humans also display an elongated nuclear phenotype, similar to what we observed in lamin A/C-mutant animals, suggesting that the mechanical properties of myonuclei may change during normal aging. Future studies will examine whether this contributes to the decline in myofiber health commonly observed with aging.

P1398
Board Number: B407
Visualization of Lamina Association Reveals Functional Organization of Chromosomes.
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Three-dimensional nuclear organization has been implicated in expression and regulation of the genome. Chromosomes are organized into territories and discrete domains of chromatin exist within the nuclear volume. These domains are suggested to be organized by patterns of gene activity, including active (A) and inactive (B) compartments. The nuclear periphery, which consists of the inner nuclear membrane and associated proteins, forms a sub-nuclear compartment that is mostly associated with transcriptionally repressed chromatin and low gene expression. These regions of chromatin that come in molecular contact with the nuclear periphery are called Lamin Associated Domains (LADs). The 3D organization of LADs has not been investigated in the context of chromosome conformation. Our current work highlights the relationship of LADs, the epigenome and 3D architecture by utilizing “chromosome conformation paints”. We find that LADs are constrained and compacted in a sub-territory in a region we define as the “peripheral zone”. The organization we observe in single cells is directly supported by analysis of both DamID and Hi-C data. Using a refined algorithm to identify active (A) and inactive (B) compartments from Hi-C data, we demonstrate that the LADs correspond to the B compartment. Integration of DamID and Hi-C data also highlights small regions within LADs that seem to escape from the B-compartment and are enriched in regulatory elements. Additionally we directly investigate the relationship of LAD organization on chromatin state and nuclear integrity.
Vesicle Docking, Fusion and Exosome Release

**P1399**
**Board Number: B409**
The bacterium Listeria monocytogenes stimulates host exocytosis to promote pathogen uptake. H. Van Ngo¹, M. Bhalla¹, D. Che n¹, K. Ireton¹; ¹Microbiology and Immunology, University of Otago, Dunedin, New Zealand

Listeria monocytogenes is a food-borne bacterium capable of causing gastroenteritis, meningitis, and abortion. Critical for disease is the ability of Listeria to induce its internalization ('entry') into human epithelial cells. One of the major pathways of Listeria entry is mediated by binding of the bacterial surface protein InIB to its host receptor the Met receptor tyrosine kinase. InIB-mediated entry requires localized polymerization of the host actin cytoskeleton. Apart from actin polymerization, roles for other host processes in Listeria entry are unknown. Here we demonstrate that exocytosis in the human cell promotes InIB-dependent internalization. Confocal microscopy imaging of a probe consisting of VAMP3 with an exofacial Green Fluorescent Protein (GFP) tag indicated that focal exocytosis is stimulated during InIB-mediated entry into HeLa cells. Experiments involving chemical inhibitors or RNA interference (RNAi) indicated that exocytosis was dependent on Met tyrosine kinase activity and the GTPase RalA. RNAi-mediated depletion of the v-SNARE protein VAMP3 or the t-SNAREs syntaxin 4 or SNAP23 demonstrated an important role for exocytosis in Listeria internalization. Depletion of SNARE proteins failed to affect actin filaments during internalization, suggesting that actin polymerization and exocytosis are separable host responses. Confocal microscopy imaging of HeLa cells depleted for SNARE proteins indicated that exocytosis was required for delivery of the human GTPase Dynamin 2 to sites of InIB-mediated entry. Importantly, RNAi experiments demonstrated a critical role for Dynamin 2 in internalization of Listeria. Our results identify exocytosis as a novel host process exploited by Listeria for infection. We propose that exocytosis might provide a general mechanism to deliver host membrane-remodeling proteins to sites of InIB-dependent entry.

**P1400**
**Board Number: B410**
Automated detection, classification, and verification of distinct modes of exocytosis. F.L. Urbina¹, S.L. Gupton¹; ¹Cell Biology and Physiology, University of North Carolina: Chapel Hill, Chapel Hill, NC

Exocytosis is a fundamental behavior found across eukaryotic cell types, which promotes the secretion of biomolecules into the extracellular space and, in some cases, the insertion of transmembrane proteins and lipids into the plasma membrane. The minimal protein machinery required for a fusion pore to open is the assembly of the SNARE complex. A pH-sensitive variant of GFP (pHluorin) fused to the luminal end of a vesicle-SNARE protein, such as VAMP2, provides a fluorescent intensity readout of fusion pore opening and the subsequent fate of VAMP2. Historically, two modes of SNARE-mediated exocytosis have been recognized. In full-vesicle-fusion (FVF), following the opening of a fusion pore, the vesicle collapses into the plasma membrane, adding both lipids and transmembrane proteins, such as VAMP2, to the plasma membrane. During kiss-and-run vesicle fusion (KNR) the fusion pore opens transiently to secrete cargo; upon closure, the vesicle retreats from the plasma membrane and reacidifies. Previously, VAMP-pHluorin exocytic fusion events have been categorized into these two modes of exocytosis by hand based on their fluorescent profiles. This analysis technique suffers from inherent biased assumptions of what modes of exocytosis exist and reliance on the human eye and

Sunday-256
human pattern recognition to categorize fluorescent profiles, as noted in the literature by the existence of fluorescent profiles that do not fit FVF or KNR. Here, we introduce a novel method to classify exocytic fusion from TIRF time-lapse images of VAMP2-phluorin in embryonic murine cortical neurons. We used an unsupervised hierarchical clustering algorithm to perform class discovery and independently suggest an appropriate number of clusters in which to partition exocytic events. We biologically manipulate the capability of vesicles to fuse using tetanus toxin to verify which clusters represent true, exocytic events. We use HEPES to manipulate vesicle reacidification upon fusion pore closure, and found this affects only specific classes of fusion, leaving others unperturbed. This classification paradigm along with the described manipulations identified two unique, bona fide exocytic fusion event behaviors in addition to FVF and KNR. Surprisingly the distribution of events was not altered by exposure to the neuronal guidance cue netrin-1, previously shown to accelerate VAMP2-mediated exocytosis. However, we found that deletion of the E3 ubiquitin ligase Trim67 biased exocytosis away from FVF and toward exocytic events that feature fusion pore closure, thus identifying TRIM67 as a novel regulator of fusion mode. Current work is investigating whether these modes are associated with other vesicle types and mediated by specific plasma membrane t-SNAREs or vesicle tethering components.

P1401
Board Number: B411
Yck3 dependent phosphorylation of Env7 and its regulation during cell cycle in Saccharomyces cerevisiae.
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Yeast vacuolar fusion and fission is a highly dynamic mechanism that responds to intracellular and extracellular changes working to keep cells intact. This mechanism has been well conserved from yeast to humans. We discovered that Env7 is a conserved vacuolar membrane kinase that is involved in negative regulation of vacuolar fusion during budding and stress, similar to that of Yck3. We have shown that these two vacuolar kinases interact genetically and double delete shows the compromised phenotype [Manandhar, S; Gharakhanian, E., FEMS Yeast Res., 472-80, 2014] Here we also show that the double delete is defective in autophagy further supporting the possible interaction between these two genes. Based on gel shift assay, we have established that the phosphorylation of native Env7 is dependent on Yck3 and can be complemented by exogenously expressed Yck3 in an expression dependent manner. Here we also show that Yck3 directly interacts and phosphorylates Env7 at specific sites. By using site-directed mutagenesis we have identified the Yck3 dependent phosphorylation sites of Env7. Our data strongly suggest that serine residues at positions 323 and 331 (S323 and S331) are the Yck3-mediated phosphorylation sites of Env7 in consistent to the prediction by Bioinformatic analysis. Phosphorylation of Env7 is required not only for the stability of Env7 but also for regulating vacuolar morphology dynamics. Since we have shown that Env7 is essential for normal vacuole dynamics during budding, studies in phosphorylation state and localization of Env7 during cell cycle arrest and progression using confocal microscopy are in progress. We are also currently working on exploring the possible downstream substrates that are likely involved in above mentioned cellular events.
**P1402**  
**Board Number: B412**  
**Exosomes transfer into osteoclasts through bone tissue.**  
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Many cells secrete vesicles with 10 to 200 nm in diameter called exosomes. It contains nucleic acids such as mRNA and miRNA, which contribute to the transfer of the nucleic acids between cells via blood and body fluids. Recently, we have revealed that exosomes are nucleated materials for mineralization. Thus, exosomes can be deposited in bone mineralized tissue. This means the deposited exosomes can be transferred into osteoclasts through bone metabolism. In this study, we examined the hypothesis of these exosomes are transported by way of bone tissue by in vitro osteoclasts assay using exosome deposited hydroxyapatite.

We extracted exosomes from human osteosarcoma HOS cells and rat mesenchymal stem cells. Mouse macrophage RAW cells were cultured and differentiated into osteoclasts by treatment with RANKL. First, we examined whether exosomes were adsorbed on the surface of hydroxyapatite. We constructed thin apatite layer on the culture dish surface follow layer-by-layer method. Then we loaded the fluorescence labeled exosomes onto the apatite layer. The fluorescence labeled exosomes clearly adsorbed on the surface of the layer. We next examined the exosome transfer from apatite surface to osteoclasts using the exosome adsorbed apatite layer. Osteoclasts were seeded on the apatite layer on which fluorescence-labeled exosomes had been adsorbed. By observation with confocal laser scanning microscopy, fluorescence of the exosomes was observed at the cytoplasm of osteoclasts. This suggests that exosomes can be transferred through bone metabolism. Our results probably indicate that exosome can transfer into cells via not only blood or body fluids but also bone and tissue metabolism. On the other hand, it is unclear how much of exosomes were deposited in the bone tissue and whether the incorporated exosomes regulate the function or activity of osteoclasts.

**P1403**  
**Board Number: B413**  
**Plasma membrane PI(4,5)P2 is critical for secretory granule exocytosis.**  
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Phosphoinositides (PIs), including PI(4)P (phosphatidylinositol-4-phosphate), PI(4,5)P2 (phosphatidylinositol-4,5-bisphosphate) and PI(3,4,5)P3 (phosphatidylinositol-3,4,5-trisphosphate), are involved in multiple signaling cascades and play an important role in the regulation of exo- and endocytosis. At least in cell free systems, PI(4,5)P2 and PI(3,4,5)P3 are enriched near docked secretory granule, where they are thought to recruit essential proteins of the exocytosis machinery, such as syntaxin-1A, to the release site. Here we have used high-resolution imaging to analyze local PI dynamics at secretory granule release sites in live insulin-secreting cells. PIs were labeled using PH domains of phospholipase C (PLC)-δ1 (high affinity PI(4,5)P2 sensor), PLC-δ4 (low affinity PI(4,5)P2 sensor), GRP1 (PI(3,4,5)P3), and EGFP-labeled P4M domains (PI(4)P). In intact cells, all tested PI markers distributed evenly across the plasma membrane, without measurable accumulation at granule release sites. In contrast, syntaxin-1A accumulated at granule sites during their docking at the plasma membrane. However, once the cells were permeabilized using α-toxin, a punctate pattern emerged that partially colocalized with docked granules. Depletion of plasma membrane PI(4,5)P2 by recruitment of a 5′-phosphatase strongly inhibited stimulated exocytosis, but had no effect on docked granules or the
distribution of syntaxin-1A. In summary, our data challenge the notion that PIs are involved in the recruitment of secretory granules or syntaxin-1A to the release site.

P1404
Board Number: B414
Plasma membrane LAT recruitment precedes vesicular LAT recruitment to reveal two phases of early T cell activation.
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When a T cell engages cells bearing foreign antigens and becomes activated, an initial event is the formation of structures called microclusters at the contact surface between the cells. Little is understood about how these microclusters form. Some have hypothesized that membrane proteins on the surface of the T cell migrate to the area of contact; others have hypothesized that proteins from cytoplasmic vesicles in the T cell are recruited to form the microclusters. To understand the key events that lead to microcluster formation, we imaged an essential transmembrane signaling molecule LAT and a vesicular SNARE VAMP7 using lattice light sheet microscopy (LLSM). We observed a kinetic lag between microcluster formation and vesicular pool recruitment to the synapse. We also looked at the activated T cell surface with higher spatial and temporal resolution using Total Internal Reflection Fluorescence Simulated Interference Microscopy (TIRF-SIM). Once vesicles were recruited, we captured directed vesicle movement between microclusters where they exhibit decreased motility. Correlative 3D light and electron microscopy revealed an absence of vesicles at microclusters at early times, but an abundance of vesicles as activation proceeded. We propose a model in which cell surface LAT is rapidly recruited and phosphorylated at sites of T cell activation, and the vesicular pool is recruited at later time points and dynamically interacts with microclusters.

P1405
Board Number: B415
EXO70 REGULATES B-CELL MIGRATION.
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B-lymphocytes are a main part of the adaptive immune system as they are responsible for producing antibodies against foreign antigens and also perform critical roles as antigen presenting cells. To become fully activated, B cells rely on their ability to capture antigens and present them as MHCII-peptide complexes to CD4+ T cells. In order for this process to be efficient, B cells are constantly scanning for antigens within lymph node follicles. Migration is highly dependent on cell polarity, which involves local cytoskeleton remodeling by proteins such as the Arp2/3 complex, as well as polarized trafficking of chemokine receptors and integrins. Interestingly, several reports show that Exo70, a protein of the Exocyst complex, controls both these processes during cell migration, however its role in B cells has not been addressed.

Methods: To address the role of Exo70 in B cell migration, silenced its expression by using two different small hairpin RNAs (shRNAs) against Exo70, that were delivered to the cells using viral vectors. Transmigration of Exo70-silenced B cells B cells was evaluated by Transwell and 2D migration assays using CXCL13 as chemoattractant factor. To investigate the role of Exo70 in the recruitment of the
Arp2/3 complex, towards the leading edge of migrating B cells we used indirect immunofluorescence (IF) in fixed cells that were exposed to CXCL13 and co-immunoprecipitation assays. Results: We show that silencing of Exo70 expression in B cells diminishes their migratory capacity compared to control cells. This was reflected by a decrease in the percentage of migrating Exo70-silenced cells in transwell experiments and an overall reduction of migratory parameters, such as velocity and accumulated distance, in 2D migration assays. Our co-immunoprecipitation assays suggest that Exo70 interacts with the Arp2/3 complex and this interaction increases when B cells are exposed to CXCL13. Accordingly, by using IF, we show that Exo70 localizes towards the migrating front of B cells exposed to the CXCL13. Conclusion: Our data suggest that Exo70 is required for efficient B cell migration, most likely by interacting with the Arp2/3 complex at the migrating front of these cells. This work was funded by FONDECYT grant N°1141182

P1406
Board Number: B416
Otoferlin is a multivalent calcium‐sensitive scaffold linking SNAREs and calcium channels.
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Sensory hair cells rely on otoferlin as the calcium sensor for exocytosis and encoding of sound preferentially over the neuronal calcium sensor synaptotagmin. Although it is established that synaptotagmin cannot rescue the otoferlin KO phenotype, the large size and low solubility of otoferlin have prohibited direct biochemical comparisons that could establish functional differences between these two proteins. To address this challenge, we have developed a singlemolecule colocalization binding titration assay (smCoBRA) that can quantitatively characterize full-length otoferlin from mammalian cell lysate. Using smCoBRA, we found that, although both otoferlin and synaptotagmin bind membrane fusion SNARE proteins, only otoferlin interacts with the L-type calcium channel Cav1.3, showing a significant difference between the synaptic proteins. Furthermore, otoferlin was found capable of interacting with multiple SNARE and Cav1.3 proteins simultaneously, forming a heterooligomer complex. We also found that a deafness-causing missense mutation in otoferlin attenuates binding between otoferlin and Cav1.3, suggesting that deficiencies in this interaction may form the basis for otoferlin-related hearing loss. Based on our results, we propose a model in which otoferlin acts as a calcium‐sensitive scaffolding protein, localizing SNARE proteins proximal to the calcium channel so as to synchronize calcium influx with membrane fusion. Our findings also provide a molecular-level explanation for the observation that synaptotagmin and otoferlin are not functionally redundant. This study also validates a generally applicable methodology for quantitatively characterizing large, multivalent membrane proteins. Portions of the presented work are from a recently published paper in the Proceedings of the National Academy of Sciences (Proc Natl Acad Sci U S A. 2017 Jul 25;114(30):8023-8028).
Resealing of tears in the sarcolemma of myofibers is a necessary step in the repair of muscle tissue. Recent work suggests a critical role for dysferlin in the membrane repair process and that mutations in dysferlin are responsible for limb girdle muscular dystrophy 2B and Miyoshi myopathy. Beyond membrane repair, dysferlin has been linked to SNARE-mediated exocytotic events including cytokine release and acid sphingomyelinase secretion. However, it is unclear whether dysferlin regulates SNARE-mediated membrane fusion. In this study we demonstrate a direct interaction between dysferlin and the SNARE proteins syntaxin 4 and SNAP-23. In addition, analysis of FRET and in vitro reconstituted lipid mixing assays indicate that dysferlin accelerates syntaxin 4/SNAP-23 heterodimer formation and SNARE-mediated lipid mixing in a calcium-sensitive manner. These results support a function for dysferlin as a calcium-sensing SNARE effector for membrane fusion events. (Portions of this presentation have been previously published (J Biol Chem. 2016 Jul 8;291(28):14575-84).

Eukaryotic cells rely on vesicle trafficking for growth, differentiation, signaling, and many other crucial cellular functions. Vesicles deliver their cargoes by fusing with cellular organelles or, in the case of exocytosis, with the plasma membrane. These fusion reactions are driven by the assembly of SNARE proteins anchored in the two membranes into membrane-bridging complexes. Other factors, notably Sec1/Munc18 (SM) proteins and multisubunit tethering complexes (MTCs), increase the efficiency and specificity of SNARE complex assembly. MTCs in particular appear to be the major orchestrators of vesicle tethering and fusion, as they interact with SNAREs and most of the other trafficking co-factors, but the underlying molecular mechanisms are incompletely understood.

We are using biochemical and structural methods to study one of the best-characterized MTCs, the HOPS (homotypic fusion and vacuolar protein sorting) complex, which is essential for the fusion of late endosomes. HOPS is a ~660 kDa, hexameric complex and contains the SM protein Vps33 as an integral subunit. Elucidating the architecture of the HOPS complex, and deciphering its interactions with SNARE proteins, will shed light on how SNAREs and MTCs work together to ensure the correct regulation and high specificity of membrane tethering and fusion. The HOPS complex interacts with SNARE proteins not only via their SNARE motifs but also via their N-terminal "regulatory" domains. We are characterizing these interactions using biochemical, crystallographic, and functional methods. In addition, we are working toward a structure of the intact HOPS complex, using the HOPS complex derived from the thermophilic eukaryote Chaetomium thermophilum as a target for cryo-EM.
P1409
Board Number: B419
Coarse-grained simulations of the synaptic neurotransmitter release machinery.
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Synapses are the basic unit of the brain. Cognition and complex behavior rely on tightly controlled release of neurotransmitters (NTs) at synapses, and synaptic misregulation is associated with neurodegenerative or neurodevelopmental disorders such as Alzheimer’s or epilepsy. Synchronized release is accomplished by a machinery that senses $Ca^{2+}$ when an action potential arrives, responds by fusing the synaptic vesicle and plasma membranes, and releases NTs through a fusion pore. SNARE proteins constitute the core of the membrane fusion machinery, and Synaptotagmin (Syt) is the $Ca^{2+}$ sensor, but the mechanisms involved are poorly understood.

Here we quantitatively describe a mechanism of NT release in a molecularly explicit mathematical model representing the detailed architecture and collective behavior of the components of the Ca$^{2+}$-sensing fusion machine. To build the pre-Ca$^{2+}$ fusion machine, our starting point is our coarse-grained (CG) model of SNARE complexes at the fusion site (Mostafavi et al., 2017), to which we now add Syt which was shown to oligomerize into ring-like oligomers in the absence of Ca$^{2+}$ that are disassembled by Ca$^{2+}$ (Wang et al., 2014). How the Syt and SNARE proteins fit together in the synaptic fusion machinery was recently described by the crystal structure of Synaptotagmin-1 (Syt1) and the neuronal SNARE complex (Zhou et al., 2015). Using this information together with an all atom structure of the Syt ring reconstructed from Cryo-EM data, we obtained an all-atom Syt ring dressed with SNAREpins. We coarse grained this structure, representing 4 residues with one CG bead with correct total charge and size, the same CG scheme as for the SNAREpins.

Our highly CG approach has the enormous advantage that we can actually build the fusion machine and run the machine on the long timescales of NT release, implementing the complex collective behavior of its many molecular components. Placing the composite Syt-SNARE structure between membranes, we first equilibrated the pre-Ca$^{2+}$ fusion machine and found that it clamps fusion, importantly.

Incorporating kinetics of Ca$^{2+}$ binding and unbinding to the Syt ring, we found that an action potential that introduces a Ca$^{2+}$ pulse disassembles the ring after a transient, unfettering the SNARE complexes. The SNARE complexes then spontaneously assemble into a circular cluster and pull the membranes into sufficiently close proximity to trigger fusion on physiological timescales. Our model simulates Ca-evoked membrane fusion for release of NTs in molecularly explicit detail for the first time, and suggests that Ca$^{2+}$-mediated disassembly of a Syt ring may control the release of NTs at neuronal synapses.

P1410
Board Number: B420
Elucidating Individual Subunit Positions within the Exocyst Tethering Complex.
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The regulation of vesicular traffic to precise intracellular compartments is essential for cell growth, homeostasis, signaling, cell division, and development. Membrane fusion between vesicles and their target membrane is carried out via SNARE proteins; however, additional regulatory control immediately prior to fusion is essential. There are two general classes of tethering proteins that provide this regulation: coiled-coiled tethers and multi-subunit tethering complexes (MTCs). While coiled-coiled
tethers are fairly well characterized, the mechanism of action of many MTCs is not well understood. The exocyst is a hetero-octameric MTC involved in the trafficking of post-Golgi secretory vesicles to sites of polarized exocytosis. Previously, we mapped the subunit connectivity of the exocyst complex and visualized the overall structure using negative stain EM. The challenge remained to map individual subunits into the structure. Using a combination of biochemical techniques including protein tagging, selective degradation of individual subunits, and nanogold labeling of subunits, we are identifying the individual subunits within the structure and modeling the intact exocyst complex. To this end, we visualized an exocyst subcomplex module containing the exocyst subunits Sec3, Sec5, Sec6, and Sec8 using negative stain EM. Additionally, GFP-tagging and SNAP-nanogold labeling are elucidating the position of the NH2 and COOH termini of individual subunits. By determining subunit placement in the three-dimensional structure and with complementary in vivo experiments, we will gain insights into the mechanisms by which exocyst coordinates several protein-protein interactions on both the secretory vesicles and the plasma membrane to mediate vesicle tethering and regulate SNARE-mediated vesicle fusion.

P1411
Board Number: B421
Intra-Endosomal Trafficking Mediated by Lysobisphosphatidic Acid Contributes to Intracellular Release of Phosphorothioate-modified Antisense Oligonucleotides.
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Chemically modified antisense oligonucleotides (ASOs) with phosphorothioate (PS) linkages are extensively studied as research and therapeutic agents. PS-ASOs can efficiently cleave target RNA through RNase H1 dependent mechanism. A number of receptor proteins and endocytotic pathways are implicated in the intracellular uptake of PS-ASOs be internalized via and are released from membrane-enclosed endocytotic organelles, mainly late endosomes (LEs). This study was focused on the details of PS-ASO trafficking through endocytic pathways. It was found that lysobisphosphatidic acid (LBPA) is required for release of PS-ASO from LEs. PS-ASOs exited early endosomes (EEs) rapidly after internalization and became co-localized with LBPA by 2 hours in LEs. Inside LEs, PS-ASOs and LBPA were co-localized in punctate, dot-like structures, likely intraluminal vesicles (ILVs). Deactivation of LBPA using anti-LBPA antibody significantly decreased PS-ASO activities without affecting total PS-ASO uptake. Reduction of Alix also substantially decreased PS-ASO activities without affecting total PS-ASO uptake. Furthermore, Alix reduction decreased LBPA levels and limited co-localization of LBPA with PS-ASOs at ILVs inside LEs. Thus, the fusion properties of ILVs, which are supported by LBPA, contribute to PS-ASO intracellular release from LEs.

P1412
Board Number: B422
Dynamics of Exocyst Subunit Assembly and Vesicle Fusion, using CRISPR-edited GFP Tagging of Endogenous Loci.
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The exocyst is an essential complex required for delivery of proteins to the plasma membrane. There is a continuing controversy about whether the exocyst is a stable octamer that binds to the membrane and tethers exocytic vesicles, or is a dynamic complex in which subcomplexes are assembled at the fusion
site. To explore the dynamics of exocyst function we created sfGFP-tagged alleles of 5 exocyst subunits (Sec3, Sec5, Sec6, Sec8 and Exo70) in a mouse mammary epithelial cell, NMuMG, using the CRISPR/Cas9 system, and tracked the arrival of these subunits and of vesicles at the plasma membrane by TIRF microscopy. We transfected VAMP-pHluorin and TFR-pHui to visualize vesicle fusion and mApple-Rab11 to visualize vesicles. We were unable to recover viable cells with fusions of 3 vesicle-associated exocyst subunits (Sec10, Sec15 and Exo84), suggesting that these constructs are non-functional. We discovered that vesicle fusion occurs about 15 seconds after arrival at the membrane. Exo70 and Sec6 arrive at a similar time and disappear immediately after fusion. Interestingly, Sec5 arrives earlier - about 19 seconds prior to fusion - and Sec3 disappears about 4 seconds before fusion occurs. These data suggest that the exocyst is not pre-assembled but instead that subunits arrive and disappear at different times. Using double-tagged cells we are now cross-correlating these events, and can begin to catalog in unprecedented detail the itinerary for vesicle delivery to the plasma membrane, and the mechanisms by which tethering and fusion are regulated.

P1413
Board Number: B423

Synaptotagmin 5 regulates Ca\(^{2+}\)-dependent Weibel-Palade body exocytosis in human endothelial cells.
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Vascular endothelial cells secrete the adhesive and procoagulant protein Von Willebrand factor (VWF) from specialised secretory organelles called Weibel-Palade bodies (WPB). A potent trigger for WPB exocytosis is an elevation of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), that can be mediated by hormone action, mechanical stresses or cell damage. Although several cytosolic Ca\(^{2+}\)-binding proteins including calmodulin and the Annexin A2:S100A10 complex have been implicated in WPB exocytosis, the existence of WPB-associated Ca\(^{2+}\)-sensors involved in detecting and transducing acute increases in [Ca\(^{2+}\)]\(_i\) into exocytosis remains unclear. Here we show that synaptotagmin 5 (SYT5) is recruited to WPBs and regulates Ca\(^{2+}\)-driven WPB exocytosis in human endothelial cells. qPCR analysis of human endothelial cells revealed the presence of mRNA for 10 of the 17 known SYT isoforms; five Ca\(^{2+}\)-dependent (1, 2, 3, 5 and 9) and five Ca\(^{2+}\)-independent (11, 14, 15, 16 and 17). Analysis of the subcellular distribution of epitope-tagged constructs of each of these 10 SYTs showed that SYT5 localized almost exclusively to WPBs. SYT17 showed partial localization to WPBs in some cells while all other SYTs localized to other subcellular compartments. Live-cell imaging of fluorescent WPB exocytosis in cells either depleted of SYT5 by shRNA or overexpressing SYT5-EGFP showed an inhibition or enhancement of histamine-evoked WPB exocytosis respectively. Similar results were obtained in biochemical studies of histamine-evoked VWF propeptide (VWFpp) secretion. Overexpression of a SYT5 Ca\(^{2+}\)-binding deficient mutant, Asp197Ser SYT5-EGFP, inhibited histamine-evoked WPB exocytosis. Depletion of SYT17 produced a very small reduction in histamine-evoked VWFpp secretion, while overexpression of SYT17 had no effect. We propose that SYT5 is a WPB-associated Ca\(^{2+}\)-sensor for regulated secretion of VWF from vascular endothelial cells.
P1414

Board Number: B424

Co-regulation of the Glycine max soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)-containing regulon occurs during defense to a root pathogen.

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Heterodera glycines, also known as Soybean Cyst Nematode (SCN) is a major pathogen of soybean (Glycine max), causes nearly one billion dollars loss in U.S. every year (Wrather et al. 2001; Wrather and Koenning, 2006; Smolik and Draper, 2007; Koenning and Wrather, 2010). Efforts to combat SCN include production of resistant soybean varieties, use of nematicides, improved crop rotation, and bio-control methods (Wrather et al. 1984; Chang et al. 2011). However, effective control has not been achieved yet. Study of host plant interactions at the cellular level is important as it may provide new species-specific means of controlling SCN (Klink et al. 2007). We are conducting various molecular approaches to find actual cellular mechanism of host resistance. Closer study of infected cells in resistant variety G. max [Peking/PI548402] and susceptible variety G. max [Williams 82(PI518671)] through laser microdissection have resulted various unique genes present in G. max [Peking/PI548402] (Klink et al. 2007; 2009). Overexpression of these genes in susceptible cultivar G. max [Williams 82(PI518671)] have resulted resistance by inducing incompatible reaction and RNA interference of these genes in resistant genotypes resulted susceptible reaction, thereby inducing compatible reaction (Matsye et al. 2012; Pant et al. 2014). In this approach we have overexpressed the components of the Soluble N-ethylmaleimide-sensitive fusion (NSF) Attachment Protein (SNAP) REceptor (SNARE) complex that helps in docking of the vesicles to the membrane and subsequent release of the vesicular contents to the apoplast (Jahn and Fasshauer et al. 2012; Matsye et al. 2012; Pant et al. 2014). There are many proteins that play significant role in this process however, the core components of this study are syntaxin 121 (SYP121), Synaptosomal-associated protein 25 (SNAP-25), Synaptotagmin (SYT), Synaptobrevin (SYB), Secretion 1/mammalian uncoordinated-18 ([Sec1]/Munc18), and N-ethylmaleimide-sensitive fusion protein (NSF). Syntaxin 121, G. max homolog of Saccharomyces cerevisiae, Suppressors of sec1 (SSO1) known as PENETRATION1 (PEN1) in Arabidopsis thaliana, (Collins et al. 2003) function in resistance to Heterodera glycines. Co-expression of SYP121 with SNARE homologs results elevated transcripts in infected cells inducing resistance reaction.

P1415

Board Number: B425

Cryo-EM Structure of the Exocyst Complex.

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The fusion of secretory vesicles to their target compartments requires the proper function of the tether family of proteins at various stages of vesicular trafficking. The exocyst is an evolutionarily conserved hetero-octameric protein complex that tethers post-Golgi secretory vesicles to the plasma membrane for exocytosis, and is implicated in many cellular processes such as cell polarization, cytokinesis, ciliogenesis and tumor invasion. Using cryo-electron microscopy (cryo-EM) and chemical cross-linking mass spectrometry (CXMS), we solved the structure of the fully assembled yeast exocyst complex. Our atomic model revealed the architecture and hierarchical interactions of the complex, and led to the
identification of helical regions that nucleate the assembly of the exocyst at its core. Sequence analysis suggest that these regions are conserved evolutionarily across eukaryotic systems. Further cell biological studies suggest a mechanism for exocyst assembly that leads to vesicle tethering at the plasma membrane.

P1416
Board Number: B426
Oxidative stress impedes trafficking and increases vesicular accumulation of amyloid precursor protein in HTB-11 neuroblastoma cells.
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Oxidative stress has been known to impede neuronal function, resulting in impediments that are associated with neurodegenerative disorders such as Alzheimer’s disease. Increased oxidants have been known to affect amyloid precursor protein (APP) localization to the membrane; however, it is unclear whether this is due to trafficking dysfunction or to downregulation of APP expression. We therefore aimed to explore this further using HTB-11 neuroblastomas as the model for this study. HTB-11 cells were grown in 100 nM retinoic acid (RA) to induce differentiation morphological and functional neuronal phenotypes. Oxidative stress was induced with tert-butyl hydroperoxide at concentrations ranging from 1 µM to 31 µM. Cells were then stained using anti-APP and conjugated to AlexaFluor 647, and a FITC-conjugated lectin to label the membranes of vesicles. Confocal microscopy was used to image samples to obtain a 3D image of the cells, and used to quantify both the expression of APP via fluorescence intensity and pixel count, and endocytosis using fluorescence of endocytotic markers. The Manders colocalization analysis (using ImageJ) was used to quantify the percentage of APP that colocalized to the membranes, and thus determine if there are changes in the association of APP to membranes due to oxidative stress. We found that increasing concentrations of tert-butyl hydroperoxide induced an increase in the expression of APP, but did not cause a change in the colocalization of APP to the vesicle membranes. Lectin staining showed that vesicles under oxidative stress tended to accumulate in the cell body rather than dispersing throughout the cell. These results suggest that oxidative stress primarily impeded vesicle traffic, resulting in concomitant accumulation of vesicular APP that did not localize to the plasma membrane. An understanding of the effects of oxidative stress on the trafficking of APP will help to increase our understanding of one of the fundamental aspects of Alzheimer’s disease pathology.

P1417
Board Number: B427
Cholangiocyte intercellular communication via polarized exosome release.
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Cholangiocytes, epithelial cells of the bile duct, separate the lumen of the bile duct (apical surface) from the peribiliary space (basolateral surface). Cholangiocyte communication via these different surfaces is distinct. Exosomes, a more recently appreciated mode of intercellular communication, are released by the fusion of multivesicular bodies with the plasma membrane. We have investigated polarized
exosome secretion in cholangiocyte communication and machinery contributing to this process. The Normal Human Cholangiocyte (NHC) cell line was cultured in transwells, and microscopy and trans-epithelial electrical resistance were used to validate the formation of a restrictive monolayer suitable for our studies. Isolation of the exosome-enriched fractions from apical and basolateral conditioned media were performed. Nanoparticle tracking analysis, immuno-electron microscopy, and western blotting supported the conclusion that NHC release exosomes both apically and basolaterally. These exosome-enriched fractions exhibited interesting differences: more particles were released apically, and the apical particles contained more RNA and cholesterol. Examination of contents by silver staining and western blotting revealed that multiple proteins are differentially enriched between the apical and basolateral exosome fractions. Similarly, profiling of miRNA contents identified species that are enriched in apical or basolateral exosomes. The signaling activities of apical and basolateral exosomes were examined in both apical and basolateral target models. Treatment of polarized NHC via the apical surface (i.e. apical model) with exosomes resulted in distinct activation profiles with apical exosomes evoking greater activation. By contrast, exosome treatment of human THP-1 macrophage cells (i.e. basolateral model) resulted in distinct activation profiles with basolateral exosomes evoking greater activation. These results supported the conclusion that cholangiocytes release different populations of exosomes from their apical and basolateral surfaces to mediate distinct intercellular communication. Perturbation of cellular machinery implicated in exosome biogenesis altered apical and basolateral exosome secretion. Our data suggest that the mechanisms driving apical and basolateral exosome biogenesis are to some extent distinct.

P1418
Board Number: B428
Unconventional release of fusogenic nonenveloped reoviruses.
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Fusogenic reoviruses are nonenveloped viruses with segmented dsRNA genomes. As with most nonenveloped viruses, virus-induced cell lysis is the primary mechanism for reovirus release from cells. Fusogenic reoviruses are the only known examples of nonenveloped viruses that induce cell-cell fusion and syncytium formation. Syncytigogenesis is mediated by a virus-encoded fusion-associated small transmembrane (FAST) protein, which are both necessary and sufficient to induce membrane fusion. FAST protein-induced syncytium formation enhances localized direct cell-cell spread of the infection, followed by eventual syncytial lysis and release of infectious virus particles for systemic dissemination of the infection. Recent findings in the field suggest that various nonenveloped viruses, including hepatitis A virus and poliovirus, can also be released non-lytically from cells inside extracellular vesicles (ECVs) that derive from endosomal sorting complexes required for transport (ESCRT)- or autophagy-dependent pathways. Interestingly, western blotting revealed FAST proteins are embedded in the membrane of ECVs, and transmission electron microscopy and cryoelectron microscopy discovered significant levels of non-enveloped virions associated with ECVs, suggesting ECV and virus release may be associated. We further investigated these processes to determine whether this virus release pathway occurred non-lytically, and whether FAST proteins or syncytium formation contribute to non-lytic virus release. Kinetic analysis of virus release using various cell lines infected with fusogenic reoviruses was coupled with live-cell imaging using the membrane impermeable small molecule dye Sytox, which fluoresces when bound to nucleic acid inside cells, to assess plasma membrane integrity. Results indicate release of substantial quantities of infectious virus particles from cells prior to any evidence of reduced plasma membrane integrity, indicative of non-lytic virus release. Genetic and pharmacologic approaches are being used to
asses the involvement of ESCRT machinery and autophagy pathways in the mechanism underlying this non-lytic virus release mechanism.

**P1419**

**Board Number: B429**

**CSF-1R SIGNALING ON INTERNAL VESICLES INVOLVES LYN TYROSI NE KINASE.**

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CSF-1R SIGNALING ON INTERNAL VESICLES INVOLVES LYN TYROSI NE KINASE Louise Monga, Lu Huang, Jared Wollman, Natalie Thiex Department of biology and microbiology, South Dakota State University, Brookings, South Dakota Colony stimulating factor-1 (CSF-1) is a growth factor that mediates growth, proliferation and differentiation of macrophages by binding to its receptor, colony stimulating factor-1 receptor (CSF-1R). Upon activation by CSF-1, CSF-1R becomes phosphorylated and rapidly internalized. During this time, CSF-1R signals through the MAPK and Akt/PI3K pathways. Previous work assumed CSF-1R signaling originated at the plasma membrane. Yet, when internalization of the receptor is prevented, the downstream signaling cascade (measured by Akt and ERK phosphorylation) is impaired, indicating that internalization of the receptor is necessary for full signaling. Using immunofluorescence and immunoblot, we show the presence of phosphorylated (active) CSF-1R on macropinosomes, suggesting that receptor signaling continues following internalization and subsequent trafficking. However, it is not known how similar or different the membership of the CSF-1R signaling complex is at the plasma membrane or internal vesicles. Using immunofluorescence, we showed that Lyn protein kinase colocalizes with CSF-1R on macropinosomes a few minutes following stimulation of macrophages with CSF-1. The duration of the colocalization correlated with the duration of the presence of phospho-CSF-1R on macropinosomes. These findings suggest that Lyn is a CSF-1R signaling partner on macropinosomes, but its mechanism of action in this context is not well understood. Spatiotemporal organization of proteins in the cell is crucial for complete and proper signaling. CSF-1R has a relatively simple signaling pathway than most receptor tyrosine kinases. Signaling from internal membranes may provide opportunities for signal integration or spatial control of signal shape and amplitude.

**ER and Golgi Transport**

**P1420**

**Board Number: B430**

**Regulation of COPI vesicle transport via Scyl1 methylation under ER-stress.**

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Cumulative evidences have shown the importance of ER-stress in pathology of neurodegenerative diseases, such as Alzheimer’s disease, Amyotrophic lateral sclerosis, etc. To elucidate the pathogenesis of neurodegenerative diseases from the viewpoint of ER-stress, we screened the altered genes in SK-N-SH cells under the condition of tunicamycin-induced ER-stress by the gene fishing method. As the result, we found that Protein arginine N-methyltransferase 1 (PRMT1) is up-regulated in SK-N-SH cells under ER-stress. Based on this result, we examined the role of PRMT1 in the ER-stress related pathway and
organelle and found that PRMT1-knockdown cells showed the abnormal Golgi formation and increased UPR. To elucidate the mechanism of these alterations, we screened the methylated proteins as substrates of PRMT1 under ER-stress condition by immunoprecipitation-mass spectroscopy, and identified Scy1-like protein 1 (Scy1). Scy1, a member of the Scy1-like family of catalytically inactive protein kinases, was recently reported to function in retrograde COPI-mediated intracellular transport. Interestingly, Scy1 has also been identified as a gene product that is lost in an animal model of motor neuron disease, the muscle-deficient mouse. In the motor neuron of the above model animal, the protein circulation system between ER and Golgi apparatus was abnormal due to dysfunction of COPI transport. In consequence, UPR may be accerelated. Thus, we present the effect of Scy1 arginine methylation on the COPI vesicle transport. This study provides novel insights into the pathogenesis of neurodegenerative diseases caused by ER stress.

P1421
Board Number: B431
Regulation of the COPII outer coat by O-GlcNAc.
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Collagen is the most abundant human protein by mass, and is the main component of bone, cartilage, and other connective tissues. Collagen is first synthesized intracellularly, then trafficked to the extracellular space via the secretory pathway. This process depends on the coat protein complex II (COPII) pathway, which is responsible for trafficking cargoes from the endoplasmic reticulum (ER) to the Golgi. Proper trafficking of proteins is essential for their function, and defects in the COPII pathway result in human diseases, including osteogenesis imperfecta and cranio-lenticulo-sutural dysplasia (CLSD) due to failed collagen export. Although the structure and function of COPII proteins have been well-studied, the regulation of COPII by cellular stressors and other signaling events is poorly understood. Recently, regulation of COPII by post-translational modifications (PTMs), such as phosphorylation and ubiquitination, has been reported. Our lab and others have also identified O-linked β-N-acetylglucosamine (O-GlcNAc) on three COPII components. O-GlcNAc is a dynamic PTM that is responsive to cellular signals, including nutrient, oxidative, and ER stressors, leading us to hypothesize that O-GlcNAc may regulate COPII activity. To test this, we manipulated intracellular O-GlcNAc levels and quantified the COPII components present in membrane and cytoplasmic fractions. Under normal conditions, COPII components were found predominantly in the membrane fraction, but the three O-GlcNAcylated COPII components—Sec23, Sec24, and Sec31—were found predominantly in the cytoplasmic fraction when we elevated O-GlcNAc levels. Because sequential recruitment of COPII components to the ER membrane is essential for their function, this result suggests that O-GlcNAc levels may impact COPII vesicle trafficking. To investigate this possibility, we monitored a GFP-tagged cargo from the ER to the Golgi in live cells and observed a marked delay in cargo delivery to the Golgi when O-GlcNAc levels were elevated. We also utilized mass spectrometry to identify 10 O-GlcNAcylated residues on Sec31A, the COPII outer coat component which permits COPII vesicles to accommodate large cargoes like collagen. Interestingly, all 10 O-GlcNAcylated residues fall within Sec31A’s flexible hinge region or a known protein-binding domain. We are currently investigating the functional impact of these O-GlcNAcylated sites on collagen export and COPII vesicle trafficking of other cargoes. These results provide a greater functional understanding of a ubiquitous but poorly understood PTM, and yield important insight into the mechanism of regulation of an essential cellular trafficking pathway.
**P1422**  
**Board Number: B432**

The large Sec7 ARF guanine nucleotide exchange factor GBF1 contains a PIP-binding domain essential for its membrane recruitment and cellular function.  
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ADP-ribosylation factors (ARF) are key components of the vesicle budding process essential for intracellular transport of a variety of cellular materials. The ARF guanine nucleotide exchange factors (GEFs) are cytosolic proteins that undergo cycles of membrane association and dissociation, with only the membrane-localized pool catalyzing ARF activation. Thus, mechanisms that recruit GEFs to membranes directly control the sites and timing of ARF activation. Small GEFs are recruited to membranes through phosphoinositide (PIP) binding by a pleckstrin homology (PH) domain and lipid binding is absolutely required to support the functionality of these GEFs. The large GEFs (GBF1, BIG1/2) lack PH domains, and their mechanisms of recruitment are poorly understood. We focused on GBF1, a GEF that localizes to and catalyzes ARF activation on compartments at the ER-Golgi interface. The PH domains of small GEFs are immediately downstream from the catalytic Sec7 domain. In the large GEFs, that position is occupied by homology downstream from Sec7 number 1 (HDS1) domain. We posited that HDS1 may act in a manner similar to PH with regard to the regulated membrane recruitment.

Thus, we did a structure-function analysis of HDS1 to assess its participation in GBF1 recruitment to Golgi membranes. The ~173 residue HDS1 domain contains 5 α-helices. We targeted highly conserved motifs for mutagenesis within α-helix 1, 3, 4 and 5. The targeting of GBF1 lacking mutations in HDS1 to Golgi membranes was assessed by immunofluorescence of fixed cells and by live cell imaging approach. Golgi recruitment was observed for all constructs, except the GBF1 with mutation in α-helix 1, which was impaired in Golgi association and was predominantly detected in a diffuse cellular pattern. We posited that HDS1 function may be analogous to a PH domain to recruit GBF1 to membranes via direct binding to one or more lipids. We purified full length GBF1 with different point mutations from mammalian cells and by the nitrocellulose strip lipid binding and liposome flotation assays we show that GBF1 binds PIPs, with preference for PI3P, PI4P and PI(4,5)P2, and that this binding is mediated by the HDS1 domain. Our results suggest that HDS1 acts in a manner similar to the PH domain in small GEFs, and by binding specific PIPs regulates GBF1 recruitment to Golgi membranes.

Considering that GBF1 activates ARF while associated with Golgi membrane, we tested the role of PIP binding in GBF1 cellular function. We document that mutation within α-helix 1 in HDS1 domain that reduce the binding of full-length GBF1 to membranes and prevent PIP binding, also inhibit the ability of GBF1 to activate ARF and support Golgi homeostasis.
P1423
Board Number: B433
The role of the N terminus, Sec7 domain, and C terminus of GBF1 in +RNA viral replication.
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All viruses are obligatory parasites and must utilize cellular factors to support their replication. The guanine nucleotide exchange factor GBF1 is essential for replication of various +RNA viruses, such as Flaviviruses and Enteroviruses. GBF1 is an enzyme that catalyzes GDP/GTP exchange on ARFs - small GTPases that regulate multiple facets of cellular physiology including cytoskeletal rearrangements, secretory and endocytic traffic, and metabolic homeostasis. In uninfected cells, GBF1 associates with Golgi membranes where it activates ARF to support Golgi homeostasis and secretory traffic. In virus-infected cells, GBF1 is recruited to replication complexes to support viral replication. The exact mechanism through which GBF1 facilitates viral replication remains enigmatic. Thus, we initiated a detailed structure-function analysis of GBF1 with the goal of identifying its activities that support viral replication. We constructed 12 GBF1 mutants, with amino acid substitutions in the most conserved domains. Four constructs contain mutations in the N terminus since deletion of this region has been shown to prevent GBF1 from supporting replication (GBF1/N5,6AA; GBF1/Y7,8AA; GBF1/Δ10; and GBF1/Δ37). Three constructs contain substitutions within the catalytic Sec7d (GBF1/E794K binds ARF but can’t catalyze GDP/GTP exchange; GBF1/7ΔA doesn’t bind ARF; and GARG contains a Sec7d from another non-Golgi factor). Four constructs contain substitutions or deletions in different domains downstream of the catalytic Sec7d (GBF1/LF926AA in HDS1; GBF1/LF1266AA in HDS2; GBF1/PLL1544AAA in HDS3; GBF1/1-1531 lacking HDS3 and the C-terminal region; and GBF1/1-1674 lacking the C-terminal proline-rich domain). We report that the GBF1 constructs exhibit different abilities to target to the Golgi and support Golgi homeostasis and secretion in uninfected cells, as well as differences in their ability to target to viral replication complexes and support viral replication in cells infected with polio-virus replicon.

P1424
Board Number: B434
Isoform Specialization Among the Sec23 Gene Family Has Significant Consequences for Polarized Growth.
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Polarized secretion is essential for cell biological processes across the tree of life. In particular walled organisms, such as yeast, filamentous fungi, and plants use polarized secretion to divide and grow thereby providing excellent model systems to unravel the molecular mechanisms controlling this process. For polarized growth, an active secretory system is required to deliver specific building materials to the growth region. As part of the secretory pathway, transport from the endoplasmic reticulum (ER) to the Golgi apparatus, which is mediated by the coat protein complex II (COPII) is therefore a prerequisite for polarized secretion. We have investigated the role of COPII in the juvenile tissue of the moss P. patens, which grows exclusively by polarized secretion. Interestingly in P. patens, small gene families encode each of the subunits of the COPII complex. To investigate whether these
small gene families are redundant, we analyzed the loss of function phenotype resulting from silencing Sec23, a component of the inner coat of COPII, which is encoded by seven highly similar genes. To our surprise, we found that silencing Sec23D cause a severe growth defect, while silencing the remaining six Sec23 genes had no significant defect. Coding sequence complementation showed partial functional redundancy between only Sec23D and Sec23E, suggesting that Sec23D and to a limited extent Sec23E play pivotal roles during polarized secretion, we found that Sec23D localizes to the ER and partially overlaps with the Golgi. In addition, Sec23D heterodimerizes with two of the seven Sec24 genes in moss, suggesting that Sec23D is a canonical COPII component. To analyze the role of Sec23D, we generated a sec23d null mutant using CRISPR-Cas9 genome editing. We found that the sec23d null mutant grows very slowly, suggesting a strong defect in secretory activity. In support of this, we observed significant defects in ER morphology, with a subset of cells accumulating large ER aggregates. The ER aggregates form on the order of days, potentially inhibiting cell growth. From these data, we hypothesize that Sec23D plays a central role in COPII function during polarized growth.

P1425
Board Number: B435
3D ultrastructural analysis of the progressive restructuring of the endoplasmic reticulum by a coronavirus provides insight into its subversion of the ERAD tuning pathway.
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Viruses commonly modify organelle membranes and subvert cellular pathways for the various stages of their lifecycles. Coronaviruses extensively utilize the endoplasmic reticulum (ER), inducing drastic architectural changes to the ER through the course of infection. ER membrane is used for both formation of double-membrane vesicles (DMVs) associated with replication, as well as for assembly and envelopment of new virions. Early in infection, the ER is utilized to form DMVs, which have been shown to contain markers for the ER-associated degradation (ERAD) tuning pathway. ERAD tuning vesicles, termed EDEMosomes, bud from the ER and are rapidly trafficked to lysosomes for degradation. Unlike EDEMosomes, DMVs are bounded by two lipid bilayers, are believed to remain connected to the ER via a membranous network, and accumulate within the cell over the course of infection. In this work, we sought to visualize coronavirus-induced restructuring of the ER as it progresses through the infection. Specifically, we investigated how this class of EDEMosome-type vesicles form from the ER, and whether they are able to evade the degradative fate of an EDEMosome. In order to observe morphological changes both at high resolution and over a large cellular volume, we used large-volume EM tomography to image the ER at different timepoints post-infection and to perform detailed 3D analysis of the structures within the volumes. In one volume (dimensions ~8 x 8 x 2 µm), we observed nearly 700 DMVs in the cytoplasmic space. Analysis of the images reveals that DMV inner vesicles form first on or within the ER, then obtain an outer bilayer by budding out of the ER, and eventually separate from the ER. At late timepoints in infection, the ER’s role in infection shifts as it accommodates the assembly and budding of new virus particles into its lumen. Concurrent to this architectural restructuring, we observed that the number of DMVs drastically decreases, and DMVs can be visualized inside lysosomal compartments. Chloroquine treatment prevented the decrease in DMVs, indicating that DMVs indeed are degraded in lysosomes and thus follow the ERAD tuning pathway to its end. Live cell fluorescence imaging experiments are underway to further characterize the dynamics of coronavirus interaction with this pathway. In conclusion, 3D ultrastructural analysis of coronavirus-infected cells allowed us to
observe the morpho-functional flux of the ER by an obligate pathogen and to move toward a functional understanding of the involvement of cellular pathways in infection.

P1426
Board Number: B436
TMEM116: A Transmembrane Protein of Unknown Function that is Regulated by a Bidirectional Promoter.
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Our group has previously shown that ERp29 (Endoplasmic Reticulum Protein of 29 kDa), a novel molecular chaperone of the Endoplasmic Reticulum, has increased expression in response to sodium 4-phenylbutyrate (4PBA, Suaud, et al., JBC, 2011), the prototype corrector of F508del CFTR trafficking (Rubenstein, et al., JCI, 1997). Our group also demonstrated that ERp29 promotes biogenesis of the cystic fibrosis transmembrane conductance regulator (CFTR, Suaud, et al., JBC, 2011), as well as regulates the biogenesis of the epithelial sodium channel, ENaC (Grumbach, et al., AJPCell, 2014). Interestingly, the expression of ERp29 is regulated by an ~1.4 kb bidirectional promoter that regulates the expression of TMEM116, a transmembrane protein of unknown function, in the opposite direction. As it is suggested that proteins found on bidirectional promoters often interact with or regulate one another, we chose to investigate the function of TMEM116. We initially compared TMEM116 expression to that of ERp29 in response to 4PBA treatment. TMEM116 expression increased over time in 4PBA-treated IB3-1 CF bronchiolar epithelial cells and in MDCK cells. This increase in TMEM116 expression temporally correlated with the time-dependent increase in ERp29 expression seen with exposure to 4PBA (Suaud, et al., JBC, 2011), suggesting that TMEM116 and ERp29 expression are coordinately regulated by 4PBA through this bidirectional promoter. We also performed initial immunofluorescence experiments with two different commercial antibodies to TMEM116 that yielded conflicting data; one antibody suggested that TMEM116 was localized to the nuclear membrane, while the other suggested its localization to endomembrane compartments such as the Golgi and endoplasmic reticulum. To address these conflicting data, we performed subcellular fractionation experiments, and observed that TMEM116 was present in both the cytosolic fraction (containing the endomembrane compartments) and in the nuclei, but with greater amounts of TMEM116 in the cytosolic fraction. These data are consistent with the hypotheses that the expression of ERp29 and TMEM116 are coordinately regulated by their shared bidirectional promoter, and that they are likely both present in similar endomembrane compartments.

P1427
Board Number: B437
Autophagosome formation is involved in unconventional secretion of CFTR.
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CFTR is a cyclic AMP-dependent chloride channel that mediates electrolyte transport across the luminal surface of epithelial cells. ΔF508-CFTR is the most common disease-causing mutation in CF patients. We have previously reported that Golgi reassembly stacking protein (GRASP) is required for an unconventional secretory pathway of core-glycosylated CFTR that bypasses the usual route for Golgi-
dependent membrane traffic. We also showed that core-glycosylated CFTR can reach the plasma membrane and function as Cl- channel by blockade of ER-to-Golgi transport or overexpression of GRASP. The aim of the present study is to demonstrate that autophagy pathway is involved in GRASP-dependent unconventional trafficking of CFTR. Blockade of ER-to-Golgi transport induced the increase of autophagic flux and relocalization of GRASP55 to ER exit sites which is known as sites for autophagosome biogenesis. Surface biotinylation assay of CFTR was performed using PI3K class3 inhibitor, siRNA of PI3KC3 or siRNA of ATG in HEK293 cells transfected with ΔF508-CFTR and GRASP55. Wortmannin and 3-methyladenine, the inhibitor of PI3KC3 which is well known for playing a crucial role in autophagy signaling pathway, inhibited the unconventional trafficking of CFTR to plasma membrane. Knock-down of PI3KC3 reduced the cell surface expression of ΔF508-CFTR in cell GRASP55 overexpressed. In addition, depletion of components which play a role in the autophagosome formation (ATG1, ATG5, ATG7 and ATG8), but not that of lysosome fusion (Vamp7), inhibited unconventional surface trafficking of ΔF508-CFTR. Taken together, our data suggest that factors involved in early autophagosome formation, but not those involved in the late steps of lysosome fusion and degradation pathway, is associated with GRASP-dependent unconventional exocytosis of CFTR in mammalian cells.

P1428
Board Number: B438
Tubular ER shaping protein Reticulon4a/NogoA influences protein trafficking through the secretory pathway.
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The endoplasmic reticulum (ER) is a complex membranous system consisting of flat sheets and interconnected curved tubules, which conventionally correspond to rough and smooth ER, respectively. The ER is also continuous with the nuclear envelope and functionally linked to nearly every other organelle in the secretory pathway. Several curvature stabilizing membrane proteins, including reticulons (RTNs) and REPs/DP1/Yop1p, shape the tubular ER. In this study, we examined the role of Rtn4a/NogoA in protein trafficking through the secretory pathway in cultured HeLa cells. Ectopic overexpression of Rtn4a by ~46 fold enhanced the N-glycosylation and maturation of two plasma membrane markers, Integrin β1 and MHC-I/HLA-A. The mature forms of Integrin β1 and MHC-I, containing complex type N-glycans, were increased by ~3-fold and ~1.6-fold respectively, compared to controls. We also observed increased trafficking of these proteins to the cell surface without changes in their overall levels. Overexpression of Rtn4a did not alter the levels of some important glycosyltransferases and ER stress related chaperones, ruling out some potential mechanisms of action. Rtn4b and REEP5 overexpression, that also affect ER morphology, did not impact cell surface amounts of Integrin β1 and MHC-I, suggesting the effect might be specific to Rtn4a and not overall ER morphology. As previously described, Rtn4a overexpression led to proliferation of the tubular ER, with a more than 2.5-fold decrease in ER sheet volume. Interestingly, we observed a more disperse Golgi morphology concomitant with a drastic increase in Golgi volume, suggesting that altered Golgi morphology might contribute to observed effects of Rtn4a overexpression. Sec31A (a binding partner of COPII in anterograde vesicles) and ERGIC 53 (a marker of ER-Golgi intermediate compartments) also exhibited more scattered distributions in contrast to their perinuclear localization in control cells. In contrast to what we observed for Integrin β1 and MHC-I, ectopically expressed secreted alkaline phosphatase activity in the media was diminished upon Rtn4a overexpression. These results suggest that Rtn4a plays important but differential roles in the trafficking of membrane-bound and secreted proteins. Our
current focus is on studying the roles of other RTNs in protein trafficking and elucidating how Rtn4a influences the secretory pathway.

P1429
Board Number: B439
Unstacking the stacking problem of Golgi.
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Shape regulation of dynamic organelles is a fundamental cell biological problem and the mechanism that regulates Golgi apparatus stacking is still not completely understood. The Golgi apparatus is an important organelle for membrane trafficking and protein processing. In Eukaryotes, Golgi has varying structure: from dispersed cisternae in S.cerevisiae to stacked cisternae in Pichia pastoris and laterally connected ribbon of cisternal stacks in higher eukaryotes like mammals. The mechanisms that generate the cisternal stack organization is not elucidated clearly yet. In mammalian cells GRASPs are reported to be the controlling factor for the cisternal stacking. However, depletion of yeast GRASP homolog Grh1 in Pichia.pastoris has no effect on Golgi stacking. This suggest potential existence for other universally conserved factors. Genes expressing Series of potential such factors were individually deleted in a two color Pichia pastoris strain with endogenous GFP tagged early Golgi cisterna and Ds-Red tagged late Golgi cisterna and consequently cisternal stacking was monitored in these knock out strains. An increased inter-cisternal distance between medial and late Golgi compartment was observed in the deletion mutants of a family of Golgi resident proteins with completely different known functions. Electron microscopy suggests that frequency of TGN peeling from the cisternal stack is highly increased in these mutants. Results suggests the factors are directly or indirectly responsible for maintaining stacking of Golgi Cisternae. These factors possibly maintain their stacking function simultaneously along with their more recognized functions although it is yet to be discovered how they accommodate so. It is possible that these factors probably play similar role in higher order eukaryotes suggesting an universal role as Golgi stacking factors.

P1430
Board Number: B440
Novel interactions of clathrin adaptors at the TGN in yeast.
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Correct membrane traffic is important to target transmembrane proteins to their planned location in the cell. Transmembrane protein traffic through the trans-Golgi Network (TGN) is a highly regulated step. From the TGN, transmembrane cargo proteins can be targeted to different compartments of the cell. Traffic of cargo proteins from the TGN depends in part on clathrin coat assembly through the coordinated action of clathrin adaptor proteins. In the budding yeast, Saccharomyces cerevisiae, TGN clathrin adaptors include the epsins (Ent3/5), Gga1/2 and the AP-1 complex. The recruitment of these clathrin adaptors to sites of clathrin coat formation is highly regulated. Correct recruitment of these clathrin adaptors is important for adequate traffic at the TGN. Recent studies in our lab suggest additional players involved in clathrin adaptor regulation for traffic at the TGN.
P1431
Board Number: B441
apKC Influences Golgi Integrity.
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A variety of signaling molecules have been shown to play a critical role in the regulation and the coordination of intracellular membrane transport. In that regard, we are actively characterizing the role of atypical Protein Kinase C (apKC) in membrane trafficking in the early secretory pathway, a bona fide oncogene that contributes to the pathogenesis of a variety of cancer-types. apKC in tandem with GAPDH, an apKC substrate, are critical downstream effectors for the small GTPase Rab2 that facilitates maturation of pre-Golgi intermediates for incorporation into the Golgi and mediates vesicle formation for cargo recycling from the cis Golgi network back to the ER. We have now found that ectopically overexpressed apKC in normal rat kidney cells caused a striking disorganization/fragmentation of the Golgi complex similar to that observed in various cancer cells, suggesting that apKC is a contributing factor to Golgi homeostasis. The observed Golgi morphological changes induced by apKC ectopic expression in NRK cells was reversed/rescued by expression of siRNA to apKC, which led to intact and compact Golgi structures and thereby confirming specificity. Because the Golgi is a major site for microtubule (MT) formation, and apKC binds directly to MTs, we evaluated the organization of the MT network by indirect immunofluorescence. The apKC transfected cells contained an unstacked/unlinked Golgi ribbon associated with and/or aligned along MT tracks that labeled with an anti-tyrosinated tubulin antibody. These results suggest that apKC overexpression affects a subpopulation of MTs, some of which are anchored to the Golgi and that support structural integrity.

P1432
Board Number: B442
SYP73 Anchors the ER to the Actin Cytoskeleton for Maintenance of ER Integrity and Streaming in Arabidopsis.
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The endoplasmic reticulum (ER) is an essential organelle that spreads throughout the cytoplasm and undergoes extensive remodeling. In plants, the remodeling and motility of the ER rely mainly on actin and to a minor extent on microtubules. Although the actomyosin system has been suggested functional for ER streaming in plant cells, the mechanisms underlying stable interaction of the ER membrane with actin are unknown. We demonstrate here that SYP73, a member of the plant Syp7 subgroup of SNARE proteins is a novel ER membrane-associated actin-binding protein. We show that overexpression of SYP73 causes a striking rearrangement of the ER over actin and that, similar to mutations of myosin-XI, loss of SYP73 reduces ER streaming and affects overall ER network morphology and plant growth. We propose a model for plant ER remodeling whereby the dynamic rearrangement and streaming of the ER network depend on the propelling action of myosin-XI over actin coupled with a SYP73-mediated bridging, which dynamically anchors the ER membrane with actin filaments.
P1433
Board Number: B443
The Microtubule-associated End Binding proteins regulate the ER exporting and trafficking of TRPM4 channels.
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TRPM4 is a Ca2+-activated non-selective cationic channel involved in a wide variety of physiological and pathophysiological processes. Altered activity, expression and localization of these channels might trigger several pathophysiological conditions. Indeed, TRPM4 plasma membrane overexpression has been related to cardiovascular and neurodegenerative diseases and cancer. Therefore, the mechanisms involved in the regulation of TRPM4 activity constitute an important area of biomedical research. Protein-Protein Interactions (PPIs) control the expression, trafficking, localization and biophysical properties of ion channels. Thus, the identification of novel TRPM4-related PPIs and their characterization might contribute to dissect the regulatory mechanisms of this channel. Interestingly, bioinformatics analyses of the primary sequence of TRPM4 allowed us to identify a putative interacting motif to End Binding (EB) proteins, novel members of the microtubule plus-end tracking proteins. These proteins bind a consensus motif (SxIP) in their substrates and are involved in a plethora of cellular processes, including growing dynamics of the microtubule cytoskeleton, focal adhesion dynamics, cell migration and protein trafficking, targeting and localization. Here, we provide novel data suggesting that TRPM4 interacts with EB proteins. Moreover, we show that the mutation (TRPM4ASWIP and TRPM4SWNN) of the putative EB-binding motif abolishes the TRPM4-EB interaction. We also found that these mutant variants show a reduced expression of the mature population of the channel and display an endoplasmic reticulum (ER)-like distribution. Furthermore, these mutant variants present a decreased expression in the plasma membrane, consistent with a reduction in the whole-cell electrophysiological recordings. Conversely, we demonstrated that the expression of a soluble fragment containing the wild type N-terminal region of TRPM4 (EGFP-N-TRPM4WT) reduces the membrane expression of TRPM4. Moreover, we show that the interference of this interaction might constitute a mechanism to modulate dynamics of Focal Adhesions (FAs), reducing cell migration and invasion. These results suggest that EB interaction is necessary for the proper trafficking and activity of TRPM4 at FAs. These findings might contribute to the understanding and characterization of novel mechanisms involved in TRP channel trafficking/localization, and could be applied to the design of therapeutic strategies against TRPM4 gain-of-function-associated pathologies.
P1434
Board Number: B444
VLDL Receptor is required for non-genomic progesterone-dependent signaling to release oocyte meiotic arrest.
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Progesterone (P4) is a steroid hormone that controls female reproductive state and other physiological processes. P4 mediates transcription-dependent signaling in target cells through its nuclear receptor, and in addition activates rapid non-genomic signaling through membrane progesterone receptors (mPR). mPRs are 7-TM integral membrane proteins, that are coupled to various signaling modules, including cAMP, MAPK, and Ca2+ signals. However, the immediate mechanisms of action downstream of mPR remain in question. One of the best studied models of P4 non-genomic action is the release of oocyte meiotic arrest in the frog. Herein we used an untargeted quantitative proteomics approach to define the mPR interactome to better delineate non-genomic P4-mPR signaling. We identify the VLDL Receptor (VLDLR) as an mPR partner protein that preferentially interacts with mPR that localizes to the plasma membrane (PM) in Xenopus oocytes. Knocking down VLDLR abolishes P4-induced oocyte maturation, a phenotype that is rescued by overexpressing VLDLR or mPR. Mechanistically, we show that the VLDLR is required for the trafficking and PM residence of mPR. mPR needs to localize to the PM to mediate its signaling function. Taken together, our data define a novel function of the VLDLR as a trafficking chaperone required for the subcellular localization of mPR and as such non-genomic P4-dependent signaling.

P1435
Board Number: B445
Arf4 is Regulated by ArfGAP1 and Facilitates Sorting of ERGIC53 on pre-Golgi Membranes.
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In humans four highly-homologous Arf proteins (Arf1, Arf3, Arf4 and Arf5) are Golgi-associated. They are divided into the Class I Arfs (Arf1 and Arf3), and the Class II Arfs (Arf4 and Arf5). The Class I Arfs have been extensively studied. However, the Class II Arfs are more poorly understood. In particular, the biological roles of Class II Arfs in the ER-Golgi system are unclear, although one study employing siRNA knockdowns (Volpicelli-Daley et al. Mol. Biol. Cell 16:4495-508; 2005) has found evidence that retrograde trafficking of KDEL receptor from Golgi to ER requires a function of the Class II Arfs (Arf4 and Arf5) that is not supplied by Class I Arfs. Additionally, the mechanisms by which Class II Arfs associate with the Golgi apparatus are unclear, with conflicting studies suggesting recruitment of GFP-tagged Class II Arfs to membrane by brefeldin A-sensitive GEFs similarly to Class I Arfs (Chun et al., Mol. Biol. Cell 19:3488-500; 2008) or recruitment of HA-GFP-tagged Class II Arfs to membrane by a distinct mechanism insensitive to brefeldin A (Duijsings et al., Traffic 10:316-23; 2009). In this study, we further investigate the functions of Class II Arfs as well as the mechanisms by which they are recruited to Golgi membranes. We find that brefeldin A-sensitive Golgi membrane association of Class II Arfs is reduced or abrogated in the presence of a C-terminal HA or HA-GFP tag unless a spacer is employed, possibly because of the presence of negatively charged residues in the HA tag. Thus we employed GFP tags separated from
Class II Arfs by spacers for further studies. Using these tagged Arfs, we find that a major fraction of Arf4-GFP is recruited to early Golgi and pre-Golgi membranes in NRK and HeLa cells, consistent with previous reports (Hamlin et al. J. Cell Sci. 127:1454-63; 2014). Further consistent with a role in the early secretory pathway, we find that siRNA depletion of Arf4 results in a loss of local recycling of GFP-ERGIC53/p58 from pre-Golgi structures in HepG2 cells, suggesting a unique role for Arf4 in sorting of recycling cargo receptors from peripheral pre-Golgi structures. Arf4-GFP, but not tagged Class I Arfs, can be distributed to pre-Golgi structures by siRNA or pharmacological perturbations directed against ArfGAP1, suggesting Arf4 is primarily regulated by ArfGAP1 on these pre-Golgi structures. Supporting this, we conduct in vitro studies in which we find that ArfGAP1, but not ArfGAP2 stimulates coatamer-dependent GTP hydrolysis by Arf4.

P1436
Board Number: B446
OS9 and FBXO6 contribute to CD147 levels.
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CD147 (Basigin, BSG, Emmprin) is part of the immunoglobulin superfamily initially discovered on the surface of tumor cells and found to induce the activity of several matrix metalloproteinases (MMPs). Because the activation of MMPs contributes to metastasis, regardless of cancer type, CD147 plays a key role in the spread and invasiveness of many types of cancer cells (Iacono, Brown, Greene, & Saouaf, 2007; Tortora, Funke, & Case, 2007; Yang & Chen, 2013). Downregulating CD147 expression by targeting the intercellular proteins that shuttle CD147 to the cell’s surface may help prevent metastasis of cancerous cells. Through a search on the BioGRID, we identified a cluster of seven proteins of interest: SEL1L, OS9, SYVN1, VCP, FBXO6, CAND1, and CUL3. All of these proteins have been identified to play roles in the endoplasmic reticulum (ER). These proteins hypothetically interact with CD147 as well as with each other (Bennett, Rush, Gygi, & Harper, 2010; Liu et al., 2012; Tyler et al., 2012; Yu et al., 2013). After overexpression of SEL1L, SYVN1, VCP, CAND1, and CUL3 had no impact on CD147 levels, we have narrowed our focus to OS9 and FBXO6. OS9 is a lectin, binding to terminally misfolded proteins and delivering them to ubiquitin complexes such as the SEL1L-HRD1 (Christianson, Shaler, Tyler, & Kopito, 2008; Tyler et al., 2012). FBXO6, an F-box protein, is a substrate recognition component of the SCF (Skp, Cullin, F-box containing) complex, a subsection of E3 ubiquitin ligases. FBXO6 is thought to bind to misfolded proteins in the ER and then bind SCF, leading to the ubiquitination and degradation of the target protein (Yoshida et al., 2003). Our western blot results show that knockdown of OS9 via small interfering RNA (siRNA) led to an increase of CD147 expression levels in HEK293 cells. Conversely, overexpression of OS9 resulted in a decrease in CD147 protein levels. After transfecting HEK293 cells with OS9 fluorescent fusion proteins, we used fluorescent microscopy to observe OS9 in many vesicles throughout the cells. FBXO6 siRNA-mediated knockdown resulted in an increase of CD147 however, overexpressing FBXO6 also led to a higher level of CD147 expression. FBXO6 fluorescent fusion proteins localized primarily in a perinuclear dot approximately 2 μm in diameter. While OS9 and FBXO6 were previously described as ER-resident, our localizations are very distinct, thus we are in the process of identifying the cellular structures to which these proteins localize. These data suggest that both OS9 and FBXO6 may play a role in trafficking CD147 to the cell’s surface.
P1437

Board Number: B447

Golgi-to-Endoplasmic reticulum retrograde transport involves Rab11-Binding-Protein.

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Rab GTPases regulate membrane trafficking at the stages of vesicle formation, movement and fusion with target compartments. Rab11 GTPase coordinates trafficking at biosynthetic and endocytic recycling routes acting at the trans-Golgi network (TGN), post-Golgi vesicles and recycling endosomes. Rab11 Binding Protein (Rab11BP) has long been described as a potential Rab11 effector but its function remains unknown. The structure of Rab11BP includes a Rab11 binding domain and several domains presumably involved in protein-protein interactions, which include an FFAT-like domain, a proline rich domain and seven WD40 repeats typical of scaffold proteins. Here we used shRNA silencing experiments to first evaluate whether Rab11BP is involved in the Rab11-dependent endocytic recycling of transferring receptor (TfR) and then assessed the protein traffic between the endoplasmic reticulum (ER) and Golgi. We silenced Rab11BP expression with shRNA using lentiviral transduction or microinjection. Rab11BP-silenced cells showed normal TfR endocytosis and recycling analyzed by FACS. However, the distribution of KDEL-GFP and the retrograde-impaired mutant KDEL(D193N)-GFP indicated that Rab11BP functions in Golgi-to-ER retrograde trafficking. Rab11BP silencing led the KDEL-GFP to change its distribution from a predominant ER location to an accumulation at the cis-Golgi, colocalizing with Giantin, while the Golgi-retained mutant KDEL(D193N)-GFP remained unaffected. This indicates an impaired retrograde Golgi-to-ER transport without affecting the anterograde transport from ER to Golgi, which likely impact on the ER function of KDEL-bearing chaperones. Rab11BP silencing decreased TGN46, furin, M6PR and calnexin protein levels and induced the characteristic fragmentation of the TGN associated with an impaired Golgi-to-ER transport. These results indicate that Rab11BP is required for retrograde transport from the Golgi-to-ER contributing to the maintenance of the Golgi structure and homeostasis. As to our knowledge Rab11 has not been involved in this step of the biosynthetic trafficking, our results suggest that Rab11BP might have functions independently of Rab11.

P1438

Board Number: B448

Temporal distribution of the Hantavirus Nucleocapsid Protein in human pulmonary cells (A549) infected with Rio Mamore hantaviruses.

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Hantaviruses are zoonotic diseases of great clinical importance that affect humans and are distributed worldwide. The transmission to humans is by inhalation of contaminated aerosolized excreta from infected rodents and can cause different pathologies. Despite its great importance, little is known about
the replicative cycle and which cell compartments the hantaviruses use for their assembly and budding. Herein, we examine the subcellular distribution of the nucleocapsid (N) protein of hantavirus (Rio Mamore Virus) in different times post infection (p.i.). The N protein is the most abundant viral protein in infected cells and plays several roles during viral assembly and budding. Using fluorescence microscopy, we show that during the replicative cycle, the N protein accumulates in the juxtanuclear region and is also found in puctate structures scattered throughout the cytoplasm that increase in size over the time of infection. These larger structures probably represent "viral factory units". To identify the compartments in which the protein N is located, we examined its localization relative to markers of Golgi compartments (Giantin and TGN46) and early endosomes (SNX2). We found that the N protein colocalize with trans-Golgi network proteins at 48, 72, 96 and 120 h p.i., suggesting that Golgi compartments are the main sites of assembly and viral budding. It was also found that the N protein partially colocalizes with SNX2, suggesting a role for early endosomes in viral assembly. Additional studies with cellular and viral markers at the ultrastructural level could provide more information about the viral assembly and budding processes. Also, it would be interesting to analyze the participation of Golgi to endosome transport factors of the host cell that may contribute to these events. A better understanding of these events will reveal important aspects for the elaboration of strategies aimed at inhibiting viral replication.

Endosomes, Lysosomes, and Lysosome-related Organelles 1

P1439
Board Number: B449
Investigating the role of VPS33A in melanosome biogenesis and function.
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Specificity in membrane fusion is essential for proper organelle maturation. The homotypic fusion and vacuolar protein sorting (HOPS) complex facilitates the tethering of apposing membranes, promotes SNARE complex assembly, and is required for membrane docking and fusion in the classical endolysosomal system, but it is not known how the HOPS complex functions in specialized cell types to facilitate the biogenesis of cell type-specific lysosome-related organelles. Buff (bf) mice, which have a missense mutation in the HOPS complex subunit Vps33a, are hypopigmented, suggesting that the HOPS complex plays a specific role in the biogenesis of melanosomes, lysosome-related organelles required for melanin synthesis and storage. To understand the function of Vps33a in the melanosomal pathway, we analyzed immortalized melanocytes from bf mice (melan-bf cells). Unexpectedly, we show by light and electron microscopy that these cells are hyperpigmented with large, mature melanosomes localized to the cell periphery. The enlarged bf melanosomes maintain an elongated shape and do not appear to represent fused melanosome clusters. This difference in hypopigmentation in the keratinocytes of bf mice and hyperpigmentation in the epithelial melanocytes of bf mice indicate that the HOPS complex may play a role in melanin transfer from melanocytes to keratinocytes. Using immunofluorescence microscopy, we show that lysosomal and melanosomal proteins exist in distinct compartments in melan-bf cells, demonstrating that the bf mutation in Vps33a does not affect protein sorting from early endosomes or the segregation of melanosomal and endolysosomal organelles. Our studies suggest that
the HOPS complex has a specific and unexpected role in regulating the maturation of melanosomes. Future studies will aim to identify a direct or indirect mechanistic role for the HOPS complex in melanosome maturation and function.

P1440
Board Number: B450
Identification of novel regulators for myosin V-mediated cargo transport.
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Transport of organelles to their correct intracellular locations is essential for cellular function and homeostasis. In most cells, long-range movement of organelles occurs on microtubules via kinesin and dynein motors. Some cargoes are then transferred to myosin V motors, which provide short-range transport on actin filaments to the final destination. Thus, the spatial and temporal regulation of cargo release from myosin V likely specifies the terminal destination. It is largely unknown how this regulation is achieved. In the budding yeast, Saccharomyces cerevisiae, the yeast vacuole, similar to the mammalian lysosome, is one of six organelles moved by a myosin V motor, Myo2. Early in the cell cycle, Myo2 binds the vacuole specific adaptor, Vac17, and transports a portion of the vacuole into the bud. Later in the cell-cycle, the vacuole detaches from Myo2 and Vac17 is degraded. Previous work identified some key genes that regulate the termination of vacuole transport. Much of this regulation requires the Vac17-PEST sequence, a motif found in rapidly degraded proteins. An E3 ubiquitin ligase, Dma1, binds to a residue in the PEST sequence, Vac17-pT240, and ubiquitylates Vac17 to target it for degradation. Notably, Dma1 activation requires that another residue in the PEST sequence, Vac17-S222, is phosphorylated by the p21-activated kinase Cla4. Since Dma1 recruitment is not sufficient to ubiquitylate Vac17, additional genes likely play a role in Vac17 degradation and the release of the vacuole from Myo2. To identify these potential genes, we performed a whole-genome, high content screen to identify mutants with elevated levels of Vac17, a phenotype that correlates with defects in vacuole transport. Interestingly, we identified two genes previously shown to regulate vacuole fusion. Notably, mutating other genes involved in vacuole fusion did not disrupt the disengagement of the vacuole from Myo2 or Vac17 turnover. This suggests that the two genes have novel, previously unknown roles in releasing Myo2 from the vacuole. Our data suggest that these two genes regulate phosphorylation of Vac17 residues outside of the PEST sequence. Previously identified regulators acted solely on the PEST sequence, suggesting that these new regulators act in a novel step in vacuole transport. Moreover, while these regulators are required for the degradation of Vac17, they are not required for Vac17 ubiquitylation. Thus, we hypothesize that these regulators are required for the detachment of Myo2 from Vac17 and the vacuole, and suggest that Vac17 must be released from Myo2 before it is degraded. Our data suggests novel roles for these genes in vacuole delivery, and provide new insights into the termination of myosin V-mediated cargo transport.
P1441
Board Number: B451
Non-endocytic clathrin adaptors control post-endocytic sorting to the vacuole.
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The degradation of cell surface proteins is important for cellular homeostasis and responses to environmental changes. This multi-step process involves regulated endocytosis followed by transit through endosomal compartments before delivery into the proteolytic compartment. Although much is known about the early steps of endocytosis at the plasma membrane and the terminal steps at the late endosome, gaps remain in our understanding of how cargo transits from the plasma membrane to the late endosome and whether active protein sorting is important during intermediate steps. Here, we report that non-endocytic clathrin adaptors, Ent5, Ent3, Gga2, and Appl4, play key roles in post-endocytic sorting of degradative cargo in response to multiple environmental signals in Saccharomyces cerevisiae. We show close proximity between non-endocytic clathrin adaptors and cargo en-route to the vacuole using bi-molecular fluorescence complementation. Furthermore, degradative cargo transits through the trans-Golgi network (TGN) after endocytosis and loss of non-endocytic clathrin adaptors causes accumulation of cargo at the TGN. Finally, inhibiting exit from the TGN also blocks degradative traffic. These data support a direct role for non-endocytic clathrin adaptors in post-endocytic sorting of cell surface proteins to the vacuole and, suggest that the TGN is a major sorting station for endocytosed plasma membrane proteins destined to the vacuole.

P1442
Board Number: B452
SNX-BAR mediated retrograde trafficking of yeast synaptobrevin/Snc1 is conferred by its transmembrane domain.
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Synaptobrevin/VAMP2 is an essential SNARE protein that has been extensively studied in its role in synaptic vesicle fusion. However, how synaptobrevin is sorted for trafficking within the cell is not well understood. A unique structural feature of synaptobrevin is a conserved aromatic motif on the cytosol-proximal interface of the juxtamembrane region that has been proposed to confer a 35° tilt angle in the transmembrane domain, allowing conserved basic residues in the transmembrane domain to interact with the membrane surface. The aromatic motif has been shown to promote binding of calmodulin and phospholipids, and to facilitate SNARE complex assembly. In yeast, the synaptobrevin homologue Snc1 has been shown to require the aromatic motif for proper trafficking. We demonstrate that the basic residues on the cytosol-proximal surface of the Snc1 transmembrane domain play an important role in promoting sorting of Snc1 into retrograde trafficking pathways. Additionally, we demonstrate that the Snx4-Agt20 SNX-BAR dimer, which functions in retrograde trafficking of Snc1, binds preferentially to anionic membranes and we propose this contributes to Snc1 recognition and sorting out of the endosome. We further characterize Snc1 trafficking pathways in yeast and propose a bifurcated trafficking model for Snc1 where endocytosed Snc1 and Snc1 on the late/pre-vacuolar endosome are returned to the Golgi via two different retrograde pathways.
P1443

Board Number: B453

Sorting nexin 3 drives phagosomal compaction of Borrelia burgdorferi in primary human macrophages.
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Borrelia burgdorferi is the causative agent of Lyme disease, a multisystemic disorder affecting primarily skin, joints and nervous system. Macrophages and dendritic cells counteract Borrelia dissemination in the human body through capturing, internalization and subsequent degradation of spirochetes. Internalization of B. burgdorferi by primary human macrophages involves compaction of helical borreliae into Rab22a-positive phagosomes that are contacted by Rab5a vesicles. Compaction of the phagosomal surface proceeds by membrane extrusion at the contact sites of Rab5a vesicles. This step is crucial for further processing and elimination of borreliae in phagolysosomes. A screen for regulators of phagosomal membrane tubulation indentified sorting nexin 3 (SNX3). Indeed, both overexpressed and endogenous SNX3 and Rab22a were found to colocalize at borreliae-containing phagosomes during early and late steps of phagosome maturation. Accordingly, compaction of borreliae is reduced in macrophages depeleted for SNX3 and can be rescued by expression of an siRNA-insensitive SNX3 wt construct, but not by a construct defective in phosphoinositide binding. Live cell imaging further showed that Rab22a-positive phagosomes are also positive for PI(3)P, and that SNX3 is recruited to these phagosomes via Rab5a positive vesicles. We propose a mechanism for SNX3 regulation of borreliae phagosome maturation through recruitment of SNX3/Rab5a vesicles to PI(3)P/Rab22a phagosomes, which leads to reduction of the phagosomal surface through membrane tubulation. Our results thus reveal SNX3, in cooperation with PI(3)P, Rab22a and Rab5a, as an important regulator of Borrelia intracellular processing by macrophages, supporting elimination of infecting spirochetes by the human immune system.

P1444

Board Number: B454

Structural basis for the hijacking of endosomal sorting nexin proteins by Chlamydia trachomatis.
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During infection chlamydial pathogens form an intracellular membrane-bound replicative niche termed the inclusion, which is enriched with bacterial transmembrane proteins called Inclusion membrane proteins (Incs). Incs bind and manipulate host cell proteins to promote inclusion expansion and provide camouflage against innate immune responses. Sorting nexin (SNX) proteins that normally function in endosomal membrane trafficking are a major class of inclusion-associated host proteins, and are recruited by one of the Chlamydial Incrs; IncE/CT116. Crystal structures of the SNX5 phox-homology (PX) domain in complex with IncE define the precise molecular basis for these interactions. The binding site is unique to SNX5 and related family members SNX6 and SNX32. Intriguingly the site is also conserved in SNX5 homologues throughout evolution, suggesting that IncE captures SNX5-related proteins by mimicking a native host protein interaction. These findings thus provide the first mechanistic insights
both into how chlamydial Incs hijack host proteins, and how SNXS-related PX domains function as scaffolds in protein complex assembly.

P1445

Board Number: B455

Salmonella exploits the host endolysosomal tethering factor HOPS complex to promote its intravacuolar replication.

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Intracellular pathogens have devised various strategies to subvert the host membrane trafficking pathways for their growth and survival inside the host cells. Salmonella is one such successful intracellular pathogen that extensively remodels the host endolysosomal compartments to establish its vacuolar niche within the host cells that is conducive for its replication, also known as the Salmonella-containing vacuole (SCV). Vacular Salmonella initiates and maintains a prolonged interaction with endosomes and lysosomes of the host cell in the form of interconnected network of tubules (Salmonella-induced filaments or SIFs), gaining access to both membrane and fluid-phase cargo from these compartments. This is essential for vacuolar membrane integrity and intravacuolar nutrition. Here, we have identified the multisubunit lysosomal tethering factor-HOPS (HOMotypic fusion and Protein Sorting) complex facilitates acquisition of endolysosomal content by SCVs, providing membrane for SIF formation, and nutrients for intravacuolar bacterial replication. HOPS subunits were enriched in the SCV fraction from 3 hr to 10 hr post infection as revealed by density gradient ultracentrifugation of Salmonella infected HeLa cell homogenates. Not surprisingly, lysosomal small GTPase Arl8b that promotes membrane localization of HOPS complex was required for HOPS recruitment to SCVs and SIFs. Depletion of various HOPS subunits significantly reduced the bacterial load in non-phagocytic and phagocytic cells as well as in a mouse model of Salmonella infection. SCV interaction with dextran-loaded lysosomes and SIF formation was significantly impaired in HOPS depleted cells. Further, using auxotrophic strains of Salmonella that are deficient in proline biosynthesis, we demonstrate that SCV access to external proline requires HOPS-mediated interaction with the endolysosomal compartment. We found that Salmonella effector SifA in complex with its binding partner; SKIP, interacts with HOPS subunit Vps39 and mediates recruitment of HOPS subunits to SCV compartments. Our findings suggest that Salmonella recruits the late endosomal and lysosomal tethering factor-HOPS complex to its intracellular vacuole for access to membrane and nutrients, ensuring its growth and survival within the host cells.

P1446

Board Number: B456

Increased biogenesis and fusion of early endosomes promotes complement activation in the retinal pigment epithelium.

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Deregulation of the complement system and consequent inflammation are strongly associated with age-related macular degeneration (AMD), which causes irreversible blindness in millions of older adults
worldwide. The RPE, a monolayer of post-mitotic cells that sits beneath the photoreceptors, is the first line of defense against abnormal complement activation. Recent work from our lab has identified two rapid mechanisms – recycling of complement regulatory proteins and membrane repair by lysosome exocytosis - that help the RPE curtail complement-mediated inflammation (Tan et al., PNAS, 2016). We have previously reported that in models of macular degeneration, accumulation of vitamin A metabolites in the RPE results in increased levels of cholesterol, which activates acid sphingomyelinase (ASMase), the enzyme that hydrolyzes sphingomyelin to ceramide (Toops et al., Mol Biol Cell, 2015). Here, using high-speed live-cell imaging of primary RPE monolayers, we show that excess ceramide on the apical plasma membrane of the RPE results in an increase in early endosome biogenesis. We also observed formation of enlarged endosomes following increased fusion between nascent endosomes, which is likely driven by non-bilayer phases formed by ceramide. Live-cell imaging revealed that these enlarged early endosomes are significantly less motile than the smaller endosomes. Our data suggest that these features are caused by cholesterol-induced elevation of ceramide on the plasma membrane, which promotes inward budding. Consistent with this hypothesis, removal of excess cholesterol in models of macular degeneration restores the early endosome size and motility. Recent studies show that early endosomes regulate intracellular complement activity by internalizing the complement protein C3, whose cleavage is the initial step that triggers complement attack. To dissect how enlargement of early endosomes might affect complement regulation in the RPE, we examined the co-localization of C3 with early endosomes in the RPE in a mouse model of inherited macular degeneration (Abca4-/-). Our data revealed that RPE in Abca4-/- mice stain more strongly for C3. Importantly, these signals co-localize with the early endosome marker EEA1. This is accompanied by increased formation of active C3 cleavage products in the RPE of Abca4-/- mice compared to age-matched wild-type. Altogether our data suggest that in models of macular degeneration, increased biogenesis and fusion of apical early endosomes in the RPE could contribute to deregulation of complement system by increasing generation of active intracellular C3.

**P1447**

**Board Number: B457**

**TFEB regulates lysosomal positioning by modulating TMEM55B expression and JIP4 recruitment to lysosomes.**

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Lysosomal distribution within cells is directly linked to the role of lysosomes in many cellular functions, including autophagosome degradation, cholesterol homeostasis, antigen presentation, and cell invasion. Moreover, alterations in lysosomal positioning contribute to different human pathologies, such as cancer, neurodegeneration and lysosomal storage diseases. Here we report the identification of a novel mechanism of lysosomal trafficking regulation. We found that the lysosomal transmembrane protein TMEM55B promotes recruitment of JIP4 to the lysosomal surface, thus inducing dynein-dependent transport of lysosomes toward the microtubules minus-end. Accordingly, over-expression of TMEM55B causes lysosomes to collapse into the cell center, whereas depletion of either TMEM55B or JIP4 results in dispersion toward the cell periphery. TMEM55B levels are transcriptionally upregulated following TFEB and TFE3 activation by starvation or cholesterol-induced lysosomal stress. TMEM55B or JIP-4 depletion abolishes starvation-induced retrograde transport of lysosomes and prevents fusion between autophagosomes and lysosomes. JIP4-mediated retrograde transport is also observed upon induction of lysosomal stress by sodium arsenite or curcumin though a mechanism that requires MAPK activation.
Overall, our data reveal a novel role of TMEM55B linking lysosomes to microtubule motors and suggest that the TFEB/TMEM55B/JIP4 pathway plays a critical role in cellular adaptation to stress by coordinating control of lysosomal movement in response to variations in nutrient and cholesterol levels.

**P1448**

**Board Number: B458**

**VMAT2 and SLC35D3 sort from endolysosomes to dense granules during megakaryocyte differentiation to proplatelets.**


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Dense granules (DGs) are lysosome-related organelles (LROs) in platelets that store small molecules like calcium, ADP, and serotonin and larger polyphosphates. Release of these contents during platelet activation is necessary for efficient clot formation during vascular injury. Hence, defects in DG formation, as occurs in Hermansky-Pudlak syndrome (HPS), result in excessive bleeding and bruising. However, how DGs are formed within the nucleated platelet precursors, megakaryocytes (MKs), is not known. In particular, while putative DG transmembrane proteins have been identified, how and when DG membrane contents are delivered is not at all known. We first sought to determine the localization of these putative DG proteins, SLC35D3 and VMAT2, in MKs. SLC35D3 was previously shown to localize to early endosomes labeled by Syntaxin-13 in multinucleated but intact MKs; our new data with live cell probes further indicate that these endosomes also harbor VMAT2 and are unusually acidic as assessed by labeling with acidotropic agents. Preliminary data further suggest that these endosomes are a type of hybrid organelle with features of both early and late endosomes. Next, we visualized SLC35D3 and VMAT2 at later stages of MK differentiation in an attempt to capture protein sorting during DG maturation. Our lab previously showed that DG biogenesis occurs at a later stage of MK differentiation than previously thought. After maturation, MKs undergo morphological changes to form proplatelets, which are long “beads on a string” structures from which platelets are shed. Using vital imaging with mepacrine, which label DGs in platelets, we observed separation of mepacrine from lysosomal markers during this stage of differentiation. By utilizing an engineered stem cell line that differentiates to MKs and mimics MK maturation to proplatelets, we show that both SLC35D3 and VMAT2 co-segregate from acidic compartments with mepacrine in proplatelets. Lastly, we tested the putative sorting signals in SLC35D3 and VMAT2 in binding AP-3, an adaptor protein complex that is mutated in HPS types 2 and 10. Using a yeast two-hybrid and GST pulldown assays, we demonstrate that SLC35D3 relies on its tyrosine-based sorting signal to bind in vitro to AP-3. By contrast, VMAT2’s putative dileucine-based sorting signal did not bind to AP-3 in vitro; nevertheless preliminary data suggest that it is required for effective cosegregation with mepacrine in proplatelets. Together, our data suggest a model in which DGs mature in proplatelets by cargo delivery from unusually acidic endo-lysosomes, somewhat akin to models of melanosome (another type of LRO) biogenesis in melanocytes.
P1449

Board Number: B459
Regulated recruitment of C9orf72 to lysosomes supports diverse signaling and degradative functions.
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Expansion of a hexanucleotide repeat in an intron of the C9orf72 gene causes amyotrophic lateral sclerosis and frontotemporal dementia (ALS-FTD), two related neurodegenerative diseases. These insights from human genetics have stimulated significant interest in understanding C9orf72 functions. However, beyond bioinformatics predictions that have suggested structural similarity to a subset of DENN domain containing proteins that include folliculin (FLCN), the Birt-Hogg-Dubé syndrome tumor suppressor, little was known until recently about the normal cellular functions of the C9orf72 protein. To address this problem, we used genome editing strategies to investigate C9orf72 interactions, subcellular localization and knockout (KO) phenotypes. We found that C9orf72 robustly interacts with SMCR8 and WDR41 (proteins of previously unknown function). Epitope tag insertion into the endogenous human C9orf72 gene locus, allowed us to establish that C9orf72 dynamically binds to the cytoplasmic surface of lysosomes and that such localization is negatively regulated by amino acid availability. Analysis of C9orf72 KO cell lines revealed diverse phenotypes that are consistent with a function for C9orf72 at lysosomes. These include abnormally swollen lysosomes in the absence of C9orf72, impaired responses of mTORC1 signaling to changes in amino acid availability (a lysosome-dependent process) and defects in lysosome-mediated recycling of both endocytic and autophagic cargoes. Through the use of additional CRISPR-Cas9 knockin and knockout strategies targeted towards SMCR8 and WDR41, we have gained new insights into specific roles for individual components of the C9orf72-SMCR8-WDR41 complex in regulating complex assembly, recruitment to lysosomes and downstream effects on mTORC1 signaling. Collectively, these results identify strong, constitutive, physical interactions between C9orf72, SMCR8 and WDR41 and support an important lysosomal site-of-action for this protein complex. Such insights are relevant for both interpreting the contributions of C9orf72 haploinsufficiency to neurodegenerative disease as well as understanding the fundamental mechanisms that match lysosome function to ongoing changes in cellular demand.

P1450

Board Number: B460
pHLARE: a ratiometric pH-biosensor for measuring lysosomal pH.
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Lysosome functions in nutrient sensing, metabolic adaptation, and quality control of proteins are central to normal cell biology, and dysregulated lysosome functions are increasingly being recognized as determinants in disease pathologies. Central to normal lysosome functions is maintaining an exceptionally low pH of ~5.0 for the activity of luminal acid-activated proteases, phosphatases, and phospholipases. Dysregulated lysosomal pH (pHlys) occurs in diseases such as neurodegenerative disorders and diabetic nephropathy, where pHlys is constitutively increased, which is predicted to decrease protein degradation and promote protein aggregation. Conversely, a current view that has not be effectively validated is that pHlys is decreased in cancers, presumably to enable increased autophagy and biomass production. Accurately measuring pHlys, however, is limited by currently available pH
sensing probes such as Lysosensor, Lysotracker, or fluorescein-conjugate dextran that are incorporated into cells by phagocytosis and accumulate not only in lysosomes but also in endosomes and other intracellular vesicles. To resolve this limitation we generated a ratiometric genetically encoded lysosomal pH-biosensor, pHLARE (pH Lysosomal Activity Reporter), which encodes the lysosome membrane protein LAMP1 tagged at the luminal domain with superfolderGFP (sfGFP) and at the cytoplasmic domain with mCherry. We confirmed that pHLARE localizes predominantly to lysosomes by showing co-localization with endogenous LAMP1 and with live-cell imaging confirming localization in vesicles with Rab7 but not Rab5. We also confirmed that between pH 5.0 and 6.5 pHLARE has pH-sensitive sfGFP and pH-insensitive mCherry emissions, determined in buffers containing the protonophore nigericin. With calibrated ratiometric values, we determined pHlys in CCL39 hamster lung fibroblast cells and MCF7 human breast cancer cells to be 5.32 +/- 0.43 and 5.13 +/- 0.21, respectively. Acute treatment with the V-ATPase inhibitor bafilomycin increased pHlys to 6.78 +/-0.42 in CCL39 cells and to 6.64 +/-0.46 in MCF7 cells. Our findings validate pHLARE as a new reagent to accurately measure pHlys dynamics that can be used for understanding lysosome function and dysfunction. pHLARE will also be useful in revealing regulators of pHlys that could be therapeutic targets, for example to retain a lower pHlys with neurodegenerative disorders and maintain activities of acid-activated proteases for protein degradation.

**P1451**

**Board Number: B461**

**The Structure of Melanoregulin Reveals a Role for Cholesterol Recognition in the Protein’s Ability to Promote Dynein Function.**

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Melanoregulin (Mreg) is the product of the dilute suppressor locus, an extragenic suppressor of the coat color defect exhibited by myosin Va null mice. Mreg is a small, highly-charged, multiply-palmitoylated protein that is present on the limiting membrane of melanosomes. Previous studies have implicated Mreg in the transfer of melanosomes from melanocytes to keratinocytes (Wu et al, PNAS 2012), and in promoting the microtubule minus end-directed transport of these and related organelles by binding to RILP, a Rab7 effector that recruits the dynein motor complex (Ohbayashi et al, JCS 2012; Wu et al BBRC 2012). Here we shed new light on the possible molecular function of Mreg by solving its structure using nuclear magnetic resonance (NMR). The structure reveals bands of positive and negative charge that occupy opposite sides of the protein’s surface, and that sandwich a putative, tyrosine-based (Y166) cholesterol recognition sequence (CRAC motif). We confirmed that cholesterol interacts with Mreg, as residues S163 and L168 within the CRAC motif show the largest NMR chemical shift change upon cholesterol addition. Importantly, Mreg containing a function blocking point mutation within its CRAC motif (Y166I) still targets to late endosomes/lysosomes, but no longer promotes their microtubule minus end-directed transport. Reversing the charge of three closely-spaced acidic residues (D177, E180, and D181) also inhibits Mreg’s ability to drive these organelles to microtubule minus ends, but only partially. We propose that cholesterol recognition alters Mreg’s orientation on the membrane in such a way as to allow it to interact with a component(s) involved in dynein recruitment (e.g. RILP), and that this interaction is further promoted by the acidic patch. Finally, we draw comparisons between Mreg and the protein ORP1L, which controls the microtubule minus end-directed transport, positioning and fate of late endosomes in part by recognizing both cholesterol and components that target dynein.

**P1452**
**Board Number: B462**

Inhibition of HDACs 1, 2, and 3 is Necessary for Clearance of Cholesterol Accumulation in NPC1 in Fibroblasts.

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Niemann-Pick disease type C (NPC) is a rare genetic cholesterol storage disorder caused by mutations in the NPC1 protein. Mutations in this trans-membrane protein lead to a block in normal cholesterol efflux from the late endosomes and lysosomes. Pan-histone deacetylase inhibitors (HDACi) such as Vorinostat (SAHA) can correct the cholesterol accumulation phenotype in NPC1 patient fibroblast cells. We screened 125 HDAC inhibitors that targeted either individual HDACs or classes of HDACs to identify which were necessary for cholesterol clearance in NPC1 patient-derived fibroblast cells. We used a high-throughput microscopy screening assay to identify compounds that resulted in cholesterol clearance from these cells. We have determined that the important targets for HDAC inhibition for cholesterol clearance in NPC1 fibroblasts are HDACs 1, 2, and 3.

**P1453**

**Board Number: B463**

A novel approach to analyze lysosomal dysfunctions through subcellular proteomics and lipidomics: the case of NPC1 deficiency.

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Superparamagnetic iron oxide nanoparticles (SPIONs) have mainly been used as cellular carriers for genes and therapeutic products, while their use in subcellular organelle isolation remains underexploited. We engineered SPIONs targeting distinct subcellular compartments. Dimercaptosuccinic acid-coated SPIONs are internalized and accumulate in late endosomes/lysosomes, while aminolipid-SPIONs reside at the plasma membrane. These features allowed us to establish standardized magnetic isolation procedures for these membrane compartments with a yield and purity permitting proteomic and lipidomic profiling. We validated our approach by comparing the biomolecular compositions of lysosomes and plasma membranes isolated from wild-type and Niemann-Pick disease type C1 (NPC1) deficient cells. Our lipidomics analysis revealed the buildup of several species of glycerophospholipids and other storage lipids in late endosomes/lysosomes of NPC1 KO cells. While the plasma membrane proteome remained largely invariable, we observed pronounced alterations in several proteins linked to autophagy and lysosomal catabolism reflecting vesicular transport obstruction and defective lysosomal turnover resulting from NPC1 deficiency. Thus the use of SPIONs provides a major advancement in fingerprinting subcellular compartments, with an increased potential to identify disease-related alterations in their biomolecular compositions.
Neuronal Degeneration - AD, PD, HD

P1454
Board Number: B465
Extracellular oligomers made from the microtubule-associated protein, tau, induce focal accumulation of endogenous neuronal tau coupled to alterations in microtubule-based fast axonal transport.
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Insoluble hyperphosphorylated aggregates of the microtubule-associated protein, tau, define a subset of neurodegenerative disorders known as tauopathies, of which Alzheimer’s Disease (AD) is the most prevalent. Extracellular tau can induce the accumulation and aggregation of intracellular tau, and tau pathology can propagate along defined neuroanatomical networks in a manner that recapitulates the temporal spread of pathology observed upon post-mortem analysis of diseased tissue. There are six splice variants of central nervous system tau, and various oligomeric and fibrillar forms are associated with neurodegeneration in vivo. The particular extracellular forms of tau capable of transferring tau pathology from neuron to neuron remain ill-defined, as do the consequences of intracellular tau aggregation on neuronal physiology. This study was undertaken to compare the effects of extracellular tau monomers, oligomers and filaments comprising various tau isoforms on the behavior of cultured wild type neurons. This work demonstrates that tau oligomers provoke accumulation of endogenous intracellular tau much more effectively than monomers or fibrils. This effect is most pronounced for oligomers comprising isoforms with 2, as opposed to 0 or 1 N-terminal inserts, and for those isoforms with 4, rather than 3 microtubule binding repeats. A mixture of all 6 isoforms most potently provokes focal, intracellular tau accumulation and extensive tau accumulation is associated with invasion of tau into the somatodendritic compartment. Finally, this work shows that 2N4R oligomers perturb fast axonal transport of membranous organelles along microtubules. Intracellular tau accumulation is often accompanied by increases in the run length, run time and instantaneous velocity of membranous cargo, and these alterations in fast axonal transport are diminished in neurons not expressing tau. This study provides a physiologically relevant model of tau propagation in neurons, and indicates that extracellular tau oligomers can disrupt normal neuronal homeostasis by triggering focal tau accumulation and loss of the polarized distribution of tau, and by hyperactivating fast axonal transport. Additionally, by identifying tau species involved in cellular dysfunction, it provides potential targets for much needed therapies in AD and non-Alzheimer’s tauopathies.

P1455
Board Number: B466
A Novel Lysosome-to-Mitochondria Signaling Pathway and its Role in Disease.
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Inter-organelle communication is an emerging topic in cell biology, however little is known about the underlying mechanisms, its functional consequences or its potential role in disease pathogenesis. We
have developed a two-photon fluorescence lifetime imaging assay that detects mitochondrial activity in live cultured cells by monitoring autofluorescence of the mitochondrial coenzyme, NAD(P)H. Variation in enzyme-bound NAD(P)H is used as a read-out for changes in mitochondrial activity. In primary mouse cortical neurons and human NPC-derived neurons, nutrient-mediated activation (i.e., insulin, or arginine plus leucine) of the lysosome-associated, multi-subunit protein kinase complex, mTORC1, stimulates perikaryal mitochondrial activity within minutes. This effect is negatively regulated by: 1) Torin1, an inhibitor of mTOR, the catalytic subunit of mTORC1 and mTORC2; 2) knocking down Raptor, an essential mTORC1 subunit; or 3) forcing mTORC1 to associate with the plasma membrane instead of lysosomes. In addition, we found that this nutrient-induced mitochondrial activity (NiMA) pathway does not involve mTORC2 activity nor expression of eIF4E, which mediates mTORC1-regulated mRNA translation. NiMA was found to occur independently of autophagy or fatty acid transport into mitochondria, but was significantly reduced by amyloid-β oligomers (AβOs), which trigger Alzheimer’s disease (AD), and which we previously found to activate mTORC1 at the plasma membrane, but not at lysosomes (Norambuena et al. 2017. Alzheimers & Dementia 13: 152-167). In addition, we observed that either forcing mTORC1 to lysosomes, or downregulating the activity of either of two endogenous, lysosome-associated mTORC1 inhibitors, the TSC or GATOR1 complexes, leaves mitochondria insensitive to nutrient stimulation. In line with these results, NiMA was also disrupted in human fibroblasts obtained from patients affected by tuberous sclerosis, a genetic disorder triggered by dysfunctions in the TCS complex. Finally, using multi-parametric photoacoustic microscopy (Ning et al, 2015. Scientific Reports 5: article # 18775), which allows high-resolution and quantitative imaging of hemodynamics and oxygen metabolism in the live mouse brain, we also found that arginine plus leucine significantly alter cerebral oxygen consumption in vivo. Collectively, these results indicate that lysosomal mTORC1 couples nutrient availability to mitochondrial activity, thus functionally connecting these two organelles. This novel pathway also represents a new mechanistic link connecting metabolic alterations to mitochondrial diseases.

P1456
Board Number: B467
--JIP3 suppresses the development of amyloid plaque pathology by promoting the axonal transport of lysosomes.
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Lysosome-filled axonal swellings are a major, but poorly understood feature of Alzheimer’s disease amyloid plaques. It is not clear what causes these axonal lysosomes to accumulate at amyloid plaques or whether they contribute to the disease pathology. Our previous characterization of these lysosomes at amyloid plaques led us to propose a model where these axonal lysosomes accumulate due to a local defect in their retrograde transport and maturation and represent pathologically meaningful sites of Aβ production. To test this hypothesis, we have sought to develop mouse genetic strategies to alter axonal lysosome abundance and examine the impact on amyloid plaque pathology. To this end, we identified JIP3 as a candidate for regulating axonal lysosome abundance and validated such a function through extensive cell biological characterization of JIP3 KO mouse neurons. These efforts revealed that JIP3 is an important regulator of the transport, abundance and maturation state of axonal lysosomes. Furthermore, we observed multiple similarities between the axonal lysosomes that accumulate in JIP3 KO neurons and those that build up around amyloid plaques. This includes a build up of amyloid precursor protein (APP), (BACE1) and presenilin 2 (PSEN2). The high levels of these proteins on axonal lysosomes from JIP3 KO neurons suggested that loss of JIP3 is accompanied by an increase in the
processing of APP into Aβ peptides. Indeed, we detected significantly elevated Aβ levels in the JIP3 KO neurons. Finally, we tested the in vivo relationship between JIP3-dependent axonal transport of lysosomes and amyloid plaque pathology by reducing JIP3 gene dosage in a mouse model of Alzheimer’s disease. These experiments revealed that loss of JIP3 was accompanied by an increase in soluble Aβ levels, amyloid plaque size, abundance and axonal dystrophy. These results collectively support a model wherein efficient retrograde axonal lysosome transport and maturation acts as a negative regulator of APP processing and the development of amyloid plaque pathology.

**P1457**

**Board Number: B468**

**Cofilin-actin rods as mediators of α-synuclein-induced synaptic dysfunction in Parkinson’s disease.**

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Cofilin, a major regulator of actin filament severing and turnover, has received increasing attention in neurodegenerative diseases due to its ability to modulate actin in synapses and in neurites form rod-shaped inclusions containing coflin and actin. These structures have been mainly studied in the context of Alzheimer’s disease (AD) as possible mediators of axonal transport alterations and synaptic dysfunction in hippocampal neurons. Cofilin-actin rods are formed upon coflin hyperactivation by dephosphorylation, leading to co-assembly in a 1:1 complex with actin, which bundle into rods under conditions of oxidative stress. A role for the cellular prion protein (PrPc) in inducing the cognitive deficits in a mouse model of AD has been previously documented, where it serves as a co-receptor for Aβ oligomers and is required for Aβ oligomer induction of coflin-actin rods. In Parkinson’s disease (PD), in addition to the characteristic motor symptoms, cognitive impairment and dementia are often present and may be associated with α-synuclein accumulation in the hippocampus. In this work we addressed whether cognitive dysfunction in PD might be mediated by coflin pathology induced by alpha synuclein. In this respect, we observed that the overexpression of α-synuclein in hippocampal neurons induces the formation of coflin-actin rods. Moreover, our results point to a partial involvement of the PrPC in the α-synuclein-induced rod formation. Currently we are assessing rod formation in vivo through the evaluation of brains from a neuronal overexpressing α-synuclein PD mouse model, which presents cognitive dysfunction. Simultaneously, we are further dissecting the impact of α-synuclein on coflin/actin pathology in hippocampal neurons as well as the underlying molecular mechanisms. Importantly, we will determine the consequences of α-synuclein-induced actin cytoskeleton damage for neuronal function. In summary, our goals aim at validating coflin/actin pathology as a novel therapeutic target for PD dementia.

This work was financed by the project Norte-01-0145-FEDER-000008 - Porto Neurosciences and Neurologic Disease Research Initiative at I3S, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER); and by Luso American Development Foundation (FLAD).
Neurodegenerative diseases are debilitating, incurable and often fatal. As our population ages, the incidence of these diseases has increased and we still know little about what causes them. Further complicating matters, the overwhelming majority of diagnoses involve no genetic component. Recent evidence points to the contributions from epigenetic mechanisms to neurodegenerative disease etiology and progression. Epigenetics is broadly defined as any change in gene expression where the underlying DNA sequence is unaltered. Chromatin, a complex primarily involving DNA and histone proteins, controls access to the DNA. Changes in chromatin structure, brought by mechanisms such as the post-translational modifications (PTM) of histones, regulate gene transcription. Here, we characterize the epigenetic landscape of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) and Parkinson’s Disease (PD). Exploiting yeast and human models of ALS and PD, we have characterized changes in histone PTM levels and find distinct histone modification profiles for each of these ailments. Our results suggest that epigenetic mechanisms play an important role in ALS and PD and are an attractive new target in the development of novel therapeutics and treatments for these devastating diseases.

Significant evidence supports a crucial role for the autophagy-lysosomal pathway in the degradation of misfolded proteins that accumulate in numerous neurodegenerative disorders, including Parkinson’s disease (PD). Lysosomes and autophagosomes, the two major degradative components of this pathway, are believed to be, in part, regulated by the action of the transcription factor EB (TFEB). TFEB is normally sequestered inactive in the cytosol but accumulates in the nucleus upon activation by various cellular and lysosomal stresses where it binds to a 10bp CLEAR (Coordinated Lysosomal Expression and Regulation) promoter motif and upregulates the transcription of numerous lysosomal and autophagy genes. Supporting a role for TFEB activity in neuroprotection, nuclear TFEB is selectively reduced in human AD, ALS, and PD brains and co-localizes with alpha-synuclein in Lewy bodies (Decressac 2013 PNAS; Wang 2016 Neurosci J). PD meta-GWAS (Nalls 2014 Nat Genet) and other studies have also identified mutations in several direct targets of TFEB (e.g. GBA, VPS35) as genetic causes or risk factors for PD. Furthermore, TFEB viral overexpression is reported to be protective in a rat model of alpha-synuclein toxicity (Decressac 2013 PNAS) and multiple rodent models addressing amyloid beta and tau pathology (Xiao 2014 & 2015 J Neurosci; Polito 2014 EMBO Mol Med). These studies highlight the current well-founded enthusiasm for targeting TFEB activation as a novel therapeutic intervention to treat diseases associated with autophagy and lysosomal dysfunction, like PD. The purpose of the present study was to therefore identify a panel of TFEB target genes that provides a reliable, CNS-relevant transcriptional profile readout for TFEB activation and can consequently serve as a valuable assay tool for research and drug development efforts. Although hundreds of TFEB target genes have been
identified, previous transcript profiling has largely been carried out in immortalized cell lines rather than in disease-relevant CNS cell types. We evaluated the mRNA expression profile of a curated list of published TFEB target genes following TFEB overexpression in human iPSC-derived astrocytes and dopaminergic neurons, rat cortical neurons, and a HeLa cell reference line. Of the 85 genes tested, 27% were significantly upregulated in response to TFEB overexpression in both the HeLa and one CNS cell type, with 5% significantly upregulated across all cell lines tested (fold-change > 1.4). These identified responsive subsets could be used to confidently demonstrate TFEB target engagement and function across both CNS and non-CNS cell types frequently used for drug discovery.

P1460
Board Number: B471
Prion-like transmission of mutant huntingtin aggregates in Drosophila brains.
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Huntington’s Disease (HD) is an inherited neurodegenerative disorder caused by an autosomal dominant mutation in the gene that encodes the protein huntingtin (Htt). This mutation results in expansion of a polyglutamine (polyQ) region located near the N-terminus of the Htt protein. Expanded polyQ stretches prevent the Htt protein from folding properly and causes it to self-assemble into protein aggregates that are visible as dense, proteinaceous inclusions within neurons and glia in HD patient brains. Mounting evidence supports the hypothesis that mutant Htt aggregates and pathogenic aggregates associated with other neurodegenerative diseases (e.g. Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis) spread between cells in a manner similar to infectious prions. These aggregates can transfer to neighboring cells and there cause nucleated aggregation of native proteins. This “prion-like” transfer is thought to contribute to pathogenesis in neurodegenerative diseases. We have previously used the model organism Drosophila melanogaster to demonstrate prion-like transfer of mutant Htt aggregates from neurons to phagocytic glia in intact brains (Pearce et al., 2015, Nat Commun). These experiments use the Gal4-UAS and QF-QUAS binary expression systems to generate transgenic flies that express mutant and wild-type Htt proteins in independent cell populations in the same brain. Our studies also exploit the ability of mutant Htt aggregates to effect prion-like conversion of wild-type Htt expressed in “recipient” cells as a reporter for cytoplasmic entry of aggregates originating in “donor” cells. Applying a similar experimental paradigm, we have recently found that mutant Htt aggregates transfer from pre-synaptic olfactory receptor neurons (ORNs) to post-synaptic projection neurons (PNs) in the Drosophila brain. Remarkably, ORN-to-PN transfer of Htt aggregates requires the glial phagocytic receptor, Draper. These results indicate that mutant Htt aggregates transfer between diverse cell types in intact brains and suggest that phagocytic glia promote trans-synaptic aggregate transmission. A better understanding of the molecular mechanisms that allow pathogenic aggregates to spread between neurons and glia will lead to the identification of novel therapeutic targets to combat these fatal disorders.
P1461
Board Number: B472
Investigating Natural Remedies to Alleviate Parkinsons Manifested Symptoms.
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Parkinson's Disease (PD) is the second leading neurodegenerative disease in the United States and currently has no treatment that targets the pathogenesis. It causes degeneration of neurons of the substantia nigra pars compacta (SNc), thereby decreasing the release of dopamine from these cells. We have employed rotenone to induce PD-like symptoms in SH-SY5Y cells and it was observed that nerve cells pretreated with the extract of *Achillea millefolium* were protected from damage induced by rotenone. To identify the neuroprotective compounds in this extract, gravity column chromatography was used to separate and collect fractions from yellow and red flowers of *A. millefolium*. These fractions were air-dried and the dried extract was dissolved in DMSO to pretreat the SH-SY5Y nerve cells and test if neuroprotective compounds in that fraction could protect from 500 nM rotenone damage. The number of cells viable or damaged was analyzed using MTT assays. It was concluded that the fraction 4 from the yellow flowers of *A. millefolium* did protect the cells from rotenone induced damage to a significant degree. Future directions is to identify the major compound in the fraction 4 and use in silico modeling analysis to determine its targets. The data from this research project would lead to development of a novel natural neuroprotective compound that could be used as a supplemental to reduce neuro-degeneration in PD patients.

P1462
Board Number: B473
ASM regulates the autophagic process by controlling lysosomal biogenesis in Alzheimer's disease.
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In Alzheimer's disease (AD) abnormal sphingolipid metabolism has been reported, although the pathogenic consequences of these changes have not been fully characterized. Here we show that acid sphingomyelinase (ASM) is increased in fibroblasts, brain and/or plasma from patients with AD and in AD mice, leading to defective autophagic degradation due to lysosomal depletion. Partial genetic inhibition of ASM (ASM+/-) in a mouse model of familial AD (APP/PS1) ameliorated the autophagocytic defect by restoring lysosomal biogenesis, resulting in improved AD clinical and pathological findings, including reduction of amyloid-beta deposition and improvement of memory impairment. Similar effects were noted after pharmacologic restoration of ASM to the normal range in APP/PS1 mice. Autophagic dysfunction in neurons derived from familial AD patient-induced pluripotent stem cells (iPSCs) was restored by partial ASM inhibition. Overall, these results reveal a novel mechanism of ASM pathogenesis in AD that leads to defective autophagy due to impaired lysosomal biogenesis, and suggests that partial ASM inhibition is a potential new therapeutic intervention for the disease.
P1463
Board Number: B474
Mechanism of glycan binding by α-Synuclein.
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α-Synuclein (α-Syn) is the key constituent of Lewy bodies, the pathological hallmark of synucleinopathies such as Parkinson’s disease. While a significant amount of research has been dedicated to studying α-Syn aggregation and disease mechanisms, the biological functions of the protein remain poorly understood. α-Syn is predominantly expressed in the brain and is likely involved in the regulation of synaptic vesicles. Our lab has recently discovered that the α-Syn is a glycan binding protein with specificity for complex N-linked glycans. Data suggests that N-terminal region of α-Syn is involved with recognition of and binding to glycans, prompting us to further investigate the mechanism of glycan binding. As all five point variants of α-Syn linked to Parkinson’s disease – A30P, E46K, A53T, H50Q, and G51D, are located in the N-terminal domain, we investigated the impact of these mutations in glycan binding. Using a combination of biophysical approaches, we probed the structural and thermodynamic features of α-Syn mutants upon binding glycans, as well as their impact in internalization of α-Syn by neuronal lineage cells. Further biophysical characterization of this novel glycan binding activity of α-Syn and its disease mutants will provide more insights into the mechanism of glycan binding and its significance. Identification of putative glycosylated protein partners will significantly advance the understanding of α-Syn’s native functions in the cell.

P1464
Board Number: B475
Effects of mild running on substantia nigra during early neurodegeneration.
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Physical exercise has been postulated as an intervention that may attenuate progression of system aging. Moderate physical exercise acts at molecular and behavioral levels, such as interfering in neuroplasticity, cell death, neurogenesis, cognition and motor functions. Considering that the effects of physical exercise in early stages of protein aggregation remains poorly understood, the objectives of the present study is to evaluate the effects of physical exercise on oxidative stress, proteostasis, mitophagy and TrkB receptors levels and trafficking in the substantia nigra of aged rats during early neurodegeneration promoted by rotenone exposure. Aged male Lewis rats (9 months old) were exposed to rotenone 1mg/kg/day (8 weeks) and 6 weeks of moderate treadmill running, beginning 4 weeks after rotenone exposure. Substantia nigra was extracted and submitted to the analysis of proteasome and antioxidant enzymes activities, hydrogen peroxide levels and Western blot to evaluate the levels of tyrosine hydroxylase (TH), alpha-synuclein, TOM-20, PINK1, TrkB, SLP1, CRMP-2, Rab-27b, Beclin-1 and LC3II. It was demonstrated that moderate treadmill running, practiced during early neurodegeneration, prevented the increase of alpha-synuclein and maintained the levels of TH unaltered in substantia nigra of aged rats. Physical activity also stimulated autophagy, measured by an upregulation of 30% in beclin-1 levels and LC3II degradation, but decreased proteasome activity, in rotenone-exposed aged rats.
Mitophagy was impaired after rotenone exposure, as TOM-20 levels increased 40% indicating mitochondria accumulation, whereas physical training during early neurodegeneration prevented that increase. PINK1 levels showed a tendency of decrease in substantia nigra of aged rats exposed to rotenone, which was not present in exercised rats. Physical activity also prevented H2O2 increase during early neurodegeneration, although the mechanism involved remains to be elucidated, since the antioxidant enzymes evaluated did not changed. TrkB levels and its anterograde trafficking in substantia nigra seem not to be influenced by moderate treadmill running during early neurodegeneration. In conclusion, moderate physical training could prevent early neurodegeneration in substantia nigra through the improvement of autophagy and mitophagy.

P1465
Board Number: B476
Decoupling the effect of mutant amyloid precursor protein (APP) from the effect of plaque on axonal transport dynamics in the living mouse brain: A correlation MRI-microscopy study.
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The parent protein for amyloid plaques, amyloid precursor protein (APP), mediates cargo-motor attachments for intracellular transport. Axonal transport is decreased and the distal location of accumulation is altered in transgenic mice expressing human APP with the Swedish and Indiana mutations (APPSwInd) linked to Familial Alzheimer’s Disease, as detected by time-lapse magnetic resonance imaging (MRI) of transport in living mouse brains (Bearer et al. 2017). Transport is also altered in brains of Down syndrome mice with 3 copies of APP gene. Questions now become whether expression of mutated APP effects transport dynamics independent of plaque, and do plaques alone contribute to transport defects? To address these we used the Tet-Off system to decouple expression of APPSwInd from presence of plaques, and then studied transport using our MRI technique in three experimental groups of transgenic mice in which the timing and duration of APPSwInd expression, and thereby plaque formation, was altered with doxycycline: Group A (+ plaques, + APPSwInd); Group B (+ plaques, no APPSwInd), and group C (no plaques, + APPSwInd). Manganese-enhanced MRI (MEMRI) allows us to perform cell biological experiments in live animals with T1-weighted MRI in a Bruker 11.7T scanner (Medina et al 2016). Time-lapse MR images were captured before and after stereotactic injection of Mn2+ (3-5nL) into CA3 of the hippocampus at successive time-points. Images of multiple individuals were aligned and processed with our automated computational pipeline (Medina et al. 2017) and statistical parametric mapping (SPM) performed. After MRI brains were harvested for histopathology or biochemistry. Results show that within group between time-point have altered transport locations as well as diminished transport in all groups compared to wildtype (p<0.05 FDR n=36). Preliminary ANOVA between-group comparisons both by SPM and by region of interest measurements of images support the visual impression that APPSwInd expression alone may compromise transport. Groups A and B displayed plaques, but not C, and Western blots showed APPSwInd expressed 3.2-fold over normal at sacrifice in Groups A and C but not B, with Aβ detected only in Groups A and B, where phospho-tau was also present in dystrophic neurites surrounding plaques. Cholinergic neurons that project to hippocampus from the medial septal nucleus were decreased in Group C (p=0.0006 by ANOVA, n=15). Isolated hippocampal vesicles contained Mn2+, as well as Trk (NGF receptor), Rab 5 and 7 (associated with transport vesicles), suggesting a distinct vesicle population is
affected by these APP mutations. These surprising results implicate mutated APPSwlnd in transport defects, separable from the effect of plaque. Supported NS062184 and MH096093.

P1466
Board Number: B477
Transcription derepression of Fuz triggers apoptosis and contributes to polyglutamine neurodegeneration.
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Planar cell polarity (PCP) describes a cell-cell communication process through which individual cells coordinate and align on a tissue plane. In this study, we demonstrated that overexpression of Fuz, the human orthologue of the Drosophila melanogaster PCP gene fuzzy (fy), triggered neuronal apoptosis via the Dishevelled (Dvl)/Rac1 GTPase/MEKK1/JNK/caspase signalling axis. We further discovered that endogenous Fuz expression was upregulated in patients with spinocerebellar ataxia type 3 (SCA3), one of nine polyglutamine (polyQ) neurodegenerative disorders. Interestingly, Fuz gene induction was consistently observed in models of other polyQ diseases, including Huntington’s disease. And downregulation of Fuz expression rescued polyQ-associated cytotoxicity, and neurodegeneration in Drosophila. At a mechanistic level, we demonstrated that the transcriptional regulator Yin Yang 1 (YY1) associates with Fuz promoter, such that overexpression of YY1 caused hypermethylation of Fuz promoter, leading to transcriptional repression of Fuz. Soluble YY1 protein level was reduced in polyQ diseases. Such reduction perturbed the function of YY1, and led to induction of Fuz expression followed by neuronal apoptosis. In summary, our findings unveil a polyQ pathogenic pathway that involves YY1-mediated Fuz induction-promoted apoptotic cell death in polyQ diseases. Targeting this novel pathway may be of therapeutic potential.

This work was supported by the CUHK Gerald Choa Neuroscience Centre (7105306) and the General Research Fund (14100714) of the Hong Kong Research Grants Council.

P1467
Board Number: B478
GPRC6A signaling impacts mTORC1 activation and tau clearance.
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Tauopathies are a group of neurodegenerative diseases characterized by pathognomonic protein inclusions formed by abnormal accumulation of microtubule-associated protein named tau. Clinical phenotypes of tauopathies manifest as cognitive impairment and behavioral disturbance. The aggregation of tau remains a central target for drug discovery, however no disease-modifying treatments exist. The mechanistic target of rapamycin (mTOR) signaling has emerging evidence in regulating cellular proteostasis through the uncovered cytosolic and lysosomal L-arginine sensing pathways. Our group has previously discovered a unique interaction between tau aggregation and mTOR signaling linked to L-arginine metabolism. In both human Alzheimer’s disease patient samples and tau transgenic mice, we observed dysregulated L-arginine metabolism and uncoupled mTOR signaling proteins associated with tau pathology. G protein coupled receptor family C, group 6 member A
(GPRC6A) was recently deorphanized by basic L-amino acids, especially L-arginine. We posit that GPRC6A mediates extracellular sensing of L-arginine to regulate mTOR complex 1 (mTORC1) signaling. We hypothesize that decreased GPRC6A signaling inhibits mTORC1 activation, thus clearing tau during tauopathies. By utilizing tau overexpressing cell lines and tau transgenic mice, we blocked GPRC6A activity with novel allosteric antagonists and downregulated GPRC6A expression via shRNA constructs. We found that both pharmacological allosteric antagonism and genetic downregulation of GPRC6A inhibited mTORC1 signaling, promoted proteostasis and significantly reduced total tau in tauopathy cells. This is the first study to uncover the mTORC1 signaling mediated by extracellular sensing of L-arginine through GPRC6A. Therapeutics that modulate GPRC6A activity may potentially provide new treatments to clear protein aggregates in tauopathies and other proteinopathies.

P1468
Board Number: B479
Aβ Oligomers Mediate its Synaptotoxic Effects through AMPK-dependent Increase in Mitochondrial Fission in Pyramidal Neurons.
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Alzheimer’s Disease (AD) is the most common form of dementia leading to socially devastating cognitive defects with currently no effective treatment. Current evidence shows that during the early stages of AD progression, oligomeric, soluble forms of Aβ42 peptide trigger the loss of excitatory synapses (synaptotoxicity) in cortical and hippocampal pyramidal neurons (PNs) even prior to the formation of insoluble Amyloid-β plaques in AD animal models. Previous work from our lab, as well as others, uncovered (1) that Aβ42 oligomers over-activate the stress-response AMP-activated kinase (AMPK) in a CAMKK2-dependent manner and (2) that pharmacological or genetic inhibition of CAMKK2 or AMPK protects hippocampal PNs from Aβ42-mediated loss of excitatory synapses observed in the hAPPswe,IND transgenic AD mouse model (J20) in vivo (Mairet-Coello et al. Neuron 2013). However, the relevant downstream effectors and the cellular processes involved in AMPK-dependent synaptotoxicity are poorly understood. We recently identified that AMPK over-activation by Aβ42 oligomers triggers spatially restricted MFF-dependent mitochondrial fission and ULK2-dependent mitophagy in dendrites, resulting in both reduction of mitochondrial length and density. In the presence of oligomeric Aβ42, knockdown of MFF prevents mitochondrial fission, whereas genetic ablation of ULK2 still allows mitochondrial fission to occur without the subsequent degradation of mitochondria. Most importantly, both of these manipulations can prevent the synaptotoxic effects of Aβ42 oligomers, suggesting that the total volume of dendritic mitochondria plays a critical role in maintaining excitatory synapses. In parallel, we observe that a profound structural remodeling occurs specifically in the apical tufts of dendrites of CA1 PNs, a portion of the dendritic arbor where we also detect dramatic synaptic loss in the J20 AD mouse model. Overall, our project elucidates the cellular and molecular mechanisms mediating the effects of Aβ42 oligomers on mitochondrial defects, synaptic maintenance and their functional impact on hippocampal circuit in AD.
**P1469**

**Board Number: B480**

**Aβ oligomers affects Blood-brain-barrier integrity in Alzheimer’s disease.**

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**Introduction:** The blood brain barrier (BBB) is a layer of tightly packed cells that regulates the entry of substances into the brain, while maintaining the removal of toxic molecules. Unfortunately, recent evidence suggests that BBB impairment may precede or even exacerbate Alzheimer’s disease pathology. Even though Aβ-oligomers are believed to play a crucial role in Alzheimer’s until today is not clear whether they are being transported through the BBB or if they cause BBB disruption. Herein, we aim to elucidate the role of Aβ oligomers in BBB breakdown related to Alzheimer’s disease. **Material and methods:** We used an in vitro BBB-model composed of brain endothelial cells and measure the transcytosis of FITC-labeled Aβ-oligomers. Also we used a PAMPA assay to evaluate different Aβ species (Aβ monomers, Aβ-oligomers and fibrils). In addition, we evaluated in vivo BBB integrity in AD-mice by following the brain penetrance of fluorescent-dyes. **Results:** Aβ-oligomers cross an in vitro BBB-model, affecting the levels of tight junctions of brain vascular cells. In transgenic mice of Alzheimer’s disease we observed increased brain penetrance of fluorescent-dyes (FITC-albumin), as well as penetrance of radioactive-labeled oligomers. In the PAMPA assay we observed differential pass of misfolded Aβ species through the membrane. In addition, we observed decreased levels of tight junctions and increased inflammation. **Discussion:** In this work, we demonstrated that Aβ-oligomers affect BBB integrity in vitro and in vivo, decreasing the levels of tight junctions. The effect of Aβ-oligomers and AD-pathology over BBB integrity markers and Aβ receptors is currently under investigation. Funding FONDECYT 11130561.

**P1470**

**Board Number: B481**

**Insulin exerts a protective effect against MPP+ induced toxicity in C6 glial cells by regulating the levels of alpha-synuclein and insulin signaling molecules.**

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Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease (AD) associated with mitochondrial dysfunction mediated by oxidative stress. Astrocytes regulate neuronal function via the modulation of synaptic transmission and plasticity, secretion of growth factors, uptake of neurotransmitters, and regulation of extracellular ion concentrations and metabolic support of neurons. Therefore, this study was undertaken to investigate the mechanism of action of insulin on a 1-methyl-4-phenylpyridinium (MPP+) induced toxicity of events associated in cell viability and toxicity to the expression profile of cell signaling pathway proteins and genes in rat C6 glial cells. The various concentrations of MPP+ alone inhibited cell viability in a dose-dependent manner. Pretreatment of insulin prevented the cell death and lowered the intracellular reactive oxygen species and calcium ion influx by MPP+. Insulin also suppressed the α-synuclein and elevated the insulin signaling pathway molecules IR, IGF-1R, IRS-1 and IRS-2 in C6 cells through phosphorylation of Akt/ERK survival pathways. Moreover, insulin inhibits MPP+-induced Bax to Bcl-2 ratio. These results suggest that insulin has a protective effect on the MPP+-toxicity in C6 glial cells.
Neuronal Organelles, Membrane Biology, Membrane Trafficking

P1471
Board Number: B482
Mitochondrial Ubiquitin Ligase Mul1 Mediates an Early Stress Protection of Neuronal Mitochondria From Degradation by Parkin-Mediated Mitophagy.
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Parkin-mediated mitophagy is an essential process for maintaining neuronal mitochondrial integrity and turnover. Identifying new molecular regulators of mitophagy is key to finding new therapeutic strategies. In this study, we show that loss of MUL1 enhances Parkin-mediated mitophagy in mouse primary cortical neurons exposed to mitophagy-inducing stress. Loss of MUL1 or over-expression of dominant negative mutants of MUL1 induces biphasic change (hyper-fusion followed by fragmentation) in mitochondrial morphology. Mitochondrial fragmentation is associated with the loss of mitochondrial membrane potential, reduced ATP synthesis and translocation of mitophagy markers (Parkin, p62 and LC3) to damaged mitochondria. We further show that removing MUL1 causes an increase in Mitofusin-2 (Mfn-2) protein levels. Over-expression of Mfn-2 but not of Mitofusin-1 (Mfn-1) induces a biphasic change in the mitochondrial morphology similar to the loss of MUL1. Over-expression of loss of function mutant of Mfn-2 (K109A) in MUL1 depleted neurons partially rescues the change in mitochondrial morphology. We find that increase in Mfn-2 levels with loss of MUL1 decreases the mitochondria-endoplasmic (mito-ER) coupling in neurons. In particular, we show Mfn-2 increase weakens the close contact between the two organelles and reduces the efficiency of IP3-induced Ca\textsuperscript{2+} transfer from ER to mitochondria. We further demonstrate that over-expressing a mito-ER tether protein tyrosine phosphatase interacting protein -51 (PTPIP51) that enhances mito-ER interaction partially rescues mitochondrial morphology, mitochondrial Ca\textsuperscript{2+} load and reduces Parkin-mediated mitophagy in MUL1 depleted neurons. Our findings reveal MUL1-Mfn2 pathway acting as a gatekeeper to maintain neuron mitochondrial quality by regulating mito-ER interaction before the activation of Parkin-mediated mitophagy.

P1472
Board Number: B483
Releasing Synaptphilin Removes Stressed Mitochondria from Axons Independent of Mitophagy under Pathophysiological Conditions.
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Chronic mitochondrial stress and impaired transport are central problems associated with neurodegenerative diseases. Long-term cumulative stress leads to axonal accumulation of damaged mitochondria that not only produce energy less efficiently, but also release harmful reactive oxygen species. Therefore, the early removal of defective mitochondria from axons constitutes a critical step of mitochondrial quality control. Here we investigate axonal mitochondrial response to mild stress in wild-
type neurons and chronic mitochondrial defects in Amyotrophic Lateral Sclerosis (ALS)- and Alzheimer’s disease (AD)-linked neurons. We show that stressed mitochondria are removed from axons triggered by the bulk release of mitochondrial anchoring protein syntaphilin via a new class of mitochondria-derived cargos independent of Parkin, Drp1 and autophagy. Immuno-electron microscopy and super-resolution imaging show the budding of syntaphilin cargos, which then share a ride on late endosomes for transport toward the soma. Releasing syntaphilin is also activated in the early pathological stages of ALS- and AD-linked mutant neurons. Our study provides new mechanistic insights into the maintenance of axonal mitochondrial quality through SNPH-mediated coordination of mitochondrial stress and motility before activation of Parkin-mediated mitophagy. (This work is supported by the Intramural Program of NINDS, NIH.)

This work was recently published in Neuron: Lin et al., (2017) http://www.sciencedirect.com/science/article/pii/S0896627317302970
Cell Press Video: https://www.youtube.com/watch?v=WIT37Y8xKOA

P1473

Board Number: B484

The effects of Shroom2 knockdown on the axonal transport of mitochondria.

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Dynamic distribution of mitochondria in axons is crucial for neuronal function. The mechanisms of axonal transport are complex and highly regulated in order to achieve the optimal distribution. Defects in the axonal transport of mitochondria have been linked with neurodegenerative diseases. Mitochondrial movement along axons is dynamic. Mitochondria display bidirectionality, travelling in both retrograde and anterograde directions. Rates vary and are inconsistent, with mitochondria often stopping, starting, and changing speed and direction. Distinct mechanisms of transport have also been identified, with transport able to be driven by kinesin and dynein motors along microtubules (MTs) as well as by myosin along F-actin. Much remains to be elucidated about the complex mechanisms of the axonal transport of mitochondria. Shroom2 is a member of the Shroom family of proteins, which has been shown to coordinate both MT and F-actin assembly and to interact with myosin. We seek to explore the role that Shroom2 plays in trafficking mitochondria in axons. Here we show that Shroom2 knockdown leads to faster mitochondrial transport speeds in both retrograde and anterograde directions in embryonic Xenopus laevis neurons, with both fast-moving and slow-moving transport rates displaying a significant increase. Mitochondria in both dissociated neurons and neural tube explants displayed this pattern in response to knockdown. In the future, we hope to determine how Shroom2 functions to inhibit mitochondrial trafficking in axons by exploring its interactions with both F-actin and MT cytoskeletons and its link with myosin in axons. The results demonstrate that Shroom2 plays an important role in modulating the essential process of axonal mitochondrial transport.
**P1474**

*Board Number: B485*

α-Synuclein is a glycan binding protein, conferred by its N-terminal acetylation.

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Fibrillar deposits of the protein α-synuclein (αS) are the defining pathological feature of Parkinson’s disease, the second most common neurodegenerative disorder. αS is abundant in the brain, where its localization to presynaptic termini and ability to stabilise curved membranes support a role in synaptic vesicle exocytosis/endocytosis, although its precise role is not known. N-terminal acetylation is universally present on αS in vivo, in samples derived from both healthy persons and Parkinson’s disease patients. Prior work from our group and others have demonstrated that N-terminal acetylation impacts the fundamental biophysical properties of αS. Here, using cell-derived proteolipid vesicles, we find that αSacetyl binds specifically to neuronal-lineage cell membranes. Cleavage of surface proteo-glycans dramatically decreases binding by αSacetyl while having a negligible impact on binding by the unmodified protein, αSunmod. An array-based screen confirms that αSacetyl interacts specifically with complex, N-linked glycans. Monomer αSacetyl selectively binds to glycans cleaved from cultured neuronal cells and removal of cell-surface glycans dramatically reduces its cellular uptake. Based on these findings we propose that N-terminal acetylation confers glycan recognition and binding to αS and that it is a crucial modification for functional interactions with cellular protein binding partners. Moreover, understanding the basis of this interaction may have critical implications in obtaining a clearer understanding in the function of monomeric αSacetyl.

**P1475**

*Board Number: B486*

Molecular interaction between 440kDa ankyrin-B and L1CAM: a proposed mechanism for high-functioning autism.

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High functioning autism, also referred to as Asperger’s syndrome is characterized by normal or above normal intellectual ability combined with impaired social interactions. Genome-wide exome sequencing has identified de novo mutations in ANK2 associated with autism in the absence of seizures or intellectual impairment. These de novo mutations in ANK2 all result in either haploinsufficiency or mutation of the 440 kDa ankyrin-B polypeptide, which contains a 6.8kb giant exon expressed only in neurons. We have generated a mouse model where the floxed giant exon is conditionally removed by Cre-recombinase expressed under a Nestin promoter. These mice lose over 90 percent of 440 kDa ankyrin-B, but retain 220 kDa ankyrin-B. We report here, using the proximity ligation assay, that adult 440 kDa ankyrin-B-deficient mice exhibit nearly complete loss of ankyrin-B interaction with L1CAM, a cell adhesion molecule which requires ankyrin-binding activity to promote normal axon pathfinding. These results were unanticipated, since 220 kDa ankyrin-B is the predominant ankyrin-B polypeptide in adult brain and 220 kDa ankyrin-B contains a binding site for L1CAM. The L1CAM gene is located on the X chromosome and its mutation in human males results in a severe neurodevelopmental syndrome. Moreover, a Y1229H L1CAM mutation that eliminates ankyrin-binding results in a full L1CAM syndrome in humans as well as axon pathfinding defects in mice. We find that cultured hippocampal neurons from 440 kDa ankyrin-B-deficient mice as well as L1CAM Y1229H mutant mice both exhibit increased axon
branching. Thus, we propose a molecular interaction between 440 kDa ankyrin-B and L1CAM on axonal plasma membranes that is necessary for normal axon function, and is impaired in high functioning autism.

P1476
Board Number: B487
Synaptic vesicle clusters in nerve terminals: an example of liquid-liquid phase separation.
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Neurotransmitter containing synaptic vesicles (SVs) form tight clusters at chemical synapses. These clusters act as a reservoir from which SVs are drawn for exocytosis during strong and prolonged activity. Several components associated with synaptic vesicles and likely form such clusters, including synapsin 1, have been identified. However, it remains unclear how SV clustering is compatible with their motility, so that release sites can be rapidly replenished after vesicle fusion events. Recently, liquid-liquid phase separation was shown to be a mechanism through which components of the cytoplasm (protein and RNA) can assemble themselves into distinct compartments without a limiting membrane. A key feature of proteins that can undergo liquid-liquid phase separation is their ability for engaging in multivalent interactions through protein-protein interacting domains and/or low complexity amino acid regions. We are exploring the possibility that SV clusters may represent an example of liquid-liquid phase separation in which one component of the phase are SVs and the other component is represented by proteins of the intervening matrix, synapsin 1 in particular. Synapsin 1 is highly abundant proteins at the nerve terminals, it binds membranes, and it contains an extended low complexity region: all of these properties make synapsin 1 an ideal candidate for mediating the phase separation.

P1477
Board Number: B488
Bulk degradation of short-lived dendritic membrane proteins requires Rab7 and transport to somatic lysosomes.
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Regulation of protein homeostasis (“proteostasis”) is necessary for maintaining healthy cells, and disturbances in proteostasis lead to aggregates, cellular stress and can result in toxicity. There is thus great interest in where proteins are degraded in cells. Neurons are very large as well as very long-lived, creating unusually high needs for effective regulation of protein turnover in time and space. We previously discovered that several dendritic membrane proteins are short-lived and have half-lives of less than two hours. These observations raised the question of whether short-lived dendritic membrane proteins are degraded by local degradative pathways in dendrites. In this work, we analyzed the spatial distribution of early endosomes, late endosomes and degradative lysosomes in neuronal dendrites, using staining against endogenous proteins residing in these compartments, as well as functional markers for acidified and degradative compartments. First, somatic compartments differ in composition from dendritic compartments further away from the soma: whereas most somatic Rab7- and LAMP1-positive compartments are degradative, few of the dendritic Rab7- and LAMP1-containing compartments are. We thus designate these Rab7-positive compartments as “post-early endosome/pre-degradative” endosomes. Secondly, inhibition of Rab7 activity impairs both retrograde transport and subsequent degradation of short-lived dendritic proteins. Rab7 activity is thus required to mobilize these
Rab7-positive dendritic endosomes for transport to the soma where they encounter degradative compartments. We thus discovered a Rab7-positive, LAMP1-positive, cathepsin-negative pre-degradative endosome in more distal portion of dendrites which might not have a clear correlate in fibroblasts.

P1478  
**Board Number: B489**  
**Axonal endoplasmic reticulum is very narrow.**  
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The endoplasmic reticulum (ER) is an interconnected network of tubules and sheets. In most mammalian tissues, ER tubules have a diameter of ~60 nm. Using new methods for serial section electron microscopy, a distinct class of very narrow, 20-30 nm diameter tubules were found in neurons of both the central and peripheral nervous system. The narrow tubules are the most abundant form of ER in axons, and are also found interspersed in the cell bodies and dendrites. At the site of branch points in axons, there is a small sheet which has a similarly narrow lumen. Free ends of narrow tubules are relatively common. The abundance of branch points and free ends was determined as a first step in analyzing how the ER network in axons is maintained at a relatively constant density down the length of the axon.

Incorporation of an excess of curvature inducing proteins such as reticulon into artificial bilayers gives rise to the formation of similarly narrow tubules (Hu et al., 2008), suggesting that the axonal ER narrowness is due to high density of curvature inducing proteins. Knockdown of reticulons in Drosophila results in widened ER tubules in axons (Yalcin et al., 2017). Since mutations in several curvature inducing proteins cause Hereditary Spastic Paraplegia, the narrowness of the ER is likely to be important for the as yet poorly characterised functions of the axonal ER.

P1479  
**Board Number: B490**  
**Elucidating the Pathophysiology of Membrin/GOSR2-mediated Progressive Myoclonus Epilepsy from Molecule to Neuron.**  
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Mutations in the Golgi SNARE protein Membrin (encoded by GOSR2) cause progressive myoclonus epilepsy (PME). Patients with this form of PME typically present with ataxia around the age of three, followed by cortical myoclonus and generalized tonic-clonic seizures. However, despite Membrin’s known role in mediating ER to Golgi and intra-Golgi membrane fusion, it is still unclear how Membrin mutations result in a selective neuronal phenotype with the clinical hallmarks of severe ataxia and hyperexcitability.
We are utilising an array of complementary approaches to elucidate the disease mechanism of GOSR2-PME. As components of this strategy, SNARE dysfunction is being experimentally assessed in liposome fusion assays; mutant Membrin localisation and Golgi trafficking investigated in patient fibroblasts; and the impact of the disease mutations upon nervous system development and function studied in a novel in vivo model of GOSR2-PME.

Liposome fusion studies revealed reduced membrane fusion rates due to the pathogenic GOSR2-PME mutations. This finding implies that anterograde cargo transport might be impaired due to the formation of fewer fusogenic cis-Golgi SNARE complexes. The seeming paradox of how a global trafficking defect might eventually cause a selective neurological disorder is being investigated in Drosophila models of this disease - the first in vivo GOSR2-PME models to date. Given the critical importance of Golgi trafficking in dendrite development, we hypothesized that dendritic growth might be impaired. Indeed, we found profoundly shortened dendritic arbors with a total length reduction of up to two thirds in normally highly elaborate larval sensory neurons. This finding may explain one key feature of this disorder – ataxia – since cerebellar Purkinje cells with their highly elaborate dendritic trees would likely be most profoundly affected by such a dendritic growth deficit. In addition, we found that motoneuron synapses in our disease models exhibit gross morphological abnormalities and synaptic retraction, combined with hyperactivity.

From our experiments a mechanistic framework emerges which understands GOSR2-PME as a neurodevelopmental disorder with dendritic arborisation defects and morphologically abnormal, hyperactive excitatory synapses. Together, these multifaceted defects may simultaneously cause ataxia and shift circuit excitation-inhibition balance towards hyperexcitation. Besides having provided a multi-level insight into GOSR2-PME’s pathophysiology, this study reiterates the importance of the early secretory pathway in dendritic development and suggests a critical role of the Golgi apparatus in presynaptic development and function.

**P1480**

**Board Number: B491**

**A microtubule-associated septin maintains neuronal polarity by directing motor-cargo traffic in dendrites.**

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Neuronal function requires axon-dendritic membrane polarity, which depends on sorting of membrane traffic during entry into axons. Dendrites receive vesicles from the axon and cell body without apparent capacity for directional sorting due to a microtubule network of mixed polarity. Here, we found that a microtubule-associated septin (SEPT9), which localizes specifically in dendrites, impedes and reverses the entry of axonally-directed cargo of the microtubule motor kinesin-1/KIF5 such as the amyloid precursor protein (APP) and Bassoon. In contrast, SEPT9 promotes anterograde transport of kinesin-3/KIF1 cargo (e.g., low density lipoprotein receptor; postsynaptic density protein 95) from proximal to distal dendrites. In live neurons and single molecule cell-free motility assays, microtubule-associated SEPT9 suppresses kinesin-1/KIF5 motility and enhances kinesin-3/KIF1 specifically, regulating the trafficking and localization of their cargo to axons-dendrites. We show that this traffic control is critical for the growth of axons and dendrites in developing neurons. Thus, membrane traffic is spatially sorted and directed in dendrites by a septin-mediated mechanism, which maintains neuronal polarity and growth.
P1481

Board Number: B492

Determining the localization and function of schizophrenia-linked protein tSNARE1b in the endolysosomal system of developing neurons.

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Schizophrenia is a severe neuropsychiatric disorder characterized by delusions and hallucinations, which lacks effective, targeted therapies, likely due in part to its polygenic etiology. Recently, the largest genome wide association study on schizophrenia to date identified 108 loci associated with the occurrence of schizophrenia. The fifth most significant hit mapped to a locus containing the gene TSNARE1, which encodes the previously unstudied protein t-SNARE domain containing 1 (tSNARE1). tSNARE1 contains a N-terminal c-Myb DNA binding domain and a C-terminal Qa SNARE domain that shares closest homology to Syntaxin 12 (Stx12), an endosomal SNARE protein. Rare variant mutations identified from patients with either schizophrenia or autism spectrum disorder suggest that the SNARE domain is critical to tSNARE1 function. Unlike canonical Qa SNARE proteins, the primary neuronal isoform of tSNARE1, tSNARE1b, lacks a transmembrane domain as well as any other predicted site for membrane attachment, which is thought to be necessary for membrane fusion. Therefore, our central hypothesis is that tSNARE1b acts as an inhibitory SNARE (i-SNARE) of specific membrane trafficking events. This hypothesis is supported by biochemical pull-down assays with recombinant proteins and embryonic brain lysates, which demonstrate that GST-tSNARE1 can replace Stx12 and assemble with the endosomal SNARE proteins Vti1a, Stx6, and VAMP4 into SNARE complexes. Because tSNARE1 shares its closest homology with Stx12, we hypothesized that tSNARE1 functions within the endosomal pathway. High resolution, live-cell confocal microscopy of tSNARE1b-GFP and a battery of spectrally distinct organelle markers in embryonic murine cortical neurons determined to which endocytic compartments tSNARE1b localizes. Colocalization of tSNARE1b and each marker was quantified using a semi-automated, quantitative image-analysis pipeline that robustly identifies colocalization based on two different measurements. Preliminary evidence suggests that tSNARE1 colocalizes the strongest with late endosome marker Rab7 and lysosome marker LAMP1, suggesting tSNARE1b may regulate trafficking between these organelles. Ongoing studies are exploring how tSNARE1b functions at the membrane trafficking between late endosomes, lysosomes, and autophagosomes with three-color, live-cell imaging.

P1482

Board Number: B493

Dynamic Spatiotemporal Organization of Exocytosis During Neuronal Morphogenesis.

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During development, neurons acquire a unique, highly polarized morphology, which establishes a high surface-to-volume ratio of plasma membrane-to-cytoplasm. Developing this elongated morphology requires significant plasma membrane expansion, primarily considered to occur via exocytic vesicle
fusion. We hypothesized that the location and timing of exocytosis in the developing neuron are both spatially and temporally non-random and that developing neurons and non-neuronal motile cells at steady state size exhibit distinct spatiotemporal organization and distribution of exocytosis. A pH-sensitive variant of GFP (pHluorin) fused to the luminal end of a vesicle-SNARE protein, such as VAMP2, provides a fluorescent intensity readout of fusion pore opening and the subsequent fate of VAMP2. Here, we exploited TIRF microscopy to image VAMP-pHluorin in embryonic cortical neurons and interphase melanoma cells, and developed computer-vision based software and statistical tools to detect and analyze exocytic events. We reveal distinct spatiotemporal distribution and organization of exocytosis in the soma and neurites that is modulated by both the developmental stage of the neuron as well as exposure to the extracellular guidance cue netrin-1. The netrin-1 dependent changes in exocytic frequency and organization in neurites are dependent on the brain-enriched E3 ubiquitin ligase, TRIM9, whereas netrin-dependent changes to exocytosis in the soma occur independently of TRIM9. Mathematical modeling of empirical data indicated that VAMP2-mediated vesicle fusion supplied excess material needed for developmental plasma membrane expansion. TIRF imaging of clathrin-light chain and additional modeling suggests that this excess delivery of membrane was balanced by clathrin-mediated endocytosis. In melanoma cells, exocytosis occurred less frequently, with distinct spatial clustering, specific to the vesicle type. This study reveals system-wide insights into spatiotemporal aspects of exocytosis in neurons that differ substantially from non-neuronal cells.

P1483
Board Number: B494
The Retromer Complex Regulates Presynaptic and Exosomal APP Trafficking at the Drosophila NMJ.
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In neurons, endosomal cargo sorting both intracellularly and between cells via endosomally-derived secreted exosomes, is necessary for growth, development, and survival, and defective cargo sorting is associated with devastating neurodegenerative diseases, including Alzheimer’s Disease (AD) and Parkinson’s Disease (PD). However, much remains unknown about the factors influencing how protein cargoes are transported and sorted within and between neurons and their associated cells. The Retromer is a membrane-associated heteromeric protein complex that is critical for endosomal cargo sorting, and loss of its activities has been heavily implicated as a cause of both AD and PD. Here, we identify Retromer as a key regulator of APP trafficking at the Drosophila neuromuscular junction (NMJ). Surprisingly, we found that APP traffic is heavily directed to exosomes in the postsynaptic muscle. Retromer loss causes an accumulation of APP presynaptically as well as in exosomes. Both retention of APP in endosomes as well as propagation of APP and its cleavage products between neurons via exosomes have been implicated in AD pathology, suggesting that these may be points where Retromer defects contribute to disease. We also show that Retromer’s effect on presynaptic protein accumulation and incorporation of cargo into exosomes extrapolates to other cargoes as well, including Synaptotagmin-4 and the antigen recognized by anti-HRP antibodies, indicating a general function for Retromer in regulating presynaptic trafficking and suggesting a novel role in regulating neuronal exosomes. Further, we demonstrate that a loss-of-function PD-causing point mutant in the Vps35 subunit of Retromer disrupts endosomal and exosomal APP trafficking, suggesting that the role of Retromer in endosomal and exosomal cargo traffic is important in PD as well as AD. Finally, we use tissue-specific manipulations of Retromer to demonstrate that Retromer’s pre- and postsynaptic cargo regulatory functions appear to be at least partially non-neuron-autonomous; depleting Retromer in the
muscle alone is sufficient to cause pre and postsynaptic accumulation of neuron-derived HRP. Taken together, our data implicate Retromer as a key regulator of both neuronal and exosomal APP, and suggests that Retromer may act in multiple tissue types to orchestrate neuronal cargo sorting.

**P1484**

**Board Number: B495**

Disruption of the epileptic encephalopathy gene DENND5A causes Golgi fragmentation and apoptotic cell death via altered TrkB signaling.

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Epileptic encephalopathies are a catastrophic group of epilepsies characterized by refractory seizures and cognitive arrest. We recently demonstrated that recessive loss-of-function mutations in DENND5A result in an epileptic encephalopathy characterized by massive alterations in neuronal development and survival. We now demonstrate that loss of DENND5A function leads to upregulation of the neurotrophin receptor TrkB, which becomes concentrated in the Golgi where it undergoes abnormal signaling leading to Golgi fragmentation and apoptotic cell death. DENND5A contains a DENN domain, an enzymatic module conferring guanine-nucleotide exchange factor (GEF) activity to multiple proteins serving as GEFs for Rabs. Interestingly, we find that DENND5A functions to activate Arf4, a member of a distinct class of small GTPases involved in membrane trafficking in the endosomal and secretory pathways. Alterations in TrkB signaling, Golgi fragmentation and neuronal development caused by DENND5A loss-of-function are phenocopied by Arf4 knockdown. Thus, we identify a novel link between an Arf GTPase, neurotrophin function and neuronal development, dysfunction of which is responsible for a new subform of epileptic encephalopathy.

**P1485**

**Board Number: B496**

Chondroitin Sulfate Proteoglycans Negatively Regulate the Positioning of Mitochondria and Endoplasmic Reticulum to Distal Axons.

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Chondroitin sulfate proteoglycans (CSPGs) are components of the extracellular matrix that inhibit the extension and regeneration of axons. However, the underlying mechanism of action remains poorly understood. Mitochondria and endoplasmic reticulum (ER) are functionally inter-linked organelles important to axon development and maintenance. We report that CSPGs impair the targeting of mitochondria and ER to the growth cones of chicken embryonic sensory axons. The effect of CSPGs on the targeting of mitochondria is blocked by inhibition of the LAR receptor for CSPGs. The regulation of the targeting of mitochondria and ER to the growth cone by CSPGs is due to attenuation of PI3K signaling, which is known to be downstream of LAR receptor activation. Dynactin is a required component of the dynein motor complex that drives the normally occurring retrograde evacuation of mitochondria from growth cones. CSPGs elevate the levels of p150-Glued dynactin found in distal axons and inhibition of the interaction of dynactin with dynein increased axon lengths on CSPGs. CSPGs also decreased the membrane potential of mitochondria and inhibition of mitochondria respiration at the growth cone impaired axon extension. Combined inhibition of dynactin and potentiation of mitochondria respiration further increased axon lengths. These data reveal that the regulation of the
localization of mitochondria and ER to growth cones is a previously unappreciated aspect of the effects of CSPGs on embryonic axons.

P1486
Board Number: B497
KIF2A Regulates the Development of Dentate Granule Cells and Postnatal Hippocampal Wiring. N. Homma1,2, R. Zhou2, M.I. Naseer1, A.G. Chaudhary3, M.H. Al-Qahtani3, N. Hirokawa2; 1Life Science, National College of Nursing, Tokyo, Japan, 2Cell Biology and Anatomy, Graduate School of Medicine, Tokyo, Japan, 3Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia

Kinesin super family protein 2A (KIF2A), an ATP-dependent microtubule (MT) destabilizer, regulates cell migration, axon elongation, and pruning in the developing nervous system. KIF2A mutations have recently been identified in patients with malformed cortical development. However, postnatal KIF2A is continuously expressed in restricted brain regions, such as the hippocampus and the olfactory bulb, in which new neurons are generated throughout an individual’s life in established neuronal circuits. In this study, we investigated KIF2A function in the postnatal hippocampus by using tamoxifen-inducible conditional knockout (cKO) mice. Despite showing no significant defects in neuronal proliferation or migration, 3w-Kif2a-cKO mice showed signs of an epileptic hippocampus. In addition to mossy fiber sprouting, the 3w-Kif2a-cKO dentate granule cells (DGCs) developed many aberrant overextended dendrites, which eventually had axonal properties. These results suggested that postnatal KIF2A is a key length regulator of the developing neurites of DGCs and is involved in establishing postnatal hippocampal wiring.

P1487
Board Number: B498
ER-shaping proteins form functionally distinct microdomains in the ER membrane of neurons. J. Nixon-Abell1,2, C.J. Obara2, J. Lippincott-Schwartz2, C. Blackstone1; 1NINDS, National Institutes of Health, Bethesda, MD, 2Janelia Research Campus, Ashburn, VA

The endoplasmic reticulum (ER) is a complex membranous structure present in all eukaryotic cells, stretching from the nuclear envelope to the plasma membrane. This remarkably versatile network plays crucial roles in numerous cellular functions while simultaneously orchestrating interactions with, and between, almost every other subcellular organelle. The regulation of ER structure and function appears to be of particular importance in neuronal tissue, as almost all neurodegenerative pathways converge on ER-associated processes. The complex structural arrangement of the ER is thought to be governed, in part, by a subset of ER-shaping proteins (ERSPs). Mutations in genes encoding many of these proteins result in abnormal ER morphologies and underlie various neurodegenerative disorders. While many of these proteins are conserved throughout eukaryotes and ubiquitously expressed across tissue types, disease phenotypes are primarily restricted to neurons. For this reason, ERSPs provide a compelling model to better understand the role ER structure plays in influencing neuronal function in health and pathogenesis.

We employed a variety of superresolution imaging techniques in conjunction with gene editing technology to study the localization and function(s) of ERSPs at endogenous levels in human iPSC-derived neurons. A single iPSC line was CRISPR-edited to target multiple representative ERSPs with small-epitope tags. Multiplexed single molecule localization microscopy (SMLM) subsequently demonstrated the existence of ERSP microdomains that correlate not only with specific structural
components of the ER, but also coincide with distinct biological processes. We suspect that these
signature microdomain localizations are disturbed in the presence of pathogenic ERSP mutations which
in turn leads to the disruption of ER-affiliated cellular processes and consequential pathogenesis. This is
a particularly appealing model as it might help to explain why such a wide range of cellular phenotypes
are observed in many neurodegenerative diseases. Studying the differentiation cascade from iPSC
through to motor neuron, we further describe a morphogenic role of ERSPs in contributing towards the
ability of a cell to generate neuronal morphology. Using a combination of correlative RNA-seq,
quantitative proteomics, and SMLM we show which ERSP-containing microdomains are important for
the differentiation process at different stages.
Here we provide the first evidence that ERSPs, at endogenous levels, exist in microdomains throughout
the ER membrane and correlate with specific biological processes. Further, our data suggests that ERSP
microdomains play a crucial role in supporting the growth, development and function of neurons.

P1488
Board Number: B499
Roles for VPS13 Family Members in the Drosophila Nervous System.
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Mutations in the human vacuolar protein sorting 13 (VPS13) gene family, VPS13 A-D, have been shown
to contribute to a range of neurological disorders. These include Parkinson’s disease (VPS13C), Chorea-
Acanthocytosis (VPS13A), Cohen’s Syndrome (VPS13B) and, recently ataxia (VPS13D). The cellular
function of these family members in the nervous system is largely unknown. Also unknown is whether
the different family members have distinct cellular functions that account for the diverse disease
phenotypes in humans. To characterize and compare the cellular function of VPS13 family members, we
are using Drosophila melanogaster as a model organism. This study focuses upon VPS13A (known as
dVPS13 in Drosophila) and VPS13D (dVPS13), and has two aims: 1) compare neuronal phenotypes in
mutant lines for the dVPS13 and dVPS13D, and 2) determine whether the VPS13A and D orthologues
can functionally substitute for each other in rescue experiments. Deletion of dVPS13 leads to a
shortened lifespan, and build-up of poly-ubiquitin containing aggregates in the brain. In contrast,
mutations in dVPS13D cause lethality in early larval stages. In preliminary studies we have noticed poly-
ubiquitin aggregates accumulate more severely dVPS13D mutants. We have also discovered severe
defects in the morphology of mitochondria in the brain for both dVPS13 and dVPS13D, however these
defects appear localized to glia for dVPS13, in contrast to both neurons and glia for dVPS13D. Our
ongoing studies will determine whether human VPS13A can rescue any of these defects when driven in
different cell types using the Gal4/UAS system, and will provide further characterization of the
mitochondrial defects. Ultimately this work may illuminate cellular functions for the VPS13 family
members and give insight into mechanisms of disease pathology.

P1489
Board Number: B500
Investigating mechanisms of STRIPAK complex regulation in neuronal transport.
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The maintenance of neural circuits is essential for cognitive function and motor system coordination. At
the cellular level, neurons are polarized, elongated cells that need to transport signals and cargoes
across vast distances. At the molecular level these cargoes and signals are transported along the microtubule cytoskeleton by molecular motor proteins. Impairment of this intracellular transport in neurons contributes to compromised synaptic and dendritic functions, which can lead to neuronal cell death, a hallmark of neurodegenerative disorders. To better understand the mechanisms that regulate motor protein function we carried out an RNAi screen in Drosophila motoneurons focusing on the transport of the retrograde specific cargo, autophagosomes. From this screen we identified the STRIPAK complex as a regulator of autophagosome and dense core vesicle transport (Neisch et al., 2017). The STRIPAK complex is a highly conserved multi-protein complex that contains kinases, the phosphatase PP2A, and other proteins. We have demonstrated that the core STRIPAK components Cka, Mob4 and Strip are required for transport regulations, as is PP2A activity within the complex. Our model suggests that the balance between PP2A and kinase activities regulate transport. Currently we are investigating the mechanism of kinase association within the complex. In mammalian culture cells, the association of kinase within the Striatin homolog of CKA is proposed to be modulated by calcium signaling (Gordon et al., 2011). We are conducting molecular genetic experiments in Drosophila to address the role of calcium signaling in regulation of vesicle transport in neurons in vivo. Moreover, targeted mutagenesis and in vivo motility assays will test the importance of phospho-binding by Mob4, a member of a family of proteins known to function as adaptors in the recruitment of protein kinases (Couzens et al., 2017).

Neuronal Cytoskeleton

P1490
Board Number: B501
Ubiquitin-dependent regulation of filopodia during axon guidance and branching.
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During development, neurons extend axons across long distances to find postsynaptic partners and create neuronal networks. Upon reaching target regions, axons branch to increase network complexity. At the distal end of an extending axon, a dynamic filopodia-rich growth cone senses extracellular guidance cues and facilitates directional extension and branching of the axon. This requires tight spatial and temporal modulation of the F-actin cytoskeleton, which is reorganized rapidly in response to extracellular guidance cues. We found that lower concentrations of the extracellular guidance cue netrin-1 increases growth cone filopodial stability and density and induces attractive axon turning and axon branching, whereas higher netrin concentrations collapse and repulse the growth cone. The mechanisms by which netrin and its receptor DCC alter cytoskeletal machinery in these distinct responses however have not been fully elucidated. Here we show that TRIM67, a brain-enriched member of the tripartite motif family of E3 ubiquitin ligases, interacts with DCC and is required for both attractive and repulsive responses to netrin. Using murine embryonic cortical neurons, we find that growth cones of Trim67⁻/⁻ axons fail to increase in size or number of filopodia in response to a low concentration of netrin, and fail to collapse in response to a high concentration of netrin. However, Trim67⁻/⁻ axons respond appropriately to other guidance cues. We find that TRIM67 regulates filopodia stability and localizes to the tips of filopodia, where it colocalizes and cotransports with the barbed end actin polymerase VASP. We show that TRIM67 and VASP interact and that TRIM67 is required to increase the dynamics of VASP at filopodial tips in response to netrin-1. We previously found that a
closely related E3 ligase TRIM9 reduces VASP dynamics and filopodial stability via ubiquitination of VASP. Surprisingly TRIM67 negatively regulates this VASP ubiquitination, potentially via ubiquitination of TRIM9. Together with investigations of Trim9<sup>−/−</sup>:Trim67<sup>−/−</sup> neurons, our results suggest that TRIM67 and TRIM9 coordinate to promote cytoskeletal reorganization in response to netrin-1 downstream of DCC. This regulation is likely critical to appropriate neuronal morphogenesis: Netrin-dependent axon branching and axon turning is absent in Trim67<sup>−/−</sup> neurons in vitro, and the netrin-dependent cortical corpus callosum projection is thinner in Trim67<sup>−/−</sup> mice in vivo.

P1491
Board Number: B502
A requirement for Mena, an actin regulator, in local mRNA translation in developing neurons.
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During development, the exquisitely regulated process of axon guidance establishes the circuitry necessary for a properly functioning nervous system (NS) in the adult. Aberrant axonal navigation results in defective connectivity and multiple neurodevelopmental disorders including, among others, epilepsy, intellectual disabilities, autism and schizophrenia. One key mechanism required for proper axon growth, guidance and maintenance is local mRNA translation. Although local protein synthesis has been extensively studied in synapse formation and plasticity, its regulation during axon development remains poorly understood. Here, we uncover a novel role for the actin-regulatory protein Mena in the formation of a ribonucleoprotein complex (RNP) that involves translational inhibitors (HnrnpK, PCBP1), and regulates local translation of specific mRNAs in developing axons. We find that translation of dyrk1a, a Down Syndrome- and Autism Spectrum Disorders- related gene, is dependent on Mena, both in steady state conditions as well as upon BDNF stimulation. In particular, after BDNF stimulation we find that the Mena-RNP dissociates, releasing the mRNA of dyrk1a for translation in the axon/growth cone. We identify hundreds of additional mRNAs that associate with the Mena-complex, suggesting it plays broader role(s) in post-transcriptional gene regulation. Interestingly, this function is Mena-specific, as we could not detect any of the other Enah/VASP family members (VASP and EVL) being associated with mRNAs in the brain. Our work establishes a dual role for Mena in neurons, raising the intriguing possibility that it could act as a regulatory node that coordinates and balances actin polymerization and local protein synthesis in response to specific cues during neuronal development and, potentially, in adult neuroplasticity.

P1492
Board Number: B503
Novel functions of LIM and SH3 domain proteins in regulating dendritic development and synapse formation.
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In the central nervous system, each neuron elaborates a dendritic arbor of varying complexity that receives and integrates thousands of synaptic inputs. The development of dendritic arbors and their postsynaptic spines depend on the actin cytoskeleton and its dynamic remodeling. However, our understanding of the mechanisms underlying actin remodeling and its regulation by synaptic activity remains limited. Here, we report that the F-actin binding LIM and SH3 domain proteins, LASP1 and LASP2, exert distinct functions on dendritic development and synapse formation. LASP proteins have
previously been shown to play a role in cell migration and tumor metastasis, but very little is known about their function in the nervous system. Here, we show that both LASP proteins are present in the dendritic compartment of cultured hippocampal neurons and, importantly, highly enriched in dendritic spines. Furthermore, we have identified the molecular domains of LASP proteins responsible for their subcellular localization in spines. Moreover, we have obtained evidence that LASP proteins exhibit isoform-specific effects on dendritic arbor, dendritic spine, and synaptic development. Down regulation of LASP proteins causes defects in dendritic arbor and dendritic spine motility and stabilization. These findings demonstrate a previously unknown role for LASP proteins in neuronal development, and suggest a function in actin-based modifications of synaptic structure and function.

P1494
Board Number: B505
Arginyltransferase ATE1 is targeted to the neuronal growth cones and regulates neurite outgrowth during brain development.
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Arginylation is an emerging protein modification mediated by arginyltransferase ATE1, shown to regulate embryogenesis and actin cytoskeleton, however its functions in different physiological systems are not well understood. Here we analyzed the role of ATE1 in brain development and neuronal growth by producing a conditional mouse knockout with Ate1 deletion in the nervous system driven by Nestin promoter (Nes-Ate1 mice). These mice were weaker than wild type, resulting in low postnatal survival rates, and had abnormalities in the brain that suggested defects in neuronal migration. Cultured Ate1 knockout neurons showed a reduction in the neurite outgrowth and the levels of doublecortin and F-actin in the growth cones. In wild type, ATE1 prominently localized to the growth cones, in addition to the cell bodies. Examination of the Ate1 mRNA sequence reveals the existence of putative zipcode-binding sequences involved in mRNA targeting to the cell periphery and local translation at the growth cones. Fluorescence in situ hybridization showed that Ate1 mRNA localized to the tips of the growth cones, likely due to zipcode-mediated targeting, and this localization coincided with spots of localization of arginylated β-actin, which disappeared in the presence of protein synthesis inhibitors. We propose that zipcode-mediated co-targeting of Ate1 and β-actin mRNA leads to localized co-translational arginylation of β-actin that drives the growth cone migration and neurite outgrowth.

P1495
Board Number: B506
A Wnt Signaling Pathway Acts as a Master Coordinator of Microtubule Regulators at Dendrite Branch Points.
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Mature neurons contain non-centrosomal microtubule arrays with different arrangements of microtubules in axons and dendrites. In Drosophila dendrites microtubule nucleation sites are concentrated at dendrite branch points, and local nucleation contributes to minus-end-out microtubule polarity. Golgi outposts have been suggested to house nucleation sites (Ori-Mckenney, Jan and Jan, Neuron 2012), however, we found that removal of the Golgi from dendrites did not reduce targeting of gamma-tubulin, the core microtubule nucleator, to dendrite branch points (Nguyen et al, MBoC 2014).
To generate an alternative hypothesis about what might localize nucleation sites to branch points, we started with the clue that another microtubule regulator, Apc2, is also concentrated at dendrite branch points. Apc2 recruits Apc so it can link the plus-tip protein EB1 to kinesin-2 and steer growing microtubules along stable ones at branch points. As Apc2 is a scaffolding protein with many known interaction partners, we performed a candidate screen to identify proteins required to position Apc2-GFP. We found that mitochondrial energy production, ankyrin2 and its membrane partner neuroglian, branched actin, heterotrimeric G proteins and axin were all required for Apc2 targeting. We next tested whether any of these proteins were also required to localized gamma-tubulin to branch points. Surprisingly we found that the membrane proteins frizzled and frizzled2 act with the heterotrimeric G protein GalphaO to localize axin to branch points, and in turn axin is required to localized gamma-tubulin. Thus a modified wnt signaling pathway is an essential regulator of gamma-tubulin localization in dendrites. To determine the functional consequences of these wnt pathway members on microtubule organization in dendrites, we performed two additional assays. First, we assayed microtubule polarity and found that these pathway members are required to maintain minus-end-out polarity. Second, we assayed the increase in microtubule nucleation triggered by axon severing, and again found that frizzleds, G proteins and axin were required for this response. Thus this alternate wnt signaling pathway acts as a master regulator to localize both Apc2 and gamma-tubulin, and is required to control local microtubule nucleation in dendrites.

**P1496**

**Board Number: B507**

Patterns of microtubule organization and dynamics determine neuronal cargo distribution.

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Microtubules (MTs) are the main cytoskeletal substrate on which axonal transport progresses. How the architecture and dynamics of the MT network are set up and how they affect the overlying transport of neuronal cargo is poorly understood. Conversely, molecular motors can also regulate MT behaviour, but the in vivo role for such modulation is not clear. We developed a rapid, fluorescence-based method for analyzing neuronal MT length, abundance and dynamics in C.elegans, and used it to explore how neuronal MTs are patterned, and how these patterns affect cargo transport. We identified roles for conserved MAPs and signalling pathways in determining microtubule length, spacing and abundance. In turn, these parameters impinge on axonal transport progression: cargo pauses at polymer termini, suggestive that switching MT tracks is rate limiting for efficient transport. Cargo run length is set by MT length, and higher MT coverage correlates with shorter pauses. We also detected a surprising role for dynein heavy chain (DHC-1) in maintaining the correct distribution and dynamics of MT polymers in the dendrite. In dhc-1 mutants, MTs shifted distally and displayed excessive growth and shrinkage, which hampered cargo transport, trapping mitochondria and synaptic vesicle precursors and depleting them from their endogenous locations. Live imaging, mutant analysis and single molecule in-vitro assays suggest a model where an anchored dynein pool at the tip of the dendrite interacts with plus-end-out MTs to stabilize them and allow efficient retrograde transport. Collectively, these results uncover the principles and mechanisms that pattern neuronal MTs to promote efficient cargo transport.
P1497
Board Number: B508
Molecular Pathogenesis of Tubulin Disorders During Neural Development.
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‘Tubulopathies’ are severe human brain malformations associated with missense mutations in the tubulin genes. Despite the identification of many tubulin mutations in patients, we do not understand how these mutations impact the microtubule cytoskeleton, how the changes to microtubule function lead to brain malformations, or how different tubulin isotypes regulate microtubules to support normal neurodevelopment. TUBA1A α-tubulin is the most commonly affected tubulin isotype, with mutations linked to diverse cortical malformations including microlissencephaly, lissencephaly, pachygyria, and polymicrogyria. We hypothesize that TUBA1A α-tubulin uniquely contributes to neuronal maturation, and distinct TUBA1A mutations may disrupt specific microtubule functions during development. Therefore, tubulopathy mutations may provide a unique window into the role of microtubules, and specifically TUBA1A, in young neurons. Here we focus on mutations affecting the conserved arginine at position 402 (R402), which is a hotspot accounting for 30% of all reported TUBA1A mutations in patients. Using yeast mutants to mimic R402C and R402H substitutions found in patients, we show that the mutant α-tubulins incorporate into microtubules but cause a dominant disruption of the microtubule motor dynein that scales with abundance of mutant α-tubulin in the cell. In addition, our preliminary experiments in cortical neurons suggest that ectopic expression of R402C/H TUBA1A mutants is sufficient to disrupt microtubule function in a manner consistent with dynein impairment. Together, our results indicate that tubulopathy mutations at R402 poison the microtubule network in young neurons by creating defective binding sites for dynein at the microtubule surface.

P1498
Board Number: B509
Tau is not a stabilizer of microtubules in the axon but rather enables axonal microtubules to have labile domains of substantial length.
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Hundreds of research papers claim that the main function of tau in the axon is to stabilize its microtubules (MTs). This claim is mainly based on findings that tau can stabilize MTs in the test tube, and that tau overexpression in cells leads to increased MT stabilization. Many researchers are so invested in the idea of tau as a MT stabilizer that they are seeking to use MT-stabilizing drugs to treat neurodegenerative diseases in which tau loses its association with MTs. However, there are reasons to be skeptical. Individual MTs in the axon consist of a stable domain and a labile domain toward the minus and plus end of the MT, respectively. On average, roughly half the microtubule mass in the axon is labile, which means that labile domains can achieve substantial lengths without being stabilized. MT stability becomes more complex in adult axons compared to developing axons, but the importance of the labile domains persists throughout life. If tau were responsible for stabilizing the stable domains, there should be relatively less MT-bound tau in the distal region of the axon (where labile domains are enriched) compared to the rest of the axon, but the opposite is true. Here we used siRNA to deplete tau from cultured fetal rat cortical neurons, and found that, over a period of four days, the MT mass in the axon diminishes by roughly 20%. In tau-depleted axons, roughly 85% of the MT mass is stable and only 15% is
labile, indicating that, not only is the lost MT mass labile, but a portion of the remaining MT mass that was labile becomes stable. Functional consequences are indicated by defects in growth cone turning, which requires labile MTs extending into the distal axon. We found that MAP6 (also called stable tubule only peptide), which is normally enriched on the stable domains of MTs, redistributes along the length of the MT after tau depletion, providing an explanation for why a higher portion of the MT mass is stable after tau depletion. When MAP6 is depleted from neurons, the portion of the MT mass that is stable drops to less than 30%, indicating that MAP6 is a genuine stabilizer of MTs in the axon. MT-bound tau becomes more evenly distributed after MAP6 depletion, further indicating that the two proteins have complementary distributions. We conclude that tau is enriched on labile domains of MTs to limit the binding to them of MAP6, thus enabling the labile domains to obtain great lengths without being stabilized by MAP6. We speculate that the process of microtubule stabilization, specifically the conversion of labile domains into stable domains, involves a switch in the proportions of tau and MAP6 bound to the MT. (LQ, XS and TOA are co-first authors. TOA is supported by an NSF GRFP. The work was funded by NIH and DOD grants to PWB and LQ.)

**P1499**

**Board Number: B510**

**Novel concepts of microtubule regulation during neuronal growth, maintenance and degeneration.**

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Axons are the slender, often meter-long processes of neurons which electrically wire the brain, and these delicate structures need to be maintained for decades. Parallel bundles of microtubules (MTs) form their structural backbones and life-sustaining transport highways. We study axon biology through deciphering the MT regulating machinery composed of different classes of MT-binding proteins as well as actin networks. For this, we use an unprecedentedly comprehensive approach capitalising on combinatorial fly genetics. We recently proposed a model in which spectraplakins (large actin-microtubule linkers) guide the extension of polymerising microtubules along cortical F-actin, thus directly laying axonal MTs out into parallel bundles. Here we propose two further novel mechanisms promoting the bundle organisation of axonal MTs: Firstly, we identified a membrane-anchored cortical collapse factor which serves as a check point by eliminating "off track" MTs that have escaped the guidance mechanism. When artificially detached from the membrane, this factor is able to deplete entire MT networks. In the absence of this factor, more MTs persist in the periphery of growth cones and looped MTs accumulate in axons. Secondly, combining super-resolution microscopy with systematic genetics, we found that evolutionary conserved periodic cortical actin rings in axons sustain MT polymerisation, hence MT bundles. To understand this phenomenon, we study the machinery of axonal MT polymerisation which is of enormous complexity. We will report about three key factors acting as a "ménage à trois" to promote MT polymerisation. So far, all our data support a model of local homeostasis in axons which provides a novel concept providing explanations for axon longevity and degeneration.
P1500  
Board Number: B511  
NEK7 regulates dendrite morphogenesis in neurons via Eg5-dependent microtubule stabilization.  
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Organization of microtubules into ordered arrays is best understood in mitotic systems, but remains poorly characterized in postmitotic cells such as neurons. By analyzing the cycling cell microtubule cytoskeleton proteome through expression profiling and targeted RNAi screening for candidates with roles in neurons, we have identified the mitotic kinase NEK7. We show that NEK7 regulates dendrite morphogenesis in vitro and in vivo. NEK7 kinase activity is required for dendrite growth and branching, as well as spine formation and morphology. NEK7 regulates these processes in part through phosphorylation of the kinesin Eg5/KIF11, promoting its accumulation on microtubules in distal dendrites. Here Eg5 limits retrograde microtubule polymerization, which is inhibitory to dendrite growth and branching. Eg5 exerts this effect through microtubule stabilization, independent of its motor activity. This work establishes NEK7 as a general regulator of the microtubule cytoskeleton, controlling essential processes in both mitotic cells and postmitotic neurons.

P1501  
Board Number: B512  
Interplay between microtubule depolymerizing kinesin KLP-7 and Wnt signaling establishes microtubule polarity in C. elegans touch neuron.  
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In the nervous system, directional flow of information depends on the polarized architecture of neuron. Neuron has a unique structure – many dendrites and one axon. Microtubule (MT) polymers are the building blocks of the polarized architecture of neuronal cell. Although MT protofilaments give stability in axon, they themselves are dynamic - undergoing polymerization and depolymerization. The dynamics of the microtubule network needs to be regulated in a context-dependent manner during polarization, maintenance or other remodeling processes. Using C. elegans touch neuron as a model, we have asked how polarity of microtubules is established and maintained in neuron. Both anterior and posterior touch receptor neurons are unipolar with a long anterior process, which, connects to its post-synaptic cell, and a short posterior process. Loss of kinesin-13 family microtubule depolymerizing enzyme klp-7 (kinesin-like posterior) leads to excess stabilization of microtubules leading to overgrowth of the short posterior process (1). We found that this phenotype in klp-7(0) can be reversed by a microtubule-stabilizing drug Colchicine or in backgrounds lacking either alpha or beta tubulin. In vivo imaging of microtubules with EBP-2:GFP reporter reveals that the short process of PLM has mixed polarity of MTs, whereas the long anterior process has all plus end unipolar MTs. The MTs in the ectopically extended posterior process in klp-7(0) becomes plus end out. Therefore, activity of KLP-7 is important to maintain the MTs with mixed polarity in the short posterior neurites. Previous study indicated that loss of Wnt signaling reverses the polarity of touch neurons in that short posterior process becomes axon and makes synapse (2, 3). We asked whether Wnt signaling through KLP-7 can establish axonal polarity.
We found that loss of klp-7 suppresses the phenotype displayed either by loss of Wnt receptor lin-17 or its ligand lin-44. This further indicates that the activation of Wnt signaling during development might directly or indirectly regulate KLP-7 to establish microtubule polarity during neuronal polarization. We will be further investigating how KLP-7 is regulated during the establishment of neuronal polarity.


P1502
Board Number: B513
Role of Formin-2 in actin-microtubule coordination during axonal pathfinding.
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Neural circuits are formed by directed translocation of axonal growth cones to their synaptic targets. This growth cone mediated movement occurs through active and coordinated remodeling of the underlying cytoskeletal components. Recent studies from our lab have implicated Formin-2 (an actin nucleator) in this growth cone migration. The speed and directionality of growth cone movement and filopodial force generation was found to be compromised. Guidance defects were also observed in spinal commissural neurons which stalled and did not cross midline (Sahasrabudhe et. al., 2016). Growth cone steering and guidance also involve microtubules. Hence, this study examines the role of Formin-2 in mediating actin-microtubule crosstalk. Knockdown of Formin-2 reduced microtubule presence in the filopodia. Directionality of filopodal initiation was also affected due to skewed innervation of microtubules in filopodia. In addition to this, even though the area occupied by microtubules in the growth cone remains unaffected, the overall microtubule morphology is altered. These preliminary results suggest that Formin-2 might be involved in co-ordination of actin and microtubule dynamics and further studies are in motion to elucidate the nature of this interaction.

P1503
Board Number: B514
Nicotine Exposure Alters Neuronal Cytoskeleton by the Gβγ/pGSK3β Mediated Pathway.
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Background: Tobacco use is a major public health concern. Nicotine is the main addictive agent in tobacco and it is well established that nicotine interferes with the ability of neurons to send, receive, and process information. However, the underlying molecular mechanisms by which nicotine alters neuronal pathways in the brain are not well understood. It is believed that changes in the neuronal cytoskeleton may provide a mechanism by which long-lasting behavioral changes occur during chronic nicotine exposure. Goal: The present study examined key molecular targets that modulate microtubules (MTs), a major cytoskeletal filament in neurons in response to nicotine exposure. Since Gβγ, an important component of GPCR pathway, and pGSK3β (phosphorylated GSK3β), a downstream effector
of tyrosine kinase receptor Trk A, coordinate to regulate MT modulation in neurons, our hypothesis is that nicotine exposure alters/disrupts microtubule structure in neurons through the Gβγ/pGSK3β pathway. Methods: We utilized a transdisciplinary approach involving an animal model of nicotine dependence and in-vitro cell culture systems to address this emerging problem. Wistar rats were exposed to chronic nicotine via osmotic pump for fourteen days (3.2 mg/kg/day). Control rats received sham surgery. Brains from control and nicotine-exposed rats were subjected to immunoblot, immunohistochemical and confocal microscopic analysis using antibodies specific for tubulin, Gβ, and pGSK3β. In addition, PC12 cells were used to conduct the study because they respond to nerve-growth-factor (NGF) with growth arrest and exhibits typical phenotype of neuronal cells. NGF differentiated PC12 cells were treated with 5 μM nicotine for 24h followed by immunoblot and confocal microscopic analysis. Results and Conclusion: We found that the nicotine exposure in rat brain altered tubulin/MTs organization in neurons located in the nucleus accumbens (NAcc), a brain region known to be associated with nicotine addictions. Immunoblot analysis reveals that chronic nicotine exposure inhibited the expression of Gβγ and subsequent phosphorylation of GSK3β by 22% and 42% respectively in NAcc. Using cultured PC12 cells, we observed that in vitro nicotine exposure significantly reduced neurite formation, altered MTs, and increased the co-localization of Gβγ, GSK3β and MTs in NGF-differentiated PC12 cells. These studies suggest that alteration of neuronal cytoskeleton by Gβγ/pGSK3β may contribute to nicotine abuse and addiction.

P1504
Board Number: BS15
Dynamic microtubules specify local delivery and capture of presynaptic cargo at en passant synapses.
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The formation and maintenance of presynaptic sites are critical for proper neuronal transmission. Presynaptic function requires the active transport and specific delivery of presynaptic cargo, including dense-core vesicles and synaptic vesicle precursors. In contrast to the classical terminal synapse at the distal end of the axon, the vast majority of synapses in the central nervous system are established along axonal processes, juxtaposed to the uniformly polarized axonal microtubule arrays ("en passant synapses"). Presynaptic cargo are generated in the soma and are transported along microtubules by KIF1A and dynein motors, however, the mechanisms specifying the targeting of transiting presynaptic cargo to en passant synapses are not well understood. Here, we investigate the role of local cytoskeletal determinants and vesicular motors in this process using rat primary hippocampal neurons, live-cell fluorescence microscopy and in vitro single-molecule approaches. We found that transiting presynaptic vesicles are preferentially retained at presynaptic sites when moving in the anterograde direction. We found that presynaptic regions are hotspots of high microtubule dynamicity, and anterogradely moving presynaptic vesicles pause preferentially at these sites. Dampening microtubule dynamics with low-dose nocodazole was sufficient to abolish the preferential retention of anterograde-moving vesicles at presynaptic sites. In contrast, sites of lysosome pausing were stochastically distributed along the axon, with low rates of capture at synaptic regions. This difference suggests that the high dynamicity of presynaptic microtubules particularly affects presynaptic cargo transport, potentially acting as a cue to halt KIF1A processivity. Using single-molecule assays, we found that kinesin-1 binds equally well to either dynamic (GTP-like) or stable (GDP-like) microtubules. In contrast, the presynaptic vesicle motor KIF1A has a significantly lower affinity to dynamic microtubules, thus revealing a mechanistic basis for the high vesicle pausing and retention rates observed at presynaptic sites in live neurons. Together, our
results unveil a model whereby the interplay between local microtubule cues and cargo-specific motors allows precise delivery of appropriate cargo to the presynapse.

**P1505**

**Board Number: B516**

**Regulation of axon initial segment cytoskeletal architecture and function by βIV-spectrin.**
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The axon initial segment (AIS) of polarized neurons is a specialized structure that, in addition to initiating action potentials, helps maintain neuron polarity by preventing the mixing of axonal and somatodendritic components. This latter function depends, in part, on a submembrane cytoskeletal network comprising ankryinG, actin filaments, and βIV-spectrin. While ankryinG functions as the master organizer of the AIS, βIV-spectrin is thought to contribute to AIS maintenance. The role of βIV-spectrin in AIS function and structure is poorly understood. AIS structure has been shown by platinum replica electron microscopy (PREM) to contain a dense mixture of fibrils and globules that coat microtubule bundles. We have proposed that this fibrillar-globular coat functions as a selective diffusion barrier that contributes to AIS polarity function. Here we investigate the structural and functional consequences of βIV-spectrin knockdown in hippocampal neurons in vitro using two independent lentiviral shRNAs, compared with scrambled and luciferase shRNA controls. βIV-spectrin depletion caused dramatic increase in AIS lengths (as determined by ankryinG immunofluorescence) and extensive spreading of AIS membrane proteins (including voltage-gated ion channels and cell adhesion molecules) throughout the axon. These data are consistent with a role for βIV-spectrin in AIS maintenance. We also examined the polarized distribution of several somatodendritic proteins and found that while many of them (e.g., PSD95, transferrin receptor, and MAP2) remained localized to dendrites, the potassium/chloride co-transporter KCC2 re-distributed to axons/AISs of neurons lacking βIV-spectrin. Interestingly, similar results were obtained following treatment with the actin depolymerizing drug latrunculin B. To determine the effects of βIV-spectrin knockdown on AIS structure, we examined AISs by PREM and discovered a fibrillar phenotype characterized by the presence of dense fibrillar bundles that (1) contain fibrils of ~100 nm in length, (2) align parallel to the AIS shaft, (3) contain globular structures at their ends, and (4) appear to repeat periodically along the AS. These fibrillar bundles likely represent ankryinG molecules since they stain positive by ankryinG immunogold and other likely candidates (e.g., all-spectrin, βII-spectrin, βIII-spectrin, septin7, and myosin II) failed to accumulate substantially in the AIS following βIV-spectrin depletion. Efforts are underway to confirm the identity of the fibrillar bundles. Together, these data indicate that βIV-spectrin is an important component of the AIS cytoskeleton that contributes to maintenance of overall AIS structure and its role in neuron polarity.

**P1506**

**Board Number: B517**

**Utilizing microtubule polarity as a tool for characterizing neuronal processes in the nerve net of an early metazoan: Nematostella vectensis.**
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Functionally polar neurons with separate axons and dendrites have been found in all bilaterian model organisms examined to date, and thus neuronal polarity appears to be a universal feature of bilaterian
nervous systems. The evolutionary origins of neuronal polarity have not been examined, so it is unclear if neuronal polarity evolved in concert with nervous system centralization in early bilaterians or was already present in the diffuse nerve nets of early metazoans. We addressed this question by looking for distinct axons and dendrites in the nerve net of a cnidarian, the starlet sea anemone Nematostella vectensis. Cnidaria is a sister lineage to Bilateria that is believed to have separated prior to the evolution of centralization. The nervous system of anthozoan cnidarians, including Nematostella, is a diffuse net that is likely similar to the nervous system of the cnidarian/bilaterian ancestor. Microtubule orientation differentiates axons (+ end out) and dendrites (- end out or mixed) in polar bilaterian neurons, and is believed to underlie trafficking differences that contribute to functional specialization of neurites. We therefore developed a live imaging strategy to examine microtubule polarity in Nematostella neurons and cnidocytes using an EB1-GFP + tip marker. Two characteristic types of neurons we identified were bipolar and tripolar neurons with unbranched neurites. These cells are non-polar with respect to the microtubule cytoskeleton because all neurites contain identical axon-like + end out microtubules. Cnidocytes, which are nervous system cells specialize for stinging, also appear non-polar but, in contrast, have branched neurites with dendrite-like mixed microtubule orientation. Cnidarians can thus make two types of neurites with axon-like and dendrite-like microtubule orientation, a prerequisite for bilaterian-style neuronal polarity. However, further studies will be needed to determine whether these cnidarian neurites are functionally unique and orthologous to axons or dendrites, or whether there are additionally polar neurons within the nerve net.

P1507
Board Number: BS18
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Microtubule-associated protein Tau (MAPT) is involved in the stability of microtubules, which maintain the cell shape. Tau protein is predominantly expressed in neurons, where it has a role in microtubule assembly and stability, axonal transport, and neurite outgrowth. In neurodegenerative diseases known as tauopathies, such as Alzheimer’s disease (AD), Tau function is compromised. Tau expression is developmentally regulated by alternative splicing, giving rise to 6 different isoforms in the human adult brain. Alternative splicing of exon 10 gives rise to two protein isoforms with either three or four microtubule-binding repeats in the 3R Tau (exon 10 exclusion) and 4R Tau (exon 10 inclusion) splice variant, respectively. Expression of both isoforms is developmentally regulated, and upon adulthood they are characterized by region- and cell type-specific neuroanatomic distribution. In this study we sought to identify the spatio-temporal pattern of Tau variant expression during postnatal mouse brain development. To achieve this we utilized a highly specific and sensitive single-molecule RNA in situ hybridization assay, BaseScope, to evaluate the age-dependent neuroanatomic expression of 3R and 4R Tau splice variants in the mouse brain at postnatal days P1, P10, P30, and P56. BaseScope probes designed to uniquely identify the exon junctions of E9/E10 (4R Tau) and E9/E11 (3R Tau) were able to specifically detect expression of the Tau variants at the single cell level within the morphological tissue context. Quantification of the 3R and 4R Tau expression levels revealed changes in the 3R:4R Tau ratio over the course of development. In addition, we found distinct subregional and layer-specific differences in the expression patterns of both isoforms. The observed layer-specific expression patterns may provide novel insight into how the two Tau isoforms differ biologically. Taken together, these results demonstrate the ability of the BaseScope assay to characterize the specific spatio-temporal expression
pattern of 3R and 4R Tau variants during mouse brain development at the single cell level. This is the first study of MAPT exon 10 alternative splicing by in situ detection which enables expression mapping of Tau variants in the morphological and spatial context, and a similar assay strategy can also be used to study Tau isoforms in relation to AD and other tauopathies.

P1508
Board Number: B519
Proteomic Insights into Cytoskeletal Mechanisms of Neurodegeneration.
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Cytoskeletal defects are hallmark of several neurodegenerative diseases. Results from our laboratory indicate that Gbeta-gamma, a major component of G protein-coupled receptor (GPCR) signaling pathway, interacts with microtubules (MTs) and plays an important role in the neuronal differentiation of PC12 cells. Inhibitors of Gbeta-gamma disrupted MTs, blocked neurite outgrowth, and induced neuronal damage, suggesting a role of Gbeta-gamma in neurodegenerative processes. Gbeta-gamma has been shown earlier to promote MT assembly in vitro and in cultured PC12 and NIH3T3 cells. In an effort to understand the mechanism of cytoskeletal disruption and neurodegeneration, we carried out a high-resolution proteomic analysis of cytoskeletal fractions (CSKs) of NGF-differentiated PC12 cells treated with agent known to trigger CSK disruption and block neuronal differentiation. Using Scaffold perSPECTives software to analyze the proteomic data, we found that neuronal differentiation of PC12 cells dramatically altered the proteomic landscape of CSK. 4-Nonylphenol (4-NP), an endocrine disruptor likely to cause neuronal damage, significantly affected the protein composition/pattern of CSK, including the association with Gbeta-gamma. String Link and Gene Ontology enrichment analysis indicated that several biological pathways are affected by 4-NP, including pathways related to Alzheimer’s disease, Parkinson’s disease, and Huntington disease. This study has potential to identify new biomarkers and/or pathways involved in disruption of CSK and the development of neurodegenerative disorders.

Establishing and Maintaining Organelle Structure 1

P1509
Board Number: B521
Identification of genes that control the formation of membrane-less organelles.
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Membrane-less organelles (MLOs) are subcellular compartments in eukaryotic cells that form through phase separation of proteins and RNAs from the surrounding milieu. The molecular composition of different types of MLOs is dynamic yet specific and determines their functional identity and biophysical properties. In human cells, almost all MLOs form de-novo after cell division. The number and size of MLOs change along the cell cycle and according to the physiological state of the cell. MLO formation is driven by many factors, such as the concentration and multivalency of organelle components. However, the mechanisms by which cells control the assembly and dissolution of MLOs remain largely unknown. Altering protein multivalency by posttranslational modifications might be key to regulating MLO formation. For instance, we recently showed that DYRK3 kinase activity is required for the dissolution of stress granules. Here, we present the results of three parallel high-resolution image-based siRNA screens that enabled us to identify kinases and phosphatases that control the formation and dissolution

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of six major MLOs in human cells: Nucleoli, Cajal bodies, splicing speckles and PML bodies in the nucleus, as well as P-bodies and stress granules in the cytoplasm. As part of our automated image analysis pipelines we employed a pixel-based classifier to segment individual MLOs in more than 12 million single cells and extracted their phenotypic features. A self-organizing map algorithm was used to assign each cell to a distinct position in the dimensionality reduced feature space according to the phenotype of two multiplexed MLO markers each. Using this powerful single cell clustering approach we were able to exclude indirect hits that only perturb the cell cycle but to detect genes with partial penetrant phenotypes on MLO formation. Computational multiplexing of all screens allowed us to identify genes with pleiotropic effects and to assess co-regulation between the six measured MLOs. We will present evidence for novel regulators of MLOs and show that perturbation of organelle homeostasis affects previously unlinked physiological processes. Together, our dataset will contribute to deciphering the regulation and biological functions of non membrane-bound organelles.

P1510

Board Number: B522

A relationship between protein mobility and organelle morphology in the endoplasmic reticulum.

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The endoplasmic reticulum (ER) is a complex membranous organelle that samples the majority of the cellular volume over very short time scales. Physically, this is achieved by a system of interconnected and highly dynamic tubes of liquid lumen enclosed in a fluid bilayer. Despite this remarkable liquid-in-a-liquid system, the diffusive properties of many membrane and luminal proteins in the ER have not been fully characterized. We thus examined three classes of membrane proteins with distinct insertion topologies: single-pass transmembrane (TM) proteins (e.g. Sec61b), hairpin shaping proteins (e.g. reticulons), and luminal spacing proteins (e.g. CLIMP63). To assess how these proteins behave within the ER membrane, we coupled fluorescence recovery after photobleaching (FRAP) to study their ensemble migration, with a single-particle tracking (SPT) approach to understand the motion of individual molecules. Each class of shaping protein exhibited differential diffusional characteristics on both the ensemble (FRAP) and single molecule (SPT) scale dependent on their membrane topology. Fascinatingly, overexpression of hairpin proteins, not TM or luminal spacing proteins, was also sufficient to dramatically decrease recovery of a luminal marker following photobleaching. This was the case for all studied hairpins, implying a unique property of this topogenic class of protein in influencing luminal diffusion. Hairpin proteins localize primarily to ER tubules and have been previously demonstrated to decrease tubular diameter in vitro and in S. cerevisiae. Thus, we proposed that a similar mechanism may be at play in mammalian cells, where a decrease in cross-sectional area limits diffusion of a luminal cargo through tubular regions of the ER. In support of this hypothesis, transmission electron microscopy reveals a dramatic and significant reduction in tubular diameter in cells overexpressing hairpin proteins, and reveals the presence of large luminal ‘sacs’ close to the nucleus, presumably where the lumen is

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“squeezed” into in order to accommodate the decreased luminal capacity of tubules. Crucially, this luminal redistribution has dramatic effects on the overall dynamics of the ER, massively proliferating the number of new tubule extensions present independent of the hairpin protein used. Additionally, when thin tubes are generated within the ER using a pharmacological treatment, hairpin proteins selectively localize to and stabilize these structures. In conclusion, we demonstrate that specific topologies of proteins inserted in the ER membrane influence diffusional properties of both membrane and luminal cargo and subsequently cause dramatic alterations in both the morphology and dynamicity of the ER.

**P1511**

**Board Number: B523**

On the Mechanism of Protein Targeting from the Endoplasmic Reticulum to Lipid Droplets.

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Lipid droplets (LDs) are organelles that possess a unique structure, consisting of an oil-phase bound by a monolayer of phospholipids. Proteins specifically target to the surface of LDs, however, our understanding of how this happens is incomplete. One class of LD proteins, including glycerol-3-phosphate O-acyltransferase 4 (GPAT4), which catalyzes the rate-limiting step of triacylglycerol (TG) synthesis, targets LDs from the endoplasmic reticulum (ER). GPAT4 re-localizes and accumulates on mature LDs through ER-LD membrane bridges. Here, we investigated the re-localization mechanism that allows for the accumulation of GPAT4 on LDs. We find that a membrane-embedded motif of GPAT4 (amino acids 160–216) forms oligomers and is sufficient to mediate protein accumulation on LDs. The amino acid sequence of this segment plays a key role in LD targeting. Specifically, mutational analysis of this sequence showed that large hydrophobic residues, in particular several tryptophans, are required for LD targeting. Conversely, several positively charged residues in the membrane-embedded sequence were not required. Our results suggest a model in which large hydrophobic residues mediate ER-LD protein targeting by detecting and binding LD surface properties, such as phospholipid packing defects resulting from the exposure of the underlying oil at the LD monolayer. These findings shed light on the mechanism of GPAT4 targeting to LDs as a model protein and suggest the critical role that the physical surface features of LDs play in the general mechanism of protein targeting from the ER to LDs.

**P1512**

**Board Number: B524**

Measurement of caveolin-1 densities in the cell membrane for quantification of caveolar deformation after exposure to hypotonic membrane tension.

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Caveolae are abundant flask-shaped invaginations of plasma membranes that buffer membrane tension through their disassembly. Few quantitative studies on the deformation of caveolae have been reported. Each caveola contains approximately 150 caveolin-1 proteins. In this study, we estimated the extent of caveolar deformation by measuring the density of caveolin-1 projected onto a two-
dimensional (2D) plane. The caveolin-1 in a flattened caveola is assumed to have approximately one-quarter the density of the caveolin-1 in a flask-shaped caveola. The one-quarter-density caveolin-1 increased after increasing the tension of the plasma membrane through hypo-osmotic treatment. The one-quarter-density caveolin-1 was soluble in detergent and formed a continuous population with the caveolin-1 in the caveolae of cells under isotonic culture. The distinct, dispersed lower-density caveolin-1 was soluble in detergent and increased after the application of tension, suggesting that the hypo-osmotic tension induced the dispersion of caveolin-1 from the caveolae possibly through flattened caveolar intermediates.

P1513
Board Number: B525
DYRK3 kinase regulates dissolution and condensation of membrane-less organelles during mitosis.
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Cell undergoes many biochemical reactions in compartments that are not surrounded by any lipid membranes called membrane-less organelles. Recently, it has been proposed that the mechanism of condensation of such compartments relies on a physical principle called phase separation, which is driven by weak molecular interactions between low-complexity domains (LCDs) within proteins and RNA. However, the cellular mechanisms regulating the dynamic condensation and dissolution of these compartments remains poorly understood. Interestingly during early mitosis, concomitant with nuclear envelope breakdown, multiple membrane-less organelles undergo dissolution, and then re-condense towards the end of mitosis. We show that the dual-specificity kinase DYRK3 interacts with, and localizes to multiple membrane-less organelles like stress granules, splicing speckles and pericentriolar satellites. Inhibition of DYRK3 kinase activity during mitosis results in co-condensation of multiple proteins from these compartments, and poly-adenylated RNA into aberrant hybrid structures. The formation of such aberrant structures in mitotic cells results in mitotic delay because of extended metaphase. We have been able to demonstrate that DYRK3 acts as a dissolvase during mitosis and increase in DYRK3 kinase to its substrate concentration ratio as a consequence of nucleo-cytoplasmic dilution post nuclear envelope breakdown allows cells to undergo rapid dissolution of organelles during early mitosis. Further, the re-condensation of organelles during late mitosis can be explained by our observation that the APC/C-CDH1 driven ubiquitination degrades DYRK3 during late mitosis, decreasing DYRK3 to substrate ratio. Thus, changes in relative concentration of the dissolvase (DYRK3) to the phase separating proteins (DYRK3 substrate) can explain the phenomenon of complete dissolution of multiple organelles during early mitosis and their re-condensation later in mitosis.

P1514
Board Number: B526
Light-dissociable membrane-less organelles maintain spatial patterns over long periods of time.
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Recent studies have shown the prevalence of membrane-less organelles inside cells, but understanding how these organelles form and dissociate remains a challenge. To better understand these issues, optogenetic tools controlling the assembly of membrane-less organelles have been developed.
However, existing optogenetic tools are only able to induce assembly upon light stimulation. Here, we report a tool for light-induced disassembly of membrane-less organelles. Our tool allows for the generation of protein droplets in the dark that dissociate upon blue light stimulation; droplets that we refer to as Reverse OptoDrops or RODs. Light-dependent assembly and disassembly of RODs are fast and reversible processes, leading to precise temporal control over protein phase separation. Moreover, we find that the spatial distribution of RODs can be tightly controlled with patterned light inputs, and that these patterns are retained for long periods of time, even after the removal of the light stimulus. Together, these results add another tool for precise spatiotemporal control over membrane-less organelles and suggest how long-term spatial information can be stored by the cell simply by the fundamental biophysics controlling protein clustering.

**P1515**  
**Board Number: B527**  
Members of the UDP-GalNAc:polypeptide N-acetylgalactosaminytransferase family of enzymes use different Golgi targeting signals.  
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Mucin-type O-glycosylation is an evolutionarily conserved protein modification in which N-Acetylgalactosamine (GalNAc) is added to the hydroxyl group of serine or threonine residues within the Golgi apparatus. This reaction is initiated by the UDP-GalNAc:polypeptide N-acetylgalactosaminytransferase (GalNAc-T) family of enzymes, with 20 family members in humans. Conceptual translation indicates that GalNAc-Ts are type II transmembrane proteins, though it is unclear how they localize to the Golgi or how their in vivo localization may affect substrate specificity. To address these questions, we sought to determine the Golgi targeting signals utilized by GalNAc-Ts. We began by identifying the minimum amino acids required for proper localization in human foreskin fibroblast (HFF) cells. We generated and transiently expressed plasmids encoding GFP fusions of various protein regions of the representative family members GalNAc-T1, -T2, -T7, and -T10. Immunofluorescence and confocal microscopy showed that -T1, -T2, and -T7 require at minimum both the cytoplasmic tail and transmembrane domain to colocalize with Giantin, a cis/medial Golgi protein, while -T10 requires the transmembrane domain and luminal stem region. These data suggest that GalNAc-Ts employ at least two distinct modes of Golgi localization—one defined by the amino acid sequence of the cytoplasmic tail and another encoded in the luminal stem. To further characterize the localization signal in the tail, we found that the -T10 tail includes a single glutamic acid while -T1, -T2, and -T7 tails lack negatively charged residues. When the glutamic acid in the -T10 tail was substituted with a lysine or alanine, the -T10 tail and transmembrane domain was directed to the Golgi, suggesting that the glutamic acid may prevent tail-directed Golgi targeting. We next performed pulldown assays using the combined tail and transmembrane domains of -T1 and -T10 and identified two Golgi-associated proteins that may interact with -T1 but not -T10: ARF1 and TIP47. Affinity purification using peptides of the -T1 and -T10 tails and subsequent western blots confirmed the interaction of TIP47 with the -T1 tail but not with the -T10 tail. Future experiments will determine if TIP47 depletion affects GalNAc-T localization. We will also attempt to identify putative binding partners for the -T10 stem and determine their importance for stem-dependent Golgi targeting. In summary, our data demonstrate that multiple Golgi targeting strategies exist in the GalNAc-T family. These studies will help determine the significance of these distinct targeting signals in GalNAc-T localization and function.
P1516
Board Number: B528
Pathogenic mechanism of human centronuclear myopathy resulted from nonsense mutations of Amphiphysin-2/Bin1.
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Centronuclear myopathy (CNM) is a genetic disorder characterized by muscle weakness that is debilitating at an early age. Autosomal recessive CNM is caused by mutations in Amphiphysin-2 (Bin1), a protein important for membrane curvature generation and critical for the formation of highly curved plasma membrane invagination in muscle cells, the T-tubules. Most of the CNM-related Bin1 mutations are located at its membrane curvature generation or membrane binding domains thus resulting in defects in T-tubule formation. Two mutations, Q434X and K436X, which result in truncated SH3 domains without membrane curvature generation perturbation, have not been well-characterized for their pathogenic mechanism. The phenotypes of these Bin1 mutants were illustrated in relation to their effects on Dynamin 2 (Dnm2) activity in our study. Dnm2 is a Bin1 interacting protein and is well-known for its membrane scission activity to release vesicles from the plasma membrane. We found the binding affinity of Dnm2 and Q434X and K436X was decreased in comparison to full-length BIN1, suggesting a diminished interaction between Bin1 and Dnm2. Surprisingly, fission activity of Dnm2 in vitro was enhanced, which was further supported by increased fission activity in vivo by co-transfection of the Bin1 mutants with Dnm2 in myoblast cells. In conclusion, we discovered the hyperactivity quality of Dnm2 membrane fission to be a key characteristic of the pathogenic mechanism of autosomal recessive CNM leading to defects in T-tubule formation. We will further investigate the effect of mutant Bin1 on Dnm2 membrane binding and assembly with electron microscopy analysis.

P1517
Board Number: B529
Cell size determines nuclear shape in Saccharomyces cerevisiae.
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Nuclei are typically round or oval, and under normal growth conditions cells maintain a constant ratio between nuclear and cell volume (N:C volume ratio). However, what aspect of nuclear size is linked to cell growth is not known. To address this, we inhibited cell growth and examined the consequences to nuclear size and shape in the yeast Saccharomyces cerevisiae. Two methods were used to inhibit cell growth: inhibition of secretion using temperature sensitive mutants in the secretory pathway, and inhibition of protein synthesis using cycloheximide. In both cases, inhibition of cell growth led to a striking nuclear phenotype, where the nucleus became bilobed with the bulk of the DNA in one lobe and the nucleolus in the other. Formation and maintenance of the bilobed nucleus was dependent on fatty acid synthesis, as the addition of a fatty acid synthesis inhibitor, cerulenin, abolished the bilobed nuclear phenotype. Thus, bilobe formation was likely a result of continued nuclear membrane expansion despite the inhibition to cell growth. Indeed, in the case of secretion mutants, the formation of the bilobe phenotype occurred predominantly in later stages of the cell cycle, when the nuclear envelope...
would have normally expanded to accommodate chromosome segregation. Importantly, the N:C volume ratio in cells containing bilobed nuclei was the same as in wild type cells as determined by three different methods. Based on this we conclude that while nuclear volume is linked to cell growth, nuclear surface area expansion is not. Therefore, in yeast under certain conditions the maintenance of a constant N:C volume ratio can come at a cost of nuclear deformation. Given that the constant N:C ratio is conserved, nuclear deformation in other systems may be caused by unregulated nuclear membrane expansion under the constraint of a nuclear volume that is linked to cell size.

P1518
Board Number: B530
Deciphering the cisternal localization of GalNAc-Ts using super-resolution imaging.
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The acquisition of mucin-type O-glycans in the Golgi is catalyzed by the family of 20 UDP-GalNAc:polypeptide N-Acetylgalactosaminyltransferases (GalNAc-Ts) which transfer N-Acetylgalactosamine (GalNAc) to selected Thr/Ser residues in substrates. The addition of GalNAc onto a substrate appears to occur in an ordered, hierarchical, manner. In vitro studies have shown two classes of GalNAcTs, the “early” GalNAc-Ts, that can modify un-glycosylated peptides (e.g. T1 and T2), and the “late” GalNAc-Ts, that can modify peptides already decorated with GalNAc (such as T7 and T10).

Conventional, diffusion limited, fluorescent imaging approaches cannot resolve the cisternal localization of Golgi proteins. Thus, to determine if the hierarchical acquisition of GalNAc is influenced by a unique spatial organization of the initiating enzymes within the Golgi we employed super-resolution microscopy approaches to localize endogenous GalNAcTs. First, STimulated Emission Depletion microscopy (STED) was used to compare the localization of GalNAc-Ts to the cis Golgi marker GM 130 and trans Golgi marker TGN 46 and to compare the localization of GalNAc-T2 with other GalNAc-Ts (T1, T5 and T7). STED imaging revealed that the four GalNAc-Ts co-localized more with GM130 than TGN 46, suggesting that they all reside close to the cis region of the Golgi. However, because the achievable resolution of STED is around 100 nm, which correspond to the size of a single Golgi cisterna, we sought to confirm these results using an additional approach. To this end, we are currently evaluating GalNAc-T localization using STochastic Optical Reconstruction Microscopy (STORM), which can achieve resolutions of up to 20nm.

Our data indicate that all four GalNAc-Ts studied, appear so far to be located in the same cis region inside the Golgi indicating that the substrate specificity of individual enzymes might be the main contributor to the hierarchical acquisition of O-glycans.

This research was supported by the Intramural Research Program of the NIH.

P1519
Board Number: B531
Novel roles for Dynamin2 (Dnm2) during ER scission and autophagy.
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The endoplasmic reticulum (ER) is a closed network of tubes and membranous sacs. Live cell imaging shows that this network is very dynamic. ER tubules routinely branch, elongate and fuse with other tubules or sacs. Elongation occurs along microtubules and fusion is mediated by the dynamin-related GTPase Atlastin. Frequent fusion events must be balanced by similarly frequent fission events or else the ER will form a continually tightening network that eventually collapses into a densely woven fabric. Mechanisms for ER fission are, however, as of yet unknown. Here, we show that Dynamin-2 (Dnm2), which severs endocytic vesicles from the plasma membrane, can also be recruited to the ER under stress-inducing conditions, coincident with the severing of ER membrane. Dnm2 knockout cells have more tightly woven ER networks and they show significant delays in ER fragmentation induced by calcium ionophores. These results suggest that Dnm2 contributes to ER scission. We also observe increased colocalization of Dnm2 with Endophilin B1 (EndoB1) and other ER markers when cells are treated with calcium ionophores or with autophagy inducing chemicals. Increased interactions are detected with immunofluorescence and proximity ligation assays for endogenous proteins and with transiently transfected fluorescent proteins. EndoB1 is a BAR domain and SH3 domain containing protein, closely related to Endophilin A1, which interacts with Dnm2 at the plasma membrane during clathrin-independent endocytosis. Although EndoB1 interacts with Dnm2 when autophagy is induced, these proteins unexpectedly have opposite effects on the progression of autophagy. Loss of EndoB1 through CRISPR knockout inhibits autophagy, but this inhibition is fully suppressed in EndoB1 - Dnm2 double knockout cells. These results suggest that Dnm2 can interfere with autophagy but this interference is relieved through binding interactions with EndoB1. We have thus uncovered novel interactions between Dnm2 and ER membrane proteins that control ER scission and autophagy.

P1520

Board Number: B532

The endoplasmic reticulum is partitioned asymmetrically during mitosis before cell fate selection in proneuronal cells in the early Drosophila embryo.
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Asymmetric cell division is the primary mechanism to generate cellular diversity, and it relies on the correct partitioning of cell fate determinants. However, the mechanism by which these determinants are delivered and positioned is poorly understood, and the upstream signal to initiate asymmetric cell division is unknown. Here we report that the endoplasmic reticulum (ER) is asymmetrically partitioned during mitosis in epithelial cells just before delamination and selection of a proneural cell fate in the early Drosophila embryo. At the start of gastrulation, the ER divides asymmetrically into a population of asynchronously dividing cells at the anterior end of the embryo. We found that this asymmetric division of the ER depends on the highly conserved ER membrane protein Jagunal (Jagn). RNA inhibition of jagn just before the start of gastrulation disrupts this asymmetric division of the ER. In addition, jagn-deficient embryos display defects in apical-basal spindle orientation in delaminated embryonic neuroblasts. Our results describe a model in which an organelle is partitioned asymmetrically in an otherwise symmetrically dividing cell population just upstream of cell fate determination and updates previous models of spindle-based selection of cell fate during mitosis.
P1521
Board Number: B533
A new pathway for membrane protein insertion at the ER.
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Membrane protein insertion is an essential cellular process. The broad biophysical and topological range of membrane proteins necessitates multiple insertion pathways, which remain incompletely defined. Here, we have discovered a new membrane protein insertion pathway, identified the class of substrates it handles, explained why other known pathways do not work for these substrates and reconstituted the pathway using purified components. We show that tail-anchored proteins with low to moderate transmembrane domain hydrophobicity fail to engage TRC40, the previously known targeting factor for this class of membrane proteins. Instead, these proteins are kept soluble in the cytosol by calmodulin. Dynamic release from calmodulin allowed sampling of the endoplasmic reticulum (ER). At the ER, the conserved ER membrane protein complex (EMC), a broadly conserved ten-subunit complex of unknown function, was shown to be essential for efficient insertion in vitro and in cells. In the absence of an intact EMC, these proteins were shown to mislocalize, aggregate, and get degraded. Purified EMC in synthetic liposomes was sufficient to catalyse insertion of its substrates in a reconstituted system. Therefore, the EMC is a transmembrane domain insertase that is necessary and sufficient for the insertion of roughly half of all tail-anchored proteins that don’t have access to the TRC40-dependent pathway.

P1522
Board Number: B534
SAC1 degrades its lipid substrate PtdIns4P in the ER to maintain a steep electrochemical gradient on donor membranes.
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Phosphatidylinositol 4-phosphate (PtdIns4P) is one of the most functionally diverse molecules utilized by eukaryotic cells. It is both a metabolic hub for other crucial signaling lipids such as PtdIns(4,5)P2 and PtdInsP3, and a key molecule for recruitment of membrane and lipid transport proteins in its own right. Most recently, it has been proposed to form a “phosphoinositide-motive force”, whereby transport of PtdIns4P molecules down their concentration gradient from the plasma membrane (PM) or Golgi to the ER drives counter transport of other lipids up their own concentration gradients. A critical requirement for this model is that the main PtdIns4P degrading enzyme, the integral ER phosphatase SAC1, hydrolyzes PtdIns4P in the ER in a “cis” configuration. Alternatively, it has been suggested that other functions of PtdIns4P are regulated by SAC1 hydrolyzing PtdIns4P directly in the PM in a “trans” configuration at membrane contact sites (MCS). However, such activity would surely disrupt lipid counter transport. Therefore, we sought to determine whether SAC1 acts in “cis”, “trans”, or both in mammalian cells. Acute chemical ablation of SAC1 activity drives ectopic accumulation of PtdIns4P in the ER, revealing “cis” activity. Furthermore, endogenous or ectopically expressed SAC1 localizes to the ER and Golgi, but does not constitutively or dynamically enrich at ER-PM MCS. Forced recruitment of SAC1 to experimentally induced MCS does not produce robust “trans” activity on PM PtdIns4P. However, “trans” activity can be induced by adding an approximately 6 nm long helical linker between the ER anchor and the catalytic domain. Together, our results reveal that SAC1 operates in a “cis” configuration. This ensures a “phosphoinositide-motive force” for lipid transport is effective, and also
implies regulation of PM phosphoinositide signaling is tightly linked to non-vesicular traffic of PtdIns4P at ER-PM MCS.

P1523
Board Number: B535
An image-based subcellular map of the human proteome.
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Compartmentalization of biological reactions is an important mechanism to allow multiple cellular reactions to occur in parallel. Resolving the spatial distribution of the human proteome at a subcellular level increases our understanding of human biology and disease. A high-resolution map of the human cell has been generated, the Cell Atlas (1), part of the open access Human Protein Atlas database (2,3). Using an antibody and imaging-based approach 12,003 proteins have been localized to 32 subcellular structures, enabling the definition of 13 major organelle proteomes. The high spatial resolution allowed identification of novel protein components of fine structures such as the midbody, nuclear bodies as well as rods & rings. An integrative approach was used including strict antibody validation criteria using gene silencing, paired antibodies, and fluorescently tagged proteins (4,5). Deep learning approaches and a citizen science approach was employed for refined pattern recognition in images, the mini-game “Project Discovery” integrated into an massively-multiplayer online game that has engaged more than 200,000 players world-wide (6).

We show that half of all proteins localize to multiple compartments. On one level, it can be a spatial confinement to control the timing of the molecular function in one compartment. On another level, such proteins may have context specific functions and ‘moonlight’ in different parts of the cell, thus increasing the functionality of the proteome and the complexity of the cell from a systems perspective. We further reveal 16% of the proteome to show single cell expression variation in terms of protein abundance or spatial distribution. Finally we show that current protein-protein network models benefit from integration of the Cell Atlas localization data as spatial boundaries.

Here we present the most comprehensive subcellular map of the human proteome and discuss the importance of spatial proteomics for single cell biology.
1. Thul (2017), Science
2. www.proteinatlas.org
3. Uhlén (2015), Science
4. Stadler (2013), Nat Methods
5. Uhlén (2016), Nat Methods

P1524
Board Number: B536
An orthogonal optogenetic toolkit to study intracellular transport and organelle positioning.
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Proper positioning of organelles by cytoskeleton-based motor proteins underlies cellular events such as signalling, polarization and growth. For many organelles, however, the precise connection between position and function has remained unclear, because strategies to control intracellular organelle positioning with spatiotemporal precision are lacking. We previously established optical control of intracellular transport by using blue light-sensitive heterodimerization to recruit specific cytoskeletal motor proteins (kinesin, dynein or myosin) to selected cargoes using LOV domains. To increase spatiotemporal control of such assays we engineered phytochrome B domains into photoswitches for intracellular transport that enable the reversible interaction between organelles and motor proteins by exposure to red and far-red light. Using patterned illumination and live-cell imaging, we demonstrate that this system provides unprecedented spatiotemporal control. We also demonstrate that it can be used in combination with a blue-light dependent system to independently control the positioning of two different organelles. Our strategy for optogenetic control of organelle positioning will be widely applicable to explore site-specific organelle functions in different model systems.

P1525
Board Number: B537
A role of an inositol 5-phosphatase in ER architecture.
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INPP5K, a member of the inositol 5-phosphatase family, consists of the phosphatase domain followed by a SKICH domain. Partial loss of function mutations of INPP5K were recently reported to result in congenital muscular dystrophy. How INPP5K localizes and functions in the ER remains unknown. Here we report that recruitment of INPP5K to the ER relies on ARL6IP1, an ER membrane protein which shares features of ER shaping proteins and whose dysfunction results in hereditary spastic paraplegia. The interaction of INPP5K with ARL6IP1 requires a cooperation of both its phosphatase and SKICH domains. Both INPP5K and ARL6IP1 are localized throughout the tubular ER network, but are not present in ER sheets. In addition, they are more concentrated, relative to generic ER proteins (e.g. Sec61b, VAPB), in newly formed tubules that undergo rapid extension along microtubule tracks. Depletion of either INPP5K or ARL6IP1 results in decreased abundance of ER tubules and in the expansion of ER sheets, possibly due to impaired formation of new ER tubules. Supporting these findings, a forward genetic screen aimed at the identification of genes controlling the ER architecture in PVD neurons of C. elegans, led to the isolation of cil-1 mutants, which are deficient in the function of the INPP5K orthologue. In these mutants, the complexity of ER tubule network and the extension of ER tubules into dendritic branches was impaired. These defects were rescued by expression of WT CIL-1, but not of catalytically inactive CIL-1 or SKICH domain-deleted CIL-1. Our results implicate an ER localized inositol-5-phosphatase in the control of the ER architecture.
Targeting of tail-anchored proteins by GET3B in Arabidopsis chloroplasts.
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Tail-anchored proteins, which have a cytosolic N-terminus, a single transmembrane domain, and a very short C-terminal tail, present a special challenge for membrane targeting. Targeting information has been shown to be primarily associated with the transmembrane domain and tail. Because these are still in the ribosome tunnel when translation stops, these proteins must be targeted post-translationally. To manage this, most eukaryotes have a dedicated system called GET (Guided Entry of Tail-anchored proteins), which includes SGT2 and GET1-5 in yeast. The central player is the targeting factor GET3, which receives the substrate protein from SGT2, GET4, and GET5 and delivers it to GET1 and 2 at the ER. This system is fairly conserved across eukaryotic lineages.

Recently, three GET3 homologs have been identified in Arabidopsis thaliana, with one, GET3B, predicted to contain a chloroplast transit peptide. However, the remaining components of the pathway do not appear to be present in chloroplasts. Chloroplasts contain multiple tail-anchored proteins, which are products of both the plastid and nuclear genomes, and play important roles in the photosystems and Sec translocases. Does GET3B play a role in targeting of these proteins? We are testing this hypothesis through a combination of genetic analysis, microscopy, and biochemistry. We will present data showing that GET3B binds to at least one tail-anchored protein in the stroma (the main aqueous space of chloroplasts) and forms a homodimer. The results from other on-going experiments will be presented. Supported by NSF DGE-1256259 and the UW-Madison Graduate School.

The AFF-1 exoplasmic fusogen is required for endocytic scission and seamless tube elongation.
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Many membranes merge during cellular trafficking, but some mechanisms involved in fusion and fission events initiating at exoplasmic (non-cytosolic) membrane surfaces are not well understood. Here we show that the C. elegans exoplasmic fusogen AFF-1 is required for endocytic scission and trafficking events during development of the intracellular lumen of a seamless unicellular tube. One proposed mechanism for intracellular lumen formation involves endocytosis to internalize basal membrane, followed by vesicle merging or exocytosis to form a lumen. An alternative mechanism involves cell wrapping to form a lumen lined by an autocellular junction, followed by membrane auto-fusion to remove that junction and become a seamless toroid. Using the C. elegans excretory duct tube as a model, we provide evidence that both mechanisms contribute to intracellular lumen formation and growth in vivo, and that both require the exoplasmic fusogen AFF-1. EGF-Ras-ERK signaling promotes duct cell fate and upregulates AFF-1 expression. AFF-1 is both necessary and sufficient to induce duct auto-fusion, and is necessary but not sufficient to promote subsequent tube elongation. Duct-specific removal of AFF-1 reduces lumen elongation independently of effects on junction removal. aff-1 mutants have an expanded apical domain and accumulate large basal inclusions with convoluted membranes, and experiments with the membrane-binding dye FM4-64 suggest that these basal inclusions correspond to a blocked endocytic intermediate. AFF-1 is an exoplasmic fusogen that localizes at or near
The basal plasma membrane during duct lumen growth, and therefore could be suitably positioned to mediate vesicle scission during endocytosis. We propose that EGF-Ras-ERK signaling stimulates both AFF-1 expression and basal endocytosis, and that AFF-1 is required for scission of basal endocytic compartments and for apically-directed exocytosis that ultimately contribute apical membrane to drive lumen growth.

P1528
Board Number: B540
Investigating the phospho-regulation of ER shaping protein RTN1A (Reticulon-1A) by the Calcineurin phosphatase.
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The endoplasmic reticulum is a dynamic organelle that performs multiple functions including intracellular Ca2+ regulation. Its structure is maintained by membrane shaping proteins such as reticulons, a group of proteins highly expressed in brain. The C-termini of reticulons are highly conserved with long hydrophobic domains while their N-termini are variable and believed to bring specific function to each protein. Interestingly, one of the reticulon family members, RTN1A, has a unique N-terminus that is highly phosphorylated, especially in serine residues. However, the functional consequence of this phosphorylation is unknown. Recent work from our lab has identified a calcineurin-binding motif in the unique N-terminus of RTN1A, suggesting that calcineurin may regulate this protein. Calcineurin (CN) is a Ca2+/calmodulin-activated serine threonine phosphatase that has been implicated in many ER-related functions including ER stress regulation. The substrates of CN that mediate these functions have remained unknown. Using a proximity-based interaction assay, we showed that RTN1A associates with CN in vivo, with the interaction being mediated by the CN-binding motif. Further, we showed that RTN1A is dephosphorylated by CN in vitro, suggesting that RTN1A is a direct substrate of CN. Recently, it was shown that the N-terminus of RTN1A is necessary and sufficient to induce ER-mitochondria contacts, which are sites for Ca2+ transfer between the ER and mitochondria. Additionally, the level of RTN1A was elevated in kidney disease and induced ER stress and apoptosis2, which increase CN activity. We are currently investigating a role for CN-dependent dephosphorylation in regulating RTN1A functions. Supported by T32GM007276. 1. Cho, I.-T., Adelmant, G., Lim, Y., Marto, J. A., Cho, G., & Golden, J. A. (2017). Ascorbate peroxidase proximity labeling coupled with biochemical fractionation identifies promoters of endoplasmic reticulum mitochondrial contacts. Journal of Biological Chemistry, jbc.M117.795286. 2. Fan, Y., Xiao, W., Li, Z., Li, X., Chuang, P. Y., Jim, B., ... He, J. C. (2015). RTN1 mediates progression of kidney disease by inducing ER stress. Nature Communications, 6, 7841.

Inter-Organelles Contact Sites and Membrane Microdomains
P1529
Board Number: B541
PI(4,5)P2 controls the level of its precursor, PI4P, in the plasma membrane by regulating PI4P/PS transport at ER-plasma membrane contact sites.
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Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] is a minor but critical regulatory phospholipid playing a role in signal transduction, endocytosis, exocytosis, actin dynamics as well as ion channel activity in the plasma membrane (PM). Little is known about how cells keep PI(4,5)P2 levels within normal range. Here we found that PM PI(4,5)P2 controls its own precursor supply at the PM by regulating the activities of the oxysterol-binding protein related proteins (ORP) 5 and ORP8 at ER-PM contact sites. ORP5/8 was previously known to transfer PI4P from the PM to the ER in exchange for phosphatidylserine (PS) which is transported in the reverse direction. We found that while ORP5 is already active at basal levels of PI4P and PI(4,5)P2, ORP8 shows little activity due to its poor membrane interaction. This difference is caused by the different membrane binding affinities of the N-terminal PH domains of ORP5/8. Importantly, interaction of ORP5 with the PM was found to depend both on PI4P and PI(4,5)P2 and decreases in either phosphoinositides levels caused dissociation of ORP5 from the PM. In contrast, PM interaction of ORP8 was strongly enhanced by expression of a PIP-5-kinase (PIP5K), which synthesizes PI(4,5)P2 in the PM. PIP5K overexpression also caused a massive reduction in PM PI4P and PS levels and only moderately increased the level of PI(4,5)P2. Thus, enhanced PI(4,5)P2 production facilitates the PI4P/PS exchange to the point where PM PI4P levels are depleted and no more able to support PS transport to the PM. This regulatory mechanism helps cells maintain PI(4,5)P2 levels by controlling the PI4P flux between PI4P/PS transfer and PI(4,5)P2 generation.

P1530
Board Number: B542
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Cells undergoing glucose restriction start to catabolize fat molecules to produce ATP through mitochondrial oxidative phosphorylation (OXPHOS). At the same time, autophagy (a conserved lysosome-mediated self-eating process) is triggered to support cellular lipid catabolic activity via redistributing stored fatty acids in lipid-droplets (LDs) to fat-metabolizing organelles such as mitochondria. However, how mitochondria and LD-specific autophagy (i.e, micro-lipophagy) interplay in starved cells for their long-term survival remains unknown. Here, we demonstrate that a regulatory feedback mechanism exists between mitochondria and micro-lipophagy in budding yeasts. We find that in response to acute glucose restriction, cells rapidly induce mitochondrial OXPHOS, followed by the upregulation of genes that control mitochondrial fusion activity. This leads to mitochondrial tubulation and prevents loss of mitochondrial DNA and respiratory capacity by protecting the organelles from being targeted to bulk autophagy during starvation. Our analysis reveals that mitochondrial respiratory activity is the key trigger for remodeling vacuolar membrane to form a micro-scale liquid-ordered (Lo)
membrane domain. We show that vacuolar Lo domain forms by the redistribution of Atg14p (a component of class III phosphatidylinositol-3-kinase (PI3K) complex I) to the vacuole surface upon the induction of mitochondrial OXPHOS. Through stabilizing other PI3K components (e.g., Atg6p) on the vacuole, Atg14p further differentiates the Lo domains to recruit LDs onto the vacuole surface for triggering micro-lipophagy. Cells lacking mitochondrial fusion activity or incapable of boosting aerobic respiration due to genetic/pharmacological stresses fail to develop vacuolar Lo domains, completely block micro-lipophagy induction, and display significantly diminished ATP production with shortened lifespans during glucose starvation. Thus, our data suggests that mitochondrial fusion plays a key role in micro-lipophagy by modulating mitochondrial aerobic respiration and vacuolar Lo domain differentiation in starved cells. This additional interplay between mitochondria and LD-specific autophagy may allow cells to operate a precise regulation of LD-stored lipid recycling in response to cellular energy demand and would help drive effective cell survival program during metabolic stress.

P1531
Board Number: B543
Reduction in the ER-mitochondria contact site plays an important role in palmitic acid-induced insulin resistance.
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Endoplasmic reticulum (ER) has physical contacts with mitochondria in its subdomain referred as mitochondria-associated ER membrane (MAM). Although reduction in crosstalk between ER and mitochondria through the MAM affects cellular homeostasis, its pathological significance in insulin resistance in type 2 diabetes mellitus remains elusive. Here, we disclosed the importance of impaired MAM formation in the fatty acid-elicited insulin resistance in hepatocytes. Palmitic acid (PA) suppressed insulin-stimulated phosphorylation of Akt in HepG2 cells within 12 hours. Neither ER stress response nor mitochondrial ROS is implicated in the repression of Akt phosphorylation as demonstrated by pharmacological inhibition. Even 3-hour-treatment of PA reduced the calcium flux from ER to mitochondria, followed by a significant decrease in MAM contact area, suggesting that PA suppressed the functional and structural interaction between ER and mitochondria. Overexpression of mitofusin2, a critical component of the MAM, substantially restored MAM contact area and partially ameliorated the PA-induced suppression of insulin sensitivity with Ser473 phosphorylation of Akt selectively improved.
These results suggest that the decline of the contact site between the ER and mitochondria, but not perturbation of homeostasis in the ER and mitochondria, plays important roles in PA-elicited Akt inactivation in hepatic insulin resistance.
P1532
Board Number: B544
STED super-resolution microscopy reveals CLIMP63-dependent domain segregation of lumenal and membrane proteins in ER tubules.
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The endoplasmic reticulum (ER) is an expansive, membrane-enclosed organelle. It plays crucial roles in numerous cellular processes including protein synthesis, stress response, calcium storage, and lipid metabolism and is classically divided between rough, ribosome-studded ER sheets and smooth, peripheral ER tubules. The spatiotemporal limitations of diffraction limited fluorescent microscopy have hindered efforts to understand microdomain organization and protein distribution within ER tubules and sheets. Here, we apply STED (stimulated emission depletion) super-resolution microscopy to characterize the morphology and microdomains of ER tubules. Live and glutaraldehyde fixed cell imaging with STED reveals that peripheral ER tubules are highly periodic, containing blobs and constrictions not observable by diffraction-limited microscopy. Lumenal (KDELmoxGFP) or membrane (Sec61β-GFP) ER reporters showed different periodicity patterns within the same ER tubule. Triple labeling 3D STED imaging of fixed cells localized endogenous ER protein (Derlin-1, Calnexin) clusters to periodic constrictions of lumenal KDELmoxGFP but not membrane Sec61β-GFP reporter in both isolated peripheral tubules and dense tubular matrices. ER lumenal periodicity and protein localization to periodic ER constrictions were disrupted by siRNA knockdown of the lumenal spacer CLIMP63. CLIMP63 localizes with lumenal KDELmoxGFP and is segregated away from Calnexin and Derlin-1 protein clusters. This suggests that ER tubules are composed of regions of dense ER membrane protein complexes that exclude lumenal ER proteins localized to CLIMP63 microdomains. These results have significant implications for our understanding of ER organization, dynamics and function in normal and diseased conditions.
Supported by the CIHR (PJ1-148698), NSERC and CFI/BCKDF. GG is the recipient of a UBC Four Year Doctoral Fellowship and CZ the recipient of an NSERC undergraduate student research award.

P1533
Board Number: B545
Membrane binding of PACSIN2 dependent on the characteristics of the membrane.
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Cell membrane tension is essential in several phenomena such as cell polarity and motility, exocytosis and endocytosis, adipogenic differentiation and so on. One of mechanisms of how cells adapt for membrane tension is mediated by flattening and disassembly of caveolae, that are small flask-shaped plasma membrane invaginations. Several membrane proteins have been identified to play important roles in caveolae formation. One of those is F-BAR domain protein PACSIN2. PACSIN2 has been reported to be colocalized in and sculpt caveolae. Under tension, PACSIN2 is released from caveolae. Mechanism of the release of PACSIN2 is not fully understood. Here we examined PACSIN2 binding to membrane phospholipids by liposome co-sedimentation assay, using liposomes of various compositions. PACSIN2 strongly binds to liposome made of bovine brain Folch fraction, which mostly contains large amount of phosphatidylserine (PS), a negatively charged phospholipid abundant in caveolae. Because the membrane tension is related to the generation of the packing defects of the membrane, we then examined the binding of PACSIN2 to saturated or unsaturated lipids, which are thought to have different
levels of packing defects. The liposomes were made of phosphatidylcholine with PS, and the two fatty acids of phospholipids was unified as 16:0-16:0, 16:0-18:1, and 18:1-18:1. Among 16:0-16:0 PC/16:0-16:0 PS (DPPC/DPPS), 16:0-18:1 PC/16:0-18:1 PS (POPC/POPS), and 18:1-18:1 PC/ 18:1-18:1 PS (DOPC/DOPS) liposomes, we found that PACSIN2 most strongly bind to DOPC/DOPS, which contain two mono-unsaturated fatty acids, suggesting that packing defects promote the membrane binding of PACSIN2. Then, we investigated the effect of tension to the PACSIN2 binding. We used NaCl buffer to create osmotically-induced tension on liposome surface made of bovine Folch fraction lipids. When concentration of solute is higher inside than outside liposome, tension occurs. However, this induced the rupture of the liposome. By performing osmotically-induced leakage assay, we show that the 0-40mM NaCl concentration differences between the inside and the outside of liposome induced negligible amount of membrane ruptures. However, PACSIN2 binding affinity under 0-40mM NaCl concentration difference remains stable. These results indicate that tension in this range is not sufficient to induce packing defects for alteration of PACSIN2 binding, or tension might not directly affect PACSIN2 binding affinity to membrane, suggesting the other mechanisms for the release of PACSIN2 from the membrane upon membrane tension.

P1534
Board Number: BS46
Single molecule and ensemble dynamics of the endoplasmic reticulum.
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The endoplasmic reticulum (ER) is an expasive, membrane-enclosed organelle that plays crucial roles in numerous cellular processes. Besides its clearly defined roles as the major site of cellular translation, the master regulator of calcium homeostasis, and origin of the secretory pathway; it makes contacts with and signals to control the behavior of many other cellular organelles. Despite this diverse set of functions, the spatial location of these processes within the ER network remain poorly characterized due to the highly intricate and dynamic nature of the organelle. Here, we utilize a variety of emerging superresolution imaging technologies to characterize the fine structure and the distribution of ER proteins within the organelle membrane and lumen. We have previously shown that the peripheral ER contains many intricate tubular clusters that cannot be distinguished from continuous sheets by diffraction-limited imaging; we further show that these structures contain diverse biological processes and proteins that are consistent with their complex structure. In fact, the ER is not as homogeneous of a structure as appears with traditional imaging approaches, instead containing a multitude of highly dynamic microdomains throughout the membrane and lumen which carry out individualized biological functions. By performing high speed, simultaneous structured illumination microscopy (SIM) and single particle tracking-photoactivation localization microscopy (sptPALM), we track the behavior of individual proteins within the dynamic membranous network of the ER. We show for the first time a strong effect of organelle structure on the behavior of single ER proteins and protein complexes, suggesting a correlation between the structure of the organelle and the localization of biological functions at the level of individual protein molecules.
P1535  
Board Number: B547  
Snx14 is a novel lipid metabolism protein at the Endoplasmic Reticulum.  
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Cells store high-energy lipids in the form of lipid droplets (LDs), which are harvested during starvation. Although it is recognized that cells also produce LDs when facing imminent starvation, how cells sense this starvation, and coordinate LD biogenesis and turnover, is unknown. Defects in this general adaptive metabolic response are associated with metabolic, cardiovascular, and neurodegenerative diseases. Here, we investigate the role of an uncharacterized protein, Snx14, in lipid metabolism and nutrient stress response. Recent studies from our lab have shown that yeast utilize the nuclear Endoplasmic Reticulum (ER)-vacuole junctions (NVJs) as dynamic metabolic platforms to coordinate LD biogenesis and turnover in response to nutritional stress. NVJ-resident protein Mdm1 localizes to distinct subdomains flanking the NVJ where LDs bud, and its loss perturbs LD biogenesis. Mdm1 has four mammalian homologs (Snx13, Snx14, Snx19 and Snx25), all of which share conserved protein architecture with Mdm1. Among them, Snx14 is associated with neuropathology characterized by cerebellar ataxia. Here, we show that Snx14 localizes to the ER in mammalian cells, where it regulates fatty acid metabolism. Indeed, we observe a significant increase in the LD number and triglyceride (TAG) levels in Snx14 overexpressed cells pulsed with oleate. Moreover, we observe a dramatic redistribution of Snx14 from the ER to LDs following oleate treatment, implying a role for Snx14 in fatty acid-to-TAG conversion. Consistent with this, global RNAseq data indicates a role for SNX14 in metabolism and cellular nutrient stress response. Current and future studies aim to understand the underlying mechanisms by which Snx14 regulates lipid metabolism and adaptive cellular responses to stress.

P1536  
Board Number: B548  
Evaluation of sterol transport from the endoplasmic reticulum to mitochondria in Saccharomyces cerevisiae.  
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Sterols are primary components of eukaryotic biological membranes and are included in plasma membranes and organelles at specific ratios. Mitochondrial membranes also contain sterols, and sterol synthesis has been shown to be critical for mitochondria morphogenesis in Saccharomyces cerevisiae. However, it remains unclear how sterol synthesized in the endoplasmic reticulum (ER) is transported to the mitochondria. In this study, we constructed a system to quantitatively measure sterol transport from the ER to mitochondria in S. cerevisiae, and explored its mechanism. In this system, sterol transport was evaluated by sterol esterification using a recombinant bacterial sterol acyltransferase SatA targeted to mitochondria (mito-SatA-EGFP) in a mutant defective in the intrinsic sterol acyltransferases Are1 and Are2 in the ER. When the are1Δare2Δ strain expressing mito-SatA-EGFP was incubated in the presence of [3H]methionine, radiolabeled sterol was converted to steryl ester by mito-SatA-EGFP in the are1Δare2Δ strain. In addition, when the ER membrane containing radiolabeled sterols were incubated with purified mitochondria containing mito-SatA-EGFP in vitro, sterol esterification was observed in a
time-dependent manner. These results suggest that this system will contribute to the elucidation of the mechanism of inter-membrane sterol transport.


P1537
Board Number: B549
Proteomics identifies organelle specific phosphorylation and reveals major subcellular reorganization in the progression of NAFLD.
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Obesity induced non-alcoholic fatty liver disease (NAFLD) is associated with the development of type 2 diabetes and constitutes a major health problem. A hallmark of the diseases is extensive lipid droplet (LD) formation and accumulation of toxic lipid species interfering with cellular signaling and functions. However, the cellular mechanisms during disease progression and cellular lipid overflow are poorly understood. Here, we develop a novel workflow for label free mass spectrometry based protein and phosphopeptide correlation profiling to systematically monitor the level and cellular distribution of ~6000 liver proteins and ~16,000 phosphosites during the development of dietary induced steatosis in mice. We see a redistribution of the secretory apparatus including a mislocalization of the COPI complex, and targeting of all analyzed Golgi apparatus proteins to LDs what leads to a general reduction of hepatic protein secretion. Further, we identify targeting of several organelle contact site proteins to LDs, accompanied by increased contacts between LDs and other organelles. Our resource provides the first systematic in vivo analysis of subcellular re-arrangements and organelle specific phosphorylation during disease development.

P1538
Board Number: B550
EHD proteins cooperate to generate caveolar clusters and to maintain caveolae during repeated mechanical stress.
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Caveolae introduce flask-shaped convolutions into the plasma membrane, and help to protect the plasma membrane from damage under stretch forces. The protein components that form the bulb of caveolae are increasingly well characterised, but less is known about the contribution of proteins that localise to the constricted neck. Here we make extensive use of multiple CRISPR/Cas9-generated gene knockout and knock-in cell lines to investigate the role of EHD (Eps15 Homology Domain) proteins at the neck of caveolae. We show that EHD1, EHD2 and EHD4 are recruited to caveolae. Recruitment of the other EHDs increases markedly when EHD2, which has been previously detected at caveolae, is absent. Construction of knockout cell lines lacking EHDs 1, 2 and 4 confirms this apparent functional redundancy. Two striking sets of phenotypes are observed in EHD1,2,4 knockout cells: 1. The
characteristic clustering of caveolae into higher order assemblies is absent. When the EHD1,2,4 knockout cells are subjected to prolonged cycles of stretch forces, caveolae are destabilised and the plasma membrane is much more likely to rupture. Our data identify the first molecular components that act to cluster caveolae into a membrane ultrastructure with the potential to extend stretch buffering capacity, and support a revised model for the function of EHDs at the caveolar neck.

P1539
Board Number: B551
Cholesterol remodeling may protect cells from pore-forming toxins by enhancing membrane repair.
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Necrotizing fasciitis and gas gangrene are caused by Streptococcus pyogenes and Clostridium perfringens, respectively. Both organisms produce virulence factors including cholesterol dependent cytolysins (CDCs), which bind to cholesterol on mammalian membranes and form lytic pores. The lethal threat these pores pose is countered by membrane repair. One mechanism of membrane repair is intrinsic repair, the sequestration and shedding of CDCs on cholesterol-enriched microvesicles. How cells sequester and shed CDCs is unknown. Toxin oligomerization, is sufficient to promote shedding, suggesting that intrinsic repair relies on membrane remodeling or the alteration of lipid domains. We hypothesize that cholesterol accessibility and cholesterol microdomain architecture drive sequestration and shedding during intrinsic repair. We tested this hypothesis by controlling the membrane cholesterol during CDC challenge in three ways: addition of hydroxypropyl-β-cyclodextrin (HPCD) to remove cholesterol, addition of monomeric, pore-deficient CDCs to sequester cholesterol, or addition of oligomeric, pore-deficient CDCs that enhance intrinsic repair. First, we carried competitive cytotoxicity assays using active (wild-type) and inactive CDCs alone or together. We found that treatment with excess inactive toxins did not impair wild-type toxin binding, but these inactive toxins protected multiple cell lines from CDCs. When we treated cells with HPCD, we found that low doses of HPCD decreased toxin activity without impairing toxin. These results suggest that cholesterol availability may regulate toxin activity to protect cells from CDCs. Thus, cholesterol remodeling may play a key role in promoting host survival during necrotizing fasciitis or gas gangrene.

P1540
Board Number: B552
Dietary fatty acids direct differentiation of mesenchymal stem cells through lipidomic remodeling, microdomain stabilization, and enhancement of Akt signaling.
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The plasma membrane is the interface between a cell and its environment and conducts the majority of extracellular signaling. These functions are strictly dependent on both membrane lipid compositions and the biophysical phenotypes (e.g. membrane fluidity and lateral organization arising from collective lipid behavior). Dietary perturbations of lipid profiles can have severe deleterious consequences (e.g. hypercholesterolemia) or be broadly therapeutic, as with -3 polyunsaturated fatty acids (PUFAs). The molecular mechanisms underlying these effects are not clear; however, an intriguing possibility is that dietary fats integrate into membrane lipids, change membrane properties, and thereby affect cell signal
transduction. We have observed extensive, cell-autonomous remodeling of both cellular and plasma membrane (PM) lipidomes during human mesenchymal stem cell (MSC) differentiation into adipocytes and osteoblasts. Lipidomic differentiation results in unique, cell-specific membrane compositions, with osteoblasts containing longer and more polyunsaturated lipids, which result in more ordered membranes and more stable raft microdomains. The distinct features of osteoblast membranes enabled rational remodeling of membrane phenotypes to direct lineage specification in MSCs. Specifically, supplementation with a lipid component characteristic to osteoblasts (the -3 PUFA docosahexaenoic acid, DHA) induces broad lipidomic remodeling in MSCs, reproducing features of an osteoblastic membrane phenotype. Specifically, DHA robustly incorporated into specific membrane lipids, eliciting compensatory lipidomic responses in the cells. Together, these lipidomic changes reproduced osteoblast membrane biophysical phenotypes, namely stabilized membrane microdomains. These changes to the membrane phenotypes in MSCs affected PM signaling, most notably resulting in Akt recruitment and activation at the plasma membrane. Finally, this DHA-mediated induction of the osteoblastic membrane phenotype significantly affected differentiation of MSCs, specifically potentiating osteogenic differentiation. These results demonstrate that membrane phenotypes are central drivers of cell function and are susceptible to remodeling by exogenous fatty acids, suggesting a novel mechanism wherein dietary fats affect cellular physiology through remodeling of membrane lipidomes, biophysical properties, and signaling.

_P1541_
**Board Number: B553**
**Phosphoinositide diffusion in the plasma membrane reveals distinct populations in COS-7 cells.**
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Polyphosphoinositides (PPIns) are key regulatory lipids that coordinate diverse cellular functions. PtdIns(4,5)P2 in particular is a pivotal lipid that regulates many plasma membrane functions, including signaling, endo/exocytosis, modulation of ion channels and the formation of cellular junctions, to name just a few. This wide range of function demands a tightly controlled spatial and temporal PtdIns(4,5)P2 distribution, to prevent cross-talk in the activation of distinct cellular processes. PtdIns(4,5)P2 has been shown to form clusters at specialized cellular structures, like phagosomes or synaptic terminals. These cellular structures are also enriched in phosphatidylinositol 4-phosphate 5-kinase (PIPSK), the enzyme that predominantly synthesizes PtdIns(4,5)P2. In addition to these structures, there are great number of potential membrane domains with important physiological functions where PtdIns(4,5)P2 is involved, but nothing is known about the dynamic of PtdIns(4,5)P2 in these domains. Here, by using genetically encoded sensors of PtdIns(4,5)P2 fused to photoconvertible fluorescent protein mEos2, we tracked individual molecules of PtdIns(4,5)P2 in the plasma membrane of living cells. The lateral mobility of PtdIns(4,5)P2 yielded an apparent diffusion coefficient of 0.15 μm² /s. To extract fine information of single molecule trajectories, we computed radial displacement distributions, which allowed the identification of two populations with different mobility. Both populations showed Brownian motion, but diffusion coefficients displayed significant differences. For the fast population the diffusion coefficient was of 0.2 μm² /s and represented a 68% of all trajectories, meanwhile for the slow population the diffusion coefficient was 0.03 μm² /s. We are attempting to correlate the spatial distribution of these populations with respect to membrane domains specialized for function, such as clathrin-coated structures and focal adhesions. These results shows that PtdIns(4,5)P2 exhibits heterogeneous motion and suggest the presence of nanodomains in the plasma membrane, which could harbor relevant physiological functions.
**P1542**  
**Board Number: B554**  
*Plasma membrane wounding and repair occurs during BCR-antigen interaction and promotes B cell activation.*  
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B lymphocytes survey antigen through the B cell receptor (BCR), which activates B cells to mount antibody responses against infectious agents. We previously showed that plasma membrane wounding and repair caused by bacterial pore-forming toxins interferes with BCR activation due to competition for lipid rafts (1). This study addresses whether the plasma membrane wounding occurs when B cells engage antigen, and if repair of such wounds impacts B cell activation. Using live cell imaging, flow cytometry and a membrane impermeable dye we found that pseudo and bone fide antigen but not transferrin, when immobilized to beads, wound the plasma membrane of B cells in an antigen dose-dependent manner. The wounding triggers lysosome exocytosis and production of ceramide at the junction between B cells and antigen-coated beads. Inhibition of lysosomal exocytosis and secretion of the lysosomal enzyme acidic sphingomyelinase, which generates ceramide at the outer leaflet of the plasma membrane, increases the number of wounded B cells. Wounded B cells show enhanced polarization of surface BCR, ceramide, and phosphorylated tyrosine toward the antigen-bead binding sites as well as internalization of the immobilized antigen, when compared to non-wounded B cells. Collectively, these results suggest that immobilized antigen can wound the plasma membrane of B cells at the antigen binding sites, and that repair of the wounds can enhance B cell activation by facilitating surface BCR/lipid raft polarization and antigen uptake.  

**P1543**  
**Board Number: B555**  
*Crosstalk between membrane lipid unsaturation and inter-organelle lipid transport.*  
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Membrane lipids are synthesized in different organelles, requiring transport of lipids between organelles. Mitochondria contain enzymes for producing phosphatidylethanolamine and cardiolipin. Their precursors, phosphatidylserine (PS) and phosphatidic acid, respectively, are synthesized in the endoplasmic reticulum (ER) and transported to mitochondria. Transport is thought to occur at regions of close contact between these organelles. In the yeast S. cerevisiae two protein complexes that tether ER and mitochondria have been identified: the ER–mitochondria encounter structure (ERMES) [1], and the ER membrane complex (EMC) [2] that are both proposed to be involved in lipid transport, however, the molecular mechanism is not known. Furthermore, a protein complex that clamps the vacuole to the mitochondria, the vacuole mitochondria patch (vCLAMP) was identified and implicated in mitochondrial lipid import [3,4].  
The efficiency of transport of newly synthesized PS into mitochondria was previously shown to be inversely related to molecular hydrophobicity, with less hydrophobic molecular species (with shorter and/or more unsaturated acyl chains) transported more efficiently into mitochondria than more hydrophobic species [5].
Recently, our lab showed that the yeast glycerol-3-phosphate acyltransferase Sct1p affects membrane lipid unsaturation by competing with the desaturase Ole1p for their shared substrate palmitoyl (C16:0)-CoA [6]. Overexpression of SCT1 decreases membrane lipid unsaturation and causes a growth phenotype. Loss of Psd1p, the mitochondrial enzyme that decarboxylates PS to PE, exacerbates the SCT1-overexpression growth phenotype and further decreases lipid unsaturation upon SCT1-overexpression, consistent with mitochondria serving as a sink for lipids with unsaturated acyl chains. This growth phenotype was recapitulated in ERMES- and vCLAMP- but not in EMC single deletion mutants, supporting a role for the ERMES and vCLAMP complexes in lipid transport. Currently we are studying the effect of inactivating ERMES- and EMC components on lipid acyl chain composition. We propose manipulation of lipid composition by overexpression of SCT1 as a tool for increasing the resolution in monitoring lipid metabolism and transport.

References:

P1544
Board Number: B556
Spatial organization of ER–PM junctions revealed by super- and high-resolution imaging.
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The endoplasmic reticulum (ER) extends widely and forms junctions with virtually all the other membrane-bound organelles in eukaryotic cells. These ER–organelle junctions enable direct interactions and transfer of molecules between the ER and its apposing organelles to fulfill various cellular functions. Particularly, ER–plasma membrane (PM) junctions mediate crucial activities ranging from Ca\(^{2+}\) signaling to lipid metabolism. The location and extent of these cellular activities may be modulated by spatial organization of ER–PM junctions in the cell cortex. Nevertheless, the morphology and distribution of ER–PM junctions are not well characterized. Using photoactivated localization microscopy (PALM), we reveal ER–PM junctions are main ablong with the dimensions at 100-nm scale. Using total internal reflection fluorescence microscopy (TIRFM) and structure illumination microscopy (SIM), we show that F-actin contributes to spatial distribution and stability of ER–PM junctions. Further functional assays suggest that intact F-actin architecture is required for phosphatidylinositol 4,5-bisphosphate [PI(4,5)P\(_2\)] homeostasis mediated by ER–PM junctions. Taken together, our study provides quantitative information about spatial organization of ER–PM junctions that is in part regulated by F-actin. We envision functions of ER–PM junctions can be differentially regulated through dynamic actin architecture during cellular processes.
P1545
Board Number: B557
Involvement of membrane rafts in acrosome reaction of avian sperm via cAMP-dependent pathway.
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Membrane rafts are specific membrane regions enriched in sterols and functional proteins, and play important roles in diverse cellular processes. Transcriptionally and translationally inactive, sperm need these membrane domains pre-assembled into specific cellular compartments where function needs later. Recently, we demonstrated in chicken sperm that membrane rafts exist in the plasma membrane overlaying sperm head region. This, combined with our previous finding of functional importance of membrane rafts in acrosome reaction (AR) in mammalian sperm, led us to investigate roles of membrane rafts in signaling pathways leading to AR in chicken sperm. Using 2-hydroxypropyl-beta-cyclodextrin (2OHCD), we showed that disruption of membrane rafts inhibited protein phosphorylation of PKA substrate and acrosome reaction in response to physiological stimulation although intra-cellular Ca2+ was increased. Furthermore, cytosolic cAMP level was decreased in 2OHCD-treated sperm. However, those inhibitions were abolished by treatment with dibutylryl cAMP, suggesting a role of membrane rafts in regulation of PKA pathway in chicken sperm. To seek an upstream cascade, we examined the involvement of soluble AC (sAC) and transmembrane AC (tmAC) in the inhibition of PKA substrate phosphorylation and AR using specific AC stimulators (NaHCO3- and Forskolin) and inhibitors (KH7 and MDL-12,330A). The results showed that both stimulators and inhibitors were effective for regulation of PKA substrate phosphorylation, suggesting presence of both proteins in chicken sperm. Furthermore, treatments with both sAC and tmAC stimulators rescued 2OHCD-induced inhibition of PKA substrate phosphorylation and AR. Conclusively, our results demonstrated that membrane rafts play an important role in acrosome reaction via regulating cAMP dependent pathway involving PKA.

P1546
Board Number: B558
Plasmodesmata defects observed in UDP-glucose:sterol glucosyltransferase B1 mutants.
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Sterol glucosides are found ubiquitously in higher plants but their biological function remains elusive. Though it is clear that the presence of these glycosylated sterol derivatives alters the biophysical properties of the membrane, how it affects membrane function is still under investigation. Here, we use transmission electron microscopy (TEM) to explore the morphology of plasmodesmata (PD) in lines of Arabidopsis thaliana carrying insertions in the UDP-Glc:sterol glucosyltransferase gene, UGT80B1. We show that the PD of the mutant line differ from those of wild-type plants. Firstly, we observed a significantly higher frequency of branched PD in ugt80B1 mutants. In addition, while the simple linear PD of wild-type plants were often dilated, those of the mutant line were not. We postulate that the altered membrane lipid profile found in ugt80B1 mutants leads to aberrant PD morphology.
The eukaryotic plasma membrane is compartmentalized into nano- and microscale domains enriched in particular lipids and proteins. However, our understanding of the molecular bases and biological roles of this partitioning remains incomplete. Here we report that clustering of the yeast Can1 permease in eisosome domains requires complex sphingolipids, is dictated by its conformation, and controls its ubiquitin-dependent turnover. In its substrate-free outward-open conformation, Can1 accumulates in eisosomes, while a substrate-induced shift to an inward-facing conformation promotes its cell surface dissipation and accessibility to the ubiquitylation machinery. Furthermore, under starvation conditions an Lsp1-dependent expansion of eisosomes provides protection for Can1 and other transporters from bulk endocytosis parallel with autophagy. Our results illustrate that the highly dynamic lateral segregation of membrane transporters is governed by the combined effects of their conformation and sphingolipids. They also highlight regulated protection from ubiquitylation and endocytosis as a novel role for protein partitioning into membrane domains.

Kinases and Phosphatases 1

The allosteric regulation of protein function plays important roles in many cellular signaling pathways, including cell metabolism, growth, division and programmed cell death. Allosteric proteins typically show a modular organization that results in segregated functional domains. Often, the communication between domains is triggered by external stimuli like ligand binding, and results in high-order functions such a cooperativity and allostery. How the protein's communication is modulated by ligand binding to enable the propagation of signals from one domain to another remains poorly understood. Here, we investigate the communication between the two cAMP binding domains (CBD) in the regulatory subunit of Protein Kinase A. We use optical tweezers to mechanically unfold each CBD alone or as part of the regulatory subunit, with and without cAMP. We find that cAMP stabilizes each individual CBD, but the magnitude of stabilization is markedly different. This result highlights that conservation in structure does not entail conservation in structural stability. The selective unfolding of each CBD within the regulatory subunit shows that cAMP triggers networks of communication that involve direct, interfacial interactions between the two CBDs as well as long-range interactions between non-contiguous structural motifs. The mutant R241A, a hotspot residue position for allosteric interaction, severely disrupts the interaction between two CBD domains. This study allows us to dissect how a ligand turns on
and off domain communication in allosteric proteins. Moreover, this study enabled the identification and characterization of aberrant allosteric events associated with mutations observed in the disease state.

P1549

Board Number: B562

Impact of Phosphorylation by Met on FAK Activation.
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Focal adhesion kinase (FAK) is a non receptor tyrosine kinase that plays a role in development, homeostasis and progression of human diseases. FAK has three structured domains; FERM, kinase, and FAT (focal adhesion targeting domain). The FERM domain covers the catalytic domain in an autoinhibited conformation, which must be released for full catalytic activity. FAK responds to multiple stimuli such as growth factor receptors and integrins. The met receptor is an example of one of the diverse activation stimuli. It relieves FAK autoinhibition in response to hepatocyte growth factor (HGF) stimulation. This stimulation leads to phosphorylation of the activation loop of FAK, which blocks the FERM domain from binding to the catalytic domain. Additional studies have shown the met receptor phosphorylates FAK on Y194 in this interaction. The effect of this phosphorylation is what we sought to investigate. A FRET-based biosensor was used to detect the conformational change of FAK upon activation. MDCK cells transfected with FAK biosensor were used to measure basal FRET levels and upon HGF stimulation. HGF binds to and activates the met receptor which in turn binds to and activates FAK. In order to study the effect of Y194 phosphorylation, two mutants were made using site directed mutagenesis. The Y194E mutation mimics the phosphorylated state of FAK. The Y194F mutation prevents phosphorylation at this site. The effects of these mutations will be observed using FRET-based and biochemical assays. This study gives a more complete understanding of FAK activation via the met receptor.

P1550

Board Number: B563

Encoding optical control in LCK kinase to study its function and effect of coreceptor binding in live cells.
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LCK is a tyrosine kinase essential for initiating T-cell antigen receptor (TCR) signaling. A complete understanding of LCK function is constrained by a paucity of methods to quantitatively investigate its activity within live cells. To address this limitation, we generated LCK*, in which a key active site lysine is replaced by a photo-caged equivalent, using genetic code expansion. This enabled fine temporal and spatial control over kinase activity, allowing us to measure phosphorylation kinetics in situ using biochemical and imaging approaches. We find that auto-phosphorylation of the active site loop of LCK is indispensable for its catalytic activity. Furthermore, LCK kinase can stimulate its own activation by adopting a more open conformation, which can be modulated by point mutations. We then show that CD4 and CD8, the T cell co-receptors, can enhance LCK activity, explaining their effect in physiological TCR signaling. Our approach also provides general insights into SRC-family kinase dynamics.
P1551
Board Number: B564
Engineered Allosteric Regulation of Protein Kinases by Light.
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A single protein kinase can initiate very distinct physiological and pathological processes depending on the timing and the location of its activation in the cell. However, interrogation of kinase-mediated processes remains challenging due to the paucity of tools that precisely regulate kinases in space and time. To overcome existing limitations, we developed a novel approach for light-mediated regulation of kinases. We engineered a light-regulated allosteric switch (LightR), a small domain based on Vivid (VVD) photoreceptor, that undergoes conformational changes upon illumination with blue light. Insertion of LightR at a specific location in the catalytic domain of Src kinase enables light-mediated control of its activity. In the dark, engineered LightR-Src is inactive. Illumination of cells with blue light leads to robust activation of the kinase. Activation of LightR-Src is specific, fast, and reversible. This allows us to mimic transient activation of the kinase in living cells. The level of activation can be controlled by changing light intensity. Specific modifications of the LightR domain allow us to modulate the activation/inactivation kinetics and the level of activity of LightR-Src, enabling additional “tuning” of the engineered kinase and its optimization for different applications. Activation of LightR-Src in living cells induces robust cell spreading and protrusive activity, which are known Src-induced phenotypes described in previous studies. Importantly, the morphological changes stop as soon as the kinase is inactivated, demonstrating reversibility of the manipulated signaling. Repeated activation/inactivation of LightR-Src induces cycles of morphodynamic activity of the cells, showing the capability of this tool to mimic oscillation of kinase signaling in living cells. Localized illumination of cells induces local formation of protrusions demonstrating spatial regulation of LightR-Src. Insertion of LightR domain at a homologous position in Abl kinase also enabled light-mediated regulation of its activity. These results, and the high structural similarity among catalytic domains of kinases, suggest broad applicability of this tool for regulation of different kinases. Furthermore, this approach could be potentially employed to control the activity of other enzymes in time and space.

P1552
Board Number: B565
Polymerization of the mitochondrial phosphatase PGAM5 underlies its biological activity.
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Phosphoglycerate mutase family member 5 (PGAM5) is an atypical protein phosphatase localized to the mitochondrial membranes through a transmembrane domain tether. PGAM5 is involved in regulation of mitochondrial fission, and has been shown to promote cell death in response to oxidative stress or mitochondrial damage. The cell death response is believed to be triggered when PGAM5 is cleaved from mitochondrial membranes and relocated to the cytosol. The physiological importance of PGAM5 is underscored by genetic studies in mice, which revealed that PGAM5 deficiency causes a Parkinson’s Disease-like disorder. The mechanism for regulation of the PGAM5 phosphatase domain activity has been poorly understood, and as a result the tools for understanding biological functions of PGAM5 or
demonstrates immunosuppressants, we evidence mitochondrial cryo approaches these predicted microscopy, identified contains select developing into phosphatases. 

Electron microscopy, we demonstrate that PGAM5 adopts the dodecameric structure also in solution and we show, for the first time, that this assembly is critical for PGAM5 phosphatase activity and its role in mitochondrial homeostasis. Our new high resolution crystal structure of the PGAM5 dodecamer demonstrates that the multimerization motif serves as an allosteric activation domain. Furthermore, our electron microscopy analysis of the purified PGAM5 reveals that the cleaved form of PGAM5 organizes into long filaments composed of the dodecameric PGAM5 rings. Using super resolution fluorescence microscopy, we demonstrate presence of these filaments in the cytoplasm of intact cells and provide evidence that PGAM5 oligomerization is necessary for function of PGAM5 as activator of cell death. In summary, our work uncovers novel structures of the multimeric PGAM5 assemblies and demonstrates their importance for PGAM5 signaling.

P1553
Board Number: B566
Mapping the human calcineurin phosphatase signaling network through global identification of short linear motifs that mediate substrate recognition.

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Systems level analyses of phospho-signaling networks requires the understanding of both kinases and phosphatases. However, current knowledge of phosphatase functions is limited by the lack of global approaches for substrate identification. Recent work has shown that many phosphatases use short linear motifs (SLiMs), which are located in intrinsically disordered regions, to recognize their binding partners and achieve specificity in spite of considerable degeneracy at the phospho-sites on which they act. Calcineurin (CN), the conserved Ca2+/calmodulin-dependent protein phosphatase and target of immunosuppressants, FK506 and Cyclosporin A, recognizes substrates via two conserved SLiMs, ‘PxIxIT’ and ‘LxVP’. CN is ubiquitously expressed, and has identified roles in the immune system, heart, and brain, but many of its substrates and functions are as yet undiscovered. We defined the CN-signaling network by integrating experimental and computational approaches to identify proteins that interact with CN throughout the proteome. We used proteomic peptide phage display (ProP-PD) to directly select calcineurin-binding SLiMs from predicted disordered regions of the human proteome, and identified PxIxIT and LxVP sequences that define 41 new candidate substrates. After confirming that these peptides bind CN with specificity in vitro, we used them, along with known CN binding motifs, to develop a Position Specific Scoring Matrix (PSSM) and defined a set of 846 human proteins containing predicted PxIxIT and/or LxVP sequences that are highly likely to be CN substrates or regulators. Further, we used proximity based BioID/MS analyses to identify CN interacting partners in HEK293 cells, of which >40% are predicted to contain CN binding SLiMs using the PSSM. This combinatorial approach has allowed us to build a high confidence CN signaling network, which connects CN to several new cellular processes such as nuclear transport, cytoskeletal organization and mitosis, and to diseases such as schizophrenia, autism and myocardial ischemia. Additionally, we show that the signaling protein Notch1 contains a CN-interacting LxVP SLiM and is a novel CN target. CN interacts with Notch1 in vivo and
regulates its phosphorylation both in vivo and in vitro. We demonstrate that CN de-phosphorylates Notch1 and enhances its steady-state levels in vivo to positively impact Notch1 signaling. Thus our analyses not only expands the role of CN in known cellular pathways and disease processes but also uncovers new roles for this enzyme in novel signaling pathways.

**P1554**

**Board Number: B567**

*The Mutation of the PTPN11 Gene that Encodes SHP-2 Protein Promotes Tumorigenic Activity of the NF1-Deficient cells.*

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The NF1 tumor suppressor gene encodes neurofibromin and is a functional Ras GTPase-activating protein (RasGAP) involved in negatively regulating the Ras signals by accelerating the conversion of activated Ras-GTP to inactive Ras-GDP. NF1 gene germline mutations cause Neurofibromatosis type 1 (NF1, von Recklinghausen disease). We inoculated the GFP-labeled human NF1-deficient cell line sNF96.2-GFP into immunodeficient mice, and sub-clonal cell lines were established from developed tumors. To determine the additional genetic alterations that promote malignancy of NF1-associated tumors, we analyzed the genomic DNA by using the next-generation sequencing. We identified 18 heterozygous variants within the coding regions of 17 genes that were present in the tumor-derived cells. Because phosphorylations of Akt and MEK were increased in the tumor-derived cells, we focused on *BRAP* (c.1109G>C, p.Gly370Ala) and *PTPN11* (c.1508G>T, p.Gly503Val), which are thought to be associated with the Ras pathway. *BRAP* and *PTPN11* genes encode the BRCA1-associated protein and the tyrosine phosphatase SHP-2, respectively. To determine the role of those gene mutations in the malignant progression of NF1-associated tumors, we established three overexpressing cell lines, *BRAP*mut, *PTPN11*mut, and *BRAP*mut/PTPN11mut. We inoculated the parental sNF96.2-GFP cells and those overexpressing cells into nude mice, and found that the *PTPN11*mut cells show a much higher tumorigenic activity than the parental sNF96.2-GFP cells. However, *BRAP*mut and *BRAP*mut/PTPN11mut cells did not form tumors, thus indicating that *BRAP*mut suppresses *PTPN11*mut-mediated tumorigenic activity. Western blot analysis revealed that the phosphorylation of MEK was increased in the *PTPN11*mut cells, but in neither the *BRAP*mut cells nor in the *BRAP*mut/PTPN11mut cells. Our data indicates that this mutation in the *PTPN11* gene promotes the malignant characteristics of NF1-associated tumors, and that this tumor promotion is canceled by the *BRAP* gene mutation. The combination of *PTPN11* gene mutations and the NF1 gene mutations appears to induce a pathological activation of the Ras pathway.

**P1555**

**Board Number: B568**

*Active-site dependent and independent roles of the lipid phosphatase Fig4 in the activation of its opposing kinase, Fab1.*

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PI lipid phosphatases modulate signaling cascades through localized dephosphorylation of specific phosphoinositides (PI) on subcellular membranes. In addition, a number of these phosphatases likely
regulate cellular processes through lipid phosphatase independent functions. For example, it has been suggested that Fig4, a PI 5-phosphatase proposed to dephosphorylate PI3,5P2, also activates its opposing kinase, Fab1. Fig4 is required to form the Fab1-Vac14-Fig4 complex and this complex is required for elevation of PI3,5P2. Here we show that Fig4 dependent activation of Fab1 is largely, but not entirely, independent of Fig4 catalytic function. The catalytically inactive Fig4-C467S mutant supports 75% - 80% of stress induced elevation of PI3,5P2. Notably, when compared with wild-type Fig4, Fig4-C467S dramatically enhances association of Fig4 and Vac14 with Fab1. One possibility is that Fig4 acts as a protein phosphatase, and that tight binding to Fab1 occurs via a substrate trapping effect. Alternatively or in addition, there may be active site dependent conformational rearrangements within the Fab1-Vac14-Fig4 complex. We found that at least two sites on opposite sides of the Fig4 active site modulate Fig4 association with the Fab1-Vac14-Fig4 complex. The Fig4 C-terminal tail was known to contact the Fab1-Vac14-Fig4 complex through direct association with Vac14. Our studies reveal an N-terminal surface on the Fig4 phosphatase domain that influences both a newly identified interaction between the phosphatase domain and the C-terminal tail of Fig4 as well as the interaction of these domains with Vac14. This surface was identified through investigation of amino acids corresponding to human neurodegenerative disease related mutations. Notably, studies of other PI-phosphatases suggest that these enzymes regulate protein partners through both phosphatase-dependent and phosphatase-independent mechanisms. We propose that the ability to behave as active site-dependent conformational switches may be a common property of PI-phosphatases.

P1556
Board Number: B569
Non-Canonical Activation of p38 During Endoplasmic Reticulum Stress.
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In all eukaryotic cells, stress signaling from the endoplasmic reticulum (ER) plays a critical role in cellular adaptation and in the maintenance of homeostasis. Severe disruption of ER function can induce dramatic changes in cellular behavior or even apoptosis (cell suicide). This study evaluates stress signaling from the ER resident signaling kinase known as PKR-like ER kinase (PERK). Prior work in our lab has shown PERK to play an essential role in the activation of apoptosis during ER stress. Downstream of PERK activity is the stress-activated kinase, p38, whose activity is required for apoptosis activation during some forms of ER stress. The mechanism of PERK and p38 interaction is not well understood. Heat Shock Protein 90 (HSP90) is a cytosolic chaperone that binds to inactive p38. Release of p38 from HSP90 is known to enable autoactivation of this kinase. In this study, we used hamster kidney fibroblasts (BHK21 cells) in cell culture to evaluate ER stress signaling. Inhibition of HSP90 (17-AAG treatment) was sufficient to induce phosphorylation of p38 on threonine 180 (T180), a marker of p38 activation. When the timing of this activation was evaluated, 17-AAG was shown to induce p38 within 5 minutes of exposure and to continue for approximately 30 minutes. Tunicamycin, an activator of ER stress, caused an activation of p38 along a similar pattern of 5-30 minutes. To examine if ER stress was sufficient to induce p38 dissociation from HSP90, immunoprecipitation assays were used. Cells overexpressing p38 (FLAG-tagged) were lysed and an anti-FLAG antibody used to pull p38 down from the lysate. HSP90 co-immunoprecipitated with p38, and this association was diminished by either HSP90 inhibition or ER stress activation. Assessment of the dependence of PERK activity upon stimulation of p38 release is ongoing and will be discussed.
P1557
Board Number: B570
The Role of SIK1 in Regulating Apical NHERF1 and Ion Transporters.
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Salt Inducible Kinase (SIK) is a Ser/Thr kinase that is activated (and induced) in response to increases in intracellular Na+ in a number of different tissues (including adrenal and kidney). SIK1 has been shown to play a role in regulating renal proximal tubule Na,KATPase, both transcriptionally and post-transcriptionally (activating basolateral Na,KATPase). In order to study the role of SIK1 in the renal proximal tubule further, we have examined the effect of a SIK1 knockdown on the expression of apical transporters in the renal proximal tubule. The laboratory of Dr. Rebecca Berdeaux (UT Houston) provided us with formalin-fixed kidneys from mice with expressing catalytically inactive SIK1 (KO SIK1), as well as from wildtype (WT) littermates. After paraffin embedding, sectioned kidney slices, we examined the sections by immunofluorescence microscopy to look at the effect of the SIK1 KO on several apical proximal tubule ion transporters (a Na+H+ antiporter, NHE3 and a Na+Pi cotransporter, NPT2a) and a regulatory protein, NHE Regulatory Factor 1 (NHERF1). NHE3 and NPT2a are essential for the reabsorption of Na+ and Pi, respectively, by the renal proximal tubule. NHERF1 plays an important role in regulating the level of NHE3 and NPT2a in the apical membrane. Thus, all of these membrane proteins are essential for Na+ and Pi homeostasis. Our results indicated that the SIK1 KO caused a reduction in the level of apical NHE3 and NPT2a, and this could be explained by a similar reduction in the level of NHERF1. These observations suggest that SIK1 likely plays a previously unknown role in regulating NHERF1 expression.

P1558
Board Number: B571
The A-kinase anchoring protein beta-synemin binds PKC epsilon.
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Synemin is a type IV intermediate filament protein. The two largest isoforms (alpha and beta) are usually co-expressed such as in muscle cells. Earlier we found that in muscle cells alpha-synemin preferentially localizes to the sarcolemma and intercalated disks while beta-synemin is concentrated at the Z-disks. Both isoforms also have an unusually long tail domain. This domain is a site for numerous protein interactions, some unique to each isoform and some common to both. For example, in the past we found that both bind protein kinase A (PKA) and are thus also classified as A-kinase anchoring proteins (AKAPs). AKAPs act to tether PKA to specific subcellular locations to maintain the specificity of the PKA signaling pathway. Excitingly, we have now found evidence that synemin also binds PKC epsilon. Specifically, co-immunoprecipitation studies show that PKC epsilon binds to beta-synemin only upon stimulation of the beta-adrenergic pathway with isoproterenol. Additionally, western analysis using cells expressing either wild type or mutant synemin indicates that, upon loss of beta-synemin anchored PKA, there is a decrease in phosphorylation of PKC substrates, including an ~22 kDa protein. Studies are ongoing to determine the identity of this protein. Based on molecular weight and known PKC epsilon substrates in muscle cells we predict this protein is either troponin I (TnI) or myosin regulatory light chain 2 (RLC2). In support of RLC2, yeast-two hybrid analysis revealed that protein-protein interaction
occurs between beta-synemin and RLC2. Thus, beta-synemin is proposed be a site of cross-talk between PKA and PKC epsilon and also may localize these kinases to the sarcomere near known PKA and PKC epsilon substrates.

**P1559**

**Board Number: B572**

Distinct mechanism of spleen tyrosine kinase activation by pITAM and Integrin cytoplasmic domain.

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Spleen tyrosine kinase (Syk) is involved in activation and development of hematopoietic cells, osteoblast differentiation, and, more generally in cellular adhesion [1]. Syk activation by genomic rearrangement is linked to certain T-cell lymphomas and Syk inhibitors have been shown to prolong survival of patients with B cell lineage malignancies [2]. Normal Syk activation is mediated either by the interaction with double-phosphorylated Immunoreceptor Tyrosine-based Activation Motif (pITAM) which induces structural rearrangements in Syk structure, or by the phosphorylation of specific tyrosine residues along the sequence [3]. Syk is activated downstream of integrin function and this is at least partially independent on pITAM. However it is not known whether integrins and pITAM directly activate Syk with the same mechanism [4]. Here we use purified Syk protein and fluorescence-based enzyme assay to study whether interaction of integrin beta3 cytoplasmic domain with the regulatory domain of Syk causes similar changes in enzyme activity as pITAM peptides. We observed no direct activation of Syk by soluble integrin peptide. Integrin did not compete with pITAM-induced activation, even though at high concentration integrin peptide was found to compete with the substrate. However, clustered integrin peptides were able to induce Syk activation, presumably via transphosphorylation mechanism. We observed a similar behaviour of integrin with Syk mutant (Y348F/Y352F), indicating that the multiple phosphorylation sites can be involved in integrin-induced activation. These studies provide valuable information about the interplay between cell adhesion and immune activation pathways.


**P1560**

**Board Number: B573**

Untangling kinase-based signalling interactions in endothelial cell migration and angiogenesis.

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Cell migration is important for embryogenesis, wound healing, angiogenesis and cancer metastasis, but how cells control their migration for above functions has remained elusive. Recent large-scale genetic
explorations have identified important signalling molecules that control cell migration, but how these signalling pathways interact with each other for proper cell motility is also unknown. We therefore started a two-hit genetic-plus-pharmacological screen to identify the significance of signalling interactions in migrating human umbilical vein endothelial cells (HUVEC). The screen identified two kinases, protein kinase C eta (PRKCH) and STE20-like kinase (SLK), both of which interact with Rho-associated kinase (ROCK) during cell migration. Interestingly, ROCK inhibition antagonized the effect of PRKCH knockdown but synergistically enhanced the effect of SLK knockdown during HUVEC migration. Suppressing the expression of PRKCH or SLK also affected the capability of HUVECs to form branching tubes in the matrigel, indicating the importance of both kinases in angiogenesis. We are currently working on the molecular mechanisms how these kinases interact with ROCK. Since ROCK inhibition has been shown effective to relieve various retinopathies, our work will improve the current treatment of angiogenesis-related eye diseases.

**Signaling Scaffolds and Microdomains**

**P1561**  
**Board Number: B574**  
**Eisosome-associated protein Slm1 regulates two downstream pathways for heat stress adaptation.**  
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*Saccharomyces cerevisiae* has three distinct plasma membrane domains that are not overlapped. One of them is called Membrane Compartment of Can1 (MCC) that corresponds to inner membrane furrows termed eisosome. Eisosome contains various membrane associated-proteins, transporters and tetraspanning membrane proteins, which are considered to contribute to normal eisosome structure and function. One of the eisosome-associated protein, Slm1 localizes to MCC/eisosome at the steady state. However, upon exposure to plasma membrane (PM) stress, such as high temperature stress or perturbation of sphingolipid metabolism, Slm1 translocates to another membrane domain called Membrane Compartment of TORC2 (MCT), where Slm1 recruits AGC kinase Ypk1, then Ypk1 is activated by TORC2. Activated Ypk1 regulates various downstream pathways, such as sphingolipid metabolism and actin organization for PM stress adaptation. In this study, we investigated how Slm1 regulates the downstream pathways during PM stress.

We found that overexpression of GFP-tagged Slm1 (GFP-Slm1 OP) causes high temperature sensitivity and hyper-phosphorylation of downstream Cell Wall Integrity (CWI) MAP kinase, Slt2. Intriguingly, deletion of SLT2 or Rho1 GEF, ROM2 in GFP-Slm1 OP cells exacerbated high temperature sensitivity, suggesting that activation of CWI pathway is indispensable for heat stress adaptation rather than the cause of high temperature sensitivity in GFP-Slm1 OP cells. Furthermore, localization of GFP-Slm1 was still observed in MCC even upon PM stress, indicating that Slm1 plays some important roles for stress adaptation in MCC that have not been well characterized. To explore it, we analyzed genetic interaction between GFP- SLM1 OP and MCC-related genes and discovered strong genetic interaction with *PIL1* that is a major MCC component. GFP-Slm1 OP in *PIL1* deletion mutant caused abnormal recycling of membrane proteins, such as v-SNARE, Snc1 and actin repolarization. We also found simultaneous deletion of both *PIL1* and *SLT2* causes PM integrity (PMI) defect upon heat stress. Taken together, we propose that Slm1 spatiotemporally regulates both PMI and CWI pathways by changing its distribution in membrane domain for heat stress adaptation.
P1562
Board Number: B575
Multi-protein Skb1 nodes control fission yeast mitotic entry.
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Protein clusters provide input into signal transduction pathways in a variety of cell types. The fission yeast Schizosaccharomyces pombe regulates mitotic entry through distinct protein clusters at the plasma membrane known as “nodes”. Protein kinases Cdr2, Cdr1 and Wee1 form nodes at the medial cortex of the plasma membrane. Wee1 inhibits CDK1 activity while Cdr2 and Cdr1 inhibit Wee1 to allow entry into mitosis. Past studies have shown type II arginine methyltransferase Skb1 forms node structures at non-growing cell regions distinct from Cdr2 nodes. Genetic epistasis revealed that Skb1 functions as a mitotic inhibitor through the Wee1 pathway. Elimination of Skb1 nodes results in altered cell size at division, making these structures crucial for correct cell cycle timing.

Similar to Cdr2 nodes, Skb1 nodes contain multiple proteins to generate cortical structures. Through proteomics and candidate-based screens, we identified the peripheral membrane-binding protein Sfl1 and the Syntaxin-like t-SNARE protein Psy1 as additional components of Skb1 nodes. Psy1 is essential for vegetative growth and exocytosis in fission yeast. The function of Psy1 at these cortical nodes is unknown. We are currently investigating the physical interactions and functional consequences of assembling these three proteins into large, megadalton-sized structures.

P1563
Board Number: B576
Single Molecule Study of Wnt Signaling Pathway Activation.
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30 years of molecular biology study on Wnt signaling pathway has yield ample knowledge of the regulation details on the molecular level. Due to the complex nature of Wnt signaling pathway, there are still open questions on how receptors and scaffold proteins orchestra to activate the pathway. In this work, we studied the membrane dynamical behavior of several important scaffold proteins in Wnt signaling pathway like Axin1 and Dishevelled2 with both biochemical and single molecule approaches. In addition to quantification of the stoichiometry of Axin1 and Dishevelled2, our results also show interesting dynamics information of how the scaffold proteins and receptors work together responding to Wnt treatment. Based on the quantitative information, we improved the existing model of canonical Wnt pathway activation.
P1564
Board Number: B577
APC regulates Wnt signaling by inhibiting a constitutive clathrin-mediated activation pathway.
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The Wnt pathway is a conserved pathway that controls many developmental processes and is mutated in many human diseases (e.g., cancer). In the absence of Wnt ligand, cytoplasmic β-catenin is phosphorylated and targeted for degradation by a complex assembled by the scaffold protein Axin, and the tumor suppressor adenomatous polyposis coli (APC). When a Wnt ligand binds to the co-receptors Frizzled (Fz) and the low-density lipoprotein receptor-related protein 6 (LRP6), a receptor complex is assembled promoting LRP6 phosphorylation. Phosphorylated LRP6 binds to the β-catenin degradation complex in a process mediated by Dishevelled (Dvl), assembling the Wnt signalosome. The signalosome is internalized which blocks β-catenin degradation, leading to the activation of a Wnt-transcription program. Mutations in the APC gene leading to constitutive Wnt pathway activation occur in over 80% of human colorectal cancers (CRC). Our lab developed a monoclonal antibody (mAb7E5) that targets the co-receptor LRP6 and inhibits Wnt signaling in vitro and in vivo. Significantly, mAb7E5 inhibits Wnt signaling in APC mutant CRC cell lines. Similarly, LRP6 knockdown inhibits Wnt signaling in APC mutant cells. Consistent with these results, activation of the Wnt pathway by knockdown of APC in human cells can be inhibited by simultaneously knocking down LRP6, or by blocking Fz or Dvl function. These results indicate that loss of APC leads to constitutive ligand-independent Wnt signalosome formation. This mechanism is conserved in Drosophila, where intestinal stem cell proliferation by loss of APC is inhibited by depletion of the receptor Arrow/LRP6 or Dvl. Using an inducible degradable form of APC, we demonstrate that APC loss is rapidly followed by Wnt receptor activation and increased β-catenin levels. Finally, clathrin inhibition blocks activation of the Wnt pathway in APC mutant cells and in organoids derived from tumors obtained from APC mutant mice. Consistent with a role for clathrin in regulating Wnt signaling upon loss of APC, we show that APC co-immunoprecipitates with clathrin. These results suggest a model in which Wnt receptor activation can occur via a constitutive, ligand independent clathrin-mediated mechanism that is normally inhibited by APC.

P1565
Board Number: B578
Molecular delineation of MST4-elicited ACAP4 phosphorylation during histamine-stimulated gastric acid secretion.
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The digestion in the stomach depends on acidification of the lumen. Histamine-elicited acid secretion is triggered by activation of PKA cascade, which ultimately results in the insertion of gastric H,K-ATPases into the apical plasma membranes of parietal cells. Our recent study revealed the functional role of PKA-MST4-ezrin signaling axis in histamine-elicited acid secretion (Jiang et al, 2015. J. Biol. Chem.). However, it remains uncharacterized how PKA-MST4-ezrin signaling axis operates the insertion of H,K-ATPases into the apical plasma membranes of gastric parietal cells. Here we show MST4 phosphorylates ACAP4, an ARF6 GTPase-activating protein (Ding et al., 2010 J. Biol. Chem.) at Thr545. Histamine stimulation
activates MST4 and promotes MST4 interaction with ACAP4. ACAP4 physically interacts with MST4 and is a cognate substrate of MST4 during parietal cell activation. The phosphorylation site of ACAP4 by MST4 was mapped to Thr545 by mass spectrometric analyses. Importantly, phosphorylation of Thr545 is essential for acid secretion in parietal cells because either suppression of ACAP4 or over-expression of non-phosphorylatable ACAP4 prevents the apical membrane reorganization and proton pump translocation elicited by histamine stimulation. In addition, persistent over-expression of MST4 phosphorylation-deficient ACAP4 results in an inhibition of gastric acid secretion and a blockage of tubulovesicle fusion to the apical membranes. Significantly, phosphorylation of Thr545 enables ACAP4 to interact with ezrin. Given the location of Thr545 between the GAP domain and the first ankyrin repeat, we reason that MST4 phosphorylation elicits a conformational change which enables ezrin-ACAP4 interaction. Taken together, these results define a novel molecular mechanism linking PKA-MST4-ACAP4 signaling cascade to polarized acid secretion in gastric parietal cells. Current effort is directed to understand the mechanisms underlying homotypic tubulovesicle fusion during histamine-elicited apical trafficking.

P1566

Board Number: B579

The mAKAP complex orchestrates the dephosphorylation of MEF2D in muscle cells to stimulate its activity.

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Myocyte Enhancer Factor 2D (MEF2D) is a transcription factor required for the development of pathological remodeling as well as the induction of cardiac hypertrophy by pressure overload. However, little is known about how this transcription factor is regulated. Previous work showed that the scaffolding protein mAKAP (muscle A-Kinase Anchoring Protein) orchestrated the calcineurin (CaN)-mediated stimulation of MEF2D in skeletal muscle cells. CaN and mAKAP immunoprecipitate with MEF2D in skeletal muscle cells and primary neonatal cardiac myocytes (RNV); MEF2D and CaN association was dependent on mAKAP expression. Furthermore, expression of anchoring disrupting peptides, which displaced CaN binding to mAKAP, blocked the induction of MEF2D gene transcription in skeletal myoblasts as well as the induction of cardiac hypertrophy in RNV, demonstrating the importance of the mAKAP complex for stimulation of MEF2D activity. The goal of this study was to elucidate the molecular mechanism that underlies the regulation of MEF2D activity by mAKAP-bound CaN. We found that stimulation of C2C12 cells with differentiation media induced the binding of active CaN to mAKAP, resulting in the dephosphorylation of MEF2D at Serine 444, which led to its desumoylation. Our current focus is on determining if MEF2D post-transcriptional modifications allows for increased gene transcription. C2C12 skeletal muscle cells will be stimulated with differentiation media, luciferase assay will be used to measure MEF2D transcriptional activity and we will also look at the expression of MEF2D target genes Nppa (atrial natriuretic factor), Acta1 (skeletal muscle α-actin), and Actc1 (cardiac muscle α-actin). Overall, our data suggests that the mAKAP complex focuses the actions of CaN onto MEF2D, to promote regulation of its post-transcriptional modifications, which we propose will allow for increased transcriptional activity in both stimulated skeletal muscle myoblasts and stressed cardiac myocytes. Understanding the regulation and activity of the transcription factor MEF2D and its associated proteins is a crucial step towards the ability to block the development of cardiac hypertrophy.
P1567
Board Number: B580
Macropinosomes Coordinate the Activation of PI3Kβ by Gβγ and Rac.
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Phosphoinositide 3-kinases (PI3Ks) are regulated by a diverse range of upstream activators, including receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs), and small GTPases from the Ras, Rho and Rab families. For the Class IA PI 3-kinase PI3Kβ, which regulates thrombosis, spermatogenesis, and tumorigenesis in vivo, two mechanisms for GPCR-mediated regulation have been described: direct binding of Gβγ dimers to the C2-helical domain linker of p110β, and Dock180/Elmo1-mediated activation of Rac1, which binds to the Ras-Binding Domain (RBD) of p110β. We now show that the integration of these dual pathways is unexpectedly complex. Gβγ and constitutively active Rac1 (CA-Rac1) additively activated PI3Kβ in breast cancer cells. GPCR-mediated activation of Akt was preserved in cells expressing a p110β RBD mutant that is deficient for Rac1 binding. However, CA-Rac1-mediated activation of PI3Kβ was blocked in cells expressing a PI3Kβ mutant that cannot bind Gβγ, and Rac1 activation of wild type PI3Kβ was inhibited by reagents that block endogenous Gβγ signaling. Moreover, Rac1 still activated PI3Kβ in cells expressing the p110β RBD mutant. These data suggest an alternative mechanism for CA-Rac1 activation of PI3Kβ that is independent of direct Rac-PI3Kβ binding. Notably, cells expressing CA-Rac1 exhibited a robust induction of macropinocytosis, which has been previously suggested to amplify signaling to PI3K by GPCRs. Consistent with this hypothesis, suppression of macropinocytosis by EIPA blocked Rac-mediated Akt activation in cells expressing wild type or Rac1-deficient p1110β. Our data suggest that Rac-stimulated macropinosomes serve as signaling platforms that enhances Gβγ coupling to PI3Kβ.

P1568
Board Number: B581
A novel YAP-binding protein Furry controls localization and activity of YAP.
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The Hippo pathway is known as a signaling pathway that restricts cell proliferation and controls organ size. MST1/2 kinases bind to their adaptor protein Sav1 and phosphorylate and activate downstream kinases, NDR1/2 and LAT51/2. LAT51/2 kinases phosphorylate transcriptional co-activators, YAP and TAZ, to promote their cytoplasmic retention or degradation, resulting in inhibition of their interaction with TEAD and TEAD-mediated transcriptional activation, and thereby leading to suppression of cell proliferation. However, the mechanisms regulating YAP phosphorylation and inactivation are not fully understood. Furry (Fry) is an evolutionarily conserved protein in eukaryotes. Fry genetically and physically interacts with NDR. Both Fry and NDR are implicated in the control of cell morphogenesis, polarization, cell cycle progression, and neurite extension. Previous studies showed that Fry plays a crucial role in mitosis by regulating chromosome alignment and mitotic spindle formation. Although a recent study revealed that NDR kinases are also involved in YAP inactivation, the role of Fry in the regulation of YAP activity is still unknown. In this study, we demonstrated that Fry is involved in YAP inactivation. To examine the role of Fry in YAP activity, we generated Fry-knockout (KO) 293A cells using
a CRISPR/Cas9 system. We observed that Fry-KO cells exhibited the accumulation of YAP into the nucleus and the elevated activity of YAP, as compared to wild-type cells. Furthermore, we showed that Fry interacts with several Hippo components, YAP, SAV1, and LATS1/2. We also showed that the ectopic expression of full-length Fry, but not its fragments, rescued the cytoplasmic localization of YAP in Fry-KO cells. We next analyzed the effect of Fry KO on actin disassembly-induced YAP inactivation, because recent studies have shown that actin disassembly induces YAP translocation to the cytoplasm in Hippo-dependent and Hippo-independent manners. We observed that YAP preferentially localized in the cytoplasm in both control and Fry KO cells upon treatment with actin-disassembling drugs, suggesting that Fry is not involved in actin disassembly-mediated YAP inactivation. Since previous studies have shown that Fry bind to microtubules, we are investigating whether Fry could regulate YAP localization through the microtubule-dependent mechanism. Taken together, these results suggest that Fry plays a crucial role in YAP inactivation through binding to YAP and scaffolding the components of the Hippo pathway.

P1569
Board Number: B582
A novel splice variant of Sab (SH3BP5) alters mitochondrial physiology.
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Mitochondria-cell communication is required for optimal cell function and to monitor mitochondrial health. Ultimately, mitochondria-cell circuits transduce signals that influence biological outcomes. However, the precise mechanisms responsible for mitochondria-cell communication are not well understood. Scaffold proteins on the mitochondrial outer membrane (MOM) are crucial hubs for mitochondria-cell communication, where the relative abundance of discrete scaffold proteins will influence the magnitude of distinct signal transduction events and determine the local outcome. Furthermore, variation in scaffold proteins may diversify signaling modules or even polarize mitochondria-cell communication towards specific biological outcomes. The MOM scaffold protein Sab facilitates mitogen-activated protein kinase (MAPK) signaling on mitochondria which affects organelle function and cell viability. Our previous work demonstrates that Sab levels alter cellular sensitivity to chemotherapy agents in gynecological cancer cells. While screening glioma cells for Sab protein concentrations, we discovered a unique variant of Sab with an N-terminal truncation that deletes the original mitochondrial localization sequence and a coiled-coil motif. Thus, our current goal is to characterize the Sab variant, distinguish the function of the shortened Sab variant from that of full-length Sab, and determine the impact of variant-mediated events on mitochondrial function. We cloned Sab-variant expressing human SF268 cells and derived clones that expressed full-length Sab alone and those that expressed both full-length Sab and the truncated variant. We next characterized the cell morphology, growth rate, respiration and drug responsiveness. We found that the shortened Sab variant localizes to mitochondria, which may be attributed to an N-terminal stretch of cationic amino acids. Cells that express the variant have an increased growth rate compared to cells with only full-length Sab. Truncated Sab expressing cells display a large, round morphology, while cells without the variant are fibroblast-like. Expression of the variant promotes a metabolic shift from respiration to a more glycolytic phenotype. The truncated Sab variant also affects stress responsiveness, as cells without the variant are more sensitive to genotoxic stress, while cells with the variant are more sensitive to mitochondrial stress. This may suggest that alternative splicing of Sab transcripts may be a means to regulate cellular responsiveness to mitochondrial health. Our work illustrates that Sab variants may have
distinct effects on mitochondrial function and, altering the structures and concentrations of MOM scaffold proteins is a novel approach to manipulate organelle function and disease pathophysiology.

P1570
Board Number: B583
Alterations in outer mitochondrial signaling promote organelle and neuronal dysfunction.
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Mitochondrial outer membranes (MOMs) are critical interfaces for mitochondria-cell communication. MOM signaling cascades influence respiratory efficiency, mitochondrial dynamics, and cellular viability. Changing the concentrations of MOM signaling components alters organelle physiology. Specifically, protein kinase A (PKA) signaling on the MOM scaffold A-kinase anchoring protein 1 (AKAP-1) promotes complex I assembly and mitochondrial fusion. Conversely, mitochondrial c-Jun N-terminal kinase (JNK) signaling on the scaffold Sab inhibits complex I, promotes ROS generation, and triggers apoptosis. The magnitude and outcomes of MOM signaling correspond to the local abundance signaling components. We found that advanced age and prolonged mitochondrial stress increase Sab concentrations in neurons; the elevation in Sab occurred concurrently with a decrease in AKAP1. The shift in MOM scaffold proteins resulted in an escalation in mitochondrial JNK concentrations and a reduction in PKA activity. To reproduce the scaffold shift, we overexpressed Sab in neurons, which reduced AKAP1 levels and fragmented the mitochondrial network. Affinity purification of epitope-tagged Sab revealed direct interactions with mitochondrial fission machinery. Inhibition of JNK/Sab signaling prevented fission and mitigated changes in organelle morphology. Additionally, inhibition of Sab-facilitated signaling prevented JNK-mediated phosphorylation and degradation of Mfn2. These studies suggest that Sab-mediated signaling is crucial for segregating dysfunctional or depolarized organelles within neurons. Moreover, enhanced Sab expression resulted in impaired neuronal activity and the accumulation of dysfunctional mitochondria at synapses. Disrupting Sab-mediated events restored neurotransmission. To determine if targeting perturbations in MOM signaling improves neurological function in diseases affected by mitochondrial stress, we analyzed mouse models of Parkinson's and mitochondrial disease. We report that Sab levels were increased and AKAP-1 levels were decreased in specific areas of the brain and reducing the Sab/AKAP-1 ratio improved performance in behavioral studies. We propose that altering mitochondria-cell communication represents a unique approach to limit mitochondrial stress and restore neuronal activity in neurological disease.

P1571
Board Number: BS84
Signalling via membrane receptors generate functional nanodomains at the plasma membrane of living cells.
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The plasma membrane (PM) of the cell is a dynamic structure that exhibits lateral heterogeneities at various length and time scales [1]. The Outer-leaflet tethered GPI-anchored proteins (GPI-APs) are one of several such components that exhibit a non-random distribution on the PM [2,3,4]. In a resting cell
membrane, about ~40% of GPI-APs form transient (<1 sec) nanoclusters driven by the dynamics of the contractile actin machinery operating close to the inner leaflet of the plasma membrane via a transbilayer acyl-chain-mediated linkage mechanism [3,5,6,7]. While an understanding of the physical principles behind the active mechanics of actin filaments and myosin that drives this behavior is emerging [8], the molecular machinery behind the generation of this dynamic pool of actin and its mode of coupling to inner-leaflet lipids is lacking. Here we show that localized activation of the β1-integrin receptor generates localized nanoclusters of GPI-APs. Surface-tethered and soluble RGD-containing ligands engage the integrin receptor and activate FAK-src kinases and the RhoGTPase, RhoA. RhoA in-turn triggers actin-nucleation via specific formins and activates myosin-II to generate contractile actin filaments. In parallel, integrin signaling results in the talin-mediated opening of vinculin, which potentiates the coupling of actin to the negatively charged lipids at the inner leaflet. This provides a regulated machinery required to create as well as couple dynamic actin activity at the inner leaflet to the organization of outer leaflet GPI-APs. Upon integrin activation, ~80% of GPI-APs from nanoclusters in its vicinity [5]. Consistent with a functional relevance of this machinery, mutant cells that are unable to generate GPI-AP nanodomains at the cell surface, despite their normal integrin activation status, exhibit severe defects in integrin-mediated cell spreading behavior.


P1572
Board Number: B585
Live cell super-resolution microscopy measures membrane-driven sorting of B cell receptor signaling partners.
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B cells are acutely sensitive to stimulation through antigen-induced clustering of the B cell receptor (BCR). Transduction of the activation signal, which is key for the adaptive immune response, involves rearrangement of membrane-resident signaling molecules relative to clustered BCR. The details and dynamics of this re-organization have been difficult to directly observe due to the small dimensions of signaling complexes and the potentially weak or transient interactions that drive their formation. To overcome these obstacles, we have utilized live-cell super-resolution localization microscopy to simultaneously image BCR and other membrane species during antigen-induced cell activation. The sensitivity of this quantitative technique has allowed us to measure the effects of subtle forces, such as those originating from lipid compositional heterogeneity, that influence the interactions of BCR with its signaling partners. We found that minimal membrane-anchored probes that partition into ordered domains of model membranes co-localize with BCR clusters, while anchors that partition into disordered domains are excluded from clusters, suggesting that BCR clusters can nucleate local enrichment of a specific lipid composition. We are exploring the effects of membrane-driven sorting on recruitment of BCR signaling proteins to receptor clusters and how it contributes to their specific role in signal
transduction. In particular, we have found that the palmitoylated transmembrane adapter proteins LAT2, LIME, and PAG, which serve distinct adapter functions in BCR signaling, are differentially recruited to BCR clusters. This differential recruitment is due to a combination of interactions between the transmembrane domains of these proteins with the membrane and to specific protein-protein interactions. Our results suggest that the membrane influences local sorting of downstream signaling proteins around BCR clusters and provide insight on the forces driving regulation of BCR signaling.

P1573
Board Number: B586
Protein sorting by phase-like domains supports emergent signaling function in B cell plasma membranes.
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Diverse cellular signaling events, including B cell receptor (BCR) activation, are hypothesized to be facilitated by domains enriched in specific plasma membrane lipids and proteins that resemble liquid-ordered phase separated domains in model membranes. However, this concept remains controversial due to the difficulty in directly observing these domains in intact cells. Here, we visualize ordered and disordered phase-like domains in intact B cell membranes using super-resolution fluorescence localization microscopy, demonstrate that clustered BCR resides within ordered phase-like domains capable of sorting key regulators of BCR activation, establish that ordered domains are local environments that favor tyrosine phosphorylation, and present a minimal and predictive model where clustering receptors leads to their collective activation by stabilizing an extended ordered domain. These results provide evidence for the role of membrane domains in BCR signaling as well as a plausible mechanism of BCR activation via receptor clustering. More generally, these studies demonstrate that lipid mediated forces can bias biochemical networks in ways that may broadly impact signal transduction.

P1574
Board Number: B587
The activation state of SRC family kinases in late endosomes determines cellular responses to Receptor Tyrosine Kinase signaling in neuroblastoma.
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Receptor Tyrosine Kinases (RTKs), a family of cell-surface receptors, activate intracellular signaling cascades that determine cellular responses such as proliferation, differentiation, migration, and apoptosis. Dysregulation of RTK signaling is known to drive the initiation and progression of several cancers and diseases. Therefore, strict spatial and temporal regulation of pathways activated by RTKs is necessary to determine appropriate cellular responses, but how this regulation is achieved remains unclear. SRC Family Kinase (SFK) signaling pathways are one of the main pathways activated by RTKs. SFKs are phosphorylated by RTKs at the plasma membrane and subsequently trafficked into endosomes and lipid rafts, where they continue to signal from endosomal surfaces. The scaffold protein PAG1 (phosphoprotein associated with glycosphingolipid enriched microdomains 1) normally regulates SFK activation in endosomes and lipid rafts by mediating an interaction with C-terminal SRC Kinase (CSK), which deactivates SFKs. Our previous research showed that the localization pattern of SFKs in different membrane fractions distinguishes signaling from different RTKs. Therefore, we hypothesized that

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disrupting SFK activation and localization within lipid rafts and endosome populations would influence cell fate decisions initiated by different RTKs. To disrupt SFK activation and trafficking, we generated a neuroblastoma cell line expressing a PAG1 fragment that lacks the membrane spanning domain (PAG1TM-). Cells expressing PAG1TM- exhibited higher global amounts of activated SFKs, higher amounts of activated SFKs in late endosome populations, and also sequestered more active SFKs in the lumen of multivesicular bodies. The sustained activation of SFKs in late endosomal populations mimics the signaling paradigms of the oncogenic receptors ALK and KIT. Accordingly, PAG1TM- cells exhibited increased proliferation, altered patterns of migration, and increased anchorage-independent growth in soft agar. PAG1TM- cells also failed to respond to signaling from the pro-differentiation receptors TRKA and RET. These results provide support for the role of PAG1 as a tumor suppressor in neuroblastoma, and suggest that localization of active SFKs in distinct endosome populations is a major method of regulating cell fate decisions triggered by RTK activation.

P1575
Board Number: B588
Exclusion of Notch from Mechanically Active Cellular Junctions.
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Notch, an essential cell communication receptor, relies on contact of ligand and receptor across cells, mechanical force, and sequential steps of proteolytic cleavage by ADAM10 and -secretase carried out within the membrane for receptor activation. As such, the spatial organization and presentation of Notch, ligand, and proteases at the membrane is a key factor in regulating Notch activation. Despite its importance, the spatial distribution of Notch at the membrane remains understudied. Here we present work mapping Notch’s cell-surface distribution and organization, particularly relative to cellular adhesions; sites of cell contact, mechanical tension, and -secretase recruitment. Surprisingly, we find Notch to be excluded from cellular adhesion structures, including E-cadherin cell-cell junctions and focal adhesions and spatially segregated from presenilin 1 (PS1), the key catalytic subunit of -secretase. We explore the mechanism of Notch’s exclusion and find it to be dependent on the Negative Regulatory Region (NRR) and Notch activation. This study reveal’s Notch’s organization at the cell surface, uncovering restrictions on Notch’s distribution and segregation of inactive Notch from its protease, implicating cellular adhesions in the regulation of Notch signaling.

P1576
Board Number: B589
TNF-α priming regulates CD82 expression of Bone Marrow Homing of Hematopoietic Stem and Progenitor cells.
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Hematopoietic stem/progenitor cell (HSPC) transplantation is a primary clinical therapy for the treatment of blood cancers, immunodeficiency disorders, and high-dose chemotherapy patients, who often develop bone marrow failure. Currently, the poor homing and engraftment of transplanted HSPCs results in reduced efficacy, which often leads to patients requiring multiple rounds of transplantation. For effective HSPC transplantation, stem cells must traffic through the blood and home to a specific microenvironment or “niche”, which is composed of soluble factors, matrix proteins, and supportive
cells. HSPC adhesion to, and signaling within the niche provide support and instructional cues to balance stem cell properties such as differentiation and self-renewal. Despite the clinical successes of HSPC transplantation, the molecular mechanisms that regulate HSPC trafficking to the bone marrow and the subsequent repopulation of the hematopoietic system remain unclear. Tetraspanins are molecular scaffolds that distribute proteins into highly organized microdomains consisting of adhesion, signaling, and adaptor proteins. As such, tetraspanins function as molecular facilitators or organizers for membrane proteins, thereby modulating cell adhesion and signal transduction. Previous work from our laboratory identified the tetraspanin, CD82, as a modulator of HSPC adhesion and bone marrow homing, where CD82 blocking antibodies and CD82 microdomain disruption inhibited niche cell adhesion and homing. Therefore, we hypothesize that the upregulation of CD82 expression may promote bone marrow homing, engraftment and improved transplantation. In the current study, we describe the use of tumor necrosis factor alpha (TNF-alpha), a cytokine produced by various tissues during inflammation, to modulate CD82 expression on the surface of HSPCs. A dose response analysis of HSPC TNF-alpha priming identified a significant increase in the surface expression of the regulatory tetraspanin, CD82. Real time-PCR analysis suggests that CD82 expression is upregulated transcriptionally along with nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), following acute treatment with TNF-alpha. In addition, our preliminary data suggest that TNF-alpha priming of HSPCs promotes the bone marrow homing of HSPCs in preclinical animal models. Future studies will evaluate the long-term engraftment potential of HSPCs primed with TNF-alpha and will further explore the mechanistic role of CD82 in the process. Taken together, we anticipate that these studies have the potential to offer new molecular targets and treatments to improve HSPC transplantation.

P1577
Board Number: B590
A mass spectrometry-based screen reveals FRMD8 as a novel regulator of iRhom/ADAM17-dependent inflammatory and growth factor signalling.
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The metalloprotease ADAM17 cleaves and releases membrane-tether forms of signalling molecules from the plasma membrane, such as the inflammatory cytokine tumour necrosis factor (TNF) and several ligands of epidermal growth factor receptor (EGFR). Thus, ADAM17 activity has to be tightly controlled. The multi-pass membrane proteins iRhom1 and iRhom2 are members of the rhomboid-like superfamily that control ADAM17 activity by trafficking ADAM17 from the endoplasmic reticulum (ER) to the Golgi apparatus, the site at which ADAM17’s inhibitory prodomain is removed. Furthermore, iRhoms also control the activity and substrate-specificity of ADAM17 on the cell surface. iRhoms ultimately regulate the release of ADAM17 substrates, and therefore TNF and most EGFR signalling. However, it remains unclear whether iRhoms require additional factors to exert their function as ADAM17 regulators. We performed a mass spectrometry-based screen for new binding partners of human iRhom2 and identified the largely uncharacterised FERM domain-containing protein 8 (FRMD8) as a novel iRhom1 and iRhom2 interactor. We mapped the binding site of FRMD8 within the cytosolic N-terminus of iRhom2. The loss of FRMD8 drastically affects the function of iRhom2 as regulators of ADAM17: knockdown and knockout of the FRMD8 led to almost undetectable levels of mature ADAM17 and significantly impaired the release of ADAM17 substrates. Using human induced pluripotent stem cell (hiPSC)-derived macrophages, we showed that FRMD8 also regulates the release of TNF in human macrophages. This shows that FRMD8 is a novel, specific regulator of the iRhom/ADAM17 pathway of
physiological relevance. To address the role of FRMD8 in vivo, we generated the first FRMD8 knockout mice and are currently analysing their phenotype.

P1578
Board Number: B591
Characterization of IQGAP1 binding to phosphoinositides and PIP kinases.
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IQ motif-containing GTPase-activating protein 1 (IQGAP1) is a multidomain protein that scaffolds multiple signalling pathways by binding to its many partners. Recent studies have suggested that IQGAP1 plays a major role in the generation of the lipid messenger PtdIns(3,4,5)P3 by scaffolding the phosphoinositide kinases PIPKis and PI(4)KII class I phosphatidylinositol-3-OH kinase (PI(3)K). It is believed to do so by allowing the PtdIns(4,5)P2 generated from phosphatidylinositol-4-phosphate by PIPKis to be selectively used by PI(3)K for PtdIns(3,4,5)P3 generation by scaffolding the kinases involved very closely. Here, we investigate the binding of IQGAP1 to these proteins and phosphoinositides both in-vitro and in cells using fluorescence techniques like FLIM and FCS. We found that IQGAP1 associates with PIP kinases and PI3K in cells. We also find evidence of IQGAP1 binding weakly to PtdIns(4,5)P2. Our results indicate that IQGAP1 plays a role in modulating the synthesis of PtdIns(4,5)P2 and PtdIns(3,4,5)P3 by scaffolding the kinases involved, as well as the phosphoinositides themselves.

P1579
Board Number: B592
The Tetraspanin CD82 Regulates Hematopoietic Stem Cell Activation within the Bone Marrow Microenvironment.
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Hematopoietic stem and progenitor cells (HSPCs) are responsible for the continued production of new blood and immune cells. The interactions between HSPCs and the surrounding bone marrow microenvironment are critical for the long-term maintenance and protection of HSPCs from biological stresses that lead to activation. Previous analysis of the contact site between HSPCs and bone marrow components identified the enrichment of the tetraspanin CD82. CD82 is a membrane scaffold protein highly expressed on the surface of HSPCs, which we have shown regulates HSPC adhesion and homing to the bone marrow. In this study, we go on to analyze the contribution of CD82 to the maintenance of HSPC quiescence and activation, which are tightly regulated by interactions with the bone marrow. More specifically, we are testing the hypothesis that CD82-mediated bone marrow interactions promote HSPC quiescence and prevent cell cycle activation. Utilizing the CD82 knock out (CD82KO) mouse, we assessed the cell cycle status of HSPCs within the bone marrow. Flow cytometry analysis of Hoechst and Ki-67 stained HSPCs identified a significant decrease in the G0 population of cells and a subsequent increase in the G1 population of CD82KO HSPCs. Additionally, we measured HSPC cell cycle status in vivo with BrdU, which labels actively cycling cells. Using flow cytometry to quantify the BrdU incorporation into HSPCs isolated from the bone marrow, we find a significant increase of BrdU within the CD82KO HSPCs when compared to control animals. Taken together, these data implicate CD82 as a critical regulator of HSPC quiescence. Next, we evaluated the role of CD82 in HSPC activation. 5-fluorouracil (5-FU) treatment ablates cycling cells within the peripheral blood resulting in activation of dormant HSPCs within the bone marrow for immune cell repopulation. A two-week analysis of control and CD82KO mice
treated with 5-FU identified a survival advantage of the CD82KO animals. In addition, we found an increase in immune cells within the blood and bone marrow of CD82KO mice compared to control animals, suggesting that CD82 contributes to HSPC activation. More recently, we completed RNA-sequencing analysis of isolated HSPCs from CD82KO and control animals to identify differentially expressed genes. Future studies will be directed toward Gene Set Enrichment analysis of cell cycle regulated genes.

P1580

Board Number: B593

Poly( L -Lactic Acid)/Gelatin Fibrous Scaffold Loaded with Simvastatin/Beta-Cyclodextrin-Modified Hydroxyapatite Inclusion Complex for Bone Tissue Regeneration.

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Recently, the application of nanostructured materials in the field of tissue engineering has garnered attention to mediate treatment and regeneration of bone defects. In this study, poly( L -lactic acid) (PLLA)/gelatin (PG) fibrous scaffolds are fabricated and β-cyclodextrin (BCD) grafted nanohydroxyapatite (HAp) is coated onto the fibrous scaffold surface via an interaction between BCD and adamantane. Simvastatin (SIM), which is known to promote osteoblast viability and differentiation, is loaded into the remaining BCD. The specimen morphologies are characterized by scanning electron microscopy. The release profile of SIM from the drug loaded scaffold is also evaluated. In vitro proliferation and osteogenic differentiation of human adipose derived stem cells on SIM/HAp coated PG composite scaffolds is characterized by alkaline phosphatase (ALP) activity, mineralization (Alizarin Red S staining), and real time Polymerase chain reaction (PCR). The scaffolds are then implanted into rabbit calvarial defects and analyzed by microcomputed tomography for bone formation after four and eight weeks. These results demonstrate that SIM loaded PLLA/gelatin/HAp-(BCD) scaffolds promote significantly higher ALP activity, mineralization, osteogenic gene expression, and bone regeneration than control scaffolds. This suggests the potential application of this material toward bone tissue engineering. This study was supported by a grant from the National Research Foundation of Korea (NRF-2014R1A1A1002630 and NRF-2016R1A2B4014600).

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP; Ministry of Science, ICT & Future Planning)(No. NRF-2017R1C1B5018358)
Cytoskeleton-Membrane Interactions

P1581
Board Number: B595
Tetraspanin 33 regulates migration, adhesion and invasion properties of human B cells.
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Adhesion molecules allow interactions with the extracellular matrix and its substrates, favoring the presence or absence of diseases such as cancer. Some of these interactions can be mediated by transmembrane proteins such as tetraspanins. Proteins belonging to this family are characterized by four transmembrane domains and two extracellular loops that allow it to interact with adhesion molecules and other proteins, forming tetraspanin enriched microdomains. Currently 33 members of the family of tetraspanins in human and mouse have been described. The newly characterized tetraspanin 33 is mostly expressed in B lymphocytes, where its expression increases upon activation of these cells, suggesting its involvement in autoimmune diseases and B-cell lymphomas.

To characterize the role of tetraspanin 33 (TSPAN33) in adhesion, migration and invasion processes, TSPAN33-EGFP was overexpressed in B lymphocytes derived from Burkitt’s lymphoma (Raji cells). The endogenous expression of adhesion molecules such as β2 and β1 integrin subunits, with their respective α subunits, was evaluated by flow cytometry. Furthermore, adhesion assays were performed on different substrates, such as fibronectin or laminin. Also, various assays were conducted to monitor cell spreading, cell migration and Matrigel invasion both depending on the response to chemoattractant CCL21.

We found that the overexpression of TSPAN33-EGFP in Raji cells generates a decrease in β integrin subunits at the cell membrane, which leads to a lower cell adhesion in substrates such as fibronectin or laminin that are part of the extracellular matrix. Consequently, we also found that these cells have greater cellular extension, greater migration and greater invasion ability.

These data suggest that TSPAN33 may represent an important molecular determinant of lymphocyte B malignancy in pathologies related to cancer.

P1582
Board Number: B596
Coordination of acto-myosin contractility and mitochondrial positioning during neutrophil migration in live animals.
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Cell migration is a fundamental process which occurs during a variety of physio-pathological events such as embryonic development, wound healing, immune response, and cancer. This multi-step process
requires an extensive remodeling of the cellular membranes, which is mediated by the constant re-arrangement of the actin cytoskeleton. Neutrophil migration has been extensively used as a paradigm to investigate cell motility, due to its important role in the innate immune response. However, most of the studies have been carried out in in-vitro model systems (2-D or 3-D cell cultures) and only limited work has been performed on dissecting the molecular mechanisms that link membrane remodeling to cell motility in-vivo.

To this end, here we use intravital subcellular microscopy (iSMIC) and focus on understanding the mechanisms underlying the coordination among acto-myosin cytoskeleton, mitochondrial metabolism, and plasma membrane remodeling during neutrophil migration in the ear skin of live mice following laser-mediated injury. Specifically, neutrophils are isolated from the bone marrow of donor mice expressing selected fluorescently-tagged proteins, labeled with additional fluorescent dyes highlighting specific sub-cellular compartments, and injected subcutaneously in the ear of recipient mice. Our preliminary results show that the actin-based motor Myosin II (NMIIA) is not only present at the rear of the migrating cells, as previously described in vitro, but also at the leading edge. Moreover, we find that sharp changes in the direction of migration are preceded by the assembly of sub-populations of NMIIA filaments on the side of the cells, perpendicularly to the new direction. Finally, we observe that mitochondria: 1) constantly reposition in order to match the localization of NMIIA, most likely in order to supply the ATP required to sustain the contractile activity; and 2) change their polarization during the various phases of migration. In summary, we have begun to highlight a novel correlation between mitochondria repositioning and activity, and the dynamic rearrangement of the actomyosin complex in vivo.

P1583

Board Number: B597

Nanofiber Curvature Enables Quantitating Single Protrusions.
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Our native extracellular matrix (ECM) is primarily comprised of fibrous proteins; the size and organization of which is now widely appreciated to be key in cellular biophysical sensing and motility. Despite decades of in vitro investigation using 2 and 3D substrates, a comprehensive description of initial protrusive events in metastatic invasion using nanofibers mimicking ECM dimensions is still critically missing. This is due to the inability of current approaches to (i) isolate single protrusions and (ii) provide controlled curvatures to quantitate protrusion sensitivity to geometry. Using contrasting diameter fibers deposited orthogonally on top of each other, we quantitate single protrusion dynamics at high spatiotemporal resolution in both breast adenocarcinoma MDA-MB-231 and brain glioblastoma DBTRG-05MG. Specifically, we constrain the cell body to the larger diameter base fiber (~2 μm), while allowing individual protrusions to form on protrusive high curvature small diameter fibers (~100, 200 and 600 nm) and low curvature flat ribbons of corresponding widths. Quantitating at ~minute temporal scales, our strategy allows us to quantitatively distinguish ('protrutype') between the protrusive dynamics of two cancer cell lines and report that the MDA-MB-231 is more sensitive to the fiber curvature compared to DBTRG-05MG. We find that in all cell types, protrusions are rich in actin and tubulin, but intermediate filament vimentin localizes only in mature protrusions. Furthermore, we show that vimentin depleted cells can still form long length protrusions, thus negating the currently understood role of vimentin in protrusion dynamics. Quantitating at ~second temporal scales, we report that protrusion initiation and maturation occurs by wrapping-around the nanofibers in a helical manner.
with the width and rotational speed of each turn regulated by curvature. Specifically, high curvature fibers (~100-300 nm) result in narrower widths (2.1 ± 0.1 µm), whereas low curvature fibers (~600-1000 nm) result in wider widths (4.4 ± 0.2 µm). Interestingly, we find that intracellular transport coincides with protrusion maturation and the kinetics of vesicle cytoplasmic trafficking occur inside protrusions at speeds of 0.7 ± 0.1, 1.0 ± 0.1, and 1.7 ± 0.1 µm/s on fiber diameters of 100, 600 and 1000 nm respectively. Mean square displacement measurements reveal a rapid shift from a sub-diffusion regime (exponents <1) for vesicles outside the protrusions to a super-diffusive regime (exponents >1) as vesicles travel within the protrusion at high persistence. In summary, we report a multiscale and integrative platform which allows the quantification of protrusion morphodynamics decoupled from cell migration and enables identifying the utility of sensory protrusions.

P1584
Board Number: B598
LINC complexes regulate cytoskeletal tension and focal adhesions through transcriptional and post-transcriptional mechanisms.
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The cytoskeleton forms a cohesive network between cellular adhesions at the plasma membrane and nuclear envelope-spanning LINC complexes. Our work demonstrates that functional crosstalk exists between these membrane domains. For example, SUN2-containing LINC complexes regulate desmosome localization and functional integrity in a microtubule-dependent manner in epidermal keratinocytes (Stewart et al., J Cell Biol (2015) 209: 403-418). Here, we have identified a role for LINC complexes in the regulation of cytoskeletal tension and focal adhesion organization. Primary keratinocytes isolated from LINC complex-deficient mice (Sun1-/-/Sun2-/- or SunDKO) display excessive actomyosin-containing stress fibers and altered active RhoA localization. These changes coincide with increased cellular traction forces, increased tension on vinculin molecules in focal adhesions as assessed using a genetically-encoded tension sensor, and increased β1-integrin engagement as assessed by immunofluorescence staining. We further observe that SunDKO cells exhibit enlarged focal adhesions that disassemble more slowly following nocodazole treatment than those in WT cells. Strikingly, SunDKO keratinocytes exhibit drastically reduced levels of vimentin at both the transcript and protein levels. As vimentin has previously been shown to modulate RhoA activity, cellular contractility, and focal adhesion dynamics, we suggest that the loss of vimentin may underlie many of the defects we observe upon LINC complex ablation. Vimentin expressed heterologously in SunDKO cells fails to organize into a typical filamentous network, suggesting that the LINC complex may additionally play a role in the polymerization of perinuclear vimentin filaments. In addition to vimentin, we similarly observe downregulation of other genes involved in the epithelial-to-mesenchymal transition (EMT) in SunDKO cells, including Zeb2 and Twist1, thereby implicating the LINC complex in EMT. Transcriptional analysis suggests that the LINC complex may exert its impact on the expression of vimentin and other EMT genes by promoting TGFβ signaling. Taking these observations together, we propose that the LINC complex acts as a critical regulator of focal adhesions and cytoskeletal tension through both transcriptional and post-transcriptional mechanisms. Moreover, we suggest that these two influences together drive the reduced migration speed and persistence consistently described for LINC complex-ablated cells in vivo and in vitro.
P1585  
Board Number: B599  
Mechanical Interaction between Macrophages and Soft Targets in Phagocytosis.  
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Macrophages are able to phagocytose targets of vastly different sizes and rigidities, ranging from bacteria during immune response to infection, to apoptotic cell fragments during tissue homeostasis, to cancer cells after blockage of CD47. At the same time, experiments have shown that phagocytosis of similar targets can be strongly affected by minor differences in target rigidity. It is currently poorly understood how phagocytes handle targets with such disparate physical properties, or how physical cues of the target feed back into the process of phagocytosis. Studying these processes is hampered by lack of tools to probe the mechanical interaction, and specifically the spatial distribution and orientation of forces, between phagocyte and target. We developed monodisperse (CV < 0.1) spherical polyacrylamide particles with tunable size (4-20 μm) and elastic properties. These model targets can be functionalized with a variety of ligands and conjugated with various fluorescent dyes. We show that IgG-functionalized hydrogel microparticles (~10 μm) are readily taken up by J774.1 macrophage-like cells. Using confocal microscopy, we show that the microparticles are deformed by the cells, implying strong (~nN) and highly localized phagocytic forces. Currently, our work focuses on the development of elasticity theory to allow calculation of the spatial distribution of cell-exerted forces from observed particle deformations. Finally, this method is expected to find broad applications in the study of in vitro and in vivo cellular forces.

P1586  
Board Number: B600  
Endothelial RhoGAP DLC1 is essential to allow leukocytes to change phenotype from rolling to spreading prior to diapedesis.  
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During immune surveillance and inflammation, leukocytes follow the well-defined stages of rolling, spreading and crawling prior to diapedesis through the endothelial cells (EC) to exit the vasculature. The essential endothelial adhesion molecules E- and P-selectin for rolling, and ICAM-1 and VCAM-1 for spreading are well known, but the intracellular mechanism that allows transition from rolling to spreading on ECs is not clear. Here we found a crucial role for the endothelial GTPase activating protein (GAP) DLC1 in regulating this transition. Using physiological flow assays, we show that silencing DLC1 in ECs prolongs the rolling phase of PMNs, evidenced by increased lateral migration distance and speed prior to the actual TEM and display of a round instead of spread adherent phenotype. Both expression and functional distribution into filopodia of EC adhesion molecules were not impaired by DLC1 depletion. Additional FRAP measurements in DLC1-deficient ECs showed no alterations in ICAM-1 surface mobility or recruitment dynamics. However, recruitment of actin adaptor proteins Filamin B, α-actinin-4 and cortactin to ICAM-1 upon clustering is perturbed in DLC1-silenced cells. The ‘rolling’-phenotype of PMNs caused by EC DLC1 depletion and the recruitment of actin adaptor proteins is rescued upon re-expression of full-length DLC1. Additional mechanistic studies showed that DLC1
functions independently from its GAP activity. In conclusion, endothelial DLC1 is crucial in providing a proper endothelial surface for leukocytes allowing them to transit from rolling to spreading by recruiting actin adapter proteins towards ICAM-1 to enhance local sub-endothelial stiffness, an essential step for leukocytes prior to diapedesis.

**P1587**

**Board Number: B601**

Model of Epithelial Tissues Based on Single Cell Mechanics.

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The generation of shape, or morphogenesis, in biological structures is frequently driven by the deformation of epithelial tissues. A variety of modeling approaches has been developed to investigate dynamic features of epithelial layers, such as the self-propelled particle model, Potts model and the vertex model. Here we develop a multicellular model for computing the geometry of epithelial monolayer based on actomyosin cortical force balance for each cell. The geometric shape of cell-cell interfaces is calculated by considering force balance between hydrostatic pressure, cytoskeleton forces and membrane tension of the two cells as well as cell-cell junction forces between them. The configuration of the complete epithelial layer can be obtained utilizing the cell volume constraint and the shape of the epithelial boundary geometry. Comparing to traditional models, this method provides an explicit connection between single cell mechanical properties to the mechanics of the epithelial layer. This model will be used to analyze mechanical properties of epithelial tissues at the single cell level.

**P1588**

**Board Number: B602**

Distinct traction stress distributions in monolayers of MDCK cells are captured by an active vertex model.

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Monolayers of epithelial cells are characterized by both “classical” material properties as well as self-responsiveness and other properties defined by their non-equilibrium status, and as such pose compelling questions at the intersection of several disciplines. I have identified, in the case of wild type and zonula occludens (ZO) 1 and 2 dKD Madin-Darby Canine Kidney cells, at least two patterns of behavior in how monolayers distribute mechanical forces to their environment, i.e., traction stresses. In the first case, ZO dKD cells distribute stresses in analogy to a homogeneous and isotropic elastic contractile gel, results that are in accordance with previous results on single cells and colonies of keratinocytes. The wild type cells, on the other hand, distribute stresses in contradiction to these results, most notably in the movement of stress peaks over time. This underlines the need for a dynamic model of traction stresses; the discipline of active matter provides natural candidates for such a model. A newly-developed active vertex model recapitulates the wild type behavior with a small set of tunable parameters. This, taken together with the experimental results, implies a spectrum of traction stress dynamics that could unify tissue-scale mechanics for several systems under one model.
P1589
Board Number: B603
Going with the Flow: Water Flux and Cell Shape During Cytokinesis.
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Cell shape changes during cytokinesis in eukaryotic cells have been attributed to contractile forces from
the actomyosin ring and the actomyosin cortex. Here we propose an additional mechanism where active
pumping of ions and water at the cell poles and the division furrow can also achieve the same type of
shape change during cytokinesis without myosin contraction. We develop a general mathematical model
to examine shape changes in a permeable object subject to boundary fluxes. We find that hydrodynamic
flows in the cytoplasm and the relative drag between the cytoskeleton network phase and the water
phase also play a role in determining the cell shape during cytokinesis. Forces from the actomyosin
contractile ring and cortex do contribute to the cell shape, and can work together with water
permeation to facilitate cytokinesis. To influence water flow, we osmotically shock the cell during cell
division, and find that the cell can actively adapt to osmotic changes and complete division.
Depolymerizing the actin cytoskeleton during cytokinesis also does not affect the contraction speed. We
also explore the role of membrane ion channels and pumps in setting up the spatially varying water flux.

P1590
Board Number: B604
Variable rescue of microtubule defects in mdx skeletal muscle expressing miniaturized
dystrophins.
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Duchenne muscular dystrophy (DMD) is a progressive and ultimately fatal loss of muscle function that
results from the absence of dystrophin protein. As a structural protein, dystrophin bridges between
the intracellular actin and microtubule cytoskeletons and the transmembrane dystrophin-glycoprotein
complex. It is believed that dystrophin provides a subsarcolemmal guide which directs the microtubule
cytoskeleton into a perpendicular lattice. When dystrophin is absent in mdx mice, the skeletal muscle
microtubule lattice becomes disorganized as characterized by the loss of 90° microtubule intersections.
Moreover, the mdx microtubule lattice exhibits increased density as compared to wildtype. We seek to
further understand the dystrophin-microtubule interface by determining which dystrophin domains
influence microtubule organization and density. A panel of truncated, therapeutic dystrophin constructs
expressed on the mdx background were utilized to quantify microtubule organization and density.
Through comparison of domain conservation across 6 transgenic mdx lines, we report here that the N-
terminus and cysteine rich domains are required for partial restoration of microtubule organization and
are competent to decrease microtubule density to wildtype levels. Full restoration of the microtubule
lattice however, appears to require additional dystrophin domains, potentially spectrin-like repeats 20-
24 which have been previously identified to influence the in vitro microtubule binding capability of
dystrophin. Additionally, the examined micro-dystrophins were capable of decreasing the elevated mdx
detyrosinated α-tubulin levels. Detyrosination is a tubulin modification that when elevated is proposed
to stiffen the microtubule cytoskeleton to drive mechanically stimulated reactive oxygen species and
disrupt Ca2+ homeostasis. We conclude that the tested therapeutic constructs, while differing in their

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truncations, are all capable of significantly improving toward wildtype the microtubule defects in \textit{mdx} skeletal muscle.

\textbf{P1591}
\textbf{Board Number: B605}
\textbf{Basal body associated striated fibers control their length to organize ciliary arrays.}
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Multi-ciliary arrays are fields of motile cilia that beat in a coordinated and polarized manner. This organization of cilia is promoted by the basal bodies (BB) that nucleate, anchor and position cilia at the cell cortex. Extending from the base of BB, striated fibers (SFs) connect BBs to each other and to the cell’s cortex. SF are structurally conserved polymers composed of striations with organism-specific periodicities. The striations in Tetrahymena SFs are spaced 27 nm apart. These ciliated cells possess a network of polarized BBs and SFs that link the approximately 500 BBs per cell. The loss of SFs causes BB and ciliary disorganization that is exacerbated by increased ciliary beating forces. Consistent with a role for SFs in resisting ciliary forces, Tetrahymena SFs lengthen when ciliary beating is elevated. Conversely, SFs shorten when ciliary forces are reduced. This suggests that SFs modulate their length in response to their hydrodynamic environment. We next studied the structure and protein dynamics of SFs to understand how SF length is controlled. The periodicity between SF striations remains the same even when the total SF length changes. The SF architecture comprises a complex network of 10 SF-assemblin-like proteins. Tetrahymena cells possess the SF-assemblin homologue that forms the core SF structure in algae. In addition, other SF-assemblin-like proteins localize to unique domains within the SF structure. As SFs lengthen during maturation or force induced elongation, the level of SF protein components increases. Thus, local SF protein component levels likely promote SF elongation. In summary, we provide a mechanistic basis for how the complex molecular architecture of SFs controls their length in response to ciliary forces.

\textbf{P1592}
\textbf{Board Number: B606}
\textbf{Using high-resolution live imaging to study a novel mechanism for axon growth.}
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We have developed a live imaging system to study the growth of a single pioneer axon, TSM1, in the developing \textit{Drosophila} wing. We find that the TSM growth cone differs from the flat, lamellar growth cones typically documented in culture, and employs a novel mechanism of motility that is fundamentally different from the familiar adhesion-clutch model of axon growth. Our lower resolution movies (1 min/frame, 3 min/frame) show that actin accumulates in the distal portion of the extending TSM1 axon, and that the axon advances by cycles of de-condensation of the actin mass, followed by re-condensation.
at a slightly more distal position. When we use higher-resolution imaging (15 sec/frame), the distal accumulation of bulk actin is still observed, and we detect both retrograde and anterograde motion of individual actin speckles. To investigate the source of this motion, we are imaging the association of actin with vesicles and microtubules. We observe some co-localization of actin speckles with motile vesicles, while there are also actin speckles that appear to move independently of vesicle motion. Looking at microtubule distribution, our data suggest that the accumulation of actin may be distal to the mass of high microtubule density, potentially consistent with models of microtubule-driven advance of the actin mass. Using these high-resolution movies, we have observed fluctuations in the width of the actin peak as TSM grows. To inspect if there is oscillatory behavior in these fluctuations, we have conducted fast fourier transform, and no periodicity is detected in the change of the actin peak width. This indicates that the TSM axon advances by taking advantage of random fluctuations of the actin mass, perhaps in a fashion similar to the “Brownian ratchet” models. Our work provides a novel picture for axon growth in vivo, which we can use to study the underlying mechanisms regulating neuron motility.

P1593
Board Number: B607
Structure, dynamics, and mechanical forces: the actin cytoskeleton in neutrophil phagocytosis.
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Immune cell phagocytosis plays a key role in the immune response, development, and tissue homeostasis, and is implicated in a wide range of disease processes from infection to cancer. Even though immune cell phagocytosis was discovered over 100 years ago, we still lack a unified, mechanistic understanding of the process, largely because of technical challenges in studying the phagocytic cup—a transient, dynamic, and topologically complex, 3D structure. This is in stark contrast to our understanding of the lamellipodium, a stable, steady-state, flat actin structure at the leading edge of migrating cells, for which we have a well-developed understanding of the interplay between actin network structure, dynamics, and mechanics. I have designed an experimental system to map the actin cytoskeleton structure, dynamics, and force generation within the neutrophil phagocytic cup at high spatiotemporal resolution. I am using this experimental pipeline to answer unsolved mechanistic questions, including how target particle properties affect phagocytosis, how the phagocytic cup is resolved into a closed compartment, and how actin is dynamically disassembled during phagocytosis.

3D Migration and Invasion

P1594
Board Number: B608
Analysis of Actin Regulators, Cell-Matrix Adhesions, and Cellular Morphodynamics in 3D Extracellular Matrix Environments.
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Actin regulators control cytoskeletal dynamics to enable critical functions such as migration and cell division. However, most detailed studies of actin regulators have been performed on cells adhered to glass coverslips. To study the role of actin regulators in a more physiological environment, we used light
sheet microscopy to enable high resolution imaging of cells deep in a 3D matrix. We systematically knocked-out a diverse set of actin regulators using CRISPR-Cas9, and evaluated the effect of each on cell spreading, cellular branching, and adhesion size and dynamics for cells embedded in stroma-like ECM environments. Early results suggest that cells require a delicate balance between actin polymerization and depolymerization to properly spread, and these effects are more evident when cells are placed in 3D ECM environments. In contrast, these phenotypes were absent when cells were seeded on 2D planar substrates. We suspect that the number and lifetime of cell-matrix adhesions, and the mechanical properties of the cortical actin network are affected in 3D (potentially inflicted by altered matrix geometry surrounding the cells), and is the subject of our ongoing investigation. Together these results emphasize the importance of the cellular environment, and suggest that subtle phenotypes may have been overlooked throughout the past several decades due to inappropriate contextual ECM cues.

P1595
Board Number: B609
Stereotyped morphological structure detection from high-resolution, live-cell, 3D images.
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3D cell migration is principally governed by environmental cues via subcellular mechanisms such as intracellular signaling and cytoskeletal dynamics. Directly observing the regulation of 3D cell migration therefore requires simultaneously imaging across multiple spatial and temporal scales, ranging from the small (<1 micrometers) and quick (<1 seconds) scales regulating cytoskeletal turnover to the large (>10 micrometers) and slow scales (>10 minutes) of the cell interacting with its environment. Recent advances in high-resolution light-sheet microscopy have begun to allow us to image cells in 3D over these scales with single molecule sensitivity. However, the large size and the difficulty of visualizing 3D objects with complex geometries on 2D computer screens prohibit interpretation of this microscopy data by eye. To facilitate automated analysis of these images, we developed a 3D morphological structure detector to locate and characterize protrusions. This detector combines techniques from computer graphics, machine learning, and computer vision to identify stereotyped protrusions, such as filopodia, blebs, and lamellipodia. The detector decomposes the cell surface into approximately convex patches, merges those patches via machine learning, and then, again via machine learning, classifies the merged patches by protrusion type. A surface patch is convex if any two points on the patch can be connected by a line that does not exit the cell. Since it relies on machine learning, to detect new protrusion types researchers do not need to mathematically describe protrusions, but rather click on examples of these structures in their own data. A support vector machine generalizes these examples to a model that can be used to detect this protrusion type in new data sets. Using this detector, we analyzed the membrane distribution of PIP2 and KRAS in blebbing melanoma cells embedded in 3D collagen. We found that PIP2 associates with individual blebs, whereas KRAS localizes to regions of high bleb surface density, but does not associate with individual blebs. This detector will facilitate the study of the subcellular regulation of 3D cell migration.
P1596
Board Number: B610
Transition between actin-driven and water-driven cell migrations depends on the external coefficient of hydraulic resistance.
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Cells in vivo can reside in diverse physical and biochemical environments. For example, epithelial cells typically live in a two-dimensional (2D) environment while metastatic cancer cells can move through dense three-dimensional (3D) matrices. These distinct environments impose different kinds of mechanical forces on cells, and thus potentially can influence the mechanism of cell migration. For example, cell movement on 2D flat surfaces is mostly driven by forces from focal adhesion and actin polymerization, while in confined geometries, it can be driven by water permeation. In this work, we develop a two-phase model of the cellular cytoplasm, where the mechanics of the cytosol and the F-actin network are treated in single unified framework. Using conservation laws and simple force balance considerations, we are able to describe the contribution of water flux, actin polymerization and flow, and focal adhesions to cell migration in both 2D surfaces and in confined spaces. The theory shows how cell migration can seamlessly transition from a focal adhesion- and actin-based mechanism on 2D surfaces to a water-based mechanism in confined geometries. Moreover, the model explains the counterintuitive experimental observation that the cell velocity can increase with increasing external coefficient of hydraulic resistance.

P1597
Board Number: B611
YAP-independent mechanotransduction drives breast cancer invasion.
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Ductal carcinoma in situ (DCIS) progresses to invasive ductal carcinoma (IDC) when carcinoma cells break through the laminin-rich basement membrane (BM) into the type-1 collagen (col-1) rich stroma, a key first step towards metastasis. The drivers of progression remain unclear, with no established biomarkers. However, increased tissue stiffness correlates with invasion and the transcriptional regulator YAP has been implicated as a mechanotransducer, largely based on 2D culture studies. To identify drivers of DCIS progression, we encapsulated mammary epithelial cells (MECs) in 3D hydrogels with a range of stiffnesses and that presented either BM-ligands or col-1 containing microenvironments to the cells. Surprisingly, enhanced stiffness induced invasion in MECs independently of YAP activation in both BM-like and col-1 rich 3D hydrogels. To provide mechanistic insight into the unexpected lack of YAP activity during MEC mechanotransduction in 3D culture, we examined YAP activation in 2D culture as a point of comparison. Interestingly, confocal microscopy and mass spectrometry showed that YAP colocalized and immunoprecipitated with actin, respectively, and identified distinct YAP binding partners depending on cell context. As actin network architecture is dramatically altered in 3D culture, this may explain differences in YAP activation in 2D versus 3D culture. Further, RNA-seq identified the global gene expression changes induced by increased 3D stiffness, and bioinformatic analysis combined with inhibition studies identified transcriptional regulators p300 and STAT3 as mediators of stiffness-induced...
invasion. RNA-seq also showed that col-1 exposure in stiff gels promotes alterations in carcinoma phenotype, inducing tumor cell expression of genes whose protein products remodel the col-1 network including FN1 and LOX. 3SEQ analysis of breast cancer patient samples revealed that genes identified by 3D culturing in hydrogels were upregulated in breast cancer patients, suggesting expression of S100A7 as a potential biomarker of cancer progression. While genetic mutations initiate transformation, these results reveal the molecular events underlying breast cancer invasion initiated by physical properties of the microenvironment, as well as alteration of the carcinoma phenotype following invasion through the BM into the col-1 stroma.

**P1598**

**Board Number: B612**

Nuclear stress dependent DNA damage and DNA repair factor mis-localization after lamin-A depletion on rigid plastic and in constricted migration.

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Lamin A has long been associated with the role of protecting the nucleus. When lamin-A is partially depleted in the nucleus, nuclear envelope becomes more susceptible to stress-induced rupture. Here, we report that multiple repair factors will mis-localize to the cytoplasm immediately after rupture but the recovery process takes ~7 hours to complete. As the recovery process takes longer than time required for DNA repair (~3hr), elevated DNA damage will then occur in cells who experienced nuclear envelope rupture. Myosin-II mediated nuclear stress is one of the causality of the nuclear stress. Releasing stress by applying soft substrate or myosin II inhibitor is sufficient to minimize rupture and therefore rescue DNA damage. In contrast, overexpressing myosin II can increase the incidence of nuclear envelope rupture and DNA damage. During constricted migration where high fraction of nuclear envelope rupture is observed, myosin II inhibition can effectively minimize nuclear envelope rupture and DNA damage, which is consistent with the result in 2D experiments. Overexpressing multiple essential repair factors at the same time is sufficient to partially rescue DNA damage caused by nuclear envelope rupture as well, indicating again the importance of DNA repair factors during nuclear envelope rupture. In vivo, cancer cells in low lamin-A tumors experience more nuclear envelope rupture than cells in Ctl tumors, hinting low lamin-A tumors may have higher chance of developing genomic variation.

**P1599**

**Board Number: B613**

DNA damage in constricted migration impairs differentiation of myoblasts.

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**Introduction:** Tissue regeneration often relies upon a stem cell’s ability to proliferate, migrate, and differentiate to form new tissue. Chronic tissue injuries are often associated with fibrosis states that poses dense connective tissue networks with small pores and compromised the tissue regenerative capacity. Recent studies show that constricted migration of cancer cells causes DNA damage, genomic instability, and phenotypic alterations. Skeletal muscle has both a local stem cell population that is highly migratory and becomes fibrotic in diseased states. Thus, we hypothesized that muscle progenitors incur DNA damage and have impaired differentiation after constricted migration, which may account for defects in regenerative capacity of fibrotic muscle.
**Methods:** Both a murine myoblast cell line (C2C12) and a human rhabomyosarcoma, muscle progenitor cancer, (Rh30) were used. Cells were placed on top of a transwell membrane with either small 3 μm or large 8 μm pores and allowed to migrate through the membrane for 24-48 hours. Immunofluorescent imaging was used to count γH2AX foci within the nucleus as a marker DNA damage as well as the presence of nuclear blebbing indicated from lamin staining. DNA damage was chemically induced with etopside. Myoblasts were differentiated with low serum and stained for the presence of sarcomeric myosin to quantify differentiation. Serial transwell experiments used migrated cells from the bottom of a membrane that were expanded and then seeded on the top of another membrane.

**Results:** We find C2C12 myoblasts and Rh30 cells accumulate DNA damage as γH2AX foci (110% C2C12 and 60%, Rh30) after migration through rigid small pores that is mitigated in migration through large pores. After constricted migration, 32% of C2C12 cells show rupture of the nuclear lamina even though replication and proliferation rate are unaffected, with similar results for Rh30. Further rounds of constricted migration cause further accumulation of DNA damage that does not recover, even after 1 week in culture. Myoblast differentiation is temporarily reduced by 55% in cells migrating through small pores and nearly eliminated after 3 rounds of transwell migration through small pores. Chemical induction of DNA damage in C2C12 myoblasts reduced differentiation by 80% demonstrating DNA damage as a plausible mechanism for impaired differentiation.

**Conclusions:** The stresses induced by constricted migration through small pores causes DNA damage and nuclear disruption in myoblasts. Constricted migration impedes the differentiation capacity of myoblasts and is compounded by serial migration. This work establishes constricted migration as a plausible mechanism for impaired differentiation, particularly in a fibrotic environment with increasingly small pores.

**P1600**

**Board Number: B614**

**Forces and cytoskeletal dynamics during collective invasion of tumor spheroids.**

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Cell movements and associated cell-generated forces are of central importance in cancer metastasis where tumor cells leave a primary tumor and invade the surrounding tissue. This invasion process is driven by cells at the tumor boundary that collectively exert physical forces on the extracellular matrix. To study the physical forces arising from these collective effects, we embed invasive HT-1080 fibrosarcoma spheroids and non-invasive MCF-7 spheroids containing 500-3000 cells in reconstituted collagen matrices. We measure the ongoing deformation of the collagen matrix over time by tracking embedded silica beads in the equatorial plane of the spheroids. We then compute the collective contractile forces from the matrix deformations with a finite element model that takes the highly non-linear mechanical properties of collagen into account. For the invasive cell line (HT-1080), we find that spheroids reach their maximal contractility after 30 min. At this time point, no cells have left the tumor. Subsequently, overall contractile forces remain constant while cells at the spheroid border invade the matrix. Surprisingly, spheroids from the non-invasive cell line (MCF-7) also generate substantial contractile forces, but these forces rise slowly over the course of 12 hours, and no cells leave the spheroid. We conclude that overall contractility is a poor indicator for cell invasiveness, and hypothesize that dynamical changes of forces and cytoskeletal reorganization may be more indicative. To measure cell dynamics, we record time-lapse images of four differently invasive cell types (A125, MDA-MB-231, HT1080, IFFUC, in increasing order of invasiveness in a 3-D collagen gel) on a fibronectin-coated, 2-dimensional plastic substrate. From the cell trajectories, we infer temporal changes in directional persistence and migration speed. We consistently find that temporal changes of directional persistence
and migration speed show a substantially higher cross-correlation in the more invasive cell lines. Together, these results suggest that cell invasiveness requires a tight coupling of cytoskeletal dynamics and force generation with cell polarization. An interesting corollary finding of this study is that collective 3-D cell invasiveness can be related to single cell behavior under standard 2-D culture conditions.

P1601
**Board Number: B615**

**WASP and WAVE activate the Arp2/3 complex for actin-based force production during basement membrane invasion.**

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Basement membrane (BM) is a dense sheet of specialized extracellular matrix that separates epithelia from underlying tissue. The penetration of cells through BM barriers, called “invasion”, is an important process during normal organ development and in cancer metastasis. Invasive protrusions are rich in actin, but it is unclear whether actin polymerization produces forces that help pierce BM or if actin simply serves as a scaffold for trafficking of components to the invasive edge.

To address the role of actin-based force production in invasion, we studied an invasion event in a developmental process, anchor cell (AC) invasion in Caenorhabditis elegans. AC breaching of the BM is known to depend on an actin-rich protrusion and matrix metalloproteases are present, similar to cancer cell invasion. When the Arp2/3 complex activator WSP-1/WASP was deleted, AC invasion was delayed, despite the fact that essential invasive components were still correctly localized at the invasive membrane. Expression of a dominant negative form of WSP-1/WASP specifically in the AC showed that AC protrusion formation and invasion depended strongly on the activity of the Arp2/3 complex. Super-resolution microscopy indicated that the AC invasive protrusion was densely packed with filaments, in keeping with the idea that the actin network in the invasive protrusion was highly branched. Another Arp2/3 complex activator, WVE-1/WAVE, colocalized with WSP-1/WASP in the invasive protrusion, as observed using worm strains carrying endogenous fluorescently labeled proteins. WVE-1/WAVE appeared to enable invasion to a lesser extent than WSP-1/WASP, perhaps due to its lower capacity to activate the Arp2/3 complex, evaluated by an in vitro pyrene actin polymerization assay.

All together these results show that the invading cell forms a dense, branched actin network via the activity of the Arp2/3 complex in order to puncture the BM, providing evidence that actin-based forces play a role in BM invasion.

P1602
**Board Number: B616**

**Transient Mechanical Strain Promotes the Maturation of Invadopodia through PAK1 Signaling.**

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Cell invasion is a process used by normal and cancerous cells and can be affected by multiple forms of biomechanical forces encountered in the cells microenvironment. Our lab has previously shown that a transient tugging force within an in vitro mechano-invasion assay enhances the invasion of fibrosarcoma cells. This tugging force mimics the magnitude of contractile forces provided by fibroblast movements in the extracellular matrix. Tugging forces down-regulated integrin β3 expression and increased active
cofilin levels in fibrosarcoma cells. The active cofilin coincided with the maturation of invadopodia, as shown by an increase in both invadopodia length and enzymatic activity, and to greater cell invasion. This study targets an established signaling pathway that results from the reduction of integrin β3 expression and tests its relevance to the cellular response to transient tugging forces. p21-Activated kinase 1 (PAK1) is a serine/threonine-kinase that is involved in cytoskeletal remodeling, cell invasion and metastasis. It is downstream from integrin β3 in a signaling pathway that culminates in increased active cofilin levels. We have found that PAK1 expression and activity is reduced by the mechanical stimulation provided by our assay. Furthermore, overexpression of PAK1 kinase mutants corroborates that PAK1 activity must be down-regulated for enhanced invasion to occur in response to stimulation. These results implicate PAK1 in the signaling pathway utilized by the cell to increase its invasion abilities when transient tugging forces are encountered. This study provides additional insight into the cellular mechanism used during the process of invasion in response to extracellular mechanical cues.

P1603
Board Number: B617
S100A4 Regulates Macrophage Invasion by Distinct Myosin-Dependent and Independent Mechanisms.
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S100A4, a member of the S100 family of Ca2+-binding proteins, is a key regulator of cell migration and invasion. Our previous studies showed that bone marrow-derived macrophages (BMMs) from S100A4−/− mice exhibit defects in directional motility and chemotaxis in vitro, as well as reduced recruitment to sites of inflammation in vivo (Li et al., Mol Biol Cell 21:2598, 2010). We have now evaluated the role of S100A4 in the invasive capacity of macrophages. Zymography and MMP9 activity assays of conditioned medium from S100A4−/− BMMs revealed an increase in MMP9 secretion that was associated with stabilization of the microtubule network and an increase in alpha-tubulin acetylation. Paradoxically, despite the increase in MMP9 secretion, S100A4−/− BMMs exhibited marked defects in 2D gelatin degradation and invasion in a Matrigel transwell assay. Furthermore, podosome rosettes were reduced in number and size, and showed a decrease in their rate of turnover. Notably, the average area of podosome rosettes and their ability to degrade gelatin was rescued by treatment of S100A4−/− BMMs with blebbistatin, an inhibitor that maintains the myosin-II motor in a weak actin binding state and counteracts the actomyosin overassembly caused by S100A4 loss. These data suggest that the effect of S100A4 loss on podosome rosettes was due in part to myosin-IIA overassembly. However, neither the enhancement in alpha-tubulin acetylation nor the defect in invasion was affected by blebbistatin. Moreover, treatment of wild type BMMs with the HDAC6-selective inhibitor tubacin led to an increase in microtubule acetylation that was similar to that seen in S100A4−/− BMMs, and caused a comparable loss of invasion. Our data demonstrate that the loss of S100A4 produces two mechanistically distinct phenotypes with regard to macrophage invasion: a defect in matrix degradation, due to a disruption of podosome rosettes caused by myosin-IIA overassembly, and a myosin-independent increase in microtubule acetylation, which increases podosome rosette stability and is sufficient to inhibit macrophage invasion. These studies highlight the role of S100A4 as a critical regulator of matrix degradation, whose actions converge on the dynamics and degradative functions of podosome rosettes.
P1604
Board Number: B618
Chemotaxis and directionally persistent migration of cancer cells is promoted by exosome secretion.
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Directional migration of cancer cells is promoted by exosome secretion but the underlying mechanisms are not well understood. Directional migration of cells could occur by responding to directional cues such as chemical, matrix, and topology of environment. Chemotaxis, directed cell motility toward chemical cues, is thought to occur in cancer cells but is poorly characterized. Here, we show that chemotaxis of cancer cells depends on exosome secretion. Migration of human fibrosarcoma cells toward a gradient of exosome-depleted serum was diminished by knocking down expression of Rab27a, an exosome secretion regulator. Rescue experiments, performed by coating extracellular vesicles and purified fibronectin onto the cell migration area of chemotaxis chambers, revealed that exosomes but not microvesicles affect both the speed and directionality of migrating cancer cells. In addition, fibronectin promoted only speed and not directionality of migrating cells. We also tested whether exosome secretion affects migration of cells in diverse topologies, using ECM-coated synthetic nanofibers to mimic in vivo environments. In these environments as well, we find that exosome secretion promotes persistent migration of cancer cells. Compared to control cells that migrate relatively persistently on aligned nanofibers, exosome secretion-inhibited cells continuously change their direction of migration in a bi-directional mode of migration. In random fiber meshworks, exosome-inhibited cells exhibit excessive pausing at the junctions of random nanofibers. Thus, exosomes promote multiple aspects of cell motility such as speed, directionality, and persistence, which are needed to navigate complex environments.

P1605
Board Number: B619
Fluctuations in directional persistence and cell speed define cell migration strategies.
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Depending on cell type and the local environment, tumor cells show a variety of different migration modes, including mesenchymal and amoeboid motion. To invade a spatially heterogeneous extracellular matrix, the cell needs to coordinate cytoskeletal remodeling, polarization and force generation. Because of this interplay of processes, cell motility indicators such as cell speed and directional persistence are not constant over time but exhibit both gradual and abrupt changes. Since conventional statistical methods average over both time and ensembles of cells, the information contained in these temporal changes is lost. Therefore, common measures of cell motility like the step width distribution or the mean squared displacement fail to distinguish between cell migration strategies in different environments, or between the migration patterns of different cell types. They also cannot explain typical features of cell migration including the exponential step width distribution and power-law mean squared displacements that are a direct consequence of temporal fluctuations in cell speed and directional persistence. Using a novel approach to time series analysis based on Bayesian statistics, we infer the directional persistence and cell speed for each time step of a cell trajectory, taking into account both gradual and abrupt changes in those parameters [1]. We demonstrate that the temporal changes in
persistence and migratory speed provide a distinct fingerprint of the strategies that cells employ to migrate through different environments. For example, persistence is positively correlated with speed in a 3D collagen matrix over much longer time periods compared to migration on 2D substrates, supporting the hypothesis that cells are able to pull themselves along collagen fibers and hence use the surrounding matrix to their advantage. In agreement with this, we find that the directional persistence of invading tumor cells is highly correlated with contractility and cell elongation [2]. The invasion behavior of these cells can thus be described by a gliding motion with alternating phases of simultaneously high or low contractility, elongation, migratory speed and persistence.


**P1606**

**Board Number: B620**

**Mechanisms of Persistent Cell Migration in Aligned Collagen Matrices.**

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Increasing evidence demonstrates that the organization of collagen in the tumor microenvironment is a key factor in breast cancer progression. Stromal collagen surrounding mouse mammary tumors is often aligned and reorganized perpendicular to the tumor boundary. Our lab has demonstrated that aligned collagen enhances tumor progression and metastasis in a mouse model, and predicts poor outcome for breast cancer patients. At the cellular level, aligned collagen increased migration persistence of breast cancer cells by limiting protrusion number. However, the cellular mechanisms involved in regulating protrusions within an aligned collagen matrix is not understood. Using multiple methods to generate various fiber arrangements, we found that the ROCK signaling pathway plays a role in limiting cellular protrusions. MDA-MB-231 breast cancer cells cultured in an aligned 3D collagen matrix had fewer protrusions, which were oriented along the fibers, compared to cells cultured in random collagen matrices. Treatment with low dose ROCK inhibitor (5μM H1152), to decrease rather than abolish ROCK activity, resulted in an increase number of protrusions in cells cultured in aligned and random collagen compared to control cells. Additionally, mammary carcinoma cells cultured in random collagen had more dynamic protrusions, which was enhanced further with ROCK treatment. Together, these results suggest a role for ROCK signaling in the regulation of protrusion number and dynamics in mammary carcinoma cells.
P1607
Board Number: B621
Myosin II governs intracellular pressure and traction force by distinct mechanisms.
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During wound healing by dermal fibroblasts and tissue invasion by metastatic cells, the actin-binding proteins tropomyosin (Tpm) and non-muscle myosin II control the cellular contractile forces that drive cell motility. Classically, two-dimensional (2D) substrate rigidity is known to promote myosin II activity to increase traction force and enlarge focal adhesions in a process coordinated by Tpm 2.1. Traction force can facilitate 3D migration by aligning and stiffening the collagen bundles surrounding moving cells. We recently discovered myosin II activity can also compresses the cytoplasm to increase intracellular hydraulic pressure and switch cells from low-pressure lamellipodia to high-pressure lobopodial protrusions during 3D migration. Both intracellular pressure and traction force require actomyosin contractility. However, it remains unclear whether these myosin II-generated cellular forces are produced simultaneously, by the same molecular machinery. To test the hypothesis that intracellular pressure and traction force are generated by two distinct mechanisms, we first compared intracellular pressure and adhesion size in cells on soft (5 kPa) versus stiff (70 kPa) 2D substrates. We found adhesion size decreased and intracellular increased on soft material, suggesting traction force and pressure can be controlled independently. To identify a potential pressure-specific regulator of actomyosin contractility, we overexpressed individual tropomyosin isoforms and discovered Tpm 1.6 expression increased intracellular pressure on 2D, while reducing traction force. In contrast, Tpm 2.1 expression increased adhesion size and decreased traction force, but had no effect on intracellular pressure. Knocking down endogenous Tpm 1.6 expression reduced intracellular pressure and switched cells migrating in 3D ECM from lobopodia to lamellipodia protrusions, consistent with inhibiting myosin II directly. Together, our results indicate that Tpm 1.6 expression is a positive-regulator of intracellular pressure, while Tpm 2.1 is a negative regulator of traction force. This suggests that actomyosin contractility generates intracellular pressure and traction force by distinct molecular mechanisms. Future work will establish how these two molecular machineries are integrated to generate complex cell behaviors on 2D and in 3D environments.

P1608
Board Number: B622
Cellular mechanics of 3D migration: Uniaxial contraction induces matrix prestress to enhance efficiency of 3D mesenchymal cell migration.
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Cell migration is a complex, integrated function that is highly dependent on attributes of the surrounding microenvironment. During cell migration, cytoskeletal contractile forces are transmitted to the microenvironment through integrin-based focal adhesions in a process known as mechanotransduction. This process provides a bidirectional pathway for local application of cellular forces to the ECM, while cells concurrently receive information about physical characteristics of the ECM. A specific characteristic of extracellular matrix (ECM) known to alter cell migration is stiffness, which many cells can discern through the process of mechanosensing. This process has classically been characterized on two-dimensional, flat surfaces where it has been shown that many cells, including
mesenchymal fibroblasts, can “sense” and migrate towards stiffer ECM. However, it remains unclear how mechanotransduction occurs within a three-dimensional (3D) microenvironment and whether this is a common process required for efficient 3D cell migration by various cell types. Here we describe a unique contractile mechanism utilized by mesenchymal fibroblasts and their malignant counterpart, HT-1080 fibrosarcoma cells, to migrate efficiently through 3D collagen gels. Analysis of ECM deformations reveal that both cell types periodically pinch the matrix locally immediately behind the leading edge. This uniaxial contractile process is obscured by a large, anterior matrix prestress generated solely in the direction of cell motility. Through quantitative temporal analysis, we find this contraction occurs prior to leading edge protrusion. Analysis of 3D cell spreading suggests initial cell polarization is highly force dependent, with cells protruding and migrating in the direction of highest ECM deformation. Myosin IIA is required for both force polarization during spreading and maintenance of matrix prestress during cell migration, while myosin IIB is only required for the latter. Local matrix severing by two-photon ablation to disrupt matrix prestress in front of a cell results in cell repolarization and an eventual change in cell trajectory, indicating mesenchymal cells can sense ECM stiffness in 3D and generate their own prestress in soft matrices to migrate efficiently.

P1609
Board Number: B623
Hierarchical influence of cell-mediated fiber movement on protrusion alignment, cell polarization, and directed migration.
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Directed cell migration plays a critical role in numerous physiological processes, such as cancer cell metastasis and tissue regeneration, and is guided by numerous factors including the physical structure of the extracellular matrix (ECM). Indeed cells have long been observed to follow anisotropic substrate features such as blood vessels and aligned fibers. However, migrating cells can also align ECM fibers, suggesting a positive feedback system in which cells simultaneously follow and align fibers, making migration persistence self-reinforcing. This phenomenon has typically been studied in the context of large cell masses and large-scale ECM deformations. Here we asked whether the same positive feedback system is able to influence individual cell directionality.

To study how the ability of cells to generate fiber alignment affects cell polarity and migration behavior, we examined scenarios in which this ability was impaired. Inhibiting non-muscle myosin II (MII) in HT-1080 fibrosarcoma cells cultured in collagen type I matrices with randomly oriented fibers reduced the cells’ overall polarization in accordance with MII’s role in establishing cell polarity. However, individual cell protrusions nevertheless exhibited contact guidance along matrix fibers. Further experimentation utilizing cells on rigid, aligned polycaprolactone (PCL) fiber scaffolds revealed that cell polarity in fibrous environments can be determined at the level of individual protrusions, which are individually guided by local fiber orientation, independent of MII. The co-alignment of multiple protrusions, such as occurs in the presence of co-aligned fibers, polarizes the entire cell. However, if the fibers are not aligned and cannot be aligned by cell-generated forces, contact guidance of individual protrusions still occurs along fibers, but does not produce overall cell polarization. This behavior is observed regardless of whether fiber reorganization is prevented mechanically (i.e. stiff fibers) or via MII inhibition.

The inability of cells to develop a persistent polarization in environments with randomly aligned fibers that cannot be aligned by the cell, led us to ask how this would affect migration persistence. We monitored the migration of HT-1080 cells in collagen matrices that were or were not photocrosslinked to reduce cell-mediated fiber movement. Our preliminary results indicate that HT-1080 cells in
crosslinked matrices are less persistent, suggesting that cells’ ability to align fibers affects migration persistence. These findings could have implications for the control of cell invasion, e.g. in cancer metastasis and in tissue engineering scaffolds, by modulating cells’ ability to generate their own contact guidance cues.

**P1610**

**Board Number: B624**


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While much research has been dedicated to identifying the cascade of specific biochemical processes involved in the recruitment of neutrophils, much less is known about the mechanical events driving their migration; in particular, how they generate the necessary traction forces to migrate across three-dimensional (3-D) extravascular spaces and the effect of matrix porosity during this process is unclear. In this study, we investigate the importance of extracellular matrix properties on the mechanics of 3-D neutrophil motility in collagen gels using Elastographic 3D Force Microscopy (E3DFM). We embedded neutrophil-like differentiated human promyelocytic leukemia (dHL-60) cells in collagen matrices of different concentrations containing fluorescent micro-beads. Neutrophil motility was induced via the introduction of the neutrophil chemokine formyl-Methionyl-Leucyl-Phenylalanine (fMLP) in a custom build device. Both Confocal and Fluorescent microscopy techniques were used to image the movement of the embedded micro-beads as well as fluorescently labeled cells. Particle Image Velocimetry (PIV) and Finite Deformation Theory were used to compute displacement fields in the collagen matrices. Stress fields in the matrices were computed using our E3DFM method. We will present data showing that morphological changes and migratory patterns differed depending on the porosity of the collagen matrices. We will also provide data showing a clear relationship between the aforementioned migratory characteristics and computed displacement and stress fields around migrating neutrophils in collagen matrices. The results from our study show that neutrophils migrating in 3-D environments employ distinct mechanical mechanisms that depend on the structure of their mechanical environments.

**P1611**

**Board Number: B625**

Achieving a unified understanding of amoeboid cell migration by analyzing the coordination of cell shapes and cytoskeletal components on a global scale.

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There is a fundamental gap in understanding how the different cytoskeletal components of amoeboid-type motile cells are coordinated and rearranged during migration, resulting in the versatility of shape change in these cells and allowing them to adapt to complex and changing environments, such as when neutrophils navigate to sites of infection, or migrating tumor cells begin their journeys through metastasis. We use the human neutrophil-like HL-60 cell line as a model system to capture the morphological changes as cells migrate through 2D confinement in vitro, decompose and map the neutrophil morphological space that contributes to motility behavior by cell shape mode and global...
protein distribution analysis. We have also developed a novel computational methodology that can decompose motile cell shapes into different cytoskeletal protein arrangements. This method allows us to objectively and quantitatively compare overall protein distributions among images of different cells and also over time as a single cell dynamically changes its shape and cytoskeletal protein distribution as it moves. We find that individual cells can explore a wide variety of shapes and cytoskeletal organizations as they undergo cycles of persistent movement, pauses, and directional changes. Many individual cells appear to follow a similar set of stereotypic state transitions as they move. We also find that amoeboid motility can be decomposed into several major shape modes, and that the shape space explored by a large population of cells is similar to the shape space explored by a single cell over time. Given this understanding, we aim to extend our analysis to make predictions on amoeboid motility behavior through the tracking of cell shapes and correlation with changes in intracellular protein distributions. Overall we aim to develop a unified quantitative understanding of how the different cytoskeleton components are coordinated on a global scale at the cellular level to give rise to directed migration.

Dynamics of Focal Adhesions and Invadosomes

P1612

Board Number: B626

RhoG regulates migration through focal adhesion dynamics and contractility.

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The integrity and development of tissues depends on the ability of cells to form physical contact with the surrounding extracellular matrix (ECM). These contacts, known as focal adhesions (FA), serve as traction points for migration by anchoring the actin stress fibers (SF) of the cytoskeleton to the ECM, thus contributing to organogenesis, wound healing, and tissue maintenance. Both assembly and disassembly of FA and SF is dependent upon forces (tension and contractility) exerted by the ECM and other cytoskeletal elements.

The Rho family of GTPases has been implicated in many processes that regulate cell migration, including the assembly and disassembly of adhesions and cytoskeletal dynamics. Here, we have characterized the lesser-studied RhoGTPase, RhoG, in adhesion dynamics and contractility, providing evidence against a correlation between focal adhesion formation and increased contractility.

Our results show that RhoG influences the number, location, and size of FA on both collagen and fibronectin and in multiple cell types. Using live imaging, we found that when RhoG is silenced (KD), FA lifetime is increased, contributing to an increase in the number and size of adhesions, and their more central location in the cell. Additionally, we found that RhoG affects protrusion dynamics, which may be a direct result of adhesion lifetime. We also noted that KD cells are more rounded with increased alignment of FA and SF, and increased number of SF, all characteristics traditionally thought to be associated with increased contractility. However, our results show reduced contractility in RhoG KD cells, both in 2D and 3D. Taken together, our results suggest that RhoG may play an important role in the signaling pathways that couple contractility with focal adhesion turnover.
P1614
Board Number: B628
Complement mediated phagocytosis involves mechanical coupling of β2 integrins to the actin cytoskeleton by a myosin-independent molecular clutch.
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αMβ2 and αXβ2 integrins (also called complement receptor 3 and 4) are highly expressed in macrophages, neutrophils and dendritic cells, and are thought to be the main phagocytic receptors for many pathogens, and participate in the clearance of dead cells and tumor cells. In other contexts, integrin functions are intimately linked to the actin cytoskeleton dynamics. For instance, at focal adhesions, coupling of the force provided by the actin retrograde flow to engaged integrins through a molecular clutch generates mechanical tension that is thought to switch integrins to a high affinity conformation and to generate traction on the substrate. Although it is established that phagocytosis requires actin, its dynamics and specific contribution to β2 integrin functions remain largely unknown. Thus, we sought to test the hypothesis that transmission of mechanical tension to β2 integrins by molecular coupling to the actin cytoskeleton is essential for particle binding, outside-in signaling, and engulfment. Using live cell imaging, we observed that engagement of complement-opsonized particles led to a dramatic reorganization of the actin cytoskeleton, characterized by the formation of large protrusions that wrapped around the particle, contrasting with the particle sinking mechanism previously proposed. Quantitative live cell imaging showed that the Arp2/3 complex, which was required for particle internalization, was recruited to the leading edge of the protrusion and drove phagosome formation. However, slow actin retrograde flow within the phagocytic cup suggested a strong coupling between engaged β2 integrins and the actin network. β2 integrins appeared to form small focal complex-like adhesions at the phagocytic cup, characterized by the recruitment of mature adhesion markers, such as Vinculin, α-Actinin, Zyxin, as well as tyrosine phosphorylation of Paxillin, FAK and Syk. We found that Src family kinases, Syk and FAK/Pyk2 activities were important for particle engulfment. Tyrosine kinases were required for the recruitment of Vinculin, independently of Myosin II, which provided higher transmission of mechanical tension to integrins. Our observations indicate that professional phagocytes co-opt the focal adhesion-lamellipodium machinery, and use signaling to rapidly achieve strong coupling of integrins with the cytoskeleton in order to protrude faster and engulf large particulate material.

P1615
Board Number: B629
IDENTIFICATION OF A RHO-GEF THAT DIRECTS SITE-SPECIFIC ASSEMBLY OF INTEGRIN ADHESION COMPLEXES IN STRIATED MUSCLE.
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The adhesion of cells to extracellular matrix is mediated by “integrin adhesion complexes” (IACs) consisting of integrin and a large number of associated proteins. Although much is known about their composition and molecular mechanisms that initiate assembly, little is known about what determines where such assemblies will form. We use the model genetic organism C. elegans to discover new and conserved aspects of muscle assembly, maintenance and regulation. Nematode striated muscle has 3 types of IACs; M-lines, dense bodies and attachment plaques at muscle cell boundaries. At the worm
muscle cell membrane, IACs begin with integrin and a number of core proteins (e.g. kindlin, ILK, PINCH, α-parvin), and then become specific for each site more distally. From a screen for mutants that disrupt the localization of PAT-6 (α-parvin), we discovered a gene that as a loss of function mutant results in the absence of PAT-6 at muscle cell boundaries, but not at the other IACs (M-lines and dense bodies). This gene encodes PIX-1, the nematode ortholog of vertebrate βPIX. PIX-1 is predicted to have 2 isoforms, PIX-1a (646 residues) and PIX-1b (450 residues); both begin with an SH3 domain followed by a RhoGEF domain; PIX-1a has a predicted coiled-coil region at its C-terminus. The PAT-6 localization defect is found in 5 pix-1 mutant alleles including 2 premature stop mutants, 2 intragenic deletions, and one missense mutation in the RhoGEF domain. In addition, for one premature stop mutant, pix-1(gk299374), at least two other IAC components, UNC-112 (kindlin) and UNC-95, are also missing at muscle cell boundaries. Based on studies of βPIX in mammals and its role in other tissues in C. elegans, we hypothesize that PIX-1 functions in the following pathway: that it activates a Rac or Cdc42 family member, perhaps via the scaffold and ArfGAP protein GIT-1, and Rac or Cdc42 acts through a PAK protein kinase. Mutants in these proteins are being examined for the PAT-6 mis-localization phenotype of pix-1 mutants; so far, we have found this phenotype in git-1 and pak-1 mutants. In both swimming and crawling assays, pix-1(gk299374), moves more slowly than wild type, suggesting that the muscle cell boundaries consisting of cell-ECM-cell contacts is important for proper transmission of lateral forces between muscle cells that occur during whole-animal locomotion. We are generating and analyzing antibodies to PIX-1, with the goal of determining the localization of endogenous PIX-1 in nematode body wall muscle. We hypothesize that PIX-1 either is located exclusively at muscle cell boundaries, or that it is localized at all three IACs in muscle, but is only activated (by an unknown mechanism) as a RhoGEF at muscle cell boundaries.

P1616
Board Number: B630
Distinct focal adhesion morphologies emerge from interplay between retrograde actin flux and stress fiber.
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Focal adhesion (FA) is an anchorage of the cell that transduces traction force on substrate for migration. FA is a dynamic structure that develops from its nascent stage into diverse morphologies. While many essential players are individually well characterized, it is unclear how these components work together in FA growth. We establish a theoretical model that bridges this gap, suggesting that the FA morphology reflects the spatiotemporal coordination between retrograde actin flux and stress fiber formation. The model thus sheds light on the underlying mechanism of FA growth.

P1617
Board Number: B631
PI 3-Kinase-β regulates invadopodial maturation and β1-integrin signaling.
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Invasive tumor cells move through the extracellular matrix by forming invadopodia, which are actin protrusions that mediate matrix degradation. Invadopodia formation is crucial for several steps of the metastatic process, including traversal of basement membranes, intravasation and extravasation. The
PI3Kβ isoform of PI3K, which is uniquely regulated by both receptor tyrosine kinases and GPCRs, and also binds to the small GTPases Rab5 and Rac1, is an important regulator of invadopodial formation. We have previously reported that in MDA-MB-231 breast cancer cells, replacement of endogenous PI3Kβ with kinase dead (110β<sup>K799R</sup>; KD) or GPCR-uncoupled (p110β<sup>526KK-DD</sup>; KKDD) mutants of PI3Kβ leads to defects in gelatin degradation. By co-immunostaining with Tks5 and cortactin antibodies, we now find that the number of invadopodial precursors is unaffected by mutant PI3Kβ. However, the number of mature invadopodia (co-localization of cortactin and Tks5 with gelatin degradation) is significantly lower in cells expressing KD-DD and KD PI3Kβ mutants. Surprisingly, we find a similar phenotype in cells expressing a mutant PI3Kβ that cannot bind to Rab5 (p110β<sup>596D-C</sup>).

Previous studies have demonstrated a role for β1 integrins in invadopodia stability and maturation, and PI3Kβ has been implicated in integrin signaling in platelets. We therefore tested if PI3Kβ is required for integrin function in breast cancer cells. In a haptotaxis assay towards collagen I, MDA-MB-231 cells expressing KD, KK-DD, and Rab5-uncoupled PI3Kβ show a significant decrease in migration as compared to cells expressing wild type PI3Kβ. Similarly, cell spreading on collagen I was significantly reduced in cells expressing KD, KK-DD, or Rab5-uncoupled PI3Kβ. Spreading was also impaired in cells treated with pertussis toxin, to block GPCR activation of PI3Kβ. Specific inhibitors of other Class I PI3Ks (PI3Kα, γ or δ) had no effect on cell spreading, which was significantly impaired by TGX221 (PI3Kβ inhibitor) or LY294002 (pan-PI3K inhibitor). Surprisingly, treatment of cells with TGX221 had no effect on Akt activation, which was completely blocked by inhibition of PI3Kα. In addition, the Akt inhibitor MK2206 had no effect on cell spreading. These data suggest that PI3Kβ has a critical Akt-independent role in integrin-mediated signaling in breast cancer cells, which may contribute to its role in invadopodial maturation and tumor cell invasion.

**P1618**

**Board Number: B632**

**Talin autoinhibition regulates cell behavior and migration in vivo.**

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Cells in multicellular organisms are arranged in highly complex three-dimensional patterns. To achieve this organization requires cells to form both transient and stable adhesive contacts with their surroundings or extracellular matrix (cell-ECM adhesion). The main family of proteins that mediate cell-ECM adhesion are the integrins, which bind ECM ligands outside the cell and build an adhesion complex inside the cell. Integrin-mediated adhesion to the ECM is precisely regulated during development and homeostasis to ensure proper tissue formation and maintenance. The mechanisms that regulate integrin-mediated cell-ECM adhesion have been the subject of intense study in both in vitro and cell culture systems, but much of their biological role in whole animal contexts remains elusive. Some of the key mechanisms that regulate integrin-mediated cell-ECM adhesion work through the adapter protein Talin. Talin is essential for the formation of the integrin adhesion complex and for linking this complex to the cytoskeleton. In particular, Talin can act as a positive regulator of integrin-based adhesion by activating integrins directly. To explore the importance of this positive mode of regulation in an in vivo context, we introduced a point mutation in mouse Talin1 that alleviates an autoinhibitory interaction within the protein. Characterization of this mutation in primary mouse embryonic fibroblasts revealed an increase in integrin activation, an increase in relative adhesive strength, and a shift to more mature focal adhesions. The upregulation of cell-ECM adhesion due to the loss of Talin autoinhibition also results in diverse cellular phenotypes such as changes in cell morphology, impaired cell spreading, and...
impaired generation of traction force. This results in delayed cell migration *in vitro* and in delayed wound healing *in vivo*. Based on our results we propose Talin autoinhibition is an important mechanism for regulating integrin-based cell-ECM adhesion *in vitro* and that autoinhibition defective mutants can be used as a general tool to investigate phenotypes resulting from wholesale activation of integrins *in vivo*.

**P1620**

**Board Number: B634**

*Ca²⁺*-dependent activation of Arf5 at ER/plasma membrane contact sites by an IQSec1/ORP3 complex controls focal adhesion turnover and cell migration.*

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Migrating cells establish focal adhesions at the leading edge that provide the traction required for motility, and disassemble them at the cell rear to allow translocation in the direction of movement. Many studies have shown that focal adhesion disassembly, which is essential for migration, requires Ca²⁺ influx. While Ca²⁺ is known to trigger multiple events related to adhesion disassembly, our understanding of this process is still incomplete. BRAG2/IQSec1 belongs to the Sec7 family of Arf-specific guanine nucleotide exchange proteins. We have previously shown that the related protein, BRAG1/IQSec2, is activated by Ca²⁺ at synapses where it drives internalization of neurotransmitter receptors. In non-neuronal cells, we have shown that BRAG2/IQSec1 modulates cell adhesion by controlling the level of surface beta1-integrins, but whether (and how) this might be regulated by Ca²⁺ is unknown. Here we demonstrate that BRAG2/IQSec1 is activated by Store-operated Calcium Entry (SOCE) via the bipartite STIM1/Orai Ca²⁺ channel, and that this is essential for focal adhesion disassembly. Depletion of BRAG2/IQSec1 from the breast cancer cell line MDA-MB-231 leads to enlarged, stable focal adhesions and decreased cell motility. Surprisingly, adhesion disassembly requires activation of Arf5, an Arf isoform typically associated with ER/Golgi transport. We found that knockdown of Arf5, but not Arf6, phenocopies knockdown of BRAG2/IQSec1, and that expression of active Arf5, but not Arf6, is sufficient to restore motility to BRAG2-depleted cells. To better understand how BRAG2/IQSec1 promotes adhesion turnover, we identified BRAG2-interacting proteins by mass spectroscopy. This analysis identified ORP3, a member of the family of oxysterol binding protein (OSBP) related proteins that mediate the direct exchange of lipids between the ER and other organelles. ORP3 has been previously reported to localize to ER-plasma membrane contact sites, although its lipid cargoes remain unknown. Importantly, like BRAG2 and Arf5 depletion, ORP3 knockdown slows adhesion turnover and impairs motility, suggesting that the three proteins act together to promote adhesion dynamics. In support of this hypothesis, we show that activation of SOCE results in rapid recruitment of ORP3 to the PM where it colocalizes with STIM1/Orai. Conversely, inhibition of STIM1/Orai-mediated Ca²⁺ influx blocks recruitment of ORP3 to the PM, and inhibits activation of Arf5. Finally, we show that knockdown of ORP3 inhibits Arf5 activation, suggesting that ORP3 acts upstream of BRAG2/IQSec1 in a Ca²⁺-dependent signaling cascade. We are currently working to identify the lipid cargoes whose exchange is mediated by ORP3, and how they may promote BRAG2-mediated Arf5 activity and subsequent focal adhesion turnover.
Structure and Function of the Extracellular Matrix

P1621
Board Number: B636
Extracellular matrix and postnatal development of GLAST+ cells.
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It is now well-known that astrocytes, GLAST+ cells, are active participants in the tripartite synapse. However, today there is an opinion that synapse represents tetrapartite structure, which includes pre- and postsynaptic neurons, astrocytes and the extracellular matrix (ECM) which is formed by glycoproteins and proteoglycans released by neighbouring neurons and glial cells. ECM does not only affect the synapse stability and function, but the development of the glial cell as well. Our aim was to study age-dependent diversity in the levels of expression of ECM-associated molecules in glial GLAST+ cells. Analysis of transcriptomic data of glial GLAST+ cells from Bst and Hip of P3 and P11/12 rats demonstrated decrease in expression values of ECM-associated molecules genes during development, but at the same time in Ctx expression values (EVs) of some genes increased to P11/12. Further studies of the most numerous gene families of ECM-receptor interaction pathway showed, that EVs of most Col family genes were increased or remained unchanged in three regions by comparison P3 and P11/12 rats. Indeed in all regions we observed increase in the EV of Col11a2 in P11/12 rat, but Col3a1 in Bst and Ctx, Col2a1 in Hip and Col1a1, Col4a1 and Col5a1 in Bst were significantly downregulated in P11/12. The similar tendency we found in P3 rats for TnC was significantly upregulated in Bst and Ctx and TnR was significantly downregulated in Bst and Hip. For Lam family genes EVs we found that in P3 rats in Bst Lam genes were upregulated, while in Ctx they were downregulated, but in Hip genes were up- and downregulated simultaneously. Itga family genes EVs showed up- and downregulation in the same region at the different ages, in addition the highestnumber of downregulated genes in P3 rats was found in Ctx, as well as Itga7 gene EV was significantly decreased. Analysis of Sdc genes EVs in P3 rats demonstrated that in Bst and Hip these genes were downregulated, but in Ctx two out of four genes were upregulated, indeed Sdc1 and Sdc4 were significantly up- and downregulated, respectively. Gene EVs of synaptic vesicle glycoprotein family were decreased in all regions of P3 rats, as well as two out of three genes of thrombospondin family had decreased EVs. Furthermore we determined that EVs of Acan, Bcan and Ncan were increased, but expressions of Agrn, Reln and Vcan were downregulated in P11/12 rats in comparison with P3. Altogether obtained data indicate on significant contribution of glial cells in releasing of ECM-associated molecules and formation ECM in the brain, as well as on an on going postnatal maturation of GLAST+ cells. This work was supported by Contract №14.575.21.0074, unique identifier of applied scientific research and experimental development RFMEFI57514X0074.
P1622
Board Number: B637
Epithelioid Osteoblasts Deposit Dense Hydroxyapatite in Type I Collagen in a pH Dependent Manner That Is Supported by Aquaporin 1.
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To form bone, osteoblasts initially secrete collagen I as a type 2 helix of 3 collagen monomers. A polarized osteoblast epithelium isolates bone matrix from extracellular space by tight junctions, communicating in the epithelial plane by gap junctions. In the matrix space, phosphate is generated by alkaline phosphatase; calcium is passively transported. Amorphous calcium phosphate crystals form on the collagen template, but must be matured into a stable hydroxyapatite—collagen composite by the established equation: 6 HPO$_4^{2-}$ + 2 H$_2$O + 10 Ca$^{2+}$ $\leftrightarrow$ Ca$_{10}$(PO$_4$)$_6$(OH)$_2$ + 8H$^+$
Since bone forms in a closed space, osteoblasts must remove protons to the extracellular space. Our genetic and transport evidence supports a model of proton movement by highly expressed CIC3 and CIC5 proteins in apical surfaces, and Type 1 Na$^+$,H$^+$ exchangers in basolateral surfaces, of osteoblasts. We generated a type I collagen layer on surface plasmon resonance chips using carboxymethyl dextran-modified gold activated by reaction with N-hydroxysuccinimide and N-ethyl-N’-(dimethylaminopropyl)-carbodiimide HCl, followed by capture of collagen I monomers in PBS at pH 7. We studied deposition of hydroxyapatite onto this surface in 0-5 mM phosphate with 1 mM calcium buffered to pH from 6.94-7.6 in increments. At pH 6.94 very little hydroxyapatite accumulated. Mineral accumulation increased >10 fold at pH 7.4. As controls, mineral was removed by EDTA with reduction to baseline RUs. In accord with earlier studies, electron microscopy of bone with mineral formation showed periodic deposition of crystals in new matrix. This has been predicted to occur in a pH dependent fashion. Measurements of pH during bone formation have been consistent with a strong pH dependence, and with H$^+$ removal by Cl/H$^+$ and Na$^+$,H$^+$ transporters. The condensation of mM calcium and phosphate ions with the sub-mM collagen into the solid composite of bone displaces huge amounts of water. For water to escape requires a novel diffusion path across the osteoblast. By cRNA screening, we detected aquaporins in human osteoblasts, aquaporin 1 being prominent. Quantitative PCR confirmed high levels of aquaporin 1. Antibody-gold labeling of newly synthesized bone showed aquaporin 1 complexes adjacent to new bone in a characteristic pattern in osteoblast membranes. We conclude that mineral deposition in bone collagen is pH dependent, in keeping with a model of H$^+$ removal by Cl/H$^+$ and Na$^+$,H$^+$ exchangers on the apical and basolateral faces of osteoblasts. Further, periodic orientation hydroxyapatite is organized on type I collagen coiled-coils, consistent with earlier work on organization of mineral during initial amorphous crystallization. Conversion to dense mature tissue is supported by aquaporin 1 in apical osteoblastic cell membrane.
P1623

Board Number: B638
Effects of aragonite particles derived from skeleton of Montipora digitata applied as a scaffold on cell proliferation and collagen fiber productivity of cultured human normal dermal fibroblasts.
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[Introduction] Collagen fiber which is extracellular matrix, functions as a scaffold of capillaries. This is an important factor in formation of neonatal bone. Coral is a bioabsorbable porous material widely known as a bone prosthetic material. On the coral transplanted into the living body, mesenchymal tissue and capillaries are formed in the lumen. Cellular response, namely the formation of collagen fiber when adding aragonite particles derived from the Montipora digitata skeleton (Aragonite particles) to cultured fibroblasts is unknown. In this experiment, we cultured human normal dermal fibroblasts (NHDF) with aragonite particles derived from Montipora digitata skeleton as a scaffold. The morphological assay of NHDF was performed with a scanning electron microscope (SEM). The biochemical assay of cell proliferation was performed with measurement of formazan. The amount of collagen fibers produced by the cultured cells was colorimetrically determined. [Materials & Methods] Aragonite particles were deproteinized with 1 N sodium hydroxide. NHDF (PromoCell, Heidelberg, Germany) was cultured aragonite particles were not added to the control group and coral were added to the experimental group. Cell proliferation of NHDF was measured by formazan (WST-1; Roche Applied Science, Indianapolis, USA). Cell proliferation was also observed with a fluorescently labeled stained EdU (EdU Alexa Fluor® 647; Thermo Fisher Schientific K. K, Kanagawa, Japan). The morphology of NHDF and collagen fibers grown inside and outside Aragonite particles was observed with SEM. The production of collagen fibers was quantified by colorimetric determination of collagen fibers by hydroxyproline (Hydroxyproline assay; Cosmo Bio Co., Ltd., Tokyo, Japan). [Results] The formazan value was significantly higher in the experimental group compared to the control group 1, 3, and 7 days after cell seeding. The proliferation of NHDF and hyperplasia of collagen fibers were observed inside and outside aragonite particles. At 28 days after aragonite particles addition, significantly higher hydroxyproline increase was observed in the experimental group than in the control group. [Discussions] The surface of aragonite particles has an uneven rough structure on the nano-level. This rough surface structure and the calcium contained in aragonite may have promoted cell adhesion and cell proliferation. [Conclusion] It was inferred that the cell proliferation ability and collagen formation ability of the cultured NHDF were enhanced by addition of aragonite particles.

P1624

Board Number: B639
Quantifying the dynamics of tissue-induced alignment of collagen fibers.
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The extracellular matrix (ECM) consists of a fibrous polymer network that provides both physical and chemical cues to cells and tissues. During tissue morphogenesis and cancer progression, cell-ECM interactions drive matrix remodeling, which can result in collagen fiber alignment. While the interplay
between collagen fibers and single cells has been reported, little is known about alignment at the tissue scale. Here, we quantified tissue-induced collagen fiber alignment and investigated the mechanisms that dictate the dynamics of the alignment process. A previously reported soft-lithography technique was used to generate three-dimensional (3D) microfabricated tissues embedded in a matrix of Matrigel and type-I collagen [1]. 3D tissues were fabricated using functionally normal mouse mammary epithelial, mammary carcinoma, fibroblast, or human breast cancer cells. A broad-spectrum matrix metalloproteinase (MMP) inhibitor was used to inhibit proteolysis, and a myosin II ATPase inhibitor, Rho kinase inhibitor, and Rho activator were used to modify cytoskeletal contractility. Collagen fibers were imaged using confocal reflection microscopy, and fiber alignment was quantified over a period of 24 hours by assessing changes in pixel intensity gradients. Collagen fiber alignment typically reached a plateau within 6 hours when ~50% of collagen fibers were aligned perpendicular to the tissue surface. Fiber alignment was observed concurrently with a decrease in tissue size, which indicated that tissues exerted strain on the surrounding ECM. Collagen fiber alignment was not observed in tissues treated with a Rho kinase or myosin II ATPase inhibitor, while an increase in the rate and extent of alignment was observed in tissues treated with a Rho activator. These results suggested that Rho-mediated contractility is important for alignment. Inhibiting MMPs had no effect on alignment, which indicated that alignment is primarily a physical process. We further found that breast cancer cells, which have weaker cell-cell adhesions, took longer to align the fibers despite having higher individual cell contractility. These results indicated that collective tissue contractility is important for dictating collagen fiber alignment. Lastly, we found that tissue geometry affected the pattern and extent of collagen fiber alignment by dictating the pattern of strain surrounding the tissue. Our results indicate that tissue-induced collagen fiber alignment is driven by Rho-mediated contractility, whereby tissues collectively contract and exert strain on the ECM. We found that alignment was dictated by cellular contractility, the level of expression of intercellular adhesions, and tissue geometry.


P1625

Board Number: B640

Low adhesive scaffold collagen prepared from type I collagen induces the osteogenic differentiation of rat bone marrow stromal cells.

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[Objective] Collagen is a major component of extracellular matrices and utilizes as a scaffold for cell culture. Cells adhere to collagen scaffold and then accelerate the proliferation. These days, we succeeded in developing low adhesive scaffold type I collagen from pig skin (LASCol, patent pending). The cells on the LASCol coated dish collide each other one after another and get to form spheroid larger. In this study, we report that LASCol markedly facilitates osteogenic differentiation of rat bone marrow stromal cells (rMSCs). [Methods] Two types of culture dish, LASCol (own coated) and Atelocollagen (commercially pre-coated), were used. Rat MSCs (4x10^4 cells/dish) were cultured on each coated-dish with osteogenic basal medium. The cell motility was monitored by time-lapse observation with a microscope. To evaluate osteogenic differentiation, we investigated a mineralization by alizarin red S staining, and measured Gla-Osteocalcin concentration of culture medium supernatant by an ELISA kit. Moreover, the quantity of mRNA of osteogenesis-related genes (Alpl, BGP, Osx, and Runx2) was analysed by RT-qPCR. [Results] Rat MSCs on LASCol coated-dish formed spheroid bodies, and each cell...
clearly showed alkaline phosphatase activity earlier than usual. In the conditions of LASCol coated-dish, Calcium deposits showed increasing (Day 3-6). Similarly, Gla-Osteocalcin of LASCol scaffold group significantly increased to 41 ng/mL (Day 6). Most of the mRNA of cells cultured on LASCol coated-dish much expressed than others. [Summary and Conclusions] We demonstrated, by analysing mRNA expression, that LASCol has the acceleration effects of osteogenic differentiation. [Funding] This work was supported by the Adaptable and Seamless Technology Transfer Program through target-driven R&D, AMED (AS2414037P to K.M.) and JST (AS2715177U to K.M.).

P1626
Board Number: B641
Regulation of collagen processing and fibrillogenesis by the fibronectin matrix.
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The extracellular matrix (ECM) is a complex network of glycoproteins, proteoglycans, and fibrous proteins that is responsible for regulating cell behaviors and developmental processes. Of the numerous members that comprise the ECM, type I collagen (coll) is the most abundant and provides the ECM with structure, stability, and tensile strength. Up-regulation of coll deposition into the ECM occurs in scar formation after tissue trauma and in chronic injury resulting in fibrosis. Fibrillar collagen is deposited once a provisional fibronectin (FN) matrix is established by cells, and only after it is proteolytically processed from its immature form, procollagen. This cleavage of the procollagen N- and C-propeptides is carried out by distinct proteases, ADAMTS-2 and BMP-1 respectively, and cleavage of the C-propeptide but not the N-propeptide is necessary for fibril assembly. Despite extensive understanding of supramolecular collagen fiber structure, the molecular interactions that initiate collagen fibrillogenesis have not been elucidated, in particular the role of FN matrix in that process. Our microscopic analyses of the ECM fibril organization by fibroblasts show co-localization of coll containing the N-propeptide with FN fibrils suggesting that coll-FN interactions occur as procollagen is being processed. When FN matrix assembly by fibroblasts was inhibited in culture, the proteolytic processing of procollagen was reduced and collagens containing one or both propeptides were enriched. Since BMP-1 has been shown to bind to FN, these results suggest that in vivo BMP-1 processing of procollagen requires binding to FN matrix. In solution, procollagen cleavage was recovered by treatment with recombinant BMP-1 and enhanced in the presence of FN. Additionally, both procollagen cleavage by BMP-1 and BMP-1 binding to FN were enhanced by the addition of heparin. Since BMP-1, its enhancer protein PCPE-1, and procollagen bind to FN and heparin, our results suggest a model in which heparin conveys these proteins to their respective binding sites on FN matrix so that FN can act as a template to initiate procollagen processing and to facilitate fibrillogenesis.

P1627
Board Number: B642
Phenotype transformation of proliferative smooth muscle cells using crosslinking collagen gel.
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Smooth muscle cells (SMCs) reveal two different phenotypes according to their external condition including contractile and proliferative phenotypes. The contractile SMCs usually exist within the normal tissue in vivo, and proliferative SMCs are found in a number of diseases, the fetal period and angiogenesis. When SMCs are explanted from normal tissue, they rapidly transform from contractile to
proliferative phenotypes in culture condition. On the other hand, it is difficult to retransform the proliferative SMCs into contractile one. It was reported that proliferative cells retransformed into contractile phenotype only when cultured on intact type IV collagen gel. Thus, we considered that physicochemical properties of the type IV collagen gel are important for the phenotype regulation of SMCs. In this study, we investigated what kind of physicochemical properties of the gel are involved in the retransformation of proliferative SMCs. We purchased pepsin treated type IV collagen gel and prepared type IV collagen gel by crosslinking with glutaraldehyde. SMCs were obtained from chick embryo gizzard and well transformed into proliferative cells by several passages. SMCs were cultured on crosslinking type IV collagen gel, cells showed a slow growth and elongated shapes. Immunostaining for SMC specific calponin was detected in cells on crosslinking type IV collagen gel. Then we measured physical properties of the crosslinking type IV collagen gel. Young's modulus of type IV collagen gel was about 0.23 kPa and this value was clearly lower than that of crosslinking or non-crosslinking type I collagen gel. We tried to evaluate a mesh size of the crosslinking type IV collagen gel using measuring the molecular diffusion coefficient in the gel by Fluorescence correlation spectroscopy. The fluorescence probes with \( r = 1.01 \) nm or 3.56 nm, the diffusion of the probes in the gel was not different from that in solution. Therefore, crosslinking type IV collagen gel mesh size was probably larger than 10 nm. This is credible much larger than the mesh of basement membrane and intact type IV collagen. We have assumed that mechanical property and other chemical properties of type IV collagen gel will be important for retransformation of proliferative SMCs.

P1628
Board Number: B643

Muscle meets immunity: Biological intersections in Drosophila melanogaster.
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Maintenance of muscle tissue during development is dependent upon the extracellular matrix (ECM) to stabilize and sense changes in the local environment. Muscle has a particularly high demand for a dynamic ECM to allow for contraction and force transmission. Inefficient contraction and/or detachment can lead to muscle tissue damage and is often seen in muscle pathologies such as muscular dystrophies and myopathies. Recent evidence shows that damaged and stressed tissues can release tissue-intrinsic and/or extracellular molecules known as damage-associated molecular patterns (DAMPs), which overactivate immune responses. Our lab is using the Drosophila muscle attachment site (MAS) as a model to understand the connection between DAMP-mediated immune activation and muscle tissue maintenance. Initially, we were focused on characterizing a novel ECM protein, Fondue (Fon), which is a critical mediator of ECM stability in the hemolymph clot. Loss of fon causes body wall muscles to detach and also creates large gaps between muscle hemisegments. Transmission electron microscopy (TEM) analysis of fon mutant MASs revealed a loss of ECM integrity through disruption of cuticle and tendon architectures, a lack of muscle-tendon interdigitation, and a depletion of electron-dense matrix accumulation. More interestingly, a sensitized background screen revealed a subset of coagulation proteins, Fon, Tiggrin, and Lsp1\(^\text{y}\), that were necessary for stabilizing muscle attachment sites. Further investigation into gene expression profiles of mutants with hypercontraction-induced muscle tissue stress indicated a clear trend of innate immune activation, suggesting a broader connection between muscle development and innate immunity. In fon mutants with muscle detachment, we also observe abnormal melanin accumulation as tumors or along the larval MASs, activation of Toll signaling in the fat body, and constitutive expression of the antimicrobial peptide (AMP), drosomycin. In a fon-sensitized
background assay, we identified genetic interactions between *fon* and Toll pathway members, including the NFκB inhibitor/κB, *cactus*. At the local level, *fon*-mediated muscle detachment and muscle hypercontraction mutants, *Mhc* and *Brkd*, cause JAK/STAT activation within muscle tissue. We propose a model where muscle tissue stress caused by altered mechanical forces of damaged MASs progresses muscle disease through overactivation of the innate immune system. Understanding the mechanisms by which these two biological processes are intertwined will advance our knowledge of how tissue stresses can be sensed and elicit multi-tissue responses.

**P1629**

**Board Number: B644**

*Triops cancriciformis;* a potential non-mammalian animal model for studies on mechanisms of kidney ultrafiltration.

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The mechanism of ultrafiltration of the blood by the kidney remains obscure. In a search for a primitive animal model that is tractable for investigation of ultrafiltration mechanisms, we carried out studies on *Triops cancriciformis*, a primitive crustacean. We hypothesized that the maxillary gland of the *Triops* is a primitive kidney. We performed structural analysis using serial sectioning and 3D reconstruction. Toluidine blue stained histology cross sections were layered to reconstruct the entire organism virtually using ImageJ. The 500 nm to 1µm thick images were aligned using the TrakEM2 plugin to correct initial deviation in alignment. Next, the maxillary gland structure was isolated using machine learning strategies set to recognize colored marks outlining each cavity. The 3D image revealed the overall architecture of the maxillary gland. When correlated with electron microscopy images, podocyte foot processes were found to surround the interior tubule, suggesting that this tubule is an ultrafiltration barrier. Interestingly, the basement membrane is exposed to the hemolymph with no cell lining in contrast to that of vertebrate animals. These findings suggest that the *Triops* maxillary gland is a “primitive kidney” that lacks endothelial cells. Potentially, *Triops* represent a tractable non-vertebrate animal model to probe the mechanisms of kidney ultrafiltration utilizing advanced molecular biology techniques.

**P1630**

**Board Number: B645**

Alpha 2 Laminin Chain Induces Cardiomyocyte Maturation In Vitro.

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Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) often remain immature expressing fetal isoforms of structural and functional proteins. Several methods to enhance maturation of hPSC-CMs have been developed with limited success. Extracellular microenvironment is critically important for several physiologic processes, such as cell growth, survival and differentiation. Laminin is a key extracellular matrix (ECM) molecule involved in cardiomyocyte-matrix interactions. The main goal of this work is to characterize the ECM isoforms synthesized by hPSC-CM and evaluate the impact of those on cell maturation in vitro. To this end, hPSC-CM culture were analyzed by western blot and
immunofluorescence to characterize their ECM content. We also performed PCR and qPCR assays to compare laminin expression between hPSC-CM and adult tissue. hPSC-CMs were fixed and processed for immunofluorescence or transmission electron microscopy. Using both immunoblotting and immunostaining, we demonstrated that hPSC-CMs expressed three of the most important ECM molecules in the heart (i.e., laminin, collagen IV, and fibronectin) in a similar amount. Since specific laminin isoforms modulate function in several tissues, we probed various laminin chains expressed by these cells. We found that hPSC-CMs, primarily expressed the laminin α1 chain whereas adult heart expressed greater amounts of laminin α2 chain. Both adult heart and hPSC-CMs expressed similar α5 laminin chain amounts. To evaluate the ability of laminin chains to induce maturation in vitro, we created three different culture substrates (laminin 111, 211 and 511). After 5 days, hPSC-CMs cultured on laminin 211 substrate showed a more organized z-line striation pattern compared to both 111 and 511 laminin substrates. To increase substrate flexibility by adding stiffness to the system, we then created a cardiac “microtissue” using layer-by-layer technique (collagen I, collagen IV, and laminin). We observed that cells attached and grew on this layered microtissue. Data regarding maturation are in process. Together, these findings suggest that specific ECM molecules, both by chemical and physical cues, can induce maturation of human PSC-CMs in vitro.

P1631
Board Number: B646
Extracellular matrix performs multi-faceted roles in ciliated sensory neurons of Caenorhabditis elegans.
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Extracellular matrix (ECM) is made up of a network of interacting proteins that surround and support cells in functions such as mechanosensation, attachment, and signaling. ECM is necessary for tissue morphogenesis and homeostasis throughout the lifespan of an organism. Alterations in ECM quality and quantity may contribute to many human diseases and disorders such as polycystic kidney disease (PKD). PKD1 and PKD2 encode polycystin proteins found in primary cilia of mammalian cells and C. elegans neurons. In humans, PKD mutations result in Autosomal Dominant PKD (ADPKD). Given the ancient and evolutionarily conserved role for polycystins in cilia, we use C. elegans as a model to identify new genes required for polycystin ciliary localization and function.

We recently found that ECM components are important for C. elegans polycystin LOV-1 and PKD2 localization and function. mec-1, mec-5, and mec-9 encode ECM components that play a role in mechanosensation and degenerin/epithelial sodium channel (DEG/ENaC) localization in non-ciliated touch neurons (Du 1996, Emtage 2004). mec-1 and mec-9 encode proteins that contain multiple EGF and Kunitz domains; mec-5 encodes a collagen (Du1996, Emtage 2004). Here, we show mec-1, mec-5, and mec-9 play multifaceted roles in ciliated sensory neurons.

These ECM components regulate polycystin localization and polycystin-mediated male mating behaviors, control ciliary and dendritic integrity, and modulate the release of ciliary extracellular vesicles. Intriguingly, mec-9 has cell-specific functions that are controlled by a short isoform that is specifically expressed in ciliated sensory neurons. Our findings reveal the promiscuity of these ECM components and activity in ciliated and non-ciliated neurons of the worm. Transmembrane and ECM proteins such as cadherins and galectins act in cilium retraction/elongation and play a role in cell-to-cell junction signaling in mammalian cells (Seeger 2012, Rondanino 2011). While the polycystins have been implicated in sensing and regulating collagen in zebrafish models, roles for ECM proteins in regulating
ciliary integrity, ciliary polycystin localization, and ciliary function have not been previously appreciated. Our study lends insight on how ECM contributes to ciliary localization and function of sensory receptors like PKD-2 and LOV-1 and expands options for treatment of ADPKD.

P1632
Board Number: B647
Determining Matrix Metalloproteinase Homology across Phylum Ctenophora.
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Matrix Metalloproteinases (MMPs) are conserved zinc-dependent endopeptidases that are found across all kingdoms of life. They are members of a diverse protein family characterized by their catalytic domain, Metzincin, and are divided into subfamilies based on sequence architecture and functional properties. Other major functional domains classically found in MMPs are the N-terminus propeptide domain which plays a regulatory role and the hemopexin domain which is thought to play a role in protein-protein interaction. MMPs play roles in tissue remodeling events, such as wound repair and regeneration, and activate other proteins through proteolytic activity, namely signaling pathways regulating immunity. This class of metalloproteinases has been widely studied in vertebrate model systems but less so in early diverging metazoans that lack “complex” extracellular matrix structures; thus, MMP’s ancestral role in metazoans has yet to be determined. Ctenophores are planktonic, marine invertebrates classically positioned as a sister taxon to Cnidaria based on morphological data; however, more recent molecular studies have placed ctenophores as the earliest diverging extant group of animals. Furthermore, ctenophores have been shown to exhibit rapid wound repair and regenerative properties alluding to the involvement of MMPs as an integral component of their wound repair machinery. In this study, we analyze transcriptomic and genomic data through bioinformatics to reveal the presence of putative archetypal MMPs amongst different ctenophore species. Primary putative homologs were filtered through a series of inclusion criteria including size and domain presence. Each of the ctenophore MMP amino acid sequences includes a highly conserved cysteine switch motif, XRCGXXD, in the N-terminus and the family-defining zinc-binding site motif, HEXXHXGX/XXD, in the catalytic domain. The hallmark “Met-turn” found downstream from the catalytic domain and signature hemopexin domain was found in the C-terminus in ctenophore MMPs. These structural consistencies with MMPs from model systems alludes to conserved function of these proteins in disparate taxa. Phylogenetic relationships amongst the ctenophore MMPs will inform the relatedness of this protein family within species of this phylum. Support: NSF EPS-0447675
Cell-Cell Junctions 1

P1633
Board Number: B648
14-3-3 sequesters the tight junction protein ZO-2 in the cytoplasm of cells cultured in low calcium.
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ZO-2 is a cytoplasmic tight junction (TJ) protein whose subcellular localization varies according to the confluence of the culture and the presence of calcium in the incubation media. In confluent monolayers incubated in low calcium media (LC, 1-5 μM), ZO-2 is absent from the cell borders and displays a diffuse cytoplasmic staining. Here, we aimed to discover how cytoplasmic ZO-2 avoids degradation in the LC condition and the mechanism involved in its arrival to TJs when the monolayers are transferred to normal calcium media (NC, 1.8 mM). We analyzed whether ZO-2 in the LC condition associates to 14-3-3 proteins that bind to phosphoserine/phosphotreonine residues in target proteins. We observed that when confluent monolayers of MDCK cells cultured in LC were treated with BV02, a inhibitor of 14-3-3, ZO-2 moved from the cytoplasm to the cell borders, thus suggesting that in the LC condition, 14-3-3 sequesters ZO-2 in the cytoplasm. The interaction between ZO-2 and 14-3-3 was then demonstrated by co-immunoprecipitation and by a proximity ligation assay. Then, we analyzed the co-localization of isoforms 14-3-3σ and 14-3-3ζ with ZO-2 before and after a Ca-switch in confocal images employing the Fluorescence Covariance Index. We found that in the LC condition ZO-2 is associated with 14-3-3ζ and 14-3-3σ in the cytoplasm, and that transfer to NC media induces recruitment of these complexes to the plasma membrane and their dissociation as ZO-2 incorporates into the nascent TJs. By in silico analysis we found that cZO-2 has 25 putative 14-3-3 binding sites and that 11 of them are located within the U2 segment of the molecule. Interestingly, we found that mutation of one of this residues (T248A), inhibited the capacity of ZO-2 to interact with 14-3-3ζ in a proximity ligation assay and reduced the stability of the protein. Overall, our results indicate that 14-3-3 sequesters ZO-2 in the cytoplasm in the LC condition.

P1634
Board Number: B649
Effect of ZO-actin complexes on tight junction barrier function.
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Tight junctions are responsible for regulating paracellular transport of ions, proteins and immune cells through cell layers. How these junctions are assembled and maintained remains a topic of significant interest, and many questions remain about the complex interactions between transmembrane proteins and the actin cytoskeleton at the junction. A critical link between tight junction transmembrane proteins and actin are the ZO proteins (ZO-1 and ZO-2). ZO proteins are scaffolding proteins that in addition to tethering proteins to actin are known to regulate barrier function, proliferation and motility. Using in vitro methods, we have identified a small actin-binding motif in ZO-1 and ZO-2 within the larger actin binding region of ZO proteins. To test the role of actin interactions through this motif and the larger
actin binding region, we created knockout cell lines for ZO-1 and ZO-2 and quantified organization, barrier function, and the dynamics of tight junction protein organization. We find that ZO lacking the actin-binding motif or actin binding region alters the barrier function suggesting that ZO-actin complexes directly influence the assembly and maintenance of tight junctions.

P1635
Board Number: B650
Tight junctions remodeling modifies polarized epithelial apical surface tension, fluidity, and intercellular adhesive forces.
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The maintenance of epithelial tissue integrity requires coordination between cell-cell adherens junctions (AJ), tight junctions (TJ), and the perijunctional actomyosin cytoskeleton. The perijunctional actomyosin cytoskeleton lies just inside the lateral plasma membrane near the apical junctions, and mainly consists of filamentous actin, non-muscle myosin II motor proteins, and proteins that regulate actin filament dynamics. Here we addressed the hypothesis that alterations in TJ structure and remodeling of the actomyosin cytoskeleton can modify the epithelial tension, fluidity, and intercellular adhesive forces. These supracellular mechanical properties are difficult to measure in intact monolayers and the current methods are disruptive. We developed a novel method to determine the epithelial tension using noncontact acoustic frequency modulation atomic force microscopy (FM-AFM) and tested it on Madin-Darby canine kidney polarized monolayers. Our results show that double knockdown (dKD) of zonula occludens ZO-1 and ZO-2 elevate the apical epithelial tension and effective viscosity in monolayers. Interestingly, epithelial tension is more sensitive to inhibition of myosin II ATPase activity than to inhibition of ROCK activity, but viscosity is highly sensitive to both. Additionally, we showed the epithelial intercellular pulling forces at tricellular junctions and adhesion forces in dKD cell lines are elevated with an increase in contractility. In conclusion, FM-AFM enables the physiological and quantitative study of relevant mechanical forces in intact biological tissues, and can be used to decipher the molecular regulation of epithelial organization and morphogenesis.

P1636
Board Number: B651
Flares of active Rho and F-actin locally reinforce the tight junction barrier in response to mechanical stress.
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The epithelial barrier is important for generating specialized compartments in multicellular organisms. The barrier property of vertebrate epithelia is dependent on tight junctions, which restrict the flow of ions, water, and small molecules between epithelial cells. Epithelia are subject to a number of cell- and tissue-scale forces, such as cell divisions, extrusions, wound healing, and morphogenetic events. During these events, cell-cell junctions must remodel to accommodate changes in cell shape and mechanical force. However, very little is known about how tight junctions are able to maintain barrier function during these events. In order to examine this question, we developed a highly sensitive barrier assay
compatible with live imaging. When we applied this barrier assay to gastrula-stage *Xenopus laevis* embryos, we found that the epithelial barrier is not uniform across space and time. Instead, small barrier breaches occur – often around dividing cells – and persist for minutes before barrier function is restored.

We have previously reported that “flares” of active Rho accumulate at cell-cell junctions, particularly in situations where the cell-cell junctions are compromised (1, 2). Because Rho flares occur on similar time scales and at similar locations as barrier breaches, we investigated whether the two may be correlated in space and time. Indeed, we found that local barrier breaches are followed by local increases in Rho activity. As Rho returns to baseline levels, the barrier function is restored. Thus, we hypothesized that Rho may be involved in restoring barrier function on subcellular scales. To further investigate this possibility, we examined fluorescently-tagged tight junction proteins ZO-1 and Occludin with respect to Rho. Intriguingly, we observed local discontinuities in ZO-1 and Occludin prior to the flare, and both proteins remained locally increased over baseline, or reinforced, following the flare. In order to investigate how active Rho contributes to reinstatement of the barrier and reinforcement of ZO-1, we perturbed actin polymerization and junction contraction using pharmacological tools. Both junction contraction and actin polymerization appear to contribute to ZO-1 reinforcement, as disruption of either results in only partial ZO-1 reinforcement. When both junction contraction and actin polymerization are lost, ZO-1 fails to be reinforced, and the barrier function is not reinstated. Taken together, these data indicate that transient breaches of the tight junction barrier arise in response to mechanical force, and Rho flares serve to rapidly repair these breaches, preserving the overall barrier function of the epithelium.

P1637

**Board Number: B652**

**Na⁺-K⁺-ATPase beta1 Subunit fortiﬁes alveolar epithelial tight junctions via ion transport-independent pathway.**

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Normal epithelial barrier function is dependent on intact tight junctions. The Na-K-ATPase, an ion transporter that regulates intracellular sodium homeostasis, has been shown to regulate cell polarity and cell junctions in multiple tissues, however, the mechanism is largely unknown. In this study, we used lipopolysaccharide-induced mouse lung injury model and isolated rat primary alveolar epithelial cells to study the Na-K-ATPase mediated tight junction regulation. Overexpression of the beta1 subunit of Na-K-ATPase in mouse lungs by electroporation results in decreased wet to dry ratio and lung permeability, suggesting an improvement of the lung barrier function. Western blot analysis indicates that overexpression of the beta1 subunit in alveolar type I cells induces the expression of occludin, zo1 and zo2 by at least 2-fold. In addition, these proteins and claudin4 show higher degree of localization to cell borders, leading to tighter epithelial monolayer measured by transepithelial electrical resistance and paracellular flux of 4 kDa FITC-dextran. Unlike beta1, the beta2 and beta3 isoform of the Na-K-ATPase are unable to enhance tight junctions, suggesting a pump independent pathway that may arise from the unique amino acid sequence of the beta1 subunit. Treatment of 1uM ouabain, which inhibits the transport activity, does not block the upregulation of tight junctions, further confirming a transport-independent pathway, which may act via protein interaction. Mass spectrometry analysis of the immunocomplex pulldown by a beta1 subunit specific antibody detects many unreported interacting
partners of the beta1 subunit, including several protein kinases that is involved in cytoskeletal dynamics and cell junctions. Knockdown experiment of these kinases reveals that MRCKalpha, a CDC42 downstream serine/threonine-protein kinase, appears to mediate the beta1 subunit induced tight junction regulation. These results suggest that the Na-K-ATPase beta1 subunit fortifies lung epithelial tight junctions through transport independent mechanism. Manipulating the Na-K-ATPase beta1 subunit levels thus provide new therapeutic target for many human disease that are characterized by a dysfunction of tight junctions.

P1638
Board Number: B653
The Mechanotransduction role of cell-cell junction in cell extrusion context.
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Extrusion is the process of cell removal from monolayer to maintain tissue homeostasis, which implies in various physiological and pathological processes such as embryonic development, intestinal tract diseases and bacterial invasion. The active role of cell-cell junction (CCJ) during extrusion process has not been extensively discusses in the literature despite a plethora of research on its role in maintaining epithelial integrity. At CCJ, alpha-Catenin (a-Cat) plays a key role as a mechanotransducer at adherent junctions of epithelial cells. In the cadherin adherent complex, a-Cat can interact with F-Actin or via vinculin in a mechano-responsive manner. Such tension-dependent vinculin recruitment process involves biphasic transition of a-Cat structure from the weak-binding state to the stabilized-binding state to Factin, and hence, stabilizes the adherent junction. Here we show that by changing CCJ strength via manipulating different forms of a-Cat mutants, the extrusion rate, global dynamics and local traction changes were observed in MDCK monolayer. We transfected two forms of a-Cat mutants into MDCK cells with a-Cat knock-down background and observed the cell extrusion from the confluent monolayer for 24-48 hours. The extrusion rate was decreased and the duration was prolonged in the mutant that binds to vinculin constitutively and vice versa, there was increased rate and duration for the mutant with deficiency of vinculin-binding domain compared to both WT and MDCK with a-Cat knock-down with rescued WT a-cat. Moreover, re-localization of traction force tissue exerted on the substrate was observed with a-Cat mutants, implying different mechanism surrounding extrusion site with different CCJ strength. Instant drop of traction followed by recovery from neighboring cells with weakened CCJ suggests a lamellipodia-forming mechanism to help extrude the cells and close the gaps. Overall, our experimental results demonstrate that cell-cell adhesion via the stabilization of cadherin-catenin-vinculin complex regulates epithelial homeostasis.
P1639
Board Number: B654
Effect of influenza infection on epithelial monolayer integrity.
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In vitro methods for studying disease dynamics have proven to be a productive and cost effective alternative to in vivo approaches. While significant effort has been committed to exploring the dynamics of virus production and distribution in epithelial sheets, less is known about how infection impacts the contractile and adhesive behavior of cells in a monolayer. Here, we use replication-competent fluorescence-expressing influenza viruses to infect monolayers of Madin-Darby Canine Kidney (MDCK) epithelial cells. Using fibronectin coated micropatterns on polyacrylamide gels to control the morphology of the epithelial cell cultures, we quantify how individual cell morphology and cytoskeletal architecture change over time in response to influenza virus infection. We found that cells steadily weaken in their adhesive contact to neighboring cells, resulting in gaps and holes in the cell monolayers. The spread area of the cells at later time points post-infection also decreased, suggesting weakened contractile behavior. Using Traction Force Microscopy (TFM) to measure the contractile work done between the cells and the substrate we found that the entire contractile output of the cell monolayer weakens during the course of viral infection. Studying fundamental dynamics of epithelial sheet mechanics during influenza virus infection will further the understanding of this important respiratory human disease, and may assist in identifying alternative therapeutic treatments in the future.

P1640
Board Number: B655
Aquaporin-5 in carcinogenesis: expression decreases levels of cell:cell adhesion proteins in MDCK cells.
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Aquaporins (AQPs) are water channels that facilitate transport of water across the plasma membrane. Moreover, increasing evidence indicates oncogenic properties of some AQPs, especially AQPS: AQP5 is overexpressed in several cancers and overexpression correlates with poor prognosis. In cell cultures, ectopic expression induces cell migration and loss of epithelial cell markers. When AQP5 is phosphorylated on S156 by protein kinase A (PKA), major signaling pathways such as NF-κB and Ras are activated. To further elucidate the role of AQP5 in carcinogenesis, we investigated the effects of AQP5 on proteins involved in cell adhesion. We found that AQP5 expression in MDCK cells affected both mRNA and protein levels of several junctional proteins. Immunofluorescence showed that AQP5 expression decreased protein levels of β-catenin, ZO1, p120 catenin and plakoglobin (γ-catenin) and fluorescent in situ hybridization analysis showed a drastic decrease in β-catenin and ZO1 transcripts. Surprisingly, the AQP5S156A mutant that is impaired in phosphorylation by PKA also promoted low levels of adhesion proteins, indicating a Ras independent pathway. The Wnt pathway feedback inhibitor
Axin2, a β-catenin/TCF-regulated gene, was also down-regulated in AQP5 expressing cells. Inhibition of glycogen synthase kinase-3β (GSK-3β) with LiCl partially restored β-catenin levels in AQP5 expressing cells. This indicates that AQP5 expression may interfere with the Wnt signaling pathway by activating or preventing inhibition of GSK-3β. Moreover, pull-down and mass spectrometry analysis revealed that AQP5 interacted with β-catenin, plakoglobin and ZO1. Thus, AQP5 may downregulate adhesion proteins via interference with the Wnt signaling pathway as well as interactions with adhesion proteins, which triggers degradation. Our findings suggest that AQP5 may contribute to carcinogenesis by several mechanisms including interference with the Wnt pathway and degradation of adhesion proteins.

P1641
Board Number: B656
The role of VASP in modulating actin architecture at adherens junctions.
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Cell-cell adhesions and cell-extracellular matrix adhesion sites (focal adhesions) are regions of intense actin remodeling. Some actin-binding proteins appear to be recruited to both of those regions to regulate the actin remodeling. Enabled/Vasodilator-stimulated phosphoproteins (Ena/Vasp) are a family of tetrameric proteins that are recruited to both adherens junctions and focal adhesions. In mammals, Ena/VASP family protein encompasses Mena, VASP and Ena/VASP-like protein (EVL). Ena/VASP is thought to promote actin assembly by two main mechanisms: protecting barbed ends from premature capping and promoting polymerization via profilin-bound g-actin. Ena/VASP proteins are also finely tuned by PKA and PKG phosphorylation. PKA specifically phosphorylates Ena/VASP at Ser 157 to regulate localization of the protein to either focal adhesions or to the cell surface. In order to isolate the contribution of VASP to adherens junctions we plated Caco2 cells on e-cadherin-Fc coated surfaces. These adherens junction plaques contain VASP that is preferentially localized at beta-catenin macroclusters. We also showed that transient knockdowns for VASP (siVASP) decrease adherens junction plaque size and the percentage of cells that have perpendicular stress fibers terminating at those plaques. siVASP monolayers are less stable and present holes. Inhibition of PKA-mediated VASP phosphorylation by H89 show a similar phenotype in comparison to siVASP Caco2 cells, with reduction in plaque size and generation of holes in the monolayers. Stabilization of PKA mediated VASP phosphorylation by NECA treatment maintained plaque size and monolayer integrity. These results indicate that VASP is important for modulating actin architecture at adherens junction plaques and play an important role in stabilizing Caco2 monolayers.

P1642
Board Number: B657
The RhoGEF Trio induces junctional F-actin bundles by locally activating Rap1 to stabilize VE-cadherin-based cell-cell junctions.
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Endothelial cell-cell junctions maintain a restrictive barrier that is tightly regulated to allow dynamic responses to permeability-inducing angiogenic factors as well as inflammatory agents and adherent leukocytes. The ability of these stimuli to transiently remodel endothelial adherens junctions (AJs)
depends on Rho- and Rap-GTPase-controlled cytoskeletal rearrangements. How activity of Rho and Rap-GTPases is spatiotemporally regulated at endothelial AJs by guanine-nucleotide exchange factors (GEFs) is incompletely understood. Here, we identify a crucial role for the RhoGEF Trio in stabilizing VE-cadherin-based junctions. Trio interacts with VE-cadherin and locally activates Rac1 at AJs during nascent contact formation. The N-terminal domain of Trio, including the spectrin-repeats and the GEF1 domain, is responsible for remodeling of junctional actin from radial to cortical actin bundles, a critical step for junction stabilization. Additional evidence using a DORA-Rap1 biosensor shows that this domain of Trio indirectly activates Rap1 through Rac1 and RhoG and the RapGEFs C3G and PDZGEF2 at cell-cell junction regions. Phenotypically using super resolution structural illumination microscopy, we show that Trio activation results in the formation of Rap1-dependent linear AJs and thick F-actin fibers that co-localize with phosphorylated Myosin Light Chain but not with VE-cadherin or beta-catenin. Functionally, we found a dramatic 4-fold increase in the endothelial monolayer resistance when expressing the N-terminus of Trio. Unexpectedly, laser ablation studies revealed low tension at junctional F-actin and high tension at cortical F-actin fibers, indicating a balanced distribution of tension between endothelial F-actin cables and peripheral F-actin cables. Reducing tension using blebbistatin in the presence of active Trio reduced the endothelial barrier resistance independently from VE-cadherin. Collectively, our data reveal that Trio can regulate the endothelial junction integrity in two ways: 1. upon formation of cell-cell junctions: Trio directly binds to the C-terminus of VE-Cadherin and locally promotes the activity of Rac1 and 2. for the increased stability of already matured endothelial cell-cell junctions by locally regulating tension of cortical actin bundles through the signaling axis Trio-Rac1-C3G/PDZGEF2-Rap1-F-Actin in a VE-cadherin-independent fashion.

P1643
Board Number: B658
Rapamycin reduces TNFα-induced VCAM-1 expression in endothelial cells by promoting VCAM-1 degradation via autophagy.
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VCAM-1 is a cell adhesion molecule and plays important roles in inflammatory responses. Rapamycin is known to reduce cytokine-induced VCAM-1 expression in endothelial cells. However, the mechanism by which rapamycin reduces VCAM-1 expression is not clear. Although it is reported that rapamycin inhibits VCAM-1 transcription by increasing ERK1/2 activation, our data does not support this mechanism. We hypothesize that rapamycin reduces VCAM-1 expression in endothelial cells by promoting VCAM-1 degradation. To test this hypothesis we treated human umbilical vein endothelial cells (HUVECs) with tumor necrosis factor α (TNFα) along with rapamycin. The results show that rapamycin had no effect on TNFα-induced MAPKs or NF-κB activation and did not reduce TNFα-induced VCAM-1 mRNA level. By time-course analysis, we found that rapamycin did not affect short-term (<16 h after TNFα stimulation) but reduced long-term (20~24 h after TNFα stimulation) TNFα-induced VCAM-1 expression. Moreover, short-term VCAM-1 expression was reduced in HUVECs pretreated with rapamycin for 16 h, which indicates that the effect of rapamycin takes time. These results are not consistent with inhibition of VCAM-1 transcription by Bay11 or SB202190 and suggest that rapamycin reduces VCAM-1 expression by promoting VCAM-1 degradation. Rapamycin is known to induce autophagy. With western blotting and fluorescence lysotracker staining, we confirmed that rapamycin induces LC3-II accumulation, p62 degradation and increases lysosomal activity in HUVECs. To test whether autophagy is involved in rapamycin-reduced VCAM-1 expression, we blocked autophagy and lysosomal degradation with Atg5

Sunday-408
siRNA and Bafilomycin A, respectively. Atg5 siRNA and Bafilomycin A blocked the effect of rapamycin on VCAM-1 expression, suggesting that reduction of VCAM-1 expression is mediated by autophagy. To further clarify the mechanism of VCAM-1 degradation, we blocked endocytosis of VCAM-1 by dynasore. Dynasore blocked VCAM-1 degradation, which suggests that the degradation is happened after VCAM-1 endocytosis. In this study, we shed light a novel mechanism by which VCAM-1 expression is regulated via autophagy-mediated degradation.

P1644
Board Number: B659
In Vitro Evaluation of Damage by Heavy Metals in Tight and Gap Junctions of Sertoli Cells.
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The Sertoli cell plays a vital role during the spermatogenesis process and has been identified as one of the main targets of the toxic action of heavy metals on the seminiferous epithelium. In the present work, the effect of lead (Pb), Arsenic (As), and Cadmium (Cd) in primary cultures of Sertoli cells was analyzed by measuring the expression of the genes Cldn11, Ocln, and Gja1 that participate in the tight and gap junctions, which are responsible for maintaining the blood-testis barrier. Sertoli cells were isolated from the testes of Wistar rats. Sertoli cell cultures were exposed separately and at the same concentrations of three heavy metals for 48 h. Subsequently, gene expression was measured by real-time polymerase chain reaction. In the morphological analysis of the cultures, after 24 h, the cultures exposed to Cd showed greatest detachment of the monolayer, followed by those exposed to As and Pb. As for gene expression patterns, As induced a decrease in the expression of the Cldn11 gene at 24 and 48 h (p < 0.01) and in that of Ocln at 24 (p < 0.001) and 48 h (p < 0.01), whereas Cd induced overexpression of the Gja1 gene from day 1 of exposure (p < 0.001) and subexpression of the Ocln gene (p < 0.05) at 24 h. Because each of these three metals generated different expression patterns in the three genes, we can postulate that the mechanisms of damage that they induce are different; therefore, the effect that they exert on the Sertoli cell occurs through different pathways, generating changes in structural proteins, altering Sertoli cell morphology, and compromising its function in the regulation of the spermatogenesis process.

P1645
Board Number: B660
Use of a Time-Resolved Fluorescence Resonance Energy Transfer-based screening assay to identify a claudin-4 binder that attenuates tight junction barrier function.
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Objectives: Claudins, a 27-member family of proteins with four transmembrane domains each, play a pivotal role in maintaining tight junction (TJ) seals in epithelial and endothelial tissue. The C-terminal fragment of Clostridium perfringens enterotoxin (C-CPE) binds to claudin-4, which is a key functional and structural component of the TJ in mucosal epithelial cells. Upon binding, C-CPE reversibly modulates TJ seals, yielding an enhancement of the paracellular transport of solutes. However, the use of C-CPE as an
In the present study, we developed a high-throughput screening system using Time-Resolved Fluorescence Resonance Energy Transfer (TR-FET) to identify small-molecule compounds that bind to claudin-4.

Methods: A europium-tagged (Eu (K)) -anti-His Antibody (Ab) and an allophycocyanin (XL665) -tagged-anti-glutathione S-transferase (GST) Ab were used for detection of the interaction between GST-C-CPE and His-claudin-4 using Eu (K) as a donor and XL655 as an acceptor in FRET. To screen for a small-molecule claudin-4 binder, a 32,560-compound library was used. The effects of thiostrepton, a candidate molecule identified in this screen, on epithelial barrier function were confirmed by measurement of transepithelial electrical resistance (TER) and by a paracellular tracer flux assay. The effect of thiostrepton on jejunal absorption was analyzed using a rat loop assay. Results: To construct the claudin-4 binder screening system, we developed a TR-FRET-based assay system for detection of the claudin-4 - C-CPE interaction. The formation of a quaternary complex containing Eu (K) -conjugated-anti-His antibody (Ab), XL-665-conjugated-anti-GST Ab, His-claudin-4, and GST-C-CPE resulted in fluorescence energy transfer between the donor and acceptor. Using this screening system, we performed a claudin-4 binder screen of a library (32,560 compounds), and identified 33 compounds, including thiostrepton, as novel claudin-4 binders. Among these binders, thiostrepton decreased the TER value and increased the flux of 4-kDa fluorescein isothiocyanate (FITC) -dextran in Caco-2 cell monolayers. Furthermore, thiostrepton enhanced rat jejunal absorption of 4-kDa FITC-dextran in a dose-dependent manner. We therefore successfully identified a novel claudin-4 binder, which has the potential to function as an absorption enhancer. Conclusion: The TR-FRET-based screening system is expected to be of use in identifying compounds that modulate the permeability of the intestinal epithelial cell barrier.

**P1646**

**Board Number: B661**

**Concatemerization of connexins - a tool to analyze the oligomerization behavior of heteromeric connexins.**

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Gap junction channels (GJCs) are built between neighboring cells by docking of two connexons (hemichannels), which are made up of six connexins (Cx). It is assumed that hemichannels can be homomeric or heteromeric, depending on whether they are composed of one or more connexin isoforms. Accordingly, the formation of gap junctions with variable connexin combinations has been postulated as a mode of fine tuning of the gap junction channels and their regulation in tissue. eGFP labelled monomeric hCx26 and hCx46, two homodimers (hCx46-hCx46, hCx26-hCx26) and two heterodimers (hCx26-hCx46, hCx46-hCx26) were expressed in communication deficient HeLa cells. Confocal laser scanning microscopy showed that the tandems formed gap junction plaques, which, however, had a reduced plaque area compared to the monomeric hCx26 or hCx46. Using the whole-cell patch-clamp configuration with pipette solution containing Lucifer Yellow or AMCA (7-Amino-4-methyl-3-coumarinylacetic acid), we found that the homodimeric and heterodimeric constructs formed metabolically coupled gap junction channels like the monomeric constructs. Expressed in Xenopus laevis oocytes, patch-clamp experiments in inside-out modus revealed that single hemichannels composed of the tandems, or the monomeric constructs were opened by depolarizing voltage steps and closed by classical hemichannel blockers, such as Ca²⁺ or CBX. Additionally, hemichannels composed of the heterodimers hCx46-hCx26 and hCx26-hCx46 showed a higher single channel fluctuation rate, as well as more open states than those composed of the monomers or the homodimers. Furthermore, we
observed similar junctional currents of whole gap junction channels in double whole-cell patch-clamp measurements using N2A cells transfected with the monomeric or homodimeric constructs. The study shows, that the concatemerization of connexins can be used to analyze biophysical properties of heteromeric connexons, as well as the heteromeric-heterotypic gap junction channels.

P1647

Board Number: B662

The interactions between β1 subunits of the Na⁺,K⁺-ATPase promote cell-cell adhesion in CHO fibroblasts and ouabain intensified it.

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Adhesion is the most important characteristic of epithelial cells to provide a crucial barrier to pathogens and substances. In polarized epithelial cells, adhesion depends on tight junctions, adherent junctions and the Na⁺, K⁺-ATPase, all these located in the basolateral membrane of the cells. The hormone ouabain, member of cardiotonic steroids binds to the α subunit of Na⁺, K⁺-ATPase and inhibits the pump activity when used at above uM concentrations. At physiologic concentrations (nM) it affects the adhesive properties of epithelial cells (Larre et al. 2010). Our group demonstrated the homotypic interactions between β1 subunits of the Na⁺, K⁺-ATPase of neighboring cells (Padilla-Benavides et al. 2010). In the present study we investigate whether the adhesion between β1 subunits is also affected by ouabain. We used CHO fibroblasts stably expressing the β1 subunit of the Na⁺, K⁺-ATPase (CHO-β1) and characterized the effect of ouabain on cell adhesion. Our previous work has shown that non-adherent CHO cells transfected with dog β1 subunit turn out to be adhesive (Shoshani et al. 2005). Here we observe, by IF and Biotinylation assays that ouabain (50 nM) increases the expression of the β1 subunits of Na⁺, K⁺-ATPase at the cell membrane. Aggregation assays show that ouabain increments the adhesion between CHO-β1 cells. It was previously demonstrated that ouabain affects cell adhesion molecules through activation of signaling pathways associated to the α subunit (Larre et al. 2010). We therefore analyzed the effect of ouabain on activation of different kinases in CHO-β1 cells and their effect on cell adhesion. Our results strongly suggest that c-Src, ERK1/2 and AKT are activated by ouabain and have a direct effect regulating the adhesive properties of the sodium pump.

P1648

Board Number: B663

Role of Connexin 32 on gap junctions in breast cancer cells with varying metastatic potential.

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Connexins (Cx) are primary components of gap junctions. Their role in gap junctional intercellular communication (GJIC) is to selectively allow molecules to pass between adjoining cells to regulate many cellular processes. Connexins play a variety of roles in different stages of tumorigenesis, both dependent and independent of gap junction channel functions. While, Cx26 and Cx43 are expressed in healthy breast tissue, they were found to be downregulated in early stages of breast cancer. On the other hand, cytoplasmic accumulation of Cx32 was shown in some breast cancers, and compared to the primary tumors Cx32 is further upregulated in metastasis. However, the complete picture for the role of Cx32 in breast cancer remains to be elusive. By overexpressing Cx32, we investigated localization of Cx32
protein as well as its effect on gap junction function, hemichannel activity and proliferation in Hs578T metastatic breast cancer cells that do not express Cx32 and MCF-7 non-metastatic breast cancer cells that endogenously express Cx32. Transient transfections of Cx32 showed cytoplasmic accumulation of Cx32 proteins in both cell lines and no gap junction plaques were observed between adjacent cells. Proliferation analysis using MTT and trypan blue analysis showed increased viability for Hs578T cells upon Cx32 infection with no significant change on MCF7 cells. In parallel with this, cell cycle analysis demonstrated significant increase in S phase in Cx32 infected Hs578T cells while not showing any effect on MCF7 cells. Further, overexpression of Cx32 in MCF-7 cells did not result in the formation of functional gap junctions but it significantly reduced dye transfer between Hs578T cells in scrape loading assay. However, Cx32 overexpression did not affect hemichannel functions in neither cell type. Our data showed that Cx32 infection does not have any effect on MCF7 cells while it reduces gap junction functions in Hs578T cells without affecting their hemichannel activity. Decrease in dye transfer upon Cx32 infection in Hs578T cells might indicate a suppressive effect of Cx32 on other connexins. Significant increase in the number of Cx32 expressing Hs578T cells in S phase may indicate a tumor promoting effect of Cx32. This effect of Cx32 needs to be confirmed by checking its effect on invasion and metastasis of both cells.

**P1649**

**Board Number: B664**

Relocalization of the adhesion molecule nectin-1 from cell junctions is induced by herpes simplex virus glycoprotein D.

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Nectins are conserved adhesion molecules found at specialized intercellular junctions including adherens junctions (AJ) in epithelia and neuronal synapses. Nectins participate to the initial organization and maintenance of these junctions through homophilic or heterophilic trans-interactions within the nectin family. Responses to nectin trans-interactions include recruitment of other adhesion molecules, connection to and remodeling of the actin cytoskeleton. Interestingly nectins act as receptors for various human viruses like measles virus, poliovirus and several herpesviruses. In particular nectin-1 is the main receptor for herpes simplex virus (HSV) infection of epithelial cells and neurons. These viruses evolved to trigger and use cellular responses that favor infection of target cells. The objective of this study is to understand whether the HSV ligand, glycoprotein D (gD), mimics a natural cellular ligand or triggers a unique response upon interaction with nectin-1. Structural data show that HSV gD binds to the canonical adhesive interface between nectin-1 homodimers. Despite structural similarities with the binding of natural nectin-1 ligands, we found that HSV gD triggers a unique response which causes endocytosis of nectin-1 together with viruses. Here we used purified soluble gD to study how nectin-1 can be displaced from cell junctions. We found that disruption of nectin-1 trans-interaction at cell contacts led to rapid relocalization of nectin-1-GFP at the cell surface of model B78H1 murine melanoma cells. Real time recording of cells exposed to gD also showed that the rapidity of this relocation correlated with the affinity of gD for nectin-1. In human ECC1 epithelial cells, gD-induced delocalization of nectin-1 from established AJ appeared less efficient than in B78H1 cells. We also found that forms of nectin-1 lacking the ability to interact with cytoplasmic adaptors to the actin cytoskeleton responded to soluble gD in similar ways. This suggests that connection of nectin-1 with known adaptors (e.g. afadin) and the cytoskeleton is not needed for gD-induced movement of nectin-1 away from junctions. Interestingly,
nectin-1-GFP failed to accumulate at contacts between B78H1 cells seeded on gD-coated glass, when gD is not intercalating between nectin-1 trans-dimers at junctions. This suggests that gD may induce a global cell response which prevents nectin-1 to accumulate at cell contacts. Altogether these data indicate that HSV gD is unique in inducing nectin-1 delocalization in target cells through binding and signaling.

Glycoproteins and Metalloproteases

P1650
Board Number: B665
Contribution of ppGalNAc transferase-1 to mucin-type O-glycosylation on the Ebola virus glycoprotein and subsequent loss of cell adhesion.
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As evidenced by the 2014-2015 outbreak in West Africa, Ebola virus (EBOV) poses a significant public health risk with mortality rates as high as 90%. As of now, no vaccine against EBOV has been approved for widespread use and few therapeutic options exist to specifically combat Ebola virus disease. Infection is characterized by uncontrolled inflammation and endothelial damage, often resulting in hypovolemic shock, multi-organ failure, and death. The EBOV surface glycoprotein (GP) is known to be instrumental in causing many of the virus’ pathogenic effects, including a dramatic loss of both cell-cell and cell-matrix adhesion in the endothelium. Significantly, this de-adhesion depends upon the extracellular mucin-like domain of GP, which contains a dense cluster of mucin-type O-glycosylation sites. O-glycosylation enzymes may therefore provide excellent therapeutic targets against Ebola virus disease. Mucin-type O-glycosylation is initiated by transfer of a single GalNAc sugar to a serine or threonine residue by the the polypeptide GalNAc transferase enzyme (GalNAcT), of which there are up to 20 distinct isoforms in humans (GalNAcT1-T20). To test which, if any, of the GalNAcTs are individually required for attachment defects, we assayed for changes in cell adhesion following expression of GP in mammalian wild-type or knockout cell lines each deficient in a single GalNAcT isoform. We found that GalNAcT1 knockout cells displayed reduced cell rounding and cell detachment, and more robust adhesiveness with GP expression as compared with wild-type cells. Moreover, we attribute the detachment resistance of GalNAcT1-deficient cells to changes in the glycosylation pattern on GP itself, as both cell lines showed similar overall adhesive properties before transfection, and expressed identical levels of mature surface GP with indistinguishable turnover rates. We are currently conducting mutational analyses to determine the specific residues that GalNAcT1 glycosylates, and how these residues contribute to de-adhesion. In conclusion, we have found that a single GalNAcT isoform is largely required for decreased cell adhesion upon Ebola GP expression, identifying GalNAcT1 as a promising therapeutic target to reduce vascular damage during EBOV infection.
P1651
Board Number: B666
MMP28 is overexpressed in bronchial and alveolar epithelial cells in Idiopathic Pulmonary Fibrosis.
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Idiopathic Pulmonary Fibrosis (IPF) is a devastating and destructive aging-associated disease of unknown etiology and limited therapeutic options. The disease is characterized by epithelial injury and activation followed by the upregulation of multiple mediators that induce the migration, proliferation and activation of fibroblasts with the consequent destruction of the lung architecture. Some matrix metalloproteinases (MMPs) are upregulated in IPF, indicating that they may be important in the pathogenesis and/or progression of the disease. MMP28 (epilysin) is expressed during development and recent evidence has demonstrated that it is upregulated in diverse pathological conditions. In this study, we examined the expression of MMP28 in IPF and evaluated its functional effects in alveolar and bronchial epithelial cells. We found that the enzyme is expressed in bronchial (apical and cytoplasmic localization) and alveolar epithelial cells (cytoplasmic and nuclear localization) in IPF lungs. In vitro, MMP28 epithelial silencing decreased the proliferation rate and delayed wound closing, while overexpression showed the opposite effects, protecting from apoptosis and enhancing epithelial-mesenchymal transition (EMT). These effects were lost when the catalytic domain was affected by site-directed mutagenesis changing the glutamate in the MMP-28 catalytic site for an alanine. These findings demonstrate that MMP28 is upregulated in the respiratory epithelium of IPF lungs where it may be implicated in its proliferative and migratory phenotype.

P1652
Board Number: B667
Matricellular Tinagl1 affects cilia function in early zebrafish embryos.
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Tinagl1 (tubulo-interstitial nephritis antigen-like protein 1) is a matricellular glycoprotein broadly associated with basement membranes in animal species having three germ layers; mammals have an additional family member, Tinag, that is largely restricted to the developing and adult kidney. Drosophila Tinagl1 binds tightly to the Wnt, Wg, and is required for Wg stability in vitro, while the mammalian proteins have been associated with a FAK-dependent cell survival pathway and with reduced metastatic potential of mammary tumor cells. However, no clear in vivo requirement or pathway mechanisms have been defined for Tinagl1 in these animal models. Using MO knockdown in zebrafish, we observed defects consistent with a ‘ciliopathy’ spectrum, including small eye, body curvature, L/R randomization of heart looping, hydrocephalus, renal cysts, and craniofacial defects (CPCI 54:381-90, 2017, and manuscript in preparation). Transient Crispr/Cas9 mosaic knockdown and an ENU truncation mutant support heart looping, renal, and craniofacial defects. With the MOs, we documented shorter and fewer cilia in Kupffer’s vesicle (KV) and pronephric duct. Interestingly, tinagl1 morphant pronephros defects were enhanced by co-injection with wnt3a MO but not control MO, and partially rescued by tinagl1 and


**P1653**

**Board Number: B668**

**Equilibrium structure and mechanics of the cellular glycocalyx.**
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Cells can employ distinct modes of cell migration, ranging from mesenchymal-like or amoeboid-like single cell motility to collective migration, where cells move as coherent strands. Each mode of migration is characterized by a unique balance of cell-matrix and cell-cell adhesion. Carbohydrates called glycans are concentrated on the cell surface in a complex structure called the glycocalyx. While the glycocalyx is known to impede cell adhesion by acting as a steric barrier, the mechanical response of the glycocalyx and its molecular constituents to force is largely unknown. Therefore, we have developed a physical model of the glycocalyx in order to understand and predict its deformation and reorganization under compressive loads, such as those that would be expected on cells in confined three-dimensional environments. Simulations of our model predict that the glycocalyx can sustain substantial pressures without undergoing significant deformations, but beyond a critical pressure it experiences a pressure-sensitive response, followed by increased resistance at high strains. These results suggest that cells could switch between a weakly adherent state and highly adherent state depending on the degree of confinement and the compression of the glycocalyx. Additionally, the simulations predict the formation of load-bearing clusters of glycoproteins in favorable regions away from the cytoskeletal membrane attachments. We elucidate the effect of the biophysical properties of the glycocalyx and the cell membrane on the mechanical response. We find that increasing the stiffness and the density of glycoproteins and the membrane bending modulus or decreasing the length of glycoproteins and the spacing between cytoskeletal filaments increases the collective stiffness of the glycocalyx and the critical pressure required to generate significant deformations. Together, our results demonstrate how the bulk material properties of the glycocalyx emerge from the physical properties of its molecular constituents and how these constituents rearrange under load. The understanding that we develop with this study has broad relevance in cell migration, cell adhesion, cell communication, and other receptor-mediated processes occurring within the glycocalyx.

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**cttnb2** mRNA but not control **egfp** mRNA. Overall, our results support a hypothesis that, in zebrafish, Tinagl1 is broadly required for motile cilia function and may interact with Wnt/beta-catenin signaling that functions upstream of the motile cilia regulator **foxj1a** in pronephros and KV. Signaling via integrins and associated focal adhesion proteins is also a consideration in how Tinagl1 may affect cilia function. We are currently focusing on knockout mutant generation in zebrafish, needed for definitive analysis of these possibilities, and on testing whether Tinagl1 can also regulate primary cilia function in mammalian cell lines. This ongoing work is relevant to deciphering the complex interplay of extracellular matrix, Wnt signaling pathways, and cilia in human developmental defects and in diseases such as cancer.
P1654
Board Number: B669
Cellular reprogramming of primary human adipocytes into brown adipose tissue (BAT)-like cells.
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Objective: To examine the molecular mechanism of transdifferentiation of primary human adipocyte cells into Brown Adipose Tissue (BAT)-like cells.

Methods: Previous results from our lab have demonstrated lipid-accumulation, lack of cellular proliferation, stimulation of genes involved in the BAT pathway and increased number of mitochondria when heparin binding EGF-like growth factor (HB-EGF) and a soluble form of a disintegrin and metalloproteinase 12 (ADAM 12S) were co-expressed in cells. HB-EGF and ADAM 12S adenoviral expression vectors were engineered in order to recapitulate these results with the goal of using these vectors in vivo and in vitro. Human primary adipocytes were infected with either mock or ADAM 12S high titer adenovirus, monitored for fluorescence and lipid accumulation, and RNA was extracted after 3 weeks of infection. Gene expression patterns will be examined using qRT-PCR for the BAT-like genes, PRDM16, PGC-1α, and UCP-1.

Results: Infection of the primary human adipocyte cells was confirmed by the presence of enhanced green fluorescent protein. Both the mock infected and the ADAM 12S adenovirus infected cells exhibited fluorescence. The ADAM 12S infected cells demonstrated noticeable lipid droplet accumulation in comparison to the mock infected cells. RT-PCR results demonstrated the presence of endogenous hHB-EGF. We would expect the BAT-like genes PRDM16, PGC-1α, and UCP-1 to be upregulated in the cells that were infected with ADAM 12S, while the mock infected cells will not be expected to exhibit this pattern of gene expression.

Conclusions: These results further support previous findings of co-expression of HB-EGF and ADAM 12S stimulating cellular reprogramming into brown adipose tissue (BAT)-like cells. We believe that ADAM 12S stimulates cellular reprogramming into BAT-like cells utilizing endogenous hHB-EGF. These novel insights may provide the first evidence demonstrating BAT-like cellular reprogramming occurs in vivo in humans. This research has possible therapeutic applications to combat obesity and type 2 diabetes.

P1655
Board Number: B670
HB-EGF and ADAM 12S co-expression of mouse fibroblasts results in increased metabolic activity.
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Objective: To examine the metabolic profile of HB-EGF/ADAM 12S BAT-like cells via oxygen consumption and extracellular acidification rate.

Methods: Previously our lab demonstrated lipid-accumulation, lack of cellular proliferation, up-regulation of genes involved in the BAT pathway and stem-cells, down-regulation of WAT pathway genes, and increased number of mitochondria when heparin binding EGF-like growth factor (HB-EGF) and a soluble form of a disintegrin and metalloproteinase 12 (ADAM 12S) were co-expressed. MLC, MLC HB-EGF, MLC ADAM 12S, MLC HB-EGF/ADAM 12S, 3T3-L1 cells were cultured and grown to confluence. 3T3-L1’s were split and differentiated into WAT-like cells and BAT-like cells according to Asano et al.,

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(2014) protocol. After 8 days Seahorse extracellular flux assays were conducted on the Seahorse XF24 according to manufacturer’s (Agilent) recommendations. Besides basal conditions, exposure to catecholamines (200 nM) and FCCP (1 µM) + Oligomycin (1 µM) and results were analyzed. The same experiments will be carried out on a mutant form of HB-EGF with truncated extracellular and intracellular domains while maintaining the processing site for ADAM 12 referred to as ΔNAC and ΔNAC co-transfected with ADAM 12S (ΔNAC/ADAM 12S).

Results: MLC HB-EGF/ADAM 12S co-transfected cells show an increased metabolic rate at basal conditions for oxygen consumption, when exposed to catecholamines, and FCCP+oligomycin when compared to controls (MLC, HB-EGF, ADAM 12S, HB-EGF/ADAM 12S, 3T3-L1, and 3T3-L1 WAT-like cells) and a very similar metabolic profile with 3T3-L1 beige/BAT-like cells. Co-transfected cells saw an increase in OCR when exposed to catecholamines 19.8% while BAT-like cells showed at 62.8% increase. However co-transfected cells demonstrated higher basal levels of metabolism and when comparing their OCR to ECAR ratio when exposed to catecholamines their ratios are nearly identical as they both only saw a 7% and 8% change respectively. It is expected that ΔNAC/ADAM 12S will mimic these results as we have previously demonstrated that ΔNAC/ADAM 12S accumulates multilocular lipid droplets recapitulating the results of HB-EGF/ADAM 12S when stained with Oil Red O.

Conclusions: These results further demonstrate that HB-EGF and ADAM 12S co-expression results in cellular reprogramming into BAT-like cells. Of particular note, HB-EGF/ADAM 12S co-expressing cells can be activated with catecholamines resulting in increased metabolic output and thus may provide a novel therapeutic approach to combating obesity and type II diabetes.

Ubiquitin and Proteasome Function

P1656
Board Number: B672
SCFSlmb mediates degradation of Survival Motor Neuron (SMN) protein.
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Spinal Muscular Atrophy (SMA) is a neuromuscular disorder caused by homozygous loss-of-function mutations in the human survival motor neuron 1 (SMN1) gene, which leads to reduced levels of functional survival motor neuron (SMN) protein. Expression of a duplicate gene (SMN2) primarily results in skipping of exon 7 and production of an unstable protein isoform, SMNΔ7. Although SMN2 exon skipping is the principal contributor to SMA severity, mechanisms governing stability of SMN isoforms are poorly understood.

We generated transgenic Drosophila melanogaster that express only flag-tagged wild-type SMN using the endogenous promoter. We collected embryos from these animals and analyzed Flag-purified lysates by ‘label-free’ mass spectrometry. We identified Flag-SMN, along with other core SMN complex components such as the Sm proteins and the Gemins.
In addition, we identified the SCF真的很 ubiquitin E3 ligase complex as a novel SMN binding partner. Each component: supernumerary limbs (Slmb), SkpA, and Cullin 1 was highly enriched (at least 10 fold) in Flag-SMN samples as compared to the control sample. SCF真的很 interacts with a phospho-degron embedded within the human and fruitfly SMN YG-box oligomerization domain. Substitution of a conserved serine (S270A) interferes with SCF真的很 binding and stabilizes SMNAΔ7. Proteins containing SMA-causing missense mutations that block multimerization of full-length SMN are also stabilized in the degron mutant background. Overexpression of SMNAΔ7S270A, but not wild-type SMNAΔ7, provides a protective effect in SMA model mice and human motor neuron cell culture systems. Our findings support a model wherein the degron is exposed when SMN is monomeric and sequestered when SMN forms higher-order multimers.

P1657

Board Number: B673

Histidine Ammonia-Lyase is a Proteasome Interacting Protein.
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The purpose of this study was to investigate histidine ammonia-lyase (HAL) (EC 4.3.1.3) interaction with the proteasome. HAL is the histidine-degrading enzyme that catalyzes the non-oxidative deamination of histidine to urocanic acid. HAL was found in the 20S proteasome fraction highly purified from the liver using FPLC-chromatography and MALDI-TOF mass spectrometry analysis. HAL gene expression was significantly decreased when proteasomes were specifically inhibited by PS-341. The role of HAL associating with the proteasome is not known. It is possible that HAL is associated with the proteasome to regulate Zinc tolerance via high availability of histidine that chelates zinc. Zinc is present in high concentrations in the eye and plays a key role in maintaining vision. However, the excess of Zinc leads to proteasome dysfunction and aggresome formation. Methods: Cornea tissue sections from rabbit model of limbal stem cell deficiency (LSCD) were analyzed for HAL expression. Oral mucosa epithelial cells (OMEC) were isolated from a small biopsy of rabbit buccal cheek, cultured and treated with 100 uM of Zn, 100 uM of carnosine (a precursor for histidine biosynthesis) and Zn+CR. Results: Healthy corneal epithelium stained for HAL in a granular pattern. However, HAL expression was weakly detected in LSCD diseased corneal epithelium but markedly detected in the invasive blood vessels in central cornea. Results showed that a high concentration of Zinc significantly inhibited proteasome chymotryptsin-like activity when OMEC were incubated with Zinc. However, when carnosine was combined in the cell culture media with Zinc, proteasome chymotryptsin-like activity was significantly recovered. These results indicated the negative role of Zinc excess on the proteasome activity and the positive effect of carnosine supplementation in the recovery. The high level of histidine was expected to chelate Zinc—allowing the proteasome chymotryptsin-like activity to recover and to inhibit HAL as a feedback mechanism. To support this observation, OMEC were cultured and treated with L-cystein (a competitive inhibitor of HAL). Results showed no proteasome inhibition, even when Zinc was added to culture media along with L-cystein, indicating the protective effects of HAL inhibition or high availability of histidine in the presence of Zinc excess. Conclusion: The present study reports HAL interaction with the proteasome and documents the role of HAL in modulating proteasome activity, providing new insights that will help our future understanding of protein degradation in healthy and diseased corneal epithelial cells. Supported by Emmaus Life Sciences, Inc.
P1658

Board Number: B674

Arkadia (RING finger protein 111) mediates sumoylation-dependent stabilization of Nrf2 through K48-linked ubiquitylation.

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The transcription factor Nrf2 is a master regulator of the antioxidant defense system that serves to protect cells from oxidative damage. We previously reported that the SUMO-targeted E3 ubiquitin ligase (STUbL), RING finger protein 4 (RNF4) accelerated the degradation rate of Nrf2 in promyelocytic leukemia-nuclear body (PML-NB)-enriched fractions and decreased Nrf2-mediated gene transcription. STUbLs contain SUMO-interaction motifs that enable it to target substrate proteins that have been modified by SUMO resulting in ubiquitylation. These ubiquitylation events help in regulating eukaryotic cellular activity. In this study, we establish the second mammalian STUbL, Arkadia/RNF111 ubiquitylates polysumoylated Nrf2 to stabilize it, but has no effect on Nrf2-mediated gene transcription. Interestingly, we discovered that the Arkadia-mediated stabilization of Nrf2 occurs through Ub-K48 linkages rather than the predicted Ub-K63 linkages. These results suggest Arkadia-mediated ubiquitylation of Nrf2 protects it from degradation, thereby allowing Nrf2-dependent gene transcription. Collectively, these findings highlight a novel mechanism to positively regulate nuclear Nrf2 levels in response to oxidative stress.

P1659

Board Number: B675

A Model Substrate whose Degradation Pathway is Determined by Aggregation Propensity.

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Cellular protein homeostasis is maintained by a hierarchy of protein quality control checkpoints, including endoplasmic reticulum-associated degradation (ERAD), which leads to the efficient degradation of misfolded proteins in the ER. Although most aberrant proteins in the ER are degraded by ERAD, some misfolded membrane proteins can escape ERAD and are degraded instead by lysosomal/vacuolar proteases. To date, it remains elusive how ERAD cooperates with post-ER quality control pathways to destroy misfolded membrane proteins. Here, we designed a novel model substrate, SZ, to investigate how ERAD substrate selection is regulated in yeast. We discovered that SZ is degraded by both the proteasome and vacuolar proteases, which occurs after ER exit and requires the multivesicular body (MVB) pathway. After interrogating cells with a variety of stresses, we found that both heat-shock and SZ* overexpression increase ERAD targeting. These events correlate with substrate aggregation. In addition, the aggregation of the large, cytosolic misfolded domain in SZ* is concentration-dependent, and both the cytosolic Hsp70 Ssa1 and Hsp40 Ydj1 molecular chaperones are required to maintain substrate solubility. Finally, fusion of this misfolded domain targets a post-ERQC substrate, which is normally routed to the vacuole, for ERAD. Together, we have identified a novel substrate that can be used to investigate ERAD and post-ERQC degradation pathway selection, and show that a misfolded membrane protein with a higher aggregation propensity is preferentially targeted for ERAD.
P1660
Board Number: B676
Sterol oxidation mediates stress-responsive Vms1 translocation to mitochondria.
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Mitochondrial function is constantly threatened by damaging agents, including mitochondria-derived reactive oxygen species. This stochastic burden damages mitochondria, which left unchecked leads to mitochondrial dysfunction. The detrimental consequences of mitochondrial dysfunction are highlighted by its presence in many human diseases including cancer, Parkinson’s Disease, and diabetes. To prevent mitochondrial dysfunction, cells have evolved mitochondrial quality control mechanisms, which include protein degradation. A critical component in mitochondrial protein quality control degradation is the protein Vms1 (VCP/Cdc48-associated mitochondrial stress-responsive 1). Vms1 serves as an adaptor to promote the mitochondrial localization of Cdc48, the S. cerevisiae p97 homolog, and its cofactor Npl4 when cells are exposed to mitochondrial stressors such as H2O2. In the absence of the Vms1-Cdc48 complex, cells accumulate ubiquitylated mitochondrial proteins, suffer progressive mitochondrial failure, have elevated levels of mitophagy, and fail to survive under mitochondrial stress conditions. Mitochondrial targeting of Vms1 is mediated by its conserved Mitochondrial Targeting Domain (MTD), which in unstressed conditions is inhibited by intramolecular binding to the Vms1 N-terminal Leucine Rich Sequence (LRS). In the absence of the Vms1-LRS, Vms1-MTD constitutively localizes to mitochondria in the presence or absence of stress, suggesting that translocation of the full-length protein is regulated by this intramolecular interaction. Localization is also regulated by mitochondrial damage because full-length wild type Vms1 localizes specifically to damaged but not to undamaged mitochondria, indicating that damaged mitochondria are uniquely marked for Vms1 recruitment. These data prompt several questions regarding the mechanism of Cdc48-Vms1 complex recruitment to damaged mitochondria in response to stress. First, how does the Vms1-LRS inhibit localization of the Vms1-MTD to mitochondria? Second, what is the mitochondrial molecule(s) that recruits Vms1-MTD to mitochondria? Third, what is the stress signal that modifies mitochondria and/or Vms1 to promote Vms1 translocation to damaged mitochondria? We here report a 2.7Å crystal structure of Vms1 that reveals that the LRS lies in a hydrophobic groove on the autoinhibited MTD. We also demonstrate that the oxidized sterol, ergosterol peroxide, is necessary and sufficient for Vms1 localization to mitochondria, apparently by binding the MTD in an interaction that is competitive with binding to the LRS. These data support a model in which stressed mitochondria generate an oxidized sterol receptor that recruits Vms1 to support mitochondrial protein homeostasis.

P1661
Board Number: B677
Bromodomains control amyloid-like aggregation of aberrantly acetylated proteins.
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Lysine acetylation is becoming increasingly recognized as a general biological principle which underpins many different aspects of cellular homeostasis, and is subject to aberrant control in different human
pathologies. Lysine acetylation is regulated through the concerted action of acetyltransferases (HAT) and deacetylases (HDAC), where bromodomain proteins read the mark and effect the functional consequence of the acetylation event.

However, it has been unclear, how protein acetylation influences the stability of the proteome, the folding and protein homeostasis at a global level. Here, we describe amyloid-like protein aggregation in human cells that result from aberrant lysine acetylation. The protein aggregation disturbs proteostasis by causing abnormalities in the ubiquitin proteasome system (UPS), autophagy and protein translation, resulting in decreased cell viability. Bromodomain reader proteins are involved in forming the aggregates and, using a chemical biology-led approach to probe their composition, we establish p300/CBP proteins as necessary to form the aggregates. Other proteins, many involved in proteostasis, are also present in the aggregates as confirmed by quantitative mass spectrometry. Further, small molecule inhibitors of bromodomain recognition impede aggregate formation, which coincides with enhanced UPS function and increased cell viability. Significantly, amyloid-like protein aggregates formed by a pathologically relevant form of huntingtin protein, causally involved with Huntington’s disease, are similarly susceptible to intervention by bromodomain inhibition.

Our results show that the amyloid-like protein aggregates which occur upon aberrant protein hyperacetylation disturb proteostasis, cause reduced cell viability and involve bromodomain recognition. Since the aggregates are susceptible to small molecule intervention, our results have important implications for treating amyloid-like pathologies and related protein folding diseases with bromodomain inhibitors. Our results also imply that aggregate formation and disturbed proteostasis contributes to the cytotoxic effect of pan-HDAC inhibitors and may explain some of their side effects.

P1662
Board Number: B678

USP3 regulates the fate of cargo proteins that enter cells by Clathrin-independent endocytosis (CIE).

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We are interested in studying the turnover of plasma membrane proteins that enter cells by clathrin-independent endocytosis (CIE). CIE cargo proteins include the major histocompatibility complex Class I protein (MHC-I), which normally traffics either for recycling back to the PM or to lysosomes for degradation and a number of cargo proteins (CD44, CD98 and CD147) that are directly recycled to the PM and not directed to lysosomes. As a consequence, CD44, CD98 and CD147 are long-lived PM proteins. Ubiquitylation of CIE cargo proteins can target them to the lysosomes for degradation. Conversely, Ubiquitin-specific proteases/deubiquitylating enzymes (DUBs) that catalyze the removal of ubiquitin allows CIE cargo proteins to recycle back to the PM. We sought to identify DUBs that might be responsible for the longer half-life of CD44, CD98 and CD147. We performed an siRNA screen using the library of human DUBs to identify DUBs in HeLa cells responsible for this longevity such that loss of these DUBs would result in downregulation of surface CD44, CD98 and CD147. We found that depletion of either USP3 or TRE17/USP6 resulted in reduced surface levels of CD44, CD98 and CD147. USP3 or TRE17 knockdown shifted the trafficking of the above proteins away from recycling tubules to lysosomal compartments, resulting in reduced surface levels. But the extent of traffic of MHC-I to the lysosomes remained unchanged in cells depleted of USP3. The specificity of the siRNA experiment was validated by the rescue of the siRNA effect by expression of an siRNA-resistant form of USP3. Expression of wild type USP3 alone in Hela cells did not change the route followed by the cargo s. Interestingly, a cysteine-to-serine substitution at 168 of the USP3 gene that inactivates the
deubiquitination activity of USP3 shifted the fate of CD98 to degradative compartments, similarly to that observed in cells depleted of USP3. Thus USP3(C168S) acts as a dominant negative and might provide a way to identify USP3 cargo and/or regulators. Our lab has shown previously (Eyster et al, 2011) that expression of an E3 ubiquitin ligase MARCH8 promotes trafficking of CIE cargo proteins to lysosomes by ubiquitylating the proteins. Here, we show that over-expression of USP3 counteracts the MARCH8 dependent targeting of CD98 to the lysosomal compartment. This study demonstrates that cycles of ubiquitylation and deubiquitylation can determine whether CIE cargo proteins are degraded or recycled.

P1663
Board Number: B679
Linking ISG15 to Cellular Stress Responses: Lessons from Listeria infection.
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ISG15 is an interferon-stimulated, ubiquitin-like protein, with anti-viral activity, however its role during bacterial infection had not been addressed. We previously found that ISG15 restricts Listeria monocytogenes infection both in vitro and in vivo and identified ISGylated proteins that could be responsible for the protective effect. Strikingly, infection or overexpression of ISG15 leads to ISGylation of ER and Golgi proteins, which correlates with increased secretion of cytokines known to counteract infection. More recently, we endeavored to map the in vivo ISGylome following Listeria infection to mechanistically understand the function of this pathway in host defense. To do so we combined a genetic approach employing a murine model of hyper-ISGylation with quantitative proteomics of immunoenriched endogenous ISG15 modification sites. In addition, our approach mapped the endogenous ubiquitylome following infection with Listeria in vivo including the identification of several modified bacterial proteins. Interestingly, we detected ISG15 sites in a number of ER and Golgi proteins, which we had previously shown to be ISG15 targets in vitro, as well as proteins known to control cellular stress responses such as ER stress and autophagy. Taken together, this work will open the door to understanding mechanisms of action of an understudied ubiquitin-like protein.

P1664
Board Number: B680
Structural and kinetic analysis of protein degradation by the 26S proteasome.
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Controlling the levels of key proteins is achieved through both synthesis and degradation, and failure of this control is implicated in a wide range of human diseases. The ubiquitin-proteasome system(UPS) is central in the regulated protein degradation, playing a key role in most cellular functions, such as cell-cycle progression and protein homeostasis. The supreme regulatory potency of the protein degradation machinery depends on its high specificity for substrates and the ability to efficiently unfold and degrade its targets, which is bestowed by the exquisite structures of the 26S proteasome holoenzyme. Specificity of ubiquitin-mediated protein degradation was thought to be simply determined by the ~600 E3 enzymes in human. However, statistical analysis on published datasets shows insignificant correlation between the steady-state ubiquitylation levels and protein stability in all comparison. We still do not understand the underlying rules by which the proteasome recognizes ubiquitin configurations during the
process of substrate engagement and degradation. Complicating this problem is that many targets of the UPS, including most substrates of the Anaphase-Promoting Complex (APC), carry multiple ubiquitylated lysine residues and therefore possess complex ubiquitin configurations. To understand these rules, we developed single-molecule fluorescent assays with enough precision to distinguish different reaction intermediates, to analyze the kinetics of the degradation of well-characterized APC substrates with defined ubiquitin configurations. Contrary to a prevailing view maintaining that a tetra-ubiquitin chain is the minimal signal for efficient proteasomal degradation, we find that, for all tested substrates, multiple di-ubiquitin chains having the same overall level of ubiquitylation as tetra-ubiquitin chains are more efficient degradation signals. A high-throughput study using proteasome trap further supports this conclusion on a wide range of substrates. Most substrates require ubiquitin chains for proteasomal degradation. We find that the chain structure of ubiquitin on substrates specifically promotes initiation of translocation through the axial channel of the proteasome, while the strength of substrate binding to the proteasome is mainly determined by the total number, but not the chain structure of ubiquitins on a substrate. To understand the molecular mechanism underlying the processive protein degradation, we resolved multiple structures of the human 26S proteasome to near-atomic resolutions using cryo-electron microscopy. Comparative analysis suggests exquisite structural transitions, coupled to the states of nucleotide binding and hydrolysis, in an allosteric network featuring long-rang interactions. Current structural and kinetic s

P1665
Board Number: B681
The ubiquitin-proteasome system regulates degradation of an anti-inflammatory receptor SIGIRR.
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Single Ig IL-1-related receptor (SIGIRR), a receptor for anti-inflammatory cytokine IL-37, negatively regulates inflammatory responses through inhibition of ILR and TLR signaling. However, the role of SIGIRR in lung inflammatory diseases has not been well studied and molecular regulation of SIGIRR stability remains unclear. In this study, we aimed to investigate the regulation of SIGIRR stability in the ubiquitin-dependent system. Consistent with other studies, overexpression of SIGIRR in mouse macrophages attenuated LPS-induced phosphorylation of JNK and Erk, suggesting that SIGIRR plays an anti-inflammatory effect. Intratracheal injection of lipopolysaccharide (LPS) for 24 h or P. aeruginosa (strain PA103) for 4 h significantly reduced SIGIRR protein levels in murine lungs. In mouse lung epithelial cells, SIGIRR degradation was prevented with a proteasome inhibitor MG-132 in a time-dependent manner, while it enhanced by ubiquitin expression. Further, we found that SIGIRR was ubiquitinated by K48-linked polyubiquitin chains on the lysine 163 residue of SIGIRR. SIGIRR lysine 163 mutant (SIGIRRK163R) exhibited an extended half-life compared to SIGIRR wild type. Our data suggest that SIGIRR degradation is mediated by the ubiquitin-proteasome system and is critical for pulmonary inflammation. In the future study, identification of the ubiquitin E3 ligases and deubiquitinating enzymes, which are responsible for regulation of SIGIRR stability, might provide potential therapeutic targets for treating pulmonary inflammatory diseases.
P1666
Board Number: B682
Histone acetyltransferase CBP increases activation of SCF FBXL19 ubiquitin E3 ligase by acetylation and stabilization of FBXL19.
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Ubiquitin E3 ligases mediate ubiquitination and degradation of intracellular proteins. We have shown that a relatively new SCF (skp, cullin, f-box) E3 ligase subunit, FBXL19, targets cell membrane receptor and small GTPases for their ubiquitination and degradation, while regulation of its stability remains unclear. Here we show that the stability of FBXL19, is mediated by a balance of its ubiquitination and acetylation. FBXL19 is an unstable protein with a half-life around 2.8 h. FBXL19 is polyubiquitinated and a proteasome inhibitor MG132 prolongs FBXL19 stability, suggesting that FBXL19 degradation is mediated by the ubiquitin-proteasome system. Our study reveal that FBXL19 also can be acetylated and enhancing acetylation of FBXL19 by a histone deacetylase inhibitor TSA reduces ubiquitination of FBXL19. Acetylation-mimic FBXL19 mutant exhibits prolonged half-life. Further, we show that an acetyltransferase CBP mediates FBXL19 acetylation. Inhibition of CBP reduces FBXL19 stability, while it is increased in CBP-overexpressed cells. The data indicates that CBP-mediated acetylation reduces ubiquitination and stabilizes FBXL19. Furthermore, ectopically expressed FBXL19 reduces small GTPase Cdc42 protein levels, while the effect is reversed by inhibition of CBP, suggesting that CBP increases activation of SCF-FBXL19 E3 ligase through stabilization of FBXL19. Our study reveals a new molecular model for regulation of SCF E3 ligase activation by acetylation and stabilization of F-box protein.

P1667
Board Number: B683
Accelerated Senescence following DNA Damage upon Loss of a BRCA1 Associated Protein Brap is Mediated through Histone Ubiquitination and Destruction.
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One of the many outcomes of persistent DNA damage is cellular senescence, a condition that irreversibly arrests the cell cycle and leads to extensive chromatin alteration. While senescence can act as a barrier to carcinogenesis, it also elicits senescence-associated secretory phenotype (SASP) with increased expression of cytokines and proteases that cause inflammation and tissue degeneration. Proper control of cellular senescence would thus protect against cancer and aging associated diseases. However, the mechanism that links DNA damage to senescence remains poorly understood. Here we show that abrogating a BRCA1 associated protein BRAP/Brap can specifically direct cells with DNA damage to senescence. Fibroblasts and neural stem cells isolated from Brap knockout mice showed high levels of DNA damage and rapid growth arrest in culture without increasing apoptosis. In addition to increased SA-b-gal and SASP, Brap deficient cells underwent senescence with massive loss of nuclear contents including core histones and lamin B1. Consistent with our previous finding of Brap’s role as a ubiquitin E3 ligase that acts downstream of Ras-Erk signaling and targets Skp2 to maintain the G1/S CKIs, DNA damage in Brap deficient cells activated p53-p19 (ARF) but did not elevate p21. Furthermore, we found that Brap knockout mice showed significantly increased Brca1 and ubiquitinated histone H2A, a substrate for the Brca1 ubiquitin E3 ligase. The high level of mono- and poly-ubiquitination of H2A in
Brap mutants led to its proteasomal degradation and was correlated with significantly increased autophagy, leading to the loss of not only core histones but also lamin B1 and mitochondrial proteins. These findings suggest that upregulating histone H2A ubiquitination by increased BRCA1 activity in Brap deficient cells can serve as a specific mechanism for tumor suppression through directing cells with unreparable DNA damage to senescence. However, the inflammatory and neurodegenerative phenotype shown by Brap mutant brain also suggest that DNA damage and abnormal histone proteolysis underlie accelerated tissue aging.

P1668
Board Number: B684
E2 and E3 Ubiquitin Ligases in the ERAD Pathway Regulate Neural Receptors in C. elegans.
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Endoplasmic Reticulum-Associated Degradation (ERAD) is a ubiquitin-dependent process that maintains cellular homeostasis by targeting and removing misfolded proteins from the endoplasmic reticulum (ER). In C. elegans, we have identified homologs for two human ERAD E2 enzymes UBE2J1 and UBE2G2 (ubc-6 and ubc-7 in C. elegans) and three human ERAD E3 ubiquitin ligases HRD-1, GP78/AMFR, and MARCH-6 (hrd-1, hrdl-1, and marc-6 in C. elegans). The genes encoding the E3 ligases have been reported to cooperate in maintaining the overall health of C. elegans during ER stress (Sasagawa, et al., 2012), but the E2 ligases have not been previously described.

We find that two neural proteins, GLR-1 and ODR-10, are regulated by the putative ERAD E2 and E3 ligases. GLR-1 is a tetrameric glutamate receptor and ion channel that is expressed in a subset of interneurons; ODR-10 is a G-protein coupled receptor (GPCR) that is activated by the odorant molecule diacetyl and is expressed in a single pair of olfactory neurons (AWA cells). Compared to wild type controls, the abundance and localization of GLR-1::GFP and ODR-10::GFP change in C. elegans that harbor mutations in the genes encoding ERAD ligases. GLR-1- and ODR-10-dependent behaviors are also impaired in mutant animals. Mutating the three E3 ligases results in phenotypes of different severities, which suggests that those enzymes do not play redundant roles in substrate recognition and targeting in C. elegans. Similarly, mutation of the E2 proteins does not result in identical phenotypes, in correlation with recent work showing that UBC6 and UBC7 in yeast play unique roles in target recognition and ubiquitylation. Our results suggest that distinct cell types and substrates may be differentially regulated by specific ERAD pathways in C. elegans. Future work will focus on defining how the E3 and E2 pathways may differ in diverse neural cell types, and on exploring under what conditions ERAD mechanisms are required for normal neural protein regulation.
P1669
Board Number: B685
KBTBD11, a novel BTB-Kelch protein, is a negative regulator of osteoclastogenesis through controlling Cullin3-mediated ubiquitination.
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Osteoclasts are bone-resorbing giant cells that formed by the fusion of mononuclear monocyte/macrophage progenitor cells. Although osteoclast differentiation is regulated by various factors, the detailed mechanisms remain to elucidated. To investigate genes involved in the osteoclast differentiation, our research group has recently performed DNA microarray analysis of osteoclast differentiation showing that 1,363 were upregulated genes, and 881 were downregulated. Among the upregulated genes, we identified a novel gene, which is possibly implicated in an E3 ubiquitin ligase-mediated event, termed as Kelch repeat and BTB domain containing protein (KBTBD11). Despite the presence of KBTBD11 genes in mammalian genomes, there is no research reports about KBTBD11 at present. In this study, we investigated the role of KBTBD11 in osteoclastogenesis using mouse macrophage-like RAW-D cells by the method of gene knockdown using small interfering RNAs (siRNA) or gene overexpression systems. We found that expression of KBTBD11 increased during osteoclastogenesis. When we performed siRNA-mediated knockdown experiments, KBTBD11 knockdown enhanced osteoclast differentiation than control cells. Upon determining the expression levels of osteoclast marker genes using quantitative RT-PCR analysis, the expression levels of NFATc1, Src, cathepsin K, osteoclast stimulatory transmembrane protein (OC-Stamp), transmembrane 7 superfamily member 4 (DC-STAMP), cathepsin K, and matrix metalloproteinase 9 (MMP9) were all significantly higher in KBTBD11-knockdown osteoclasts than in control cells. On the contrary, KBTBD11 overexpression inhibited osteoclast differentiation. Also, the expression levels of the osteoclast differentiation marker genes in KBTBD11-overexpressing osteoclasts were all decreased compared with control cells. Concerning six major signaling pathways for osteoclast differentiation such as, nuclear factor of activated T cells cytoplasmic-1 (NFATc1), p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K)/Akt, and nuclear factor kappa B (NF-κB), western blot analysis revealed that KBTBD11 predominantly influenced NFATc1 signaling. Moreover, to determine whether KBTBD11 interacts with the E3 ubiquitin ligase Cullin3, immunoprecipitation experiments were performed. Consequently, we found that KBTBD11 specifically interacts with Cullin3. These results indicate that KBTBD11 negatively modulates osteoclast differentiation by interacting with Cullin3. It is likely that the KBTBD11-Cullin3 complex probably mediates ubiquitination of important factors for osteoclast differentiation through the proteasomal-dependent pathway.
P1670

Board Number: B686

Cullin-3 is required for normal skeletal muscle development.

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E3-ubiquitin ligases are protein complexes that are essential for the recognition and the degradation of specific substrates by the ubiquitin-proteasome pathway. Cullin-RING ligases represent the largest E3-ubiquitin ligase family in mammals. The Cullins constitute the backbone of the E3-ubiquitin ligase complex, and interact with specific substrate adaptors that are necessary for the recruitment of their individual substrates for degradation. While Cullin-RING ligase roles were intensively studied in the context of cancer, very little is known about their functions in muscle biology. Recently, several genes of the BTB-domain family (KBTBD13, KLHL40 and KLHL41) were found mutated in severe and early onset forms of Nemaline Myopathy (NM). Due to their BTB-domains, these proteins are thought to be substrate adaptors for Cullin-3. These findings argue in favor of important and yet uncharacterized roles for Cullin-3 mediated protein turnover for muscle development, maintenance and function.

We have shown the absolute necessity of Cullin E3-ligase activity for the induction of myogenesis, and revealed that treatment of myoblasts with MLN4924, an inhibitor of Cullin-RING ligase activity used in clinical trials for cancer therapy, has a dramatic effect on myoblast differentiation and muscle formation.

Using conditional knockout mice for Cullin-3 in skeletal muscles, we demonstrate the pivotal role of this protein for postnatal life. Indeed, our data reveal that absence of Cullin-3 leads to respiratory defects at birth, associated with a strong skeletal muscle atrophy and neuromuscular junction disorganization, mimicking severe cases of NM. Large-scale proteome analysis of embryonic skeletal muscles that lack Cullin-3 revealed an accumulation of several proteins, presumably representing novel substrates of this E3-ligase. Our results suggest that Cullin-3 mediated protein turnover is required for proper early skeletal muscle development. Future experiments will decipher the molecular mechanisms contributing to the severe myopathy observed in these animals.

P1671

Board Number: B687

The effects of YopJ on respiratory growth due to downregulation of a mitochondria ubiquitin protease in S.cerevisiae.

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YopJ is a virulence factor secreted from Yersinia that inhibits host immune responses and induces apoptosis. Learning more about how YopJ affects eukaryotic cells is crucial to understand how the Black Plague caused one of the worst pandemics in human history. The objective of our study was to examine if YopJ affected ubiquitin proteases in yeast cells. To examine this, we compared the expression of various ubiquitin proteases in both wild type cells and YopJ-expressing cells. We found that a mitochondria-associated ubiquitin protease had decreased level of expression in YopJ cells. To follow-up this result, we tested whether YopJ affected respiratory growth in yeast cells. Specifically, we compared the growth of cells with or without YopJ in the media, which use lactate as the sole carbon source. We found that the growth of YopJ-expressing cells on the lactate plate was significantly diminished when comparing it to wild type cells. Together, our results suggest that the ubiquitin protease that was
diminished in YopJ cells had a significant effect on respiratory growth in the cells and this enzyme may be a new target of YopJ.

P1672

Board Number: B688

Proteasome activity and protein oxidation levels in the skeletal muscles of cultivated rainbow trout.

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Proteasomes, calpains, and lysosomal cathepsins are the main proteolytic pathways in any eukaryotic cell. In fish, calpain-dependent reactions dominate in the hydrolysis of myofibrillar proteins while a contribution of ubiquitin-proteasome system (UPS) to total protein degradation recognized to be minor. However, UPS is a main path of protein quality control machinery utilizing abnormal proteins including oxidatively damaged ones. Oxidative damage of biomolecules, particularly oxidative post-translational modifications of proteins, affects cell signaling, behavior, and survival. Rainbow trout, Salmo gairdneri, is a typically cold-water species highly susceptible to hypoxia and environmentally-induced oxidative stress followed by high lethality and fish growth depression under rearing conditions. Antioxidant supplementation to fish diet presumably would stimulate fish growth and resistance to stress-inducing factors. In our study we used dihydroquercetin, a polyphenolic flavonoid compound produced from larch raw materials. Dihydroquercetin (Taxifolin, diquertin) widely exploring in medicine, cosmetology and veterinary practice (but not in fish farming yet) behaves as powerful antioxidant and free radical scavenger. To describe oxidative stress manifestation and a possible effect of dihydroquercetin on cultivated rainbow trout growth and viability we have compared (1) a control group of fish fed by a commercial diet and (2) an experimental group fed by commercial diet mixed with dihydroquercetin (50 mg per kg) up to three months. Protein reactive carbonyl products concentration as a marker of oxidative damage and cellular stress in fish has been measured using 2,4-dinitrophenylhydrazine-based assay. Chymotrypsin-like proteasome activity has been assayed in order to evaluate possible correlation between proteasome proteolytic capacity and protein carbonyl content as well as to prove an idea on ubiquitin-proteasome pathway contribution to oxidized protein pool utilization. In the control group, an increase in the protein carbonyl concentration in the muscles associated with proteasome activation has been noted. In the presence of dihydroquercetin, partial decrease in protein oxidation without stimulation of proteasome system has been detected. Significant effect of dihydroquercetin on protein oxidation has developed since a month of experimental diet consumption while decreased proteasome activity has been detected in antioxidant-fed group throughout the experiment. Besides, fish fed by the antioxidant has been characterized by substantial increase in individual growth rate. This work was supported by the Russian Science Foundation, project no. 17-74-20098.

P1673

Board Number: B689

Hyperphosphorylation repurposes the CRL4B E3 ligase to coordinate mitotic progression.

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Ubiquitylation depends on an enzyme cascade that allows transfer of ubiquitin to a target protein by an E3 ligase. The E3 ligase family Cullin-RING ligases (CRLs) include Cullin-4A (CUL4A) and CUL4B which
together regulate multiple cellular events, in particular chromatin-associated processes such as chromatin remodeling and DNA repair. However, CUL4B mutations result in syndromic X-linked intellectual disability (XLID), never reported for CUL4A, which denotes a non-overlapping function. Through cell cycle analysis in HeLa cells, we discovered that CUL4B is important for mitotic entry. CUL4B is subject to mitotic phosphorylation in its unique N-terminal sequence, mediated by CDK1, PLK1 and possibly other kinases and consequently no longer localizes to the chromatin. Interestingly, proliferation assays and live-cell imaging experiments revealed that the overexpression of the phosphomimetic CUL4B mutant is lethal in HeLa cells due to aberrant metaphase progression and mitotic exit, with a subset of cells failing cytokinesis. While further phenotypic and mechanistic characterization is under investigation, our data suggests that CUL4B phosphorylation repurposes this E3 ligase for a non-chromatin function that is both important for the early mitotic stages and must be abolished by mitotic exit. Our study establishes a clear regulatory distinction between CUL4B and CUL4A, with functional implications, which may aid in clarifying why CUL4A cannot compensate for CUL4B loss in human XLID patients.

P1674
Board Number: B690
Deciphering the Ubiquitin Code with Poly-Ubiquitin Chain Selective Affinity Matrices.
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Post-translational modification of proteins by ubiquitin (Ub) is a versatile process, highly dynamic, and involved in nearly all aspects of biological functions in eukaryotes. Dysregulation of ubiquitylation has been implicated in plethora of pathological conditions such as developmental abnormalities, neurodegenerative diseases, and cancers. Ub is attached, via isopeptide bonds, to lysine residues in the target protein or to another Ub to form poly-Ub chains. The reversibility, heterogeneity, and diversity of these modifications combined with the lack of suitable tools have made it difficult to properly isolate and characterize poly-ubiquitylated cellular proteins. However, in 2009, a novel technology called TUBEs (Tandem-repeated Ub-Binding Entities) was developed. TUBEs have revolutionized the Ub field by allowing poly-ubiquitylated proteins to be enriched/purified from cellular extracts. Development of TUBEs that selectively bind K48-, K63-, and M1-linked poly-ubiquitylated proteins has helped understand the role of modified proteins in cell physiology. However, the roles of other Ub-linkages remain obscured mainly due to the lack of tools that specifically recognize them. To overcome this, we recently developed a novel microarray platform that facilitates the identification of unique Ub binding domains (UBDs) for rare Ub-linkages. This array contains GST-tagged versions of 140 known and predicted UBDs from over 8 different Ub interacting families. Our initial screen with mono-Ub as well as K48-linked diUb revealed several unique binders. For example, Ub-binding motif (UBM) of REV1L bound uniquely to mono-Ub. On the other hand, Ub-associated (UBA)-like domain of NSFL1C bound preferably to K48-linked diUb. Moreover, UBA domains of RAD23A and RAD23B interacted uniquely with K48-linked diUb, which is concordant with the published data. We also identified several proteins that non-selectively bind mono-Ub and K48-linked diUb, such as the UBA domain of NBR1. The complete array results obtained for mono-Ub and K48-linked diUb will be presented in the poster. We are currently working on to screen the array against other Ub-linkages and we will present data in the poster. To conclude, the array allowed us to identify unique binding partners for mono-Ub and K48-linked diUb, and we strongly believe that our future screens with other Ub-linkages will identify several selective binding partners for rare Ub-linkages. Domains that display selective binding specificity will be utilized to construct linkage-specific TUBEs. The expansion of the TUBE toolkit to include recombinant proteins that can discriminate
between each of the Ub linkages would dramatically accelerate the pace of discovery in this important area of biology.

P1675
Board Number: B691
OTUB1 regulation of E2 ubiquitin conjugating enzyme levels in vivo.
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Ubiquitin signaling plays an essential role in nearly all aspects of biology. Substrate ubiquitination is tightly regulated temporally and in response to different stimuli through the opposing actions of E2/E3 enzymes, which attach mono and polyubiquitin to substrate proteins, and by deubiquitinating enzymes (DUBs), which remove ubiquitin modifications from proteins and cleave polyubiquitin chains. Imbalanced ubiquitin signaling lies at the heart of many cancers and genetic disorders. OTUB1 is an OTU-class DUB that cleaves K48-linked polyubiquitin chains and is one of the most abundant DUBs in human cells. In addition to this deubiquitinating activity, OTUB1 also inhibits the activity of a subset of E2 ubiquitin conjugating enzymes by binding to the charged E2~Ub thioester intermediate and inhibiting ubiquitin transfer both in vitro and in vivo. When the E2 partners of OTUB1 are not charged with ubiquitin, the E2 enzymes stimulate K48 polyubiquitin cleavage by OTUB1 in vitro, although the physiological significance of this is unknown. In order to study the effects of OTUB1-E2 pairs in cells, we searched for potential OTUB1 substrates in OTUB1 knockout MEF cells using tandem mass tag proteomics. Surprisingly, we saw lower protein levels for some E2 conjugating enzymes when OTUB1 was knocked out, specifically isoforms of the Ube2E family, UbcH10, Ube2S, and UbcHSC. We confirmed this result by Western blotting in the OTUB1 knockout MEFs as well as in U2OS cells with a CRISPR knockout or siRNA knockdown. OTUB1 does not affect E2 expression levels by any transcriptional mechanism because RT-PCR results show similar mRNA levels for these E2s in the siRNA knockdown and CRISPR knockout cells. We followed protein levels for Ube2E isoforms in a cycloheximide chase experiment and found that OTUB1 may be affecting the stability of Ube2E isoforms. We are currently exploring whether this is due to OTUB1’s catalytic activity, or through non-catalytic inhibition of Ube2E isoforms. Interestingly, the Ube2E isoform that is most rapidly turned over in cycloheximide chase experiments follows a cell cycle-dependent expression pattern in thymidine release time course experiments as well as by immunofluorescence. We see that OTUB1 knockout causes a delay of entry into mitosis in synchronized HeLa cells both by Western blotting for cyclin B and by live cell imaging. We discuss these findings in light of the known interaction of OTUB1 with E2 enzymes in vivo.

Autophagy

P1676
Board Number: B692
ATG5 deletion results in organelle dysfunction and confers enhanced cytotoxicity following the induction of autophagy.
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The advent of CRISPR-cas9 has revolutionized the ease with which precise manipulations, either insertions or deletions, can be made within a cell’s genome. These approaches are already creating
disease models as well as controls for various pathways and processes. We used one such process, autophagy, and created the gold standard ATG5 knock-out model for autophagy using CRISPR-CAS9. Following deletion, we confirmed successful editing at the protein level through western blot analysis of ATG5 expression levels. Furthermore, we used cell-based assays to show that the ATG5 knock-out cells were deficient in macroautophagy, confirming that no redundant or response mechanism was able to compensate for ATG5 loss. Given the importance of macroautophagy to cellular homeostasis, we sought to understand the dynamic effects of ameliorating this pathway by performing extensive phenotypic profiling in this model using automated, high-throughput imaging. Using cell-based, phenotypic assays, we show that ATG5 knock-out cells display enhanced susceptibility to cytotoxic stressors involved in mTOR signaling and the unfolded protein response. However, general cytotoxic stressors or those that target mitochondrial function or the ubiquitin-proteasome pathway were no more cytotoxic to ATG5 knock-out cells than to wild type cells. We also show perturbation to organelle structure and function, notably mitochondria and lysosomes, following ATG5 knock-out. The above observations are not a function of perturbed general cellular function as analysis of other cellular processes such as proliferation, protein synthesis, reactive oxygen species production, and endocytosis showed no difference between wild type and ATG5 knock-out cells. We present the use of cell-based phenotypic assays as a to probe down-stream effects of gene knockouts using CRISPR-cas9.

P1677

Board Number: B693

Autophagosomal closure is mediated by the ESCRT machinery.

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Autophagy is an intracellular degradation process that delivers cytoplasmic contents to the lysosome. During the autophagic process, a single-membraned sac termed the isolation membrane elongates, bend, and engulfs a part of the cytoplasm. Finally, the edge of the isolation membrane is closed to form a double-membraned structure, the autophagosome. The autophagosome fuses with lysosomes to degrade its contents. Although many molecules such as ATG proteins are involved in this process, the molecular mechanism of the closure of the autophagosomal edge remains to be elucidated.

In this study, in order to identify novel autophagy-related factors, a genome-wide screen was performed using the CRISPR-Cas9 system and the novel autophagic flux probe GFP-LC3-RFP. As a result, in addition to many conventional ATGs, VPS37A was identified as one of the positive regulators of autophagy. VPS37A is a member of the ESCRT (endosomal sorting complex required for transport) machinery, which is involved in membrane scission processes such as the formation of multivesicular bodies, viral budding, and cytokinesis. Because the autophagosomal closure is also a scission process, we hypothesized that it is mediated by the ESCRT machinery. Our protease protection assays suggested that autophagosomes are not closed when VPS37A as well as other ESCRT proteins were depleted. The endocytosis and lysosomal function may be partially affected but almost normal in VPS37A knockout cells. Furthermore, live-cell imaging showed that ESCRT proteins were recruited to the edge of autophagosomes immediately before spherical change, which is likely the timing of the closure of the autophagosome. In conclusion, we have identified VPS37A as a novel autophagy-related factor by a non-biased forward genetic screen. Our further analysis suggests that the autophagosomal edge is closed by the ESCRT-mediated membrane fission.
P1678

Board Number: B694
Autophagy controls steroid hormone synthesis and developmental timing by regulating cholesterol trafficking in Drosophila melanogaster.
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Autophagy is a common cellular response to nutrient deficiency in a wide spectrum of cell and tissue types. Previous studies show that autophagy occurs in various endocrine organs, but no straight evidence demonstrating the function of autophagy in steroid hormone producing cells has been provided so far. In development of fruit fly Drosophila melanogaster, the timing of metamorphosis is directly controlled by steroid hormone ecdysone (E) which is predominantly produced in larva endocrine organ prothoracic gland (PG). It is well known that both E synthesis in PG and metamorphic timing of animals are finely coordinated with nutritional condition during larva stage, so in this study we try to uncover the potential role that autophagy performs in the PG cells. Firstly, we monitor autophagy in PG under fed and starvation conditions. Autophagy is significantly induced by starvation treatment during early 3rd larva instar (L3) stage, which correlates with the time window that starvation arrests larval development before metamorphosis. Suppression of autophagy results in elevated E level and precocious onset of metamorphosis during starvation, while forced induction of autophagy causes decreased E content and developmental delay/arrest under well fed condition. These results demonstrate that autophagy suppresses E synthesis in PG and consequently blocks metamorphosis. We then study the mechanism on how autophagy attenuates E production and surprisingly find that autophagy regulates cholesterol metabolism in PG cells. In an ex vivo incubation assay we observe that the autophagosomes in PG interact with cholesterol containing lipid droplets. In vivo autophagy induction results in decreased number of lipid droplet, while cholesterol feeding rescues the autophagy induced developmental defects. When testing the interaction between autophagy and endolysosomal pathway, we find that the autophagosomes overlap with Rab7-positive but not LAMP-positive vesicles, suggesting a non-canonical autophagic flux. Further, using live imaging approach we see dynamic formation and mobilization of tubule-like structures marked by Atg8, which to our knowledge is also rarely observed in previous studies. In all, our findings uncover a novel type of autophagic process that controls cholesterol trafficking and hormone synthesis in Drosophila PG cells, which may also represent a common function that autophagy exerts in steroid hormone producing cells.
P1679  
**Board Number: B695**  
The Autophagy Conjugation Machinery Specifies The Loading of RNA-Binding Proteins Into Extracellular Microvesicles.  
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Autophagy is an autodegrative pathway in which cytoplasmic material is sequestered into double-membrane vesicles and delivered to the lysosome for breakdown. Predominantly viewed as a catabolic mechanism, we are beginning to appreciate that the autophagy pathway also promotes unconventional secretion of proteins lacking N-terminal signal sequences. However, the underlying mechanisms and full repertoire of proteins released via autophagy-dependent secretion remains unknown. To further address the role of autophagy in secretion, we developed a novel strategy using proximity-specific biotinylation to label proteins that engage the autophagy regulator MAP1LC3B (LC3), and subsequently, are secreted outside of the cell. Quantitative proteomic analysis of the LC3-labelled secretome revealed a highly interconnected network enriched in RNA-binding proteins (RBPs) and the cargoes of extracellular microvesicles (EMVs, exosomes). Indicating that specific proteins are loaded into EMVs via secretory autophagy. Focusing on a number of RBP candidates for further mechanistic study, including heterogeneous nuclear ribonucleoprotein K (HNRNPK) and scaffold-attachment factor B (SAFB), we demonstrate these proteins biochemically interact with LC3 and are secreted within EMVs highly enriched with lipidated LC3 (LC3-II). Secretion of LC3-II and LC3-binding RBPs requires essential components of the autophagy conjugation machinery and neutral sphingomyelinase 2 (nSMase2)-mediated ceramide production. Collectively, our data demonstrates a new role for the autophagy pathway in specifying RBPs that are packaged into EMVs and highlights a novel mechanism by which autophagy controls the secretion of RBPs and RNAs outside of the cell.

P1680  
**Board Number: B696**  
Stress-Induced Cdk5 Activity Enhances Cytoprotective Basal Autophagy by Phosphorylating Acinus at Serine⁴³⁷.  
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In the defense against the long-term consequences of accumulating protein aggregates and dysfunctional organelles, neurons rely on basal autophagy. Little is known, however, how the levels of basal, starvation-independent autophagy are adjusted to the specific challenges that neurons face. Here, we explore the role of the Cdk5 kinase in that process. Cdk5, a member of the family of cycling-dependent kinases, has important functions in the post-mitotic maintenance of neuronal health. Cdk5 dysregulation has been linked to multiple neurological diseases, but the various mechanisms by which Cdk5 inhibits and promotes neurodegeneration are still poorly understood. We show that Cdk5 regulates basal autophagy, a key mechanism suppressing neurodegeneration. In a targeted screen in Drosophila, Cdk5 genetically interacted with Acinus (Acn), a primarily nuclear protein, which promotes starvation-independent, basal autophagy. Loss of Cdk5, or its required cofactor p35, reduces S437-Acn phosphorylation, whereas Cdk5 gain-of-function increases pS437-Acn levels. The phospho-mimetic
S437D mutation stabilizes Acn and promotes basal autophagy. In p35 mutant flies, basal autophagy and lifespan are reduced, but restored to near wild-type levels in the presence of activated Acn\textsuperscript{S437D}. Expression of aggregation-prone polyQ-containing proteins or the Amyloid-beta 42 peptide, but not alpha-Synuclein, enhance Cdk5-dependent phosphorylation of S437-Acn. Our data indicate that Cdk5 is required to maintain and adjust the protective role of basal autophagy in the initial responses to a subset of neurodegenerative challenges.

P1681
Board Number: B697
RXR-PPAR-delta agonist therapy achieves neuroprotection by autophagic induction.
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Formation of aggregates, a common feature of neurodegenerative disorders, is indicative of insufficient protection provided by the protein quality control system. Improvement of protein quality control has therefore become a strategy to developing therapeutics to ameliorate pathological symptoms and impede neurodegeneration. Recently, our group tested the neuroprotective potential of the Peroxisome-Proliferator-Activated-Receptor-delta (PPARd) agonist KD3010. Preclinical trials of KD3010 resulted in rescue of behavioral symptoms, metabolic deficits, and mitochondrial abnormalities in an aggressive Huntington’s Disease (HD) mouse model with a truncated N-terminal huntingtin (htt) fragment containing a 82 CAG repeat (N171-82Q). Neuropathology indicated reduction of polyQ-expanded htt aggregates in striatal medium spiny neurons, suggesting a role for PPARd in proteostasis. To directly evaluate the effect of PPARd activation on intracellular waste management, we assayed autophagy flux in Neuro2a cells subjected to PPARd shRNA knock-down or PPARd agonist activation, and noted a significant reduction in autophagy flux upon PPARd knock-down, and a significant increase in autophagy flux with PPARd agonist activation. PPARd transcriptional activity is dependent on dimerization with the Retinoic X Receptor (RXR). A preclinical trial of bexarotene in the N171-82Q model gave rise to similar results to those seen with KD3010. Here we evaluated the potential for bexarotene, an FDA approved agonist of RXR, to promote PPARd-RXR dimerization and subsequent activation of autophagy. The ability of bexarotene to induce expression of PPARd target genes was confirmed by qRT-PCR. Repeating the autophagy flux experiments, we found that bexarotene increased autophagy flux similar to that seen with PPARd-specific activation. Spautin-1 inhibition of (macro)autophagy significantly blunted this effect as did ATG7 knockdown, while treatment with lactacystin, a Ubiquitin Proteasome Pathway inhibitor, did not. To determine if PPAR required TFEB to activate autophagy, we measured autophagy flux in HeLa cells lacking TFEB. We detected an increase in autophagy flux upon PPARd agonist treatment in TFEB KO cells comparable to the autophagy flux increase in WT cells, indicating little role for TFEB in PPARd-mediated autophagy activation. Finally, to determine if bexarotene amelioration of mutant htt protein aggregation requires PPARd, we quantified htt-Q104 aggregation in Neuro2a cells treated with bexarotene +/- a PPARd inhibitor. Indeed, PPARd inhibition abrogated bexarotene amelioration of htt protein aggregation. These findings indicate that PPARd activation achieves neuroprotection at least in part by improving proteostasis through autophagy induction.
P1682

Board Number: B698

The Unfolded Protein Response Maintains Lipid Homeostasis by Selective Autophagy during Lipid Perturbation-Induced ER Stress.

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Metabolic disorders such as obesity and nonalcoholic fatty liver disease (NAFLD) are emerging disorders that affect the global population. One facet of the disorders is attributed to the disturbance of membrane lipid homeostasis. Perturbation of endoplasmic reticulum (ER) homeostasis through changes in membrane phospholipid composition results in activation of the unfolded protein response (UPR) and causes dramatic translational and transcriptional changes in cell. To restore cellular homeostasis, the three highly conserved UPR transducers ATF6, IRE1, and PERK mediate cellular processes upon ER stress. The roles of the UPR in proteostatic stress caused by the accumulation of misfolded protein is well understood but lipid perturbation-induced UPR remains elusive. We found that genetically attenuated PC synthesis in C. elegans causes lipid droplets accumulation if not for the intervention of the UPR program. Transcriptional profiling of lipid perturbation-induced ER stress animals shows a unique subset of genes modulated in an UPR-dependent manner that are unaffected by proteostatic stress. Among these, we identified IRE1-modulated autophagy genes that trigger liberation of free fatty acids from excess lipid droplets suggesting a stress release mechanism by which free fatty acids are rechanneling to restore lipid homeostasis. Considering the important role of lipid homeostasis and how its impairment contributes to the pathologies in metabolic diseases, our data uncovers the indispensable role of a fully functional UPR program in regulating lipid homeostasis in the face of chronic ER stress.

P1683

Board Number: B699

Ribosome profiling reveals that autophagy impacts DNA damage repair, cell cycle progression and centrosome maintenance through protein translation regulation.

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Autophagy is a cellular recycling system that degrades large proteins and organelles by delivery to the lysosome. Protein translation is tightly tuned to the metabolic state of the cell by sensing lysosomal amino acid levels and signaling through mTORC1. During metabolic stress, protein translation is abrogated while autophagy is concurrently induced. Accordingly, autophagy is proposed as a principal regulator of the protein translational landscape, especially during starvation, yet to date evidence supporting this hypothesis is lacking. Here, we utilize ribosome profiling to dissect how the autophagy pathway impacts translation, both at baseline and in response to starvation. Although the prevailing viewpoint is that the recycling functions associated with autophagy sustain de novo protein translation, we discovered that in both nutrient replete conditions and in nutrient starvation, global translation rates as well as intracellular levels of most amino acids were intact in autophagy deficient cells. Instead, we observed that specific proteins were translated in an autophagy dependent manner. To identify these products of autophagy-dependent translation, we employed ribosome profiling in autophagy competent vs. deficient fibroblasts, achieved via genetic deletion of the key autophagy regulator ATG12, in both fed and starved conditions. Using biochemical approaches, we
validated that autophagy controls the translation of multiple targets. Most strikingly, we found that basal autophagy is important for the translation of a group of targets involved in centrosome clustering, DNA repair and cell cycle control, including BRCA2, both in vitro and in vivo. While increased DNA damage in autophagy deficient cells has been largely attributed to an increase in defective mitochondria and production of reactive oxygen species, our results reveal translational control as another critical pathway through which autophagy regulates DNA damage levels. We are working to identify how autophagy controls this program of protein translation, hypothesizing that it acts through targeted degradation or sequestration of key RNA binding proteins. The increase in baseline DNA damage and decreased BRCA2 levels means that the autophagy impaired cells have increased sensitivity to various cytotoxic reagents, including PARP inhibitors. These data broach the hypothesis that tumors with high rates of chromosome instability may rely more heavily on autophagy-dependent translation of proteins. Overall, these results expand the functions of autophagy beyond its traditional role as an intracellular degradation pathway, and reveal new roles for autophagy an anabolic pathway that instructs that translation of specific proteins in cell cycle control.

P1684
Board Number: B700
Herbal medicine for resolution of cancers.
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Cancers such as hepatocellular carcinoma and pancreatic cancer remain the leading cause of mortality with limited therapeutic targets. Herbal medicines have been considered for treating or preventing cancers but require systemic scientific evidence. We conduct in exploration of traditional Chinese herbal medicines, as an attempt to discover new candidates and mechanisms of phytomedicines for treating or preventing cancers. Several in vitro and in vivo gene- and cell-based assays, comparative transcriptional analyses, as well as hepatocellular carcinoma and pancreatic cancer xenografts are employed to validate the pharmacological effects and the underlying mechanisms for the identified bioactive phytomedicines. In addition, how phytomedicines modulate proteostasis to achieve their preventive or therapeutic effects against cancer cell growth are investigated. Furthermore, we identify the molecule targets of bioactive compounds i.e. gallic acid, a secondary metabolite present in most plants, by chemical synthesis in combined with proteomics analyses. Nevertheless, we also investigate phytomedicines, alone or in combination, in sensitizing the chemotherapeutic drugs efficacy in tumor-bearing mice, as a means to evaluate the potential of using natural phytomedicines or compounds in complementary medicine in cancers.
P1685
Board Number: B701
Filamin and Valosin Containing Protein (VCP) interaction in Inclusion Body Myositis.
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Sporadic Inclusion Body Myositis (IBM) is the most common acquired myopathy in individuals over the age of 50, and is characterized by slowly progressive weakness in both proximal and distal muscles. One pressing question in the field is whether IBM is primarily an autoimmune disease or a degenerative disease with secondary inflammation. The association with aging, lack of response to immunotherapy, and presence of pathological features seen in neurodegenerative disease (e.g. protein aggregation) suggest that degeneration may drive disease progression. Furthermore, mutations in valosin-containing protein (VCP) cause inclusion body myopathy with Paget’s disease and frontotemporal dementia (IBMPFD), and recent evidence suggests that VCP variants may cause or be risk factors for developing IBM. However, there is an increased association of IBM with specific HLA haplotypes and other autoimmune diseases. Thus, the relationship between inflammation and protein aggregation in IBM is poorly understood. Recently, we performed whole exome sequencing on a large family with several members that meet diagnostic criteria for sporadic IBM (i.e. autoimmune invasion of CD8+ T cells and typical clinical features). In addition to identifying a known pathogenic VCP variant (R159C), we found a novel variant in the Filamin C (FLNC) gene (F2616S) that cosegregates with disease. Since FLNC mutations are associated with myofibrillar myopathy, we hypothesize that digenic inheritance of VCP and FLNC mutations causes IBM in this family.
To determine whether Filamin C may play a role in IBM pathogenesis, we first investigated FLNC localization in IBM muscle tissue and find that filamin C is mislocalized to large aggregates within human IBM muscle fibers. We next looked for a genetic interaction between FLNC and VCP using a previously characterized Drosophila IBMPFD model (Ritson et al, J Neurosci 2010). Indeed, RNAi-mediated knockdown of Drosophila filamin (cheerio) suppresses muscle degeneration and impaired climbing performance of IBMPFD flies. These data suggest that FLNC may play a role in IBM pathogenesis, potentially through a recently described role in autophagy.

P1686
Board Number: B702
Compartment-specific regulation of neuronal autophagy during homeostasis and stress.
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Autophagy is an essential degradative pathway that maintains neuronal homeostasis and prevents axon degeneration, but neuron-specific mechanisms are poorly understood. Here, we use live-cell imaging in mouse hippocampal neurons to establish the compartment-specific mechanisms of autophagy under basal conditions, and during stress induced by nutrient deprivation. We find that at steady state, axonal autophagy is a vectorial process that delivers cargo from the distal axon to the soma. The soma, however, contains multiple populations of autophagosomes at different maturation states, including
input received from the axon combined with locally generated autophagosomes. Once in the soma, autophagosomes are confined within the somatodendritic domain, which likely facilitates cargo degradation by promoting fusion with proteolytically-active lysosomes that are enriched in this region. Surprisingly, canonical autophagy inducers such as starvation or mTOR-inhibition that robustly activate autophagy in other cell types (e.g. hepatocytes and HeLa cells), do not markedly upregulate autophagy in neurons, in either the axonal or somatodendritic compartments. Interestingly, we find that autophagy in glial cells is regulated differently as compared with neurons, suggesting alternative mechanisms of coordinating autophagy in the brain. Together, these observations suggest that the primary physiological function of autophagy in neurons, unlike in other cell types, may not be to mobilize amino acids and other biosynthetic building blocks in response to nutrient deprivation. Rather, constitutive autophagy in neurons may function to maintain cellular homeostasis and regulate the quality of the neuronal proteome by balancing synthesis and degradation, especially within distal axonal processes far removed from the soma.

P1687
Board Number: B703
Comparison of autophagy and mTOR pathways in mouse embryonic stem cell, lung cancer and somatic fibroblast cell lines in molecular analysis base.
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Regulations during the process of the embryonic development have always been appealing. Since the discovery of cancer stem cells, which are particularly involved in cancer development and recurrence, embryonic stem cells and regulatory mechanisms as well as cancer cells and cancer stem cell control mechanisms have become fundamental research. Aging and aging-related diseases also constitute a major part of today's health problems. Studies that centers on aging related to mTOR and autophagy are available and current research topic in the literature. Autophagy is one of the main mechanisms in eukaryotic cells, for the removal of damaged organelles, macromolecules and the recycling of aminoacids, and with aging, autophagic degradation also reduces resulting with accumulation of damage. In recent years, there have been many studies on how the inhibition of especially mTORC1 is associated with aging. mTORC1 inhibition has both positive and negative effects on age-related diseases. At the same time, it is believed that the reduction of stem cell functions has a role in the pathology of aging and age-related diseases. There is evidence that mTORC1 inhibition can protect stem cell function in many tissues, and even restore this decreased function. Understanding the similarities and differences between somatic cells and cancer cells, which are prospective forms of embryonic stem cells divisions and determinations, led to the rise of medicinal and pharmaceutical sciences. For this purpose, mouse embryonic stem cells (mESC), mouse skin fibroblast cells (MSF) and mouse lung squamous cancer cells (SqLCC) were grown in vitro. We examined autophagy and mTOR related gene expressions; compared them between each groups and support the results with immunofluorescence staining. The study found that mTOR and autophagic regulators are particularly active in embryonic stem cells and generally silent in somatic cells and showing variety in cancer cells. Cellular stabilization mechanisms and damage removal pathways were particularly active in mESCs. Optimum intracellular balance may be in cooperation with protection of embryonic development and preservation of stem cell function. These
results suggest that the normal functioning of the mechanisms of embryonic stem cell regulation results in the formation of somatic tissues whereas that these cells may be the causative agents of cancer as a result of any deterioration.

P1688
Board Number: B704
Systematic analysis of human cells lacking ATG8 proteins uncovers roles for GABARAPs and the CC21/MON1 regulator C18orf8/RMC1 in macro and selective autophagic flux.
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Selective and macro autophagy sequester specific organelles/substrates or bulk cytoplasm, respectively, inside autophagosomes as cargo for delivery to lysosomes. The mammalian ATG8 orthologues (MAP1LC3A/B/C and GABARAP/L1/L2) are ubiquitin (UB)-like proteins conjugated to the autophagosome membrane and are thought to facilitate cargo receptor recruitment, vesicle maturation, and lysosomal fusion. To elucidate the molecular functions of the ATG8 proteins, we engineered cells lacking genes for each subfamily as well as all six mammalian ATG8s. Loss of GABARAPs alone attenuates autophagic flux basally and in response to macro or selective autophagic stimuli including PARKIN-dependent mitophagy, and cells lacking all ATG8 proteins accumulate cytoplasmic UB aggregates, which are resolved following ectopic expression of individual GABARAPs. Autophagosomes from cells lacking GABARAPs exhibit reduced lysosomal content by quantitative proteomics, consistent with fusion defects, but also accumulate regulators of late endosome (LE)/autophagosome maturation. Through interaction proteomics of proteins accumulating in GABARAP/L1/L2-deficient cells, we identified C18orf8/RMC1 as a new subunit of the CC21-MON1 RAB7 guanine exchange factor (GEF) that positively regulates RAB7 recruitment to LE/autophagosomes. This work defines unique roles for GABARAP and LC3 subfamilies in macro and selective autophagy and demonstrates how analysis of autophagic machinery in the absence of flux can identify new regulatory circuits.

P1689
Board Number: B705
Microtubule-Associated Protein 1 Light Chain 3B (LC3B) is Necessary to Maintain Lipid Homeostasis in the Retinal Pigment Epithelium.
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Objective: Microtubule-associated protein 1 light chain 3 (LC3) is an essential component of selective autophagy including LC3-associated phagocytosis (LAP), a hybrid degradation pathway utilizing components of canonical autophagy and phagocytosis. These autophagy associated processes play an important role in homeostasis of phagocytic cells such as retinal pigment epithelium (RPE) cell that are tasked with a daily burden of degrading lipid rich outer segments from the photoreceptors. LC3 exists as three isoforms in human (LC3A, B, and C), with two of these (LC3A and B) in mouse. Here, we tested the hypothesis that LC3B plays a key role in maintaining lipid homeostasis in the RPE. Methods: LC3BKO and wild-type mice ranging from 2 month to >2 years were used. LC3 isoform expression was analyzed by RT-PCR and immunoblotting. Biopotenig Spectral Domain Optical Coherence Tomography at 840nm was used to image retina. 4-hydroxynonenal (HNE) lipid oxidation adducts were evaluated by ELISA and immunofluorescence. Fixed frozen retinal sections were stained for neutral lipids with Bodipy 493/503,
or immunostained for various antibodies followed by confocal imaging. Lipid turnover as reflected in, β-Hydroxybutyrate (β-HB) released from mouse RPE explants was measured using LiquiColor reagent kit (StanBio). Results: Quantitative RT-PCR experiments showed similar levels of LC3A and LC3B transcripts in the WT mouse RPE. In the LC3B KO, there was no detectable LC3B transcript and the level of LC3A transcript was similar to that in the WT suggesting lack of potential compensatory upregulation in the absence of LC3B. There was an elevation in phagosome numbers for up to 11 h after light onset in the LC3BKO RPE as compared to the a sharp peak within one hour of light onset followed by a decline in the WT. The lipid turn-over, as inferred from mitochondrial ketogenesis was also altered: RPE explants from WT animals show a peak of β-HB at 2 hours after light onset, whereas in LC3BKO, this peak was delayed by 4 hours relative to the WT. Consistent with this, there appeared to be an increase in neutral lipid staining and a decrease in free fatty acids. In addition, there was an accumulation of lipid peroxidation products (HNE-adducts) in the LC3BKO, accompanied by an increase in Iba1 immuno-reactive cells in the sub-retinal space in the LC3B KO mice. The LCBKO RPE also appeared hypertrophic with multinucleated cells in several areas especially in the older mice. Conclusions: Our study provides evidence for a critical role played by LC3B in lipid homeostasis, as lack of LC3B causes defective phagocytosis, leading to metabolic imbalance, lipid deposition, pro-inflammatory microenvironment, and RPE hypertrophy.

P1690
Board Number: B706
Dhh1 regulates autophagy protein expression under long time starvation.
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Autophagy is an important degradation system that is required for cellular homeostasis in eukaryotes. Fine regulation of autophagy on both transcriptional and translational level is essential to maintain proper autophagy level in cells. Using yeast as a model system, we investigate the role of Dhh1, a cytoplasmic helicase, in the translational control of autophagy proteins (Atg proteins) under long-time starvation. In contrast to function as a post-transcriptional repressor in the early autophagy, Dhh1 serves as a positive regulator in long time starvation. Several Atg proteins showed decreased protein level in the dhh1 mutant under long-time starvation while the mRNA levels remain similar to wild type. As a result, Autophagy level is decreased in the dhh1 mutant during long time starvation. More evidence indicates Dhh1 regulates autophagy via interaction with the translational machinery. Our study reveals a novel role of Dhh1 in regulating autophagy, providing details of translational control of autophagy.

P1691
Board Number: B707
Regulation of autophagy through post-translational modifications.
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Macroautophagy/autophagy is primarily a self-eating process that recycles cytosolic components such as misfolded or aggregated proteins and dysfunctional organelles for homeostasis and survival in unfavorable conditions. This highly conserved and constitutive pathway has to be tightly regulated; either too much or too little autophagy can be detrimental. Dysregulation of this pathway is related to various diseases that include neurodegeneration, cancer and infection, as well as aging-related disorders. Autophagy is stringently regulated at different levels: Transcriptionally, post-transcriptionally,
translationally and post-translationally. A thorough understanding of the mechanisms involved is crucial to allow the manipulation of autophagy for the treatment of diseases. Although 41 autophagy-related (ATG) genes have been identified, we have a limited understanding of the complex network of regulatory factors that control this process. Post-translational modifications (PTMs) represent a subset of regulatory mechanisms that are critical for modulating autophagy in order to adapt to different types of environmental stress. Recent studies have reported a function of the CUL3-KLHL20 ubiquitin ligase in feedback regulation, leading to the downregulation of autophagy through the degradation of the ULK1 and PIK3C3/VPS34 complexes. Based on a large-scale analysis predicting that yeast Atg9 is ubiquitinated, we studied the regulation of Atg9 through PTM. Atg9 is the only transmembrane protein in the core autophagy machinery, which is absolutely required for autophagosome biogenesis and autophagy activity. Unlike other Atg proteins, Atg9 has a distinctive feature with regard to its subcellular localization: This protein travels between peripheral sites and the phagophore assembly site (PAS) where the autophagosome is formed, presumably delivering membranes from different donors to the PAS for autophagosome biogenesis. In this study, we show that Atg9 is ubiquitinated and targeted for degradation in a proteasome-dependent manner during nutrient-rich conditions, therefore limiting autophagy to a basal level. However, when cells are nutritionally deprived, autophagy is highly induced, necessitating an increase in the amount of Atg9. During these conditions, we show that the proteasome-dependent reduction of Atg9 protein levels is reduced. Thus, the post-translational ubiquitination of Atg9 provides an additional mechanism that allows cells to maintain appropriate levels of autophagy, and to rapidly respond and adapt to environmental stresses.

**P1692**
**Board Number: B708**

**Natural genetic variation modifies polyglutamine aggregation via an imbalance in autophagy.**

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Many late-onset neurodegenerative diseases, such as Huntington’s disease (HD), are caused by aggregation of misfolded proteins. Individuals stricken with these diseases succumb to the detrimental symptoms associated with them. Discovering the cellular and genetic pathways that are protective against protein aggregation will improve our understanding of potential treatments for these diseases. Huntington’s disease (HD) is associated with aggregation of mutant huntingtin protein, containing an expanded polyglutamine tract. In HD patients, individual’s genetic backgrounds can modulate the severity of the disease. However, the variants responsible for modulating disease phenotypes are not known. To ask what natural variant(s) play a role in modifying protein aggregation, we use a *Caenorhabditis elegans* polyglutamine model expressing a fluorescent 40-glutamine expansion (Q40-YFP) in muscle cells. We have shown that introduction of Q40-YFP into genetically diverse wild strains of *C. elegans* results in a wide range of phenotypes, from suppression to strong enhancement of Q40-YFP aggregation and toxicity. Here, we have identified a 400kb genetic interval derived from one of the wild strains (DR1350) that contains variants that increase polyglutamine aggregation. A RNAi survey of 24 candidate genes in that interval identified *atg-5*, an important component necessary for the proper formation of autophagic vesicles (autophagosomes) during autophagy. When *atg-5* is knocked down we observed a 33% decrease in polyglutamine aggregation. Interestingly, genome sequencing identified 2 variants within the 3’UTR of *atg-5* in the DR1350-derived interval. Animals carrying this interval have increased ATG-5 expression and deficient autophagosome formation. Because autophagy is important for degradation of protein aggregates, we hypothesize that natural variants in the DR1350 genetic
of background increase polyglutamine aggregation by causing increased levels of ATG-5 expression and thus misregulating autophagosome formation.

Computational Cell Biology

P1693
Board Number: B710
Decoupling global biases and local interactions between cell biological variables.
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Analysis of coupled variables is a core concept of cell biological inference, with co-localization of two molecules as a proxy for protein interaction being a ubiquitous example. However, external effectors may influence the observed co-localization independently from the local interaction of two proteins. Such global bias, although biologically meaningful, is often neglected when interpreting co-localization. Here, we describe DeBias, a computational method to quantify and decouple global bias from local interactions between variables by modeling the observed co-localization as the cumulative contribution of a global and a local component. We showcase five applications of DeBias in different areas of cell biology, and demonstrate that the global bias encapsulates fundamental mechanistic insight into cellular behavior. The DeBias software package is freely accessible online via a web-server at https://debias.biohpc.swmed.edu.

P1695
Board Number: B712
Inferring cell state by quantitative motility analysis reveals a dynamic state system and broken detailed balance.
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Cell populations display heterogeneous phenotypic states at multiple scales. Similar to molecular features commonly used to explore cell heterogeneity, cell behavior is a rich phenotypic space that may allow for identification of relevant cell states. Inference of cell state from cell behavior across a time course may enable the investigation of dynamics of transitions between heterogeneous cell states, a task difficult to perform with destructive molecular observations. Cell motility is one such easily observed cell behavior with known biomedical relevance. To investigate cell heterogeneity through the lens of cell behavior, we developed Heteromotility, a software tool to extract quantitative motility features from timelapse cell images. In mouse embryonic fibroblasts (MEFs), myoblasts, and muscle stem cells (MuSCs), Heteromotility analysis identifies multiple motility phenotypes within the population. In all three systems, the motility state identity of individual cells is dynamic. Quantification of state transitions reveals that MuSCs undergoing activation transition through progressive motility states toward the myoblast phenotype. By probability flux analysis, we find that this MuSC motility state system breaks detailed balance, while the MEF and myoblast systems do not. Our data indicate that the system regulating cell behavior can be decomposed into a set of attractor states which depend on the identity of the cell, together with a set of transitions between states governed by inputs from signaling.

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pathways such as oncogenes and growth factors. Within one state, equilibrium formalisms can capture variation in behavior, while switching between states violates equilibrium conditions and would require an external driving force. These results support a conceptual view of the cell as a non-deterministic state automaton, responding to inputs from signaling pathways and generating outputs in the form of observable motile behaviors.

**P1696**

**Board Number: B713**

Identification of gene expression variability with phenotypic consequences using Luria-Delbrück-seq.

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Single cell gene expression measurements have shown that individual cells can have highly variable levels of mRNA for many genes. In cancer, even a single cell expanded in a culture dish can show tremendous plasticity such that the resulting population of cells will drastically different gene expression patterns at the single cell level. It is also known that individual cancer cells can exhibit different phenotypes, such as proliferation rates and drug sensitivities, and these phenotypes are thought to underlie important transformations in cancer including drug resistance and metastasis. However, making the direct connection between gene expression variability and phenotypes at the single cell level has been huge challenge in this field. Specifically, it largely unknown how to separate meaningful biological variability from “noise” in gene expression.

We hypothesized that variable genes associated with different behaviors at the single cell level would show memory of gene expression across cellular division, as genes with this behavior would be part of a coherent “cell state”. Thus, we developed an unbiased method for quantifying single cell gene expression memory by using the design of Luria and Delbrück’s “fluctuation analysis” and combining it with RNA sequencing. Specifically, we isolated individual cells, allowed them to expand, and then performed RNA sequencing on each of the single-cell derived cultures. For genes with uniform expression in every cell, all the samples should have similar RNA sequencing counts; however, for genes that occasionally turn on in a subset of cells, the RNA sequencing counts across all cultures should show high variability. By looking at the coefficient of variation for each gene across all of the samples, we have a metric for the heritability of each gene.

We applied this method to two melanoma cell lines and one triple negative breast cancer cell line, and identified sets of hundreds of genes in each cell line with transcriptional memory. We next used single molecule RNA FISH and time-lapse imaging to directly demonstrate that the genes with transcriptional memory are indeed expressed in related cells. In melanoma, we identified a gene expression program present in a rare subpopulation of cells and found that these cells are more resistant to targeted therapy. Similarly, in breast cancer, we identified subpopulation of cells expressing a different transcriptional program that confers resistance to chemotherapy. Taken together, Luria-Delbruck-seq is a method for quantification of genome-wide transcriptional memory which can enable de novo identification of functionally important rare subpopulations with differential sensitivities to therapy.
P1697
Board Number: B714
Monte Carlo simulations of *Listeria monocytogenes* cell-cell spread predict a stratified spreading behavior crucial for survival in the intestinal epithelium.
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Bacterial pathogens use diverse mechanisms to colonize their hosts. After invading an intestinal epithelial cell during an oral infection, the pathogenic bacterium *Listeria monocytogenes* can spread to neighboring cells without exposing itself to the bactericidal extracellular milieu. This mechanism, known as cell-cell spread, enables *L. monocytogenes* to breach epithelial and endothelial barriers, and to reach distant organs such as the liver, placenta, and brain. To learn about the dynamics of *L. monocytogenes* cell-cell spread, we established a live microscopy system which allowed us to track fluorescent intracellular *L. monocytogenes* as they spread through polarized epithelial monolayers. Qualitatively, we observed that the dynamics of *L. monocytogenes* cell-cell spread resembled those of a random walk. To test whether a random walk alone could explain bacterial movement during cell-cell spread, we performed a Monte Carlo simulation in which these movements were modeled based on the Normal distribution. Surprisingly, we discovered that the observed speed of spread in our experimental data was higher than that predicted by our simulated data, suggesting that we cannot use a random walk alone to model *L. monocytogenes* cell-cell spread. However, Monte Carlo data generated by simulating stratified spread, a form of spread characterized by stochastic dispersal events over longer distances, agreed well with our experimental data. Importantly, further simulation demonstrated that stratified spread increases the probability that *L. monocytogenes* will mount a successful infection of the epithelium, which depends on the bacterium’s ability to spread and replicate efficiently without killing the host cell.

P1698
Board Number: B715
Semi-automatic Segmentation and Frequency Mapping of Murine Hair Cells in Multi-Channel Light Microscopy Images.
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A place-frequency map correlates the location along the cochlea to the frequencies interpreted by inner and outer hair cells (IHC, OHC) at that location. This map can be generated from microscopy images showing the organ of Corti. Current solutions for creating the place-frequency map depend on researchers manually tracing a line through the basal membrane in a microscopy image. Here we propose a workflow to automate this manual tracing step in Amira 6.4 (Thermo Fisher Scientific, Waltham, MA, USA) and thus accelerate the throughput of images. **The ultimate goal of this on-going research is to achieve semi-automatic segmentation of the basilar membrane, IHC, and OHC to correlate hair cell characteristics to given frequencies of the murine cochlear map.**

The whole volume of each cochlea is imaged in 2-8 sections using multi-channel fluorescent microscopy. Each section implies a piece of the basal membrane, so that the traced line must continue through all sections as one continuous object from the most basal to the most apical location.

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Membrane binarization begins with a difference of boxes filter and Otsu thresholding to extract highly-expressed areas in a single channel. The entire section’s structure is masked using morphological operations. The binarized structures surrounding the implied membrane are grown together using a watershed algorithm, creating a map of “boundaries” expressing potential membrane space. This membrane space is masked by the whole section’s volume and filtered by measurements (volume, surface area, etc.) to exclude all structures except the true membrane. A line is then extracted and smoothed from the binary voxel map to define the frequency map.

IHCs and OHCs are segmented from appropriately-stained channels using Otsu criteria and morphologically cleaned before being separated using watershed. False positives are removed from potential cell candidates using thresholded maximum intensity projections from each channel as a mask. The centroid from each unique cell is then correlated to the closest point along the extracted frequency line. Qualitative evaluation of the frequency line segmentation routine shows agreeable results in test images. Relevant IHC and OHC are shown to be successfully thresholded and individually labelled. Determination of the points describing the frequency line throughout the cochlea allows successful correlation to the centroid of each segmented cell which, in turn, allows correlation of any measurement calculated for any cell to a given frequency according to the species-specific place-frequency function. Future work includes testing processing routines against ground truth segmentation and improving the morphological cleaning to allow more robust detection of the frequency line.

P1699
Board Number: B716
Dealing with SNP’s - a Hurdle in Renin and ACE Inhibition.
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Hypertension refers to the consistent and chronic blood pressure elevations which result in a pathological state of the body. Blood pressure remains less than 120/80 mmHg under normal conditions. However, in hypertension it rises up to 140/90 mmHg or even above. According to WHO, around 18% of Pakistani population suffering from hypertension which tragically is not considered as a problem by majority of the public. Many genes have been found to play their role in regulation of the blood pressure and hence, mutation in any of them may directly or indirectly result in hypertension. Nonetheless, certain genes are considered more significant than the others and the two targets (Renin and Angiotensin-Converting Enzyme (ACE)) selected in this study were among such critical blood pressure regulators. Amino acid sequences and the 3D structures of selected proteins were retrieved from PDB database. Three dimensionastructural structures of active ingredients present in 05 FDA approved drugs (targeting renin and ACE) and 40 phytochemicals (source plants used in herbal medicines) were retrieved from commonly used ligand databases. Efficiency of ligands was determined in term of docking scores and binding energies. Mutated forms of renin were generated focusing the SNP’s influencing the amino acid residues of their target sites. Molecular docking among normal targets and mutated forms of renin with respective ligands including antihypertensive phytochemicals and synthetic drugs was performed. Substantial binding affinity was found among the phytochemicals and the selected targets even stronger than that of their respective in use synthetic drugs. Chinese isoliensinnine was found to be more than 10% effective against renin; whereas, tannin was more than 40% effective against ACE, as compared to their respective drugs. Furthermore, the tested FDA approved drugs showed significantly less (~20%) binding energy with mutated proteins as compared to selected phytochemicals. Theobromine and Allicin showed ~20% higher binding scores as compared to respective drugs in all ten mutated proteins. Some compounds were found better to be used in case of certain
SNPs. Hence, genetic data should be considered while treating hypertension, moreover, plants can also be considered as a cheaper source of cure.

P1700
Board Number: B717
**Rational design of anti-diabetic agent.**
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Glucagon Like Peptide-1 Receptor (GLP-1R) is the major drug target in the treatment of Type II Diabetes (T2D). The failure of injectable insulin or Glucagon Like Peptide -1 (GLP-1) mimetic to cure T2D is believed to underlie the high incidences of T2D worldwide. Moreover, the discovery of small molecule drugs remains a challenge due to the nature of the orthosteric binding site in GLP-1R. Therefore using the allostERIC sites of GLP-1R to design drugs represents a novel approach to the discovery of anti-diabetic agents. Allosteric sites in G-Protein Coupled Receptors (GPCRs) are less conserved than the orthosteric site, show spatiotemporal specificity, saturation and exhibit the noncompetitive inhibitory effect. We hypothesize that if we design small molecule positive allosteric modulator (PAM) targeting GLP-1R, then the synergy between PAM and endogenous GLP-1 would enhance insulin production. For this study, in house homology modeling method and Sitemap was used to predict the GLP-1R model and allosteric site in model respectively. In order to find an anti-diabetic small molecule, we generated a library of 5689 compounds and docked in the predicted allosteric site using computational tools. Based on the docking scores, top 9 molecules were screened in vitro using luciferase assay. From the preliminary screening, we found two GLP-1R agonists. Furthermore, synergistic studies confirmed that one of the two GLP-1R agonists bind to the allosteric site in GLP-1R. This study has identified novel small molecules PAM of GLP-1R. In future, we will perform rational design to improve the binding affinity and absorption, distribution, metabolism and excretion (ADME) properties of the hit compound. The innovative aspect of this work is the structure based small molecule allosteric drug designing for GLP-1R activation. The significance of this work to public health is getting an anti-diabetic oral molecule that would induce insulin production in the presence of low levels of an endogenous GLP-1 in T2D patients.

P1701
Board Number: B718
**Computational analysis of the membrane targeting domains of the phospholipase D family in Arabidopsis thaliana.**
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The phospholipase D (PLD) class of enzymes is key to several cellular functions that require an association with the cellular membranes, including cell signaling, growth, vesicular trafficking, secretion, and endocytosis. Although well studied in animals, the mechanism and behavior of the membrane targeting domains that mediate these interactions is largely unknown in plants. To this end, we have characterized the structure of membrane targeting domains of the six classes of PLD isoforms in the model organism, *Arabidopsis thaliana*. Utilizing sequence and structural analysis including template based modeling, electrostatics analysis, and docking studies, we have analyzed the membrane binding mechanism for the different PLD classes in *Arabidopsis thaliana*. Domain architecture analysis has
revealed the presence of C2 domains coupled with the catalytic domains of the alpha, beta, gamma, delta, and epsilon isoforms. Only one class of PLDs in Arabidopsis contain Pleckstrin Homology (PH) and Phox homology (PX) domains coupled with their catalytic domains similar to the animal PLDs. Our results show that Arabidopsis PLDs largely control their localization via C2 domains that appear to be structurally analogous to their mammalian counterparts in other protein families. Here we present the modeled membrane targeting domains of all the six classes and elaborate their membrane targeting behavior discerning differences within individual members of this expanded PLD family and compare them with mammalian PLDs. The results from this study lay the foundation for understanding the detailed membrane targeting behavior of the plant PLDs and the consequences of the expansion of this gene family in plants.

P1702
Board Number: B719
Incoherent Inputs Enhance the Robustness of Biological Oscillators.
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Robust biological oscillators retain the critical ability to function in the presence of environmental perturbations. Although central architectures that support robust oscillations have been extensively studied, networks containing the same core vary drastically in their potential to oscillate, and it remains elusive what peripheral modifications to the core contribute to this functional variation. Here, we have generated a complete atlas of two- and three-node oscillators computationally, then systematically analyzed the association between network structure and robustness. We found that, while certain core topologies are essential for producing a robust oscillator, local structures can substantially modulate the robustness of oscillations. Notably, local nodes receiving incoherent or coherent inputs respectively promote or attenuate the overall network robustness in an additive manner. We validated these relationships in larger-scale networks reflective of real biological oscillators. In addition, we built Xenopus extract droplet system to track cell cycle circuit in parallel while manipulating molecular circuit to verify our hypothesis. Our findings provide an explanation for why auxiliary structures not required for oscillation are evolutionarily conserved and suggest simple ways to evolve or design robust oscillators.

P1703
Board Number: B720
3D Computational Modeling of Bleb Initiation Dynamics.
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Blebbing occurs in cells under high cortical tension when the membrane locally detaches from the actin cortex, resulting in pressure-driven flow of the cytosol and membrane expansion. Some cells use blebs as leading edge protrusions during cell migration, particularly in 3D environments. Blebs can be initiated through either a localized loss of membrane-cortex adhesion or ablation of the cortex in a region. Bleb morphologies resulting from different initiation mechanisms have not been studied in detail, either experimentally or with theoretical models. Results from experiments have suggested that cytoplasmic elasticity is important for limiting bleb size. A 3D dynamic computational model of the cell is presented that includes mechanics of and the interactions among the cytoplasm, the actin cortex, the cell
membrane, and the cytoskeleton. The model is used to quantify bleb expansion dynamics and shapes that result from simulations using different initiation mechanisms. The cytoplasm is modeled as a viscous fluid and as a poroelastic material. Results from simulations using a viscous fluid cytoplasm model show much smaller and broader blebs when they are initiated via cortical ablation than when they are initiated by removing membrane-cortex adhesion. Simulation results using the poroelastic model of the cytoplasm provide qualitatively similar bleb morphologies regardless of the initiation mechanism. The resulting cell shapes also are qualitatively similar to those from experimental data.

P1704
Board Number: B721
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Mathematical models are important to guide synthetic biology experiments, including those using Phage-Assisted Continuous Evolution (PACE). Our aim was to use PACE to evolve a riboswitch sequence in an M13 phage, selecting for the ability of the riboswitch to bind to a new small molecule. We modeled random mutations in the phage, accounting for lethal mutations (in functional noncoding regions such as promoters), non-lethal mutations (in nonessential genes and unannotated regions of the phage genome, as well as synonymous mutations in coding regions) and potentially lethal mutations (nonsynonymous mutations in coding regions). The lethality probability of a nonsynonymous mutation in a coding region is a user input to the model. We calculated the expected value and 90% confidence interval for the number of phage required to observe all possible single, double and triple base substitutions in the riboswitch sequence of the M13 phage, after removing from the population all phage with one or more lethal mutations. This probability model was used to ensure that the spatial and temporal scale of the wet lab experiment was sufficient to explore the extremely large sample space of all possible riboswitch sequences of a given length. Further, we used differential equations to predict the time required for the small molecule anhydrotetracycline (aTc) to reach each of its target concentrations, and for negative selection host cells to dominate the population in each compartment of the PACE chemostat. Generalized versions of our probability and differential equation models were coded into web tools that biologists can use for future experimentation.

P1705
Board Number: B722
Graph Fingerprints of Mitochondria and Mitochondrial-Like Networks.
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Mitochondria are the respiratory powerhouses of eukaryotic cells. These organelles form a variety of physical networks in many biological contexts, ranging from multi-cell structures in skeletal muscle tissue to highly-fused networks in single cells of S. cerevisiae. Though mitochondrial networks are quite common, the reason for maintaining a network structure is unclear. Here we begin to test mathematically the similarity between mitochondrial networks in S. cerevisiae and network structures that have been optimized for a variety of tasks, such as for macromolecular transport and rejection of
low-quality components. To do this, I develop a “fingerprint” using a variety of mathematical signatures of a target graph, which characterizes the major properties of the graph. Preliminary data suggest that this fingerprint is quite different for graphs that are superficially similar, though built using distinct sets of construction rules. By computationally simulating mitochondrial-like networks incorporating different types of construction rules, then comparing their fingerprints, I will be able to differentiate between networks optimized for different tasks. I will then compare these fingerprints to those of true mitochondrial networks in S. cerevisiae. Through analyzing fingerprints for time-courses of mitochondrial networks, I will be able to determine conserved characteristics of true mitochondrial networks, allowing a more acute approach toward understanding the properties for which these networks are optimized.

P1706  
**Board Number: B723**  
Protein docking and molecular dynamics simulations of the extracellular domain of Na⁺,K⁺-ATPase β₁ subunit reveals a reliable binding model for epithelial Na⁺,K⁺-ATPases on adjacent cells.  
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In addition to its ion transporting function, Na⁺,K⁺-ATPase (sodium pump) acts as a homotypic epithelial cell adhesion molecule via its glycosylated β₁ subunit ¹,². The β subunit extracellular domain is not homologous to any other family of cell adhesion molecules and, when expressed as a soluble recombinant ectodomain, maintains its adhesive properties. We have performed Molecular Dynamics (MD) simulations and protein-protein docking of the truncated β₁ subunit ectodomain (ED) in order to gain insight into the recognition mode between Na⁺,K⁺-ATPase β₁ subunits. Our MD results show that the N-terminal half of β₁ subunit ED suffers important structural rearrangements, probably as a result of loss of interactions with Na⁺,K⁺-ATPase alpha subunit but nonetheless the C-terminal half of β₁ subunit ED remains stable during the simulations thanks to its β-sheet sandwich structure imposing movement restrictions in the ectodomain. We submitted several conformers to directed protein-protein docking and have obtained a robust model of β₁ ectodomain binding. In such a model two Na⁺,K⁺-ATPase recognize each other via its β₁ subunit by means of a large, complex interface in which one of the three N-glycosylation sites is involved. Our model reconciles experimental data suggesting that both N-glycans and loop residues are involved in the trans dimerization of Na⁺,K⁺-ATPase in epithelia ³,⁴.


P1707  
**Board Number: B724**  
Structural and functional analysis of key proteins involved in ESX-1 protein secretion system of M. tuberculosis: novel targets for drug developments.  
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Tuberculosis is a chronic infectious disease caused by M. tuberculosis. M. tuberculosis contains ESX protein secretion system for the transport of intracellular proteins across their highly impermeable cell wall. Out of five ESX pathways, ESX-1 and ESX-3 are essential for virulence and growth of M.
tuberculosis. ESX-1 pathway contains at least 10 genes that encode T-cell antigens as well as AAA-ATPases. Proper functioning of ESX-1 pathway requires the knowledge of structure and interaction of multiple ESX-1 substrates and other component proteins prior to their secretion. We have expressed, purified and determined the X-ray structure of M. tuberculosis EspR protein. EspR is a transcriptional activator that promotes ESX-1 secretion by activating transcription of espACD operon immediately upon macrophage infection. Currently, we are involved in structure analysis of complexes of EspR with promoter DNA sequences and specifically designed molecular inhibitors. EspC is small protein that strongly recognized by T cells isolated from patients with M. tuberculosis. EspC is immunodominant in active and latent tuberculosis infection. The high immunodominance characteristics of EspC, makes it an attractive vaccine candidate for M. tuberculosis. We have expressed, purified and crystallized and looking for x-ray structure of M. tuberculosis EspC and EccA1 proteins. We have also characterized the biological activities and mechanism of interaction between EspC and EccA1. The structure-function analysis of EspR and EspC/EccA1 complex proteins will contribute significantly in understanding the mechanism of ESX-1 secretion pathway and drug development against M. tuberculosis.

P1708
Board Number: B725
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Genomic sequencing is the cutting-edge in advancing technology and the understanding of how the world functions through DNA, specifically how gene interactions and function lead to successful cell functioning and survival. Currently, 10% of open reading frames (ORFs) in Saccharomyces cerevisiae, remain uncharacterized after many years of analysis. The purpose of this study is aimed at analyzing and gaining data surrounding currently uncharacterized ORFs (ORFans) within S. cerevisiae. Two such ORFan proteins of interest are YGL101W and YLR407W. YGL101W is a yeast protein of unknown function that interacts with DNA Helicase Hpr5p and has a paralog from whole genome duplication, YBR242W, and YLR407W has been observed to include an abnormal phenotype of elongated buds and containment of a single nucleus among mutants. The ORFan proteins were first tested for sequence similarity comparisons utilizing the BLAST algorithm to determine the presence of a viable protein. Structure based evidence was then accumulated and multiple sequence alignment searches were run to formulate predictions on function through homology between the ORFan proteins and different protein families and motifs. Cellular localization data algorithms were used to predict protein membrane topology and cellular localization. The yeast deletion collection and ORFan protein-deleted genetic and physical interaction and expression data were also for any viable information to further characterize protein function. Through these analyses, it has been revealed that YGL101W may function as a phosphohydrolase with homology to the HD domain, while YLR407W contains a nuclear localization signal, an ER membrane retention-like signal, and the appearance of a peroxisomal targeting sequence. This information leads towards the cytoplasm as the cellular location of YLR407W. Both proteins may be involved in cell proliferation, however, further analysis using GFP tagging and knockout-experimentation is required to determine the validity of this hypothesis.
systems and Synthetic Biology and Tissue Engineering

P1709
Board Number: B726
Pigment epithelium derived factor facilitates cornea limbal regeneration in a mouse model through the activation of STAT3 and sonic hedgehog (SHH) signaling.
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We previously reported the mitogenic effect of pigment epithelium derived factor (PEDF) on limbal epithelial stem cells (LSCs) and further demonstrated that PEDF successfully induced limbal regeneration in rabbit partial limbal deficiency. This study was conducted to investigate the mechanism of PEDF-mediated limbal regeneration. In the present study, 120 degree limbal excision of mice was followed by synthetic 44-mer PEDF treatment. Compared with the control, 44-mer PEDF promoted limbus repair with evidence of expression of △P63α and Lrig1 positive cells within limbal wound at 2 weeks and accelerating central corneal epithelium healing after wound challenging. Inhibitors for sonic hedgehog (SHH) and STAT3 significantly decreased the PEDF effect on △P63α and Lrig1 expression in mice as well as the LSC expansion in cell study. Using organ culture and cell study, PEDF sustained the upregulation of SHH activity accompanied with nuclear translocation of Gli3 at least for 6 hours. STAT3 inhibitor blocked the SHH-Gli signaling sustained by PEDF. Together, we demonstrate that LSC expansion and limbal regeneration promoted by PEDF was mediated by STAT3 and SHH-Gli signaling pathway. We hypothesize that limbal regeneration was dependent on the sustained SHH-Gli pathway activity through phosphorylation of STAT3 promoted by PEDF.

P1710
Board Number: B727
The physical microenvironment influences plexus self-assembly in a 3D in vitro model of vasculogenesis.
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Developing large-scale tissue grafts for soft tissue defects remains an ongoing challenge in bioengineering largely due to an inability to fully vascularize these constructs. The current standard of care is to use autologous grafts; however, critical flaws include donor-site trauma and volume reduction due to ischemia and slow revascularization. Thus, an engineered graft is ideal. Current bioengineered approaches mainly rely on the fabrication of patterned structures; however, they are difficult to endothelialize and prone to collapse. Our approach instead relies on the de novo formation of a 3D, perfusable, stable vascular plexus within a collagen gel in a process termed vasculogenesis. Although vasculogenesis-based approaches do not lend themselves to controlling vessel phenotype, altering the physical properties of the collagen scaffold allows us to influence cell behavior and dictate the morphology of the resulting network. Engineered vascular plexuses were formed by seeding human umbilical vein endothelial cells at a range of cell densities into collagen gels of varying mechanical properties and allowing them to self-assemble into networks over seven days of culture. The collagen gels were then fixed, stained, and imaged via confocal microscopy. Custom MATLAB algorithms were used to segment the images and quantification of the vascular network phenotype was characterized by vessel diameter, vessel length between junctions, and network connectivity via vessel lumens. To understand how the physical variables tested affect plexus self-assembly, we developed a quantitative
framework equivalent to a “state diagram” where emergent properties of the morphology could be mapped from the variable space. Additional experiments modulating cell contractility via nonmuscle myosin II and ROCK inhibition were performed, and the resulting phenotypes were also mapped onto this framework. Using this framework to understand the role of collagen density, collagen stiffness, cell density, and cell contractility, we can generate lumenized vascular plexuses to fill collagen gels in a variety of regular and irregular shapes and sizes with networks that span centimeters in length. Vessel perfusion was confirmed by flowing fluorescent microspheres throughout the self-assembled architecture. The platform and quantitative framework discussed herein has several advantages, including versatility, stability, and ease of fabrication. By adjusting physical parameters, a fully interconnected, perfusable vascular network with a desired morphology could be incorporated into large-scale grafts via self-assembly and presents an opportunity to implant fully vascularized constructs with minimal risk of volume reduction.

P1711
Board Number: B728
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Patients that have lost major sections of tissue from events like major trauma, may require clinical intervention including amputation, skin grafting, transplantation, or, the most interesting option that is not available to doctors yet, tissue engineering (TE), growing a new section of tissue in vitro [1]. TE methods grow cells well in flat sheets, but when the sheets are layered the center mass is poorly diffused decreasing cell viability. If pores were introduced into the hydrogel, then the cell viability will increase, while the introduction of tension and over the counter muscle supplements will increase maturation rates to better mimic native tissue. A 10% (w/v) gelatin solution was used for the experiments [2]. Microbial transglutaminase (mTG) at a concentration of 1% (w/v) was used as a cross-linking agent for the hydrogels. Chloroform was added (0.02% v/v) to sanitize the hydrogels [2]. The hydrogel scaffolds maintained their 3D shape for two weeks after successful cross-linking in PBS at 37°C. Pores were induced with nails, 3D printed structures, and stainless-steel (ss) wire with the ss wire being the most effective. Several different pin configurations were tested including squares, circles, and triangles. There is a dilemma between inducing more pores to increase diffusion without weakening the gel to the point it cannot stretch without tearing; a triangle formation seemed to be the most effective. C2C12 cells with randomly incorporated mCherry fluorescent tags were used for this investigation. C2C12 cells were embedded in the hydrogel at a concentration of 1 million cells/well. The cells were imaged every three days for two weeks. Overall, a viable porous cross-linked hydrogel scaffold was developed; however, the cells failed to orient themselves properly in the hydrogel. Ongoing research includes applying tension to the C2C12-gelatin scaffold to help the myoblasts orient in a unified direction. Additionally, over-the-counter muscle supplements marketed as having the ability to improve muscle growth during workouts are being investigated on decreasing time to cell maturation in vitro. The ability to rapidly grow thick and viable skeletal muscle for introduction will help patients have an increased quality of life by restoring function to their injured muscle.
P1712
Board Number: B729
Acss2 controls mode of acetate utilization.
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The current knowledge of Acss2 (acyl-CoA short chain synthetase-2) is expanding and has taken center stage in acetate metabolism. In mammals, cytoplasmic and nuclear acetyl-CoA are replenished by ATP-citrate lyase (Acly) and Acss2. Up until recently, Acly was alone thought to be the major source of extramitochondrial acetyl-CoA but accumulating data indicate that both enzymes are critically involved in acetate metabolism. Acss2 -/- mice develop and reproduce normally, but exhibit behavioral differences from wild type mice. Acss2 has been implicated in lipid synthesis, but additional functions of Acss2 remain mostly unexplored. Therefore we examined gene expression changes in the liver and brain under conditions in which exogenous acetate is increased (acetate loading by oral GTA) vs. when endogenous acetate and ketone bodies are increased (48 hour starvation) and compared the responses between wild type and Acss2 -/- mice. Initial bioinformatics analysis of the data indicates that endogenous and exogenous increases in acetate are not equivalent, and in fact, result in vastly different changes in gene expression. Following acetate loading, wild type and Acss2 -/- mice exhibited significant differences in the expression of 151 genes in the liver. In contrast, 48 hr fasting resulted in the differential expression of 2675 genes in the liver. Interestingly, when GTA fed mice were compared to 48 hour fasted Acss2 -/- mice, the number of differentially expressed genes in liver increased to 4768. In brain, the main pathways perturbed were PI3K-AKT and serotonergic responses. Alcohol induced organ damage showed strong correlation to the gene function loss. Previously unknown roles for Acss2 such as cell signaling cascades, specific chromosomal regulatory networks, cell cycle, immune regulation, apoptosis and others were uncovered indicating the diverse functional roles played by Acss2, particularly during nutrient deprivation and negative nitrogen balance. Supported by: CHIRP grant no: APG-70-3917

P1713
Board Number: B730
The Integration of Cellular and Subcellular Dynamics for Cell Migration.
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Integrated biology requires real-time cell information to establish comprehensive cellular-molecular connections. The lack of appropriate cellular assessments will hinder a systematic understanding of cell physiology. Hence, we develop a novel cellular signature — the CN correlation profile — to quantify the contributions of the momentary subcellular migratory activities in the cell migration pattern of a cell type. We found that the CN correlation profile can uniquely and consistently represent the cell migration pattern of the cell types probed. The effects of molecular perturbations, such as Y27632 and Cdc42 knockdown, also can be clearly revealed on each subcellular migratory activity. As a result, the CN correlation approach can effectively promote integrated biology through bridging cell dynamics and
migration pattern with their underlying subcellular migratory activities and elucidated molecular mechanisms.

P1714
Board Number: B731
Coupled control of mRNA and protein variability in single mammalian cells.
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Central dogma of biology is an intriguing question since decades, mainly due to the lack of clarity concerning the stochastic or deterministic nature of single cell variability in both, mRNA and protein level. While various bulk approaches tried to address to which extend do the mRNA levels predict the protein levels, the variability within single cell population that can lead to a different time scale regulation of mRNA production and translation remained vaguely addressed. We have recently shown that cytoplasmic transcript abundance in single mammalian cells is minimally stochastic, and absolute mRNA quantities are largely predictable by the cellular features. Now, using ~20 mammalian cell lines where gene of interest has been tagged on all alleles with mEGFP by CRISPR/Cas9 or ZnF technology combined with antibody staining approach, we provide very accurate measurements of both protein and mRNA (using single molecule mRNA FISH), together with ~200 single cell features (cell size/shape, nucleus size/shape, DNA content, total protein content, neighbor relations and activity...) and ~35 features that describe mRNA spatial patterns (distance from the nucleus, plasma membrane, each other, relation to the neighboring cells...). Importantly, pairwise correlations of mRNA and protein for all genes across thousands of measured single cells are very high, irrespective of the expression distribution that is gene-specific (uni- or multimodal, multimodal-inducible). mRNA content largely explains the variance in the measured protein levels, however, when cellular features are taken into account, we are able to further map the cells that deviate in their kinetics from the bulk of the population. Using mathematical modelling we can accurately predict protein levels of all single cells, pointing that the kinetics of the translation/transcription processes is determined by a particular cell cycle stage, cellular phenotype or mRNA pattern, and is also gene-specific. Contribution of the mRNA patterning to the explained variance of the protein levels further suggests cytoplasmic compartmentalization (apart from nuclear retention) as one of the mechanisms by which cells buffer the transcriptional and translational noise, by directly modulating kinetics of mRNA stability and translation.

P1715
Board Number: B732
Chemotropism in yeast.
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Eukaryotic cells like migrating cancer cells, developing neurons, and mating yeast move (chemotaxis) or grow (chemotropism) in a specific direction by using a chemical gradient for directional reference. Existing models for how eukaryotic cells sense chemical gradients generally involve comparing concentrations of ligand-bound receptors across the cell surface to infer the gradient. However, our studies, using yeast chemotropism mating as a model, suggest that cells may sense a chemical gradient with a pheromone-sensing patch of the cell cortex (a "nose") that is mobile. The nose is set up by
polarity factors and moves in response to vesicle traffic. We have modeled the dynamics of this moving nose with stochastically perturbed reaction-diffusion equations. In this study, we show that an in silico cell based on this computational model can track pheromone gradients. However, the model is much worse at tracking shallow gradients than yeast cells in in vivo experiments. Interestingly, an additional pathway, linking receptor-coupled G-alpha to a formin, was implicated in gradient sensing in yeast. When we incorporate the potential effects of this pathway into our mathematical model, the model's ability to track pheromone gradients improves significantly if, and only if, we assume that G-alpha (unexpectedly) inhibits the formin. Furthermore, we experimentally demonstrate that the change in the moving nose's dynamics in G-alpha mutants recapitulate the mathematical model's predictions. In summary, we propose a mechanistic basis for a novel pathway that improves the accuracy of gradient tracking in yeast.

P1716
Board Number: B733
Visualizing and controlling calcium signaling dynamics after wounding in engineered stromal microtissues.
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How cellular populations coordinate their behaviors to drive tissue morphogenesis and organ function has been a long-standing question. The most familiar paradigm is having a group of control cells, such as pacemaker cells in a beating heart, that dictate the tissue's action. However, in the context of wound healing, no specific ``pacemaker'' cells driving tissue closure have been identified. Instead, the cells need to communicate and coordinate the tissue response after injury. In a wounded tissue, the multicellular response is driven by several key signaling molecules, the earliest of which is calcium. In 2D scratch assays the calcium response has been characterized as an initial wave followed by periodic oscillations and it has been demonstrated that these periodic oscillations change based on the mechanical environment. As the mechanical environment in 3D tissues is complex and changes in these mechanics are critical to the wound closure process, we hypothesize that the calcium response is different and that these differences could be amplified down the signaling cascade, ultimately leading to a different healing response. To address this hypothesis, we have implemented a toolbox of genetically-encoded fluorescent calcium sensors and optogenetic calcium actuators to permit the visualization and control of calcium signaling in 3D microtissues composed of fibroblasts and collagen type I matrix. We have found that some cells in these 3D tissues display calcium oscillations prior to wounding and that the oscillations after wounding are faster than those observed in 2D scratch assays, both of which we believe are due to the different forces cells experience in these microtissues. Using our actuators, we have begun to manipulate calcium signaling to test our hypothesis that these differences in the initial calcium signaling dynamics manipulate the downstream healing response. This toolbox will allow us to causally link signaling dynamics to their mechanical environment and the downstream responses they control, both permitting us to potentially reprogram cellular communication to optimize the wound healing response and more generally understand how non-hierarchical cellular populations make decisions.
P1717
Board Number: B734
Using phage assisted continuous evolution (PACE) to evolve riboswitches that function reliably in vivo.
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Phage assisted continuous evolution (PACE) is a method first developed by the David Liu laboratory at Harvard University. PACE uses both positive and negative selection of filamentous phage M13 that infect E. coli. A chemostat is used to direct mutations to phage populations instead of E. coli host cells. A two-campus team of Missouri Western State University and Davidson College undergraduates adapted PACE to evolve a new riboswitch that functions reliably in vivo. Riboswitches are RNA elements within mRNAs that control protein production by binding ligands of interest and regulating either transcription or translation. Discovering new riboswitches that work in vivo has proven to be very difficult, and incorporating a published aptamer into existing riboswitches rarely succeeds. Our PACE experiments aimed to evolve transcriptional and translational riboswitches known to bind theophylline into xanthine-specific riboswitches. We carried out positive selection in the presence of xanthine, and negative selection in the presence of theophylline. After PACE, a variant of the translational theophylline riboswitch, which contained a seven base pair deletion in the aptamer, dominated the phage population. This variant was tested to ascertain the effect of the mutation on riboswitch function. Populations of new riboswitches taken from different M13 samples after PACE were subjected to next generation sequencing to obtain a library of variants that had evolved over the course of PACE. The populations will be tested further to determine if xanthine-specific riboswitches evolved through PACE.

P1718
Board Number: B735
Tunig DNA- and Membrane-binding proteins to sense cellular geometry.
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Adenocarcinoma samples from the Penn Medicine BioBank show evidence of oversized cells. These cells may affect cancer progression. However, testing whether large cells are relevant to pathology in a mouse requires a way to target only oversized cells. Targeting this subpopulation cannot be accomplished with drugs or any other existing method. Therefore, we are building synthetic genetic circuits to target only oversized cells. This synthesis approach has another benefit: it teaches us about the evolutionary constraints upon size-sensing proteins. To synthesize size-sensing, I am building proteins to sense cellular geometry. I describe how DNA- and membrane lipids titrate with cell volume, and simulate binding of a protein to DNA or membranes as a function of volume. Simulations of this “binding-with-volume” predict an increase the free activity of a protein at larger cell sizes. This effect could be employed to activate a cell-death program. To account for nuclear transport effects on binding, I solve partial differential equations for nuclear binding and transport as a function of size. The results show that altering import and export rates of a DNA-binding protein will shift its binding curve. Overall, increasing any of the parameters of KD, binding specificity, protein concentration, or nuclear export all shift size-response curves to smaller sizes.
I apply this theory to experiments in cell-sized droplets by showing binding vs. volume of HMG proteins to DNA-coated beads, and an amphipathic helix to phospholipids. As expected, protein fluorescence in the lumen of droplets is depleted at smaller sizes. To show this behavior is relevant in cells, I demonstrate the binding-with-volume effect in cell-like compartments containing Xenopus laevis oocyte extracts and reconstituted sperm nuclei. Finally, I describe the differences between theory and experiment and differences between the buffer and extract-based system.

P1719  
**Board Number: B736**  
Optogenetic control of protein activity in cell-like compartments.  
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The spatial organization and temporal activation of proteins are critical for cellular function. For example, the Rho family of GTPases act as spatiotemporal ‘switches’ to control organelle development, cell motility, and cytoskeletal dynamics. To characterize the organizing principles that guide intracellular signaling, it is necessary to control initiation and spatial patterning of these pathways. To this end, we previously developed cell-like compartments that enable broad control of cellular contents and dimensions. However, this minimal system lacked tools to control subcellular localization and spatiotemporal triggering of enzymatic activities. We chose light as an optimal trigger to augment our system because the area of illumination can be precisely controlled and because it circumvents the difficulty of diffusing small molecules through the continuous phase into water-in-oil emulsions. To introduce optogenetic control, we employed cTMP paired to Halo-DHFR and dRAP coupled with FRB-FKBP. We implemented these photocaged dimerization systems with split proteins to enable reconstitution of biochemical activity with light. Here, we demonstrate this scheme by activating split tripartite GFP with light through the cTMP-Halo system and by reconstituting split TEV (Tobacco Etch Virus) protease with light through the dRap and FRB/FKBP system. In pairing split proteins with light inducible dimerization systems, we demonstrate the potential for a myriad of light-inducible protein activities using previously-developed split proteins inside of synthetic cell-like compartments. These novel optogonetic tools can aide in studies linking cell geometry, subcellular localization, and signaling within synthetic cells.

P1720  
**Board Number: B737**  
Controllable phase separation and modular recruitment to form synthetic membraneless organelles.  
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Many intrinsically disordered proteins (IDPs) self-assemble intracellularly into liquid droplets that function as membraneless organelles. Because of their importance in biology and unique chemical characteristics, these protein compartments represent a compelling target for synthetic biology research. Here, we manipulated the intrinsically disordered, arginine/glycine-rich RGG domain from the P granule protein LAF-1, to demonstrate controllable phase separation and cargo recruitment to a synthetic membraneless compartment. First, we demonstrated methods to control phase behavior, externally triggering droplet assembly and disassembly by using specific proteases to manipulate the...
valency of IDP domains and presence of solubility-enhancing domains. Second, we characterized permeability of these compartments to soluble macromolecules and devised strategies to target and colocalize cargo molecules into the droplets. Cargos were recruited using either RGG domains or coiled-coiled interaction domains as recruitment modules, and cargo release was triggered by proteolytic removal of the recruitment domains. Droplet assembly and cargo recruitment were robust and occurred in cytoplasm. Our results using this platform suggest it is now possible to recruit multiple enzymes and substrates to stimulus-responsive membraneless organelles. This system provides a much-needed experimental framework to investigate the biochemical consequences of localizing enzymes and substrates to membraneless organelles and to harness IDP compartments for synthetic biology applications.

P1721
Board Number: B738
A forward genetic screen identifies host factors that influence the lysis-lysogeny decision in phage lambda.
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The lysis-lysogeny decision made by bacteriophage lambda is one of the classic problems of molecular biology. Shortly after infecting a cell, the virus can either go down the lytic pathway and make more viruses, or go down the lysogenic pathway and integrate itself into the host genome. While much is known about how this decision takes place, the extent to which host physiology influences this decision and the mechanisms by which this influence takes place has remained mysterious. To answer this question, we performed a forward genetic screen to systematically identify all of the genes in E. coli that influence the lysis-lysogeny decision. Our results demonstrate previously unknown links between host physiology and viral decision making and shed new light on this classic system.

P1722
Board Number: B739
Characterization of the Gain-of-function Toxicity of Optineurin in Yeast.
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Optineurin is an autophagy adaptor protein associated with two distinct types of neurodegenerative diseases: Normal Tension Glaucoma (NTG) (Rezaie et al., 2002) and Amyotrophic Lateral Sclerosis (ALS) (Maruyama et al., 2010). NTG is a sub-type of Glaucoma, which may lead to complete blindness following progressive optic nerve degeneration. ALS is a fatal neurodegenerative disorder, attacking mainly the upper and lower motor neurons. Although overexpression (Chi et al., 2010) and deletion (Ito et al., 2016) of optineurin were shown to induce degenerative phenotypes in mice similar to those observed in disease, the underlying mechanisms remain unclear. Overexpression of optineurin leads to growth defects in yeast (Kryndushkin et al., 2012). Using yeast genetic approaches, the human homolog of a yeast gene, MKK1, was identified as a potential drug target that suppresses the toxicity of optineurin in mammalian cells and in zebra fish models (Jo et al., 2017). Overexpression of Ypt1, the yeast homolog of a human interactor protein of optineurin, Rab8, also alleviates optineurin mediated...
toxicity (Kryndushkin et al., 2012). We found that Ypt1 is an interacting protein of optineurin (Zhong et al., 2016). Based on this observation, we hypothesized that other interacting proteins of optineurin in yeast could be involved in its toxicity. We therefore expressed the wild-type and six mutated forms of optineurin in yeast. Each allele alters a specific interaction domain of optineurin: the NTG-associated E50K allele in the Nemo-like domain, the ALS-associated V295F and E478G alleles in the coiled-coil and the ubiquitin-binding domains, the F178W and F178A alleles specifically altering the binding motif of the autophagy protein ATG8/LC3 and the C555X allele truncating the Zinc finger domain. We determined overexpression phenotypes of optineurin alleles and systematically tested these alleles against ~6000 yeast proteins for interactions using the yeast two-hybrid method. We identified 128 interactors including three reported suppressors of the optineurin induced toxicity, Ypt1, Btn2 and Mdv1 (Kryndushkin et al., 2012, Jo et al., 2017). The mapped interaction network reflects the interplay of multiple cellular processes including autophagy, protein trafficking and mitochondrial functions. Different optineurin alleles change the interaction network differently. Strikingly, the ALS-associated E478G allele appears to gain a large number of new interactions. Our current work focuses on examining the modifier effect of each optineurin interactor protein on its toxicity. Such allele-specific physical and genetic interaction networks, if demonstrated to be conserved in human, may provide new insights in optineurin-associated neurodegenerative processes.

P1723
Board Number: B740
Causes and consequences of slow-cycling cells within isogenic populations.
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The subpopulation of slow-cycling cells has been well characterized in bacteria, which often give rise to antibiotic resistance. In mammalian cells, it has been proposed that cancer drug resistance is mainly conferred by a small subpopulation of slow-cycling cells, also referred as dormant cancer cells. Yet, a clear connection between slow growth and adaptability to drug environment has not been established, mainly due to the lack of tools to identify and follow this small subset of slow-cycling cells throughout the process. The Spencer lab has recently developed several live-cell sensors to distinguish quiescent cells from proliferating cells. These tools enable flow-cytometry-based isolation of naturally slow-cycling cells within a heterogeneous population, as well as tracking the dynamics of slow-cycling cells at the single-cell level. By profiling the sorted slow-cycling cells by RNA-seq, we present the molecular network underlying the slow-cycling subpopulation. Using dynamical and systems-level information, we show that stress is the fundamental cause of spontaneous entry into the slow-cycling state and that, in turn, the slow-cycling state may protect cells from future stress.

P1724
Board Number: B741
Mathematical models for tissue structure based on asymmetric cell division.
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While the life of any multicellular organism is dependent on maintaining organization of its tissues, the laws that govern the organization of cells in tissues have not been elucidated. Hypothesis: Simple mathematical laws involving temporal and spatial rules explain how the organization of tissues is
maintained during tissue regeneration. Our goal was to discover the code that regulates normal tissue renewal and organization. Models (discrete and continuous) of asymmetric cell division were used to determine rules that explain, at the cellular scale, how tissues maintain their organization in an emergent fashion. Five mathematical rules were found to generate symmetric geometric patterns having self-renewing branches with cell numbers as described by generalized Fibonacci-recursive sequences. The dynamics of the system are described by rate equations, rate constant values for division of mature and immature cells in the system population, eigenvalues and eigenvectors. Our discrete, asymmetric cell division model involving simple mathematical laws based on temporal and spatial rules generates symmetric patterns of cell populations that maintain their organization while continuing to self-renew. This model provides a first step toward understanding how rules that regulate organization of healthy tissues might be modified in diseases of tissue disorganization such as cancer.

P1725
Board Number: B742
Optogenetic platform to probe cytokinesis signaling in vitro.
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While proper cell division is critical to the development of living cells, the timing and mechanistic details of this dynamic process remain unresolved. To gain fundamental insights on disease and development, we must first understand how intracellular signals that direct cell division are organized in space and time. Here we present a minimal in vitro platform to decode the molecular mechanisms that link spindle positioning and cell cycle progression. This simplified system and the perturbations it enables offer benefits over the complexity of living cells and in vivo genetic studies which preclude the analysis of short, dynamic processes. Precise spatial regulation of the mitotic spindle – a macromolecular machine that separates chromosomes - is necessary for proper cell division, particularly during the rapid cell cycles present in early embryo development. Once positioned, the spindle initiates other mitotic events such as cytokinesis through cleavage furrow signaling. By engineering proteins that can sense activity of key regulators of cell division, as well as proteins that can localized by light, we can reconstitute and observe many of these complex processes. Our goal is to reconstitute these mitotic processes in vitro by encapsulating Xenopus egg extract along with these engineered proteins within a synthetic boundary and tuning compartment geometries. By doing so it should be possible to investigate how cell geometry affects the transport and activation of cell cycle signals. Currently, we are developing optogenetic tools to enable us to position proteins of interest as well as proteins that interact with microtubule ends of spindles formed in vitro. Here I describe our progress toward these goals. We have expressed and purified protein pairs that dimerize upon illumination by blue light. By merging optogenetic recruitment with a boundary tethering mechanism, we are poised to recruit proteins to specific regions of the cell-like compartment. We plan to characterize how known key players in cytokinesis, such as small GTPase RhoA, are spatially activated throughout the cell. With this platform, we can determine how the rates of signaling vary in cells with extreme geometries and further examine link between spindle positioning and the molecular pathways associated with cell division.
Vascularization of engineered tissue constructs is crucial to recapitulating fluid flow and transport necessary for cell survival and to create accurate in vitro disease models for drug discovery applications. Methods to engineer vasculature currently rely on either self-assembly or microfabrication approaches which are often limited in their ability to generate vasculature with repeatable and controlled architecture or to generate 3D biomimetic networks respectively. To overcome these limitations, we developed and implemented laser-based hydrogel degradation to recapitulate the native architecture of in vivo microvasculature in engineered hydrogels. We also demonstrate the ability to quantify transport between two adjacent and intertwining microfluidic channels, similar to transport between the cardiovascular and lymphatic systems in vivo, and to fabricate planar vascular constructs to study interstitial and luminal fluid flow within tissue constructs. By tracking particle movement through vascular networks, we can replicate physiologically relevant capillary wall shear stress of 45 dyne/cm2. Our hydrogels are housed within larger pre-fabricated microfluidic (PFM) devices to conveniently enable pump-driven or pressure-driven fluid flow. For culture of microvascular endothelial cells, channel networks are created in both synthetic (poly(ethylene glycol) diacrylate; PEGDA) and natural (collagen) hydrogels, with channels ranging from 3.3 to 100 μm in diameter. Due to differing degradation mechanisms (photodegradation via plasma ionization and photocavitation in PEGDA, and via multiphoton ionization in collagen), networks can be generated two times faster in collagen than in PEGDA, albeit with similar resolution. For these studies, PEGDA hydrogels were photopolymerized within PFM devices and functionalized with AF633 for visualization and RGDS peptide for cell adhesion. Rat tail collagen type 1 at 2.5 mg/mL was incubated at 37 °C to induce polymerization within AIM Biotech PFM devices. Using a 20X(NA1.0) water immersion objective, virtual masks guide the position of a 140 fs pulsed Ti:S laser operating at 790 nm at 37.7 nJ/μm2 to induce PEGDA degradation, and 900 nm at 18.0 nJ/μm2 to induce collagen degradation. Channels were seeded with mouse brain endothelial cells, human umbilical vein endothelial cells, or human lung microvascular endothelial cells and stained and immunofluorescently labeled for nuclei, actin, and VE-cadherin. Applicable to both synthetic and natural hydrogels, laser-based degradation can be used to generate fluidic networks with controlled and engineered vascular architecture and cellular microenvironment. Currently, we are applying these techniques to study circulating tumor cell lodging in, and extravasation from, capillaries.

Germ Cells, Gametogenesis, and Fertilization

In some rare Australian species like Solanum asymmetriphylum, two types of flowers are found: one with only male reproductive structures, or male flowers, and another with both male and female structures, or hermaphroditic flowers. Pollen grains have apertures in their cell walls to facilitate
initiation of the pollen tube, which enables pollination. Grains (pollen) from the androecium of hermaphroditic flowers are inaperturate, therefore the flowers are functionally pistillate (making species with these breeding systems functionally dioecious). We propose a molecular study of the differences between pollen grains from the male flowers and the hermaphroditic flowers in two different dioecious taxa: S. ossicruetem and S. dioicum, which have male and female flowers on separate plants and we compare these to one andromonoecious taxon, S. ultraspinosum, which has inflorescences that produce one hermaphroditic flower and several male flowers. Since we know that the structure of pollen grains is different between the two breeding systems, we will use protein quantification to compare the amount of total protein in each population. We aim to quantify the differences in protein concentration between the two pollen types in the Solanum genus. After protein quantification, protein extraction will allow us to perform 2-dimensional gel electrophoresis technique (2D-GE). 2D-GE will facilitate the determination of a protein expression profile for each of the pollen grains. This analysis will give a new insight into this unusual reproductive condition.

P1728
Board Number: B746
Proteomics of phosphorylation and protein dynamics during fertilization, activation, and meiotic exit in the Xenopus egg.
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Fertilization triggers release from meiotic arrest and initiates events that prepare the egg for the ensuing developmental program. Protein degradation and phosphorylation are known to regulate protein activity during this process, especially for meiotic exit. However, the full extent of protein loss and phospho-regulation is still unknown. We examined absolute protein and phospho-site dynamics after fertilization by mass spectrometry-based proteomics at high temporal resolution. To do this, we developed a new approach for calculating the stoichiometry of phospho-sites from multiplexed proteomics for improved interpretability of the data. We measure the endogenous degradation rates of known cell cycle proteins (e.g., Cyclin B, securin, EMI2) and identified two putative degradation targets. Overall, the data suggest that degradation is limited to a few low abundance proteins in the egg. However, this degradation promotes extensive dephosphorylation that occurs over a wide range (~1,000 fold) of abundances during meiotic exit. One specific observation is the differential dephosphorylation rates of several nucleoporins which corresponds to the proposed sequence of nuclear pore complex reassembly. This gives insight to the post-translational mechanisms that may regulate the post-mitotic reassembly of the nuclear pore. The data also reveal insights related to fertilization and egg activation. We show that eggs release a large amount of protein into the medium just after fertilization, with ~1% of the non-yolk protein mass lost within minutes. This is most likely related to the blocks to polyspermy. There is also a substantial increase in phosphorylation, likely tied to calcium activated kinases, in parallel to the cell cycle-related dephosphorylation. The analytical approaches demonstrated here are broadly applicable to studies of dynamic biological systems.
P1729
Board Number: B747
Cell cycle–coupled changes of redistribution of inositol 1,4,5-trisphosphate receptor-1 and Ca2+- oscillatory activity in mouse zygotes.
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BACKGROUND Ca2+ oscillations during fertilization induce eggs activation and embryonic development in mammalian eggs. The type 1 inositol 1,4,5-trisphosphate receptor (IP3R1) is in charge of Ca2+ oscillations for the release of stored Ca2+ from the endoplasmic reticulum. The capacity of this oscillation is obtained during egg maturation and corresponds with an increase in the sensitivity of the IP3R1 and their localization in cytoplasm. Cluster formation of IP3R1 in the egg cortex is important to initiation of Ca2+ oscillations during egg and sperm fusion. In this study, we investigated that cell cycle–coupled changes of redistribution of IP3R1 and Ca2+- oscillatory activity in mouse zygotes. MATERIALS AND METHODS Metaphase II arrested eggs were collected from CD-1 female mouse after super ovulation induction. At 14 hr post hCG, MII eggs were collected, and artificially activated in Ca2+ free CZB medium with 10mM SrCl2 for 2 hrs. Pronuclear zygotes (PN) were collected from Strontium activated eggs at 8 hr post activation, and the first mitotic eggs were collected at 16~17 hr post activation. To identify cell cycle coupled IP3R1 redistribution, MII eggs, zygotes, and first mitotic eggs were fixed for immunostaining with anti-IP3R1antibody (CT-1) and observed on CLSM. Ca2+-oscillatory activity was monitored with fluorescence microscope with fura 2 AM after injection of cRNA of mouse phospholipase C zeta (mPLCZ). To evaluate the phosphorylation of IP3R1, anti-MPM2 and anti-IP3R1 were used for western blotting. RESULT IP3R1 were exhibited clusters with 1-2 um in diameter, near cortex area of ovulated MII eggs and highly phosphorylated, and these MII eggs represent high Ca2+ oscillatory activity more than 6 spikes per 60 min by mPLCZ injection. However, IP3R1 clusters were disappeared in PN eggs and these eggs showed very low Ca2+-oscillatory activity by mPLCZ. In mitosis I stage eggs, clusters of IP3R1 were appeared and Ca2+-oscillatory activity was reactivated slightly (2 spikes per 60 min). Less phosphorylation of IP3R1 in PN and mitosis I eggs than in MII eggs were observed. CONCLUSIONS This study introduced the redistribution of IP3R1 clusters were occurred in egg activation according to cell cycle dependent manner. Also, functional modification of IP3R1 was associated with cortical clustering of IP3R1 in cell cycle coupled Ca2+ oscillatory activity. ACKNOWLEDGEMENT This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A1A2056914/2017R1D1A1B03028155).

P1730
Board Number: B748
A proteomics approach identifies novel protein components of the Balbiani body.
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Oocytes maintain precise cytoplasmic organization, making them an excellent system for studying cell polarity. Oocyte polarity defines the pole of the egg that is fertilized in mouse, frog, and fish, and establishes the anterior-posterior axis of frog and fish embryos. In zebrafish, oocyte polarity is initiated by the Balbiani body, a transient aggregate of mRNA, ribonucleoprotein particles (RNP), mitochondria
and other membrane bound organelles. The Balbiani body carries the germ line determinants and polarized mRNAs from the nucleus to the cortex, defining the vegetal half of the developing oocyte. The Balbiani body is conserved across many vertebrate and invertebrate species including the mouse and the fly, but its components remain poorly characterized. Only a handful of resident Balbiani body proteins have previously been identified. Analysis of these resident proteins is insufficient to explain how the Balbiani body forms and polarizes the oocyte. We isolated Balbiani bodies from zebrafish oocytes and performed mass spectrometry to define the Balbiani body proteome. Using this technique, we successfully identified over 70 replicable hits representing Balbiani body protein components. Our list includes known Balbiani body proteins and many novel proteins. This library of Balbiani body proteins is a prerequisite for a comprehensive mechanism for Balbiani body function at a protein level. Furthermore, the number of proteins identified indicates that the Balbiani body is a complex structure that requires many proteins working in concert to generate oocyte polarity. We have validated selected proteins from our proteomics library by injecting mRNAs encoding fluorescently tagged fusion proteins directly into stage I oocytes. These proteins localize to the Balbiani body, supporting our proteomics results. The ability to easily inject very early stage oocytes with mRNAs and other reagents gives us an opportunity to test additional Balbiani body properties. By injecting fluorescently tagged dextran molecules of different sizes, we found that the Balbiani body has a partial size exclusion barrier, which we speculate could aid in mRNA cargo selection. In the future, we will use this oocyte injection assay to perform structural analysis of known and novel Balbiani body proteins and to continue validating our proteomics library.

P1731
Board Number: B749
Determining the function and regulation of polymers of nucleotide biosynthetic enzymes during Drosophila oogenesis.
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CTP synthase (CTPS) and inosine monophosphate dehydrogenase (IMPDH) are two rate-limiting enzymes in de novo pyrimidine and purine nucleotide biosynthesis, respectively. They have been found to polymerize into filaments under conditions of nucleotide depletion or elevated demand for nucleotides in many different species and cell types. For example, these enzymes polymerize in nutrient-starved mammalian cell lines and CTPS polymerizes during normal Drosophila oogenesis, where germ cells undergo rapid cycles of endoreplication and rRNA synthesis. Our lab and other have demonstrated that CTPS assemblies are present during Drosophila egg development and we are using this model system to understand their function and regulation. We also recently reported that, IMPDH filament assembly has no effect on its biosynthetic activity, either as a purified protein or cultured cells. By contrast, others have found that filament assembly of CTPS can either inhibit or enhance activity of the enzyme, depending on the species. We hypothesize that assembly of these enzymes into filaments may regulate some other aspect of their biological function unrelated to their catalytic activity. We are currently examining this question in different ways for each of these two enzymes. For IMPDH, I am utilizing transgenic flies expressing human IMPDH2 constructs that either inhibit or promote filament assembly without altering enzyme activity to functionally rescue mutants of the Drosophila IMPDH gene. Human IMPDH rescues the impaired fertility of Drosophila IMPDH mutant flies equally whether it can form filaments or not, which means that its ability to form filaments is not necessary for its role in Drosophila egg development. Along with our recent discovery that IMPDH mutants that cannot form filaments have similar catalytic activity to those that do, this indicates that both in vitro and in vivo
filament formation has no effect on the catalytic activity of IMPDH. For CTPS we are screening genes involved in nucleotide biosynthesis, endoreplication, cell proliferation, and growth to find regulators of CTPS filament formation during Drosophila oogenesis in order to understand how CTPS assembly and disassembly is regulated and what is its biological function during oogenesis.

P1732
Board Number: B750
Membrane rafts regulate acrosome reaction via glucose signaling pathways in chicken sperm.
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Sperm possess compartmentalized metabolic and signaling pathways in specific regions of cells poised to function when needed. However, little is known about how multiple different pathways are orchestrated to function in different spatial and temporal scales for achieving fertilization. Previous studies of ours and others demonstrated in mammalian sperm that membrane rafts play roles in the multistage process of fertilization, including acrosome reaction. Beyond class, we recently demonstrated the presence of membrane rafts in sperm head region of avian sperm. This motivated us to perform quantitative proteome comparison between rafts and non-rafts in chicken sperm, which enabled us to find prominent association of glucose transporter 3 (GLUT3) with membrane rafts. In fact, glucose has been known to be beneficial to sperm of some mammalian species for supporting flagellar motility and optimal acrosome reaction. However, the effect of glucose on these functions are not known in any avian sperm. These combined with the fact of extremely high glucose content in chicken uterine fluid, led us to investigate the roles of glucose in regulation of chicken sperm functions with focus on the functional interaction of GLUT3 with membrane rafts. Localization and western blot experiments showed the presence of GLUT3 in the flagellum and acrosomal region. Sharp association of GLUT3 with membrane rafts was confirmed by comparison of low density detergent-resistant membrane protein from sperm depleted with/without sterols. Characterization of the roles of glucose on sperm functions showed that glucose stimulated acrosome reaction in response to inner peri-vitelline layer treatment by taken-up via glucose transporter(s) while flagellar motility was not influenced. However, glucose uptake was significantly inhibited by AMPK inhibitor treatment or alteration of membrane rafts by sterol depletion treatment, which resulted in diminution of acrosome reaction. APMKα is known to be abundant in the midpiece and acrosomal regions. To gain mechanistic insight into the regulation of AMPK activity, comparison of phosphorylation status of AMPKα(Thr172) was performed following by treatment with several stimulators and inhibitors under the presence of glucose, showing that glucose uptake occurs via glucose transporter(s) associated with membrane rafts and it stimulates phosphorylation of AMPKα. These results demonstrate in chicken sperm that membrane rafts regulate glucose uptake, thereby stimulating acrosome reaction via AMPK signaling pathway, and suggest its possible involvement in GLUT3 regulation. Our results provide new insight into avian sperm function as well as the roles of membrane rafts in sperm.
P1733  
**Board Number: B751**  
Intercellular communication in the mouse ovarian follicle analyzed by serial section electron microscopy.  
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Somatic cells in the mammalian ovarian follicle are intimately connected to the oocyte throughout follicular development through gap junctions. As the oocyte grows in response to gonadotropin hormones, the zona pellucida is deposited, creating a physical barrier 3-4 um-thick between the oocyte and the surrounding cells. Cumulus cells maintain their connection with the oocyte through cytoplasmic projections known as transzonal projections (TZPs), which transverse the zona pellucida and make gap junctions and adherens junctions with the oocyte surface. We used serial section electron microscopy to analyze TZPs and their relationship to other structures in mouse preovulatory follicles. We found that TZPs frequently interact with each other, and with oocyte microvilli. On average, each cumulus cell sends 9 TZPs that connect to the oocyte and 33 that are free-ended. Interestingly, cumulus cells send most of their cytoplasmic projections toward the oocyte, and virtually none away from it. Additionally, we found that mural granulosa cells in preovulatory follicles have cytoplasmic projections that resemble TZPs morphologically but are oriented toward other mural granulosa cells with no apparent preferred orientation. These projections are thin (76.1 nm in diameter), short, of approximately the width of the zona pellucida (3.3 um in length), and abundant (24 per cell). They often end on the surface or inside invaginations of neighboring cells, and can make gap junctions or annular junctions at the endings. In cell culture, mural granulosa and cumulus cells can interconvert depending on their proximity to an oocyte. We suggest that the somatic cells of the follicle use filopodia that are ~3-4 um in length to determine where they are in relation to the oocyte. The default state is the mural granulosa cell. If the cell contacts the oocyte, it directs its filopodia towards the oocyte and acquires the characteristic phenotype of the cumulus cell.

P1734  
**Board Number: B752**  
Guidance of stem cell niche assembly, position, and architecture.  
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Stem cells are required for renewal and regeneration of damaged or aging tissue. Intricate signaling between the niche and its resident stem cells is necessary to accomplish these tasks. Signaling requires intimate contact with a well-designed niche: one positioned accurately in the tissue, and with its constituent cells well-organized. Unfortunately, there are few cases where niche morphogenesis can be studied at the necessary resolution. **For this reason I have chosen to study niche morphogenesis in the Drosophila male gonad.** The eventual function of this niche is well defined, and has served as a paradigm in niche-stem cell biology. Furthermore, our lab recently pioneered live-imaging morphogenesis of the niche during embryogenesis. We capitalized on visualizing niche assembly to uncover candidate tissues that could be the source for signals that organize niche formation. Imaging revealed that the niche was located near the trachea, an alary muscle, and the visceral mesoderm, all of which are known to send developmental regulatory signals. We genetically ablated each of these tissues in turn, and discovered that the visceral mesoderm (Vm) was the tissue producing a guidance cue. To identify the potential guidance signal, I screened an extant database (http://fly-fish.ccbr.utoronto.ca/)
for signaling genes expressed within the Vm, and identified candidates such as Netrin, Wunen, and Neurotrophic factor. I am currently engineering strains that will allow me to knockdown these candidates to test for roles in guiding niche placement. In summary, I have identified a tissue source that guides niche assembly, and I have identified candidate signals that might be used for this purpose. This research represents the first work describing how the stem cell niche is positioned correctly during its development, and thus will bridge an important gap in our knowledge of stem cell-niche biology.

P1735
Board Number: B753
The Misshapen kinase is Essential for Normal Expansion and Stability of the Germline Ring Canals in the Developing Drosophila Egg Chamber.
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Intercellular bridges allow for the transfer of materials between neighboring cells. These connections are essential for the normal development of gametes in organisms from fruit flies to vertebrates; defects in intercellular bridges can lead to infertility. The developing Drosophila egg is an excellent model system to study intercellular bridges. Each fly egg develops from a multicellular structure called an egg chamber; the egg chambers is composed of a central cluster of germ cells surrounded by a layer of somatic epithelial cells. The germ cell cluster contains 16 cells (1 oocyte and 15 nurse cells) that are connected to each other through intercellular bridges called ring canals. Ring canals are actin-rich structures that arise through incomplete cytokinesis during formation of the germ cell cluster, and they are essential to allow the transfer of materials from the nurse cells to the developing oocyte. During stage 11 of oogenesis, the nurse cells rapidly transfer their cytoplasmic contents to the oocyte, doubling its volume in 30 minutes. To accommodate this transfer, the ring canals undergo a significant expansion to reach a final diameter of ~10 µm. Although a number of structural and regulatory components have been identified, there is still much to be learned about ring canal formation, stabilization, and expansion. We have identified a novel role for the Misshapen (Msn) kinase in regulating the growth and stability of the ring canals. Depletion of Msn by RNAi leads to over-expansion and instability/collapse of the ring canals without disrupting the recruitment of other ring canal components, such as Cheerio, Hts-RC, and Kelch. Over-expression of Msn leads to reduced ring canal diameters, whereas over-expression of a membrane-tethered form of Msn leads to ring canal collapse and the formation of multinucleate nurse cells. Therefore, regulation of Msn levels and localization are essential for the stability and expansion of the germline ring canals. Future studies will determine whether Msn acts with other known ring canal components to promote proper growth and stability of these intercellular bridges.

P1736
Board Number: B754
Mitochondrial protein ATAD-3 facilitates germ granule formation in C. elegans embryo.
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Germ granules are RNA/protein assemblages that contribute to the specification and proliferation of germ cells in animals (Voronina, 2011). Germ granules are not membrane bound, are enriched in intrinsically disordered proteins and exhibit properties of phase separated liquid droplets (Brangwynne, 2009). Interestingly, germ granules are commonly found in association mitochondria and the
endoplasmic reticulum, although neither the molecular basis nor the importance of these associations are understood (Voronina, 2011; Kloc 2014). In the C. elegans zygote, germ granules (called P granules in C. elegans) concentrate in the posterior cytoplasm due to their disassembly in the anterior and assembly/stabilization in the posterior (Brangwynne, 2009; Gallo, 2010). In order to begin to dissect the relationship between P granules and the ER and mitochondria, we followed the dynamics of these organelles in live embryos using both spinning disk confocal microscopy and lattice light sheet microscopy (at the Janelia Farm Advance Imaging Center). We find that P granules are persistently associated with and move in accordance with both mitochondria and the ER in the zygotic cytoplasm. Through a candidate RNAi screen, we identified the conserved mitochondrial AAA+ ATPase, ATAD-3, as a regulator of P granule dynamics. Mammalian ATAD-3 spans both the inner and outer mitochondrial membranes exposing a ~50 amino acid, intrinsically disordered N-terminal domain to the cytoplasm, where it has been proposed to mediate interactions with the ER (Gilquin, 2010; Issop, 2014). We find that endogenously tagged ATAD-3::OLLAS is uniformly distributed on mitochondria in the worm zygote. In atad-3(rna) embryos, we find that both the number and size of P granules are significantly reduced. ATAD-3 was previously shown to co-precipitate with MBK-2 (Chen, 2016), a kinase that stimulates the disassembly of P granules (Smith, 2016; Wang, 2014). Mutation of a predicted MBK-2 phosphorylation site in the N-terminus of ATAD-3 (T12A) results in a significant increase in the size and number of P granules and causes their distribution to spread further towards the anterior relative to wildtype embryos. Strikingly, when expressed as a transgene, the ATAD-3 N-terminus (ATAD-3(aa1-46)::mKate) is diffusely cytoplasmic in wild-type embryos but concentrates on the ER in mbk-2(rna) embryos. Based on these findings, we conclude that the mitochondrial protein ATAD-3 promotes the growth/stabilization of P granules and speculate that this activity may be inhibited by MBK-2 phosphorylation. We are currently testing whether ATAD-3 regulates P granule dynamics through direct interaction with P granule components and/or by mediating ER/mitochondrial contacts that could, in turn, help stabilize P granules.

P1737
Board Number: B755
Utilization of the auxin-degradation system to eliminate P granules in C. elegans.
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In C. elegans, germ granules, known as P granules, play a critical role in maintaining germline pluripotency. However, much remains unknown about how P granules carry out this function. One limitation has been the ability to deplete P granules completely due to redundancy in P granule proteins and technical limitations of RNAi. Recently our lab has tried to circumvent this issue by using the auxin-degradation system to degrade essential P granule proteins in C. elegans. Normally, in plants, Auxin combines with TIR1 to degrade any proteins with a degron sequence. For our research, we obtained a C. elegans strain with Tir1 under the control of a germline promoter. Then, using CRISPR, we engineered degron sequences onto critical P granule genes. Degradation of targeted P granule proteins was seen within four hours of auxin exposure, confirming that the auxin-degradation system can be used to target P granules in the germline of C. elegans. This technique now enables us to improve our research into how P granules help maintain germline pluripotency.
Ovaries from diabetic mice exhibit loss of follicles leading to reproductive failure.
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Non-insulin dependent (type 2) diabetes is characterized by a cell’s inability to efficiently process insulin, which results in high blood glucose levels, also known as hyperglycemia. While glucose is necessary for the maturation of oocytes, high glucose concentrations accelerate maturation of the oocyte (Chang, Dale and Moley, 2005). These increased, yet fluctuating, glucose levels impair meiotic development and cause lower rates of ovulation by inhibiting extracellular communication (Colton, Pieper and Downs, 2002). Diabetes has been shown to affect the meiotic maturation of oocytes during folliculogenesis, resulting in reduced preimplantation embryo development and increased rates of infertility (Chang, Dale and Moley, 2005).

In the ovarian follicle, both granulosa cells and cumulus cells surround the developing oocyte. Granulosa cells support oocyte growth by secreting hormones through paracrine signaling and gap junctions within the follicle. Decreases in oocyte-granulosa cell communication impair oocyte maturation (Chang, Dale and Moley, 2005). Cumulus cells aid in glycolysis by metabolizing glucose into products that promote oocyte maturation such as pyruvate and ATP (Wang, Chi, and Moley, 2012). These products are transferred to the oocytes via gap junctions, but when there are insufficient amounts of these byproducts, oocyte development is impaired. Previous studies have shown that hyperglycemia induces mitochondrial dysfunction and apoptosis within oocytes via cumulus cells; in the ovaries of diabetic mice, less pyruvate and ATP is produced by the cumulus cells (Wang et al., 2010). The decrease in glucose uptake from the cumulus cells has been associated with meiotic defects in oocytes such as spindle abnormalities, chromosome misalignment, and double strand breaks (Blasiak et al., 2004). These genetic breakages trigger the apoptosis of the cells, which reduces the ovulation rate and decreases fertility. This study examines the correlation between non-insulin dependent diabetes and the formation of oocytes by comparing the number and size of follicles in both wild type and diabetic mice.

Through histological analysis of whole mount ovaries, we have observed gross morphological defects at the organ level in diabetic ovaries compared to control littermates. Upon closer examination, we also identified a significant loss of primordial follicles (often referred to as the fertility pool) in diabetic mice. Future studies aim to look at earlier time points in hopes of identifying the stage where the oocytes are lost.

Only Sertoli cells cultured at high density mimic in vivo conditions.
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The seminiferous epithelium lining the seminiferous tubules of mammalian testes is comprised of Sertoli cells and germ cells. Sertoli cells are tall, columnar cells that extend from the base of the seminiferous epithelium to the tubule lumen. They play a key role in spermatogenesis by providing support and nourishment for the developing spermatogenic cells. We have been working to establish a primary culture system for studying junction turnover in morphologically differentiated Sertoli cells. The purpose of this study is to determine the optimal density of Sertoli cells in vitro for co-culture with spermatogenic cells. Sertoli cells were enzymatically dissociated from the testes of four 20-day-old
Sprague-Dawley rats and plated at low (1.3 x 10^5 cells/cm²), medium (4.1 x 10^5 cells/cm²) or high (1.3x10^6 cells/cm²) cell densities on Matrigel-coated Transwell® culture inserts. Sertoli cells were cultured in serum-free defined media (SFDM) containing hormones (FSH and testosterone), vitamins and antibiotics, and incubated at 34°C in 95% air and 5% CO₂ for a week. The cultured cells were treated with a brief hypotonic wash after two days to lyse any residual germ cells. The culture media was replenished every 48 hours. Finally, the cells were fixed and processed for TEM to determine if the Sertoli cells had a differentiated morphology as indicated by the presence of polarized cells with basally located junctions. Primary cultures of Sertoli cells cultured at high density in vitro formed a confluent layer within 24-48 hours with cells having a cuboidal to columnar shape. These cells formed areas with a monolayer interrupted by areas where cells formed focal clumps. Sertoli cells within this culture system were polarized with nuclei and junction networks located basally. The presence of ectoplasmic specializations and tight junctions as part of basal junction complexes indicated Sertoli cell differentiation in vitro. Sertoli cells cultured at low or medium cell densities formed clumps, but did not have cells between the clumps. These results suggest that a high Sertoli cell density is required for the cells to form a confluent monolayer with specialized junctions that closely mimic the blood-testis barrier in vivo.

P1740
Board Number: B758
Positive and Negative Regulation of Cell Fusion in Budding Yeast.
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Cell fusion is ubiquitous in eukaryotic organisms. We are studying cell fusion during mating of the budding yeast, S. cerevisiae. Haploid yeast cells detect each other by pheromone signaling, polarize growth toward the partner, and then fuse to form a diploid zygote. Fusion requires removal of the cell wall between the mating cells to allow plasma membrane fusion. Because premature or mislocalized cell wall removal would lead to lysis and death, the initiation of cell fusion must be highly regulated. The signal(s) that regulates when and where cell wall degradation occurs has not been identified. Rho-like GTPases regulate cell fusion in many systems, including myoblast fusion. In yeast, the highly conserved Cdc42p promotes yeast fusion via binding to Fus2p/Rvs161p, a pheromone-induced heterodimeric amphipath. Early in mating, Cdc42p functions in pheromone signaling and morphogenesis, independent of Fus2p, and the proteins do not colocalize. Later, in prezygotes, Fus2p recruits Cdc42p to a novel focus at the center of the zone of cell fusion (ZCF). After fusion, the proteins remain colocalized on the edge of the eroding ZCF remnant. Recruitment of Cdc42p by Fus2p was dependent on the flatness of the ZCF. In fps1 mutants mating to wild-type, increased osmotic pressure caused the ZCF to be curved and Cdc42p was mislocalized. In fps1 matings to fps1, the osmotic pressure was balanced, the ZCF was flat, and Cdc42p was localized. These observations suggest that Fus2p/Rvs161p recruits Cdc42p specifically to the flattened area of the ZCF that forms between two mating cells, promoting cell wall degradation and fusion.

On the other hand, evidence suggests that the cell wall integrity (CWI) pathway negatively regulates cell fusion. Five transmembrane sensors (Wsc1-3p, Mid2p, and Mtl1p) monitor cell wall damage and activate CWI signaling. Pheromone-induced morphogenesis activates CWI signaling, and activation of the downstream kinase, Pkc1p, blocks cell fusion. Loss of one CWI-sensor, Mid2p, causes death in response to pheromone. We hypothesize that, during mating, cell-cell contact must relieve CWI signaling to allow cell wall degradation. In support, we find pheromone caused mid2 death by loss of cell wall and plasma membrane integrity at the tip of the mating projection. In mid2, Cdc42p was recruited to a focus at the tip of the shmoo, dependent on Fus2p. Mutations in cell fusion genes (FUS1, FUS2,
**P1741**

**Board Number: B759**

**Finding required genes for proper sp-ut valve function in *C. elegans***

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The *Caenorhabditis elegans* (*C. elegans*) spermatheca, the site for embryo fertilization, is a tube composed of a single layer of cells that undergo cyclic stretching, constriction, and relaxation as ~150 oocytes pass through the gonad and into the spermatheca. This feature makes the *C. elegans* spermatheca an ideal model in which to explore mechanotransduction machinery in smooth muscle-like cells. The spermatheca is made up of three regions: the distal constriction, the bag, and the spermatheca-uterine (sp-ut) valve. Immediately after oocyte entry, the sp-ut valve constricts. After ~10 minutes, the bag begins to constrict, and the valve relaxes allowing the fertilized embryo to exit the spermatheca and enter the uterus. Although we have identified several genes required for transit of oocytes through the spermatheca, very little is known about the genes required for proper sp-ut valve function. To address this, we created a sp-ut valve specific RNAi strain by expressing rde-1 under a sp-ut valve specific promoter (tag-312). Additionally, we co-expressed GCaMP, a genetically-encoded calcium sensor in the sp-ut valve, to analyze calcium dynamics. Using this strain, we have conducted a candidate RNAi screen, in which we tested possible required genes and analyzed for defects in dilation or constriction timing in the sp-ut valve. We've identified several genes necessary for sp-ut valve function, including cytoskeletal regulators, gap junction proteins, and signal transduction proteins. This work will help us better understand communication between different cell types in tissue.

**P1742**

**Board Number: B760**

**Complexes regulating *C. elegans* eggshell formation and egg activation are scaffolded by a common protein.**

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Fertilization triggers rapid remodeling of the zygote surface to create an extracellular barrier that protects the developing embryo and prevents polyspermic fertilization. In nematodes, this barrier is a multi-layered eggshell that assembles in a hierarchical manner. The outermost layer of the eggshell is called the vitelline layer, which is present on the oocyte before fertilization. Immediately following fertilization, the vitelline layer lifts off the zygote and initiates remodeling of the extracellular surface. Despite its importance, little is known about the composition of the vitelline layer or how it is remodeled to form the outermost eggshell layer. A previous RNAi screen in the nematode *C. elegans* identified PERM-2 and PERM-4 as proteins required for eggshell formation. PERM-2 and PERM-4 localize to the vitelline layer and are co-dependent for proper localization based on RNAi and CRISPR null allele studies. PERM-2 and PERM-4 localization also depends on CBD-1, a protein previously shown to stabilize the EGG/CHS-1/MBK-2 complex involved in the oocyte-to-embryo transition. Interestingly, CBD-1 appears to scaffold the PERM complex and the EGG/CHS/MBK complex independently, suggesting dual roles for...
CBD-1 in eggshell formation and regulation of egg activation. Depletion of PERM-2/4 compromises the structural integrity of the outermost vitelline layer of the eggshell, but also disrupts subsequent assembly of the innermost permeability barrier that serves as the major line of defense in protecting the newly fertilized embryo.

P1743
Board Number: B761
Comparison of fertilization in the invasive zebra and quagga mussels.
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The zebra mussel (*Dreissena polymorpha*) and quagga mussel (*Dreissena rostriformis bugensis*) are two invasive freshwater mussels infesting many waterways of the eastern, central, and southwestern United States. Key to their remarkable spreading is their reproductive strategy. Unlike most North American freshwater bivalves, dreissenid mussels broadcast spawn gametes directly into the water column where fertilization and larval development occurs – a strategy common among marine bivalves. This objective of this study is a direct comparison of fertilization and early development between these two closely related species. Light, fluorescent, and electron microscopy techniques are used to observe the various stages of early development. Reproductive features compared include egg and sperm morphology, sperm-egg binding, sperm entry and egg activation, pronuclear formation, cleavage and early larval development. As previously reported, there are observable differences in sperm morphology, particularly the curved cell body of quagga mussel sperm. We observed numerous differences in egg morphology as well, including egg surface carbohydrates, cortical granules, and egg jelly layer. Sperm binding occurs perpendicular to and uniformly across the egg surface in both species. Sperm rotation occurs during entry with quagga mussels exhibiting a more dramatic wobble along the perpendicular axis due to their curved sperm morphology. Sperm entry and rotation within the egg cortex is similar. A microtubule bundle is observed linking the polar body with the female pronucleus. Timing of pronuclear formation and early cleavage events is reported. The question of hybridization potential between the species is also addressed. Fertilization and early development had many similarities between these two congeneric species, however, several prominent morphological and mechanistic differences were also observed.

P1744
Board Number: B762
Localization of N-terminally arginylated beta-actin in mouse oocytes.
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Protein arginylation is a posttranslational modification mediated by arginylationtransferase (Ate1), and plays essential roles in functions of cells, tissues, and organs. Gametogenesis is not an exception. In conditional knockout studies, Ate1 depletion affected the reproductive ability of mice (Leu et al., PLoS One, 2009; Brower et al., PLoS One, 2009; Liu et al., J Biol Chem, 2016). While it is reported that male infertility is due to apoptosis in Ate1-depleted germ cells (Liu et al. J Biol Chem, 2016), the effect of Ate1-depletion on female germ cells is still unknown, though it is suggested that premeiotic depletion of Ate1 causes the production of defective gametes (Leu et al. PLoS One, 2009). A hypothesis is that arginylation play a role in female gametogenesis via cytoskeleton for following reasons: 1) abnormal chromosome segregation during the first meiosis is the major cause for uneuploidy in oocytes, 2) While
microtubule is the main component of meiotic spindle, actin filament has an important role in meiosis as well, 3) lack of arginylation affects actin cytoskeleton in mouse embryonic fibroblast (MEF) (Karakozova et al., Science, 2005), and 4) aneuploidy occurs frequently in Ate1 knockout MEF, suggesting that arginylation affects chromosome segregation machinery (Rai et al., Oncogene, 2015). Here, for the first step to investigate the role of arginylation in female gametogenesis, we assessed the expression and localization of Ate1 and arginylated beta-actin (R-b-Actin) are by immunocytochemistry in oocytes at germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) stages. Diffusing signal for Ate1 is detected in the entire cytoplasm of oocytes at all stages examined, suggesting that proteins can be arginylated in oocytes at these stages. Short filamentous signal for R-b-actin is detected in the entire cytoplasm of GV stage oocytes, as well as non-arginylated beta actin. At MI stage, the R-b-actin signal accumulated and formed spindle shape around chromosomes, and this localization at the spindle is more dramatic compared to non-arginylated beta-actin, strongly suggests that R-b-actin has a distinctive role at the first meiosis. At MII stage, diffusing and non-filamentous signal for R-b-actin was observed in entire cytoplasm, and accumulation at meiotic spindles was not as clear as M1 stage. These results suggest that protein arginylation is involved in the mechanisms for normal chromosome segregation during first meiosis in oocytes, and could be the initial step of novel understanding of germ cell research toward arginylation-based treatment for female infertility and improvement of animal production.

**P1745**

**Board Number: B763**

Sugar Cane Extract (SCE) influence steroidogenesis in the testicular interstitial cell of Japanese quail.

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Sugar Cane Extract (SCE) is the residue after removing glucose, fructose and sucrose from molasses, which has been proved wide range of biological effects, including deodorization, anti-inflammatory and anti-oxidative, and already used in animal feed. Our pervious studies found SCE feed decrease male quail testis weight and cloaca gland size, however the mechanism underlying this phenomenon is still unknown. Here we show the effects of SCE on gonad function in male quails with testosterone concentration in serum decreased, and corticosterone increased. Steroidogenic enzymes P450c17, 17βHSD and 3βHSD expression in the testis showed dose dependent decrease. Immunofluorescence staining showed decreased aromatase and 3β-HSD after feeding SCE in the testis. Later, testicular interstitial cells were isolated and cultured with SCE and oLH; testosterone secretion as well as 3βHSD gene expression was suppressed by SCE. We propose a model in which SCE influences male quail gonadal function by suppressing the expression of 3β-HSD in testicular interstitial cells.
Patagonian blenny (Eleginops maclovinus) spermatozoa characterization and quality markers evaluation under chilling storage.

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Patagonian blenny (Eleginops maclovinus; known in Chile as robalo) population has been decreasing due to overexploitation. Little is known about the reproduction behavior of robalo, and even less about its reproduction biology. Fish sperm of several other teleost are described in literature, but research regarding fish sperm from native species from Chile is only beginning. Understanding the ability for Patagonian blenny sperm to sustain short-term storage is a key step to carry out artificial propagation of this species. In this aspect, characterization of the spermatozoon, evaluation of sperm function markers (membrane and DNA integrity; reactive oxygen species generation; mitochondrial membrane potential; dynamics of the ATP content; oxygen consumption), and also evaluation of the pH and osmolarity of the extracellular medium during chilling storage could be highly helpful for designing an in vitro management protocol for Patagonian blenny. The objective of this study was first to characterize the male gametes of this marine fish species with potential for aquaculture and then to evaluate the sperm function markers under chilling storage. Patagonian blenny spermatozoon structure and ultrastructure are consistent with spermatozoon from modern teleost with external fertilization, measuring ~40 μM length, a head of ~0.8 μM diameter, and containing 3 to 5 spherical mitochondria. Semen contains ~15×10⁹ spz mL⁻¹, an osmolarity of ~345 mOsm kg⁻¹ and pH of ~7.5. Analyses of sperm function were done during 14 day cold storage, under diluted (1:1 with Cortland solution) and undiluted conditions. The use of Cortland solution do improve the storage time from 3 to 7 days approximately, allowing better gas exchange, preventing desiccation and keeping membrane integrity better. Factors that affect most the storage time are reactive oxygen species (ROS) generation and unwanted motility process activation during storage, produced probably by osmolarity and ion content differences. In conclusion, Patagonian blenny semen is obtainable and manageable in vitro without sacrificing males, and can be stored to aid aquaculture of this species. Tools used in this research are useful for fish sperm analysis to include new species to aquaculture.

Acknowledgements: FONDECYT 1151315 (Farias, JG). Beca CONICYT Doctorado Nacional 21140852 (Ulloa, P).

A universal feature of early oocyte differentiation is formation of the Balbiani body (Bb), a large prion-like amyloid aggregate of specific mRNA-protein (mRNP) granules and organelles. The zebrafish Bb
establishes oocyte animal-vegetal polarity by specifying the oocyte vegetal pole. The Bb has been observed for two centuries, but how it forms and is asymmetrically positioned was unknown. Using quantitative image analysis, we traced oocyte symmetry breaking to a nuclear asymmetry at the onset of meiosis called the chromosomal bouquet. The bouquet is a universal feature of meiosis where all telomeres cluster to one pole on the nuclear envelope (NE). Telomere movements and clustering on the NE facilitate chromosomal pairing and meiotic recombination. We show that Bb granules first localize with the centrosome to the cytoplasm apposing the bouquet telomere cluster. They then aggregate around the centrosome in a specialized nuclear cleft that we identified, assembling the early Bb. We found that the bouquet nuclear events and the cytoplasmic Bb granule localization are mechanistically coordinated by microtubule. Thus, the animal-vegetal axis of the oocyte is aligned to the nuclear axis of the bouquet. This symmetry breaking lays upstream to Bucky ball, the only known regulator of Bb formation. However, Bucky ball, a prion-like protein, is then required for Bb granule nucleation around the centrosome, forming the mature Bb, and showing that oocyte polarization utilizes prion-like mechanisms. We link two universal features of oogenesis, the Bb and the chromosomal bouquet, to oocyte polarization, and propose that the bouquet centrosome functions as a cellular organizer that we term the Centrosome Organizing Center (COC), coupling meiosis and oocyte patterning and controlling prion-like nucleation. We next revealed that oocytes are organized in cysts with synchronized COCs. We discovered bouquet specific primary cilia that may synchronize and mechanically regulate cyst COCs. Moreover, the COC localizes near cytoplasmic bridges that connect sister cyst oocytes, suggesting its positioning by a previous mitotic division plane. These results link oocyte polarity to cyst organization. We uncovered a cellular organizer that integrates multiple processes of mitosis, meiosis, cilia biology and prion-like mechanisms in the early differentiating oocyte.

**P1748**

**Board Number: B766**

High saturated-fat diet induces hypercholesterolemia and impairs sperm motility in the Mongolian gerbil.

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Long term high-fat diet consumption leads to hyperlipidemia and obesity. Clinical and experimental data have shown that high fat intake or obesity alters steroid hormones and affects sperm motility and reproductive capacity. However, the influence of a particular lipid composition on testicular function is largely unknown. The present study investigated the effects of a high-saturated-fat diet (HSF) on the sperm parameters and steroidogenic function of gerbils (Meriones unguiculatus) and their relationship with metabolic alterations. Male gerbils (4w old, n=10 animals/group) were fed a balanced diet (C group) or a HSF containing 31% of lard (D group), for 12 weeks. The main endpoints evaluated were the adiposity index, blood lipid profile, serum and intratesticular testosterone (T) and 17β-estradiol (E₂) levels, sperm parameters, testicular apoptosis index and testicular expression of liver X receptor α (LXRα) and peroxisome proliferation activated receptor γ (PPARγ). HSF raised body weight by about 7% and the adiposity index by 26%. These alterations were due to an increase in retroperitoneal (C: 794±300; D: 1015±351mg) and visceral (C: 140±67; D: 213±122mg) fat deposits. HSF also altered the lipid profile, increasing total cholesterol and non-HDL cholesterol levels (C: 60.8±11.5; D: 99±15.3mg/dL and C: 25.5±5.2; D: 53.1±14mg/dL, respectively). No changes were detected in serum T and E₂ levels. However, intratesticular T level was lower (C: 6.4±2.7; D: 3.9±1pg/mL) and intratesticular E₂ level was

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Sunday-475
higher in D group (C: 18.2±11; D: 33.3±9.9). HSF did not affect daily sperm production nor sperm reserve in the cauda of the epididymis, but impaired sperm motility, reducing the number of spermatozoa with progressive movement (C: 60.7±5.7; D: 48.9±5.8) and increasing the number of spermatozoa with non-progressive movement (C: 17.5±3.7; D: 25.7±5.8) and immotile spermatozoa (C: 19.9±5.4; D: 25.4±4.4). The number of apoptotic cells in the testes increased in D group (C: 2.7±0.4; D: 3.7±0.5cells/mm²). Additionally, HSF did not alter the testicular expression of PPARγ but enhanced LXRα by about 63%, an important regulator of lipid homeostasis and cholesterol uptake. These findings suggest that other mechanisms are involved in steroidogenesis regulation under HSF that not those mediated by PPARγ and LXRα. In conclusion, HSF causes perturbations in testicular steroidogenesis of the gerbil, favoring estrogen production without impacting on circulating steroid levels. Moreover, HSF does not alter sperm production in this rodent but impairs sperm motility, an important parameter of semen quality, indicating that alterations in cholesterol metabolism are prejudicial to reproductive capacity. Financial support: FAPESP (2014/04146-2), CAPES, CNPq (308367/2014-6).

Embryogenesis

P1749
Board Number: B767
Focal adhesion proteins, vinculin and integrin β5, during early pregnancy in rat uterine epithelial cells: Anastrozole favors their normal distribution.
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An alternative superovulator to replace clomiphene citrate is needed as clomiphene citrate is associated with low pregnancy rates. Anastrozole is an effective superovulator, but it has not been well researched. In order to determine the effectiveness of anastrozole as a superovulator and to compare it with clomiphene citrate in similar situations, this study ascertained the effects of these drugs on the expression of the focal adhesion proteins, vinculin and integrin β5, which are uterine receptivity markers, in the uterine epithelial cells of day 1 and day 6 pregnant Wistar rats. The results show that vinculin and integrin β5 are co-localized at the base of the uterine epithelium at day 1 of pregnancy whereas at day 6, they disassemble from the basal focal adhesions and co-localize and significantly increase their expression apically (p<0.0001). Moreover, there is a significant difference in the protein expression levels of vinculin and integrin β5 in uterine luminal epithelial cells between untreated (control) and clomiphene citrate treated rats (p<0.0001), anastrozole and clomiphene citrate treated rats at day 6 (p<0.0001) suggesting the interpretation that anastrozole seems to enhance their expression in order to perhaps assist in the implantation process of the blastocyst. The immunofluorescence experiments agree with the vinculin and integrin β5 gene expression findings in which at day 6 of pregnancy, vinculin and integrin β5 gene expression are significantly up-regulated in uterine luminal epithelial cells in the anastrozole treated group relative to the calibrator sample (p<0.0001). These findings suggest that anastrozole is implantation friendly.
P1750
Board Number: B768
Role of integrin-linked kinase in melanocyte development.
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Integrin linked kinase (ILK) is a scaffold protein essential for epidermal integrity and permeability barrier function, as well as hair follicle development. However, the role of ILK in other epidermal cell types, such as melanocytes, remains unexplored. To determine the roles of ILK in melanocytic lineage cells, we generated a reporter mouse model that allows conditional Ilk gene inactivation in melanocytic cells, and their identification through green fluorescent protein (GFP) expression. During mouse embryogenesis, melanocyte precursors, termed melanoblasts, are formed from neural crest cells which begin to migrate towards the skin around embryonic day (E) 9. By E16.5, melanoblasts have homed to the epidermis and to developing hair follicles. To investigate if ILK is necessary for melanoblast migration and homing to the skin, we purified and quantified cutaneous melanoblasts from E15.5 embryos, using fluorescence activated cell sorting. We found a 60% reduction in the number of ILK-deficient melanoblasts compared to the abundance of ILK-expressing cells. Similarly, analysis of interfollicular epidermis in E17.5 embryos revealed a 50% decrease in the number of ILK-deficient cells relative to ILK-expressing melanoblasts. Significantly, ILK-deficient melanocytes were virtually undetectable in the interfollicular epidermis of E20.5 animals, indicating that ILK is implicated in embryonic melanoblast proliferation, migration, and/or survival. To further explore this possibility, we used primary melanocyte cultures to determine the effects of Ilk gene inactivation on proliferation and motility. ILK deficiency results in a decrease in the fraction of melanocytes that express the proliferation marker Ki67, from 45% in normal melanocytes, to 17% in ILK-deficient cells. Using live-cell videomicroscopy to assess motility over a 16-h interval, we determined that ILK-deficient cells exhibited a 3.5-fold decrease in distance travelled and speed, compared to ILK-expressing cells. Melanocytes respond to integrin stimulation by laminin-332 by forming dendrites, which are necessary for the transfer of melanin granules to adjacent cells. In the presence of laminin-332, 83% of ILK-expressing cells formed dendrites, with an average of 4 dendrites/cell. In contrast, 60% of ILK-deficient cells were dendritic, but with an average of 2 dendrites/cell. Together, our results show that ILK is essential for normal migration and/or maintenance of embryonic melanoblast populations in vivo, and for postnatal melanocyte proliferation, motility, and ability to respond to laminin-332.
Supported with funds from CIHR, NSERC and LHRI.

P1751
Board Number: B769
The role of Wolf-Hirschhorn Syndrome related genes in Xenopus development.
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Wolf-Hirschhorn Syndrome (WHS) is a neurodevelopmental disorder caused by a deletion or microduplication on the short arm of human chromosome 4. Mutations within this critical region result in craniofacial malformations, heart and skeletal defects, intellectual deficits, seizures, and microcephaly. Five genes are contained within this region; TACC3, NELFA, WHSC1, LETM1 and FGFR3. While the function of many of these genes has already been characterized, their role in early embryonic development and in relation to WHS still remain unknown. In order to investigate the function of each gene we have designed knockdown strategies to reduce gene function and observe phenotypic
differences in the context of neurodevelopment using Xenopus laevis. First, we performed whole-mount in situ hybridization in order to show the localization of each gene during different developmental stages. Here, we demonstrate that these five genes show variable expression during embryonic development with enrichment in the pharyngeal arches, craniofacial region and neural tube. Previously, we hypothesized that several of the symptoms associated with WHS may be due to disrupted neural crest cell migration, leading to the craniofacial abnormalities seen in WHS patients. Thus far, we have found that knockdown of each individual gene causes variable craniofacial malformations, as well as aberrant neural crest cell migration. We have also previously characterized the function of TACC3 as a microtubule plus TIP (+TIP), which promotes axon elongation and regulates microtubule dynamics, and unpublished work from our lab suggests that TACC3 may also function during neural crest cell migration. Further, we have also performed whole-mount immunohistochemistry in order to observe brain morphology and we have found that knockdown of WHSC1, TACC3, and NELFA results in decreased forebrain size. Together, these results help to further characterize the role of these five genes during early embryonic development, as well as elucidate their role in relation to Wolf-Hirschhorn Syndrome and neural crest cell migration.

P1752
Board Number: B770
Using Drosophila denticles as a model system to investigate the role of cytoskeletal proteins in the formation of actin-based protrusions.
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The formation of actin-based protrusions, such as the denticles in Drosophila melanogaster, requires the coordination of extensive actin-associated proteins to crosslink and bundle actin filaments. Mutations in such proteins can cause defects in the shape, structure, and function of actin-based protrusions and provide us with information on the molecular mechanisms of their function. Our goal was to establish a method for systematically examining denticle size and shape in order to compare the function of a variety of genes in the formation of these protrusions. Here, we examined the mutants of several genes known to cause bristle and wing hair defects for their effect on denticle morphology. Cuticle preps of late stage embryos from loss of function alleles of singed, forked, twinfilin, flare, and ck/MyoVIIA were examined for their overall denticle morphology (shape) as well as for the height and widths of the denticles. Single mutants, ck; twinfilin and ck; flare double mutants, and snf; ck triple mutants were compared to determine if combinations of the genes might show genetic interactions. Although severe phenotypes were observed in bristles and wing hairs, singed, forked, twinfilin and flare mutants showed mild denticle defects suggesting that the stage of development and potential interactors present at that stage might influence the effect on the actin-based protrusions. Combination of the alleles with the loss of ck did not enhance all of the phenotypes and denticles of multiple mutants often looked similar to ck mutants.
The patterns of earliest cell divisions in a vertebrate embryo define later developmental events such as gastrulation, organogenesis, and the establishment of an overall body plan. Thus, understanding how early division patterns are regulated is critical for the study of vertebrate development. Here, we utilized an easily tractable vertebrate model, zebrafish, for single cell lineage tracing to examine the role of spindle placement in embryonic cellular patterning. Our studies identified a relationship between cell size and spindle positioning that regulates cell placement. We first found that embryonic cells decrease in size by approximately half following each round of division for the first six cell cycles post-fertilization. Throughout these divisions, spindle size remains relatively constant. We found this to be conserved in an invertebrate embryo, C. elegans, where cell diameter decreased to a greater degree compared to spindle length during the first ten cell divisions. We then found that during the first five rounds of zebrafish cell division a 2-D pattern of one-tiered cell arrays are formed. These cells are primarily elongated along the X-Y axis, which the spindles align to, with minimal elongation along the Z-axis. However during the sixth round of division, spindles position along the cells’ Z-axis, resulting in a two-tiered cell structure. During the sixth round, the cellular X-Y axis is no longer elongated compared to the Z-axis. Thus, our studies suggest a shape-sensing mechanism realigns the spindle from an X-Y axis alignment to the Z-axis. We find that astral microtubules, microtubules that extend from mitotic spindle poles towards the cell cortex, play a pivotal role in this process. A metaphase cell astral microtubules do not contact the cell cortex during the first five-rounds of division, but as the cell diameter decreases, astral microtubules from one mitotic spindle pole asymmetrically contacts the cell cortex closer to the yolk. These studies suggest that asymmetric astral microtubule contact at the cell cortex allows for spindle orientation away from the yolk boundary creating a two-tiered cell array.

Fundamental mechanisms of spindle assembly and subcellular scaling have been elucidated using cell-free extracts prepared from Xenopus eggs and embryos. By generating hybrids between related but different-sized Xenopus species, we are also investigating size control mechanisms at the cellular and organismal levels, as well as the barriers that lead to hybrid inviability. X. laevis (~10 cm adult body length) are larger than X. borealis (~7 cm), but both possess diploid genomes of 36 chromosomes. In contrast, X. tropicalis has 20 chromosomes and is significantly smaller (~4 cm adults). Scaling at the organismal and genome levels is accompanied by differences in the size of the egg as well as nuclei and spindles formed in egg extracts. Despite these size differences, the close phylogenetic relationship among these species allows the production of hybrid embryos by cross-fertilization. While the hybrid produced when X. laevis eggs are fertilized by X. tropicalis sperm (l×t) is viable, the reverse hybrid (t×l) is inviable and dies at the late blastula stage. Similarly, while the hybrid produced when X. borealis eggs are fertilized by X. tropicalis sperm (b×t) is viable, the reverse hybrid (t×b) is inviable. Cross-
fertilizations between *X. laevis* and *X. borealis* are viable in both directions. We applied cell biological tools and high-throughput methods to study the mechanisms underlying hybrid inviability. Strikingly, we observed mitotic defects in both *t*.xl* and *t*.xb* hybrids starting early in development that include chromosome bridges and lagging chromosomes in anaphase. Using egg extracts, we found that paternal genomes do not impair spindle assembly. Instead, DNA sequencing revealed that specific paternal chromosomes are incompatible with the *X. tropicalis* cytoplasm and are mis-segregated during mitosis in *t*.xl* and *t*.xb* hybrids. Remarkably, these chromosomes differ between the *X. laevis* and the *X. borealis* genomes. In *t*.xl* hybrid embryos, we found that specific chromosomal loss leads to death due to unbalanced gene expression at the maternal to zygotic transition, resulting in a metabolic crisis and cell-autonomous cell death. In contrast, the different chromosomal loss in *t*.xb* hybrid embryos does not impair metabolism, but leads to exogastrulation. Mechanisms underlying chromosome loss are currently under investigation.

**P1755**

**Board Number: B773**

**DDX3 induces neural crest through activation of an Akt-Wnt signaling axis.**

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DDX3 is a DEAD-box RNA helicase critical for normal development. Recently, mutations in DDX3 were found to be associated with multiple abnormalities including cleft lip/palate, phenotypes that are often caused by aberrant neural crest development. Here we show that knockdown of DDX3 inhibits neural crest induction in Xenopus embryos. This is accompanied by reduction in Akt activity and canonical Wnt signaling. Blocking Akt activity phenocopies DDX3 loss of function in inhibiting Wnt signaling and neural crest induction, and a constitutively active mutant of Akt rescues the neural crest induction phenotypes caused by DDX3 knockdown. These results show for the first time that a DDX3-Akt-Wnt axis is required for neural crest induction.

**P1756**

**Board Number: B774**

**Vg1 ortholog Gdf3 is required for Nodal dependent developmental processes in zebrafish.**

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The formation of mesendoderm and the placement of organs are conserved events within vertebrates that depend on the proper temporal and spatial activation of the Nodal signaling pathway—a Smad2/3 based pathway activated by the TGFβ superfamily member Nodal. In zebrafish, the two *nodal* orthologs *squint* (*sqt*, or *nodal-related 1*) and *cycllops* (*cyc*, or *nodal-related 2*) are required during blastula and gastrula stages for proper mesendoderm formation. Expression of a third *nodal* ortholog, *southpaw* (*spaw*), in the left-lateral plate mesoderm during somitogenesis is required for the proper asymmetric organization of organs such as the heart and liver. Here, we present evidence that another TGFβ ligand, Growth differentiation factor 3 (*Gdf3* or *Vg1*) is required for proper Nodal signaling during mesendoderm formation and left-right patterning in zebrafish. Using CRISPR/Cas9 techniques we have generated several mutant alleles of *gdf3*. *gdf3* is maternally supplied with restricted zygotic expression
domains emerging during gastrulation and somitogenesis. However, our gdf3 mutants demonstrate that maternally supplied gdf3 is capable of functioning for both mesendoderm and left-right patterning without being spatially restricted. Embryos lacking maternal and zygotic gdf3 lack most mesendodermal structures, resembling the phenotypes observed in cyc; sqt double mutants, or mutants lacking the Nodal coreceptor one-eyed pinhead (oeo, or EGF-CFC). While these results show Gdf3 is required for robust Nodal signaling, we show Nodal signaling is not completely abrogated, but severely attenuated. Utilizing mutants and morpholinos, we demonstrate that Gdf3 is also required for proper development of the laterality organ in zebrafish called Kupffer’s vesicle and for robust expression of spaw during left-right patterning. Collectively, our data indicate that gdf3 is important for multiple Nodal dependent developmental processes in the zebrafish embryo. We hypothesize that Gdf3:Nodal heterodimers are more potent signaling molecules than Nodal or Gdf3 homodimers.

**P1757**

**Board Number: B775**

*Drosophila Importin-7 is required for proper muscle attachment site formation.*

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The proper localization of proteins within cells is essential for tissue formation during the development of all organisms. Mutations that prevent or alter this normal subcellular localization may affect organismal survival and/or fitness. For example, the attachment of muscles to epidermally-derived tendon cells in embryogenesis requires the deposition of extracellular matrix (ECM) components tethered to integral complexes on the opposing surface of muscle and tendon cells. Incorrect formation these muscle attachment sites (MASs) results in muscle detachment, a loss of locomotor ability, and lethality. We previously published that embryos with mutations in moleskin (msk), which encodes for Drosophila Importin 7 (Dim7), exhibit defects in muscle-tendon attachment. Dim7 is a dynamic protein that regulates the nucleocytoplasmic shuttling of proteins in the early embryo, but also accumulates at MASs later during the process of myogenesis. Dim7 has an N-terminal Importin-beta, (IBNN) domain, an internal exportin Cse1-like (Cse1) domain, followed by an uncharacterized C-terminal region with no identifiable domains. To determine how Dim7 gets recruited into the nucleus or MAS, we generated YFP-tagged deletion mutants that remove regions of Dim7. Briefly, the Dim7-YFP deletion constructs under UAS control (UAS-Dim7ΔA-F) were recombined into a msk mutant background and examined for the distribution of Dim7 in muscle tissue under the mef2 promoter. Expression of the IBNN domain (Dim7ΔA) alone in msk mutants resulted in the strong nuclear accumulation of Dim7 without apparent muscle attachment defects. Removal of the entire N-terminus, including the IBN_N and Cse1 domains (Dim7ΔE) did not alter the overall distribution of Dim7 in muscles, but reduced the ability of the attachment site to form. These data, taken together suggest that the nuclear function of Msk may be sufficient to initiate muscle-tendon attachment, possibly through the previously identified epidermal growth factor (Egf) signaling pathway. Future experiments will test this idea by preventing the nuclear translocation of Dim7.
P1758
Board Number: B776
Using Xenopus laevis as a model for characterizing the function of C16orf52 during early embryonic development.
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The various processes that occur during early embryonic development are extremely complex and require thousands of different genes, each of which encodes for a protein that has a distinct function. Due to the vast amount of genes within the genome, many still have not yet been characterized in the context of early embryonic development. One such gene is C16orf52, which is a protein coding gene located specifically at 16p12.1. The cell type specific functions of this gene still have not been investigated in relation to development. In order to elucidate the function of this uncharacterized gene, we have created an antisense morpholino oligonucleotide to knock down this genes function and observe phenotypic differences in Xenopus laevis. Importantly, this gene is highly conserved between humans and Xenopus laevis, making it an ideal model organism to understand the developmental mechanisms of this gene. Here, we investigate the functions of C16orf52 by knocking it down and quantifying phenotypic differences in terms of craniofacial morphology, brain morphology, and axon outgrowth patterns. Thus far, we have found that reduction of this gene results in severe craniofacial and cartilage defects, such as abnormal pigmentation, smaller eyes, smaller face, as well as abnormal mouth and nostril formation. Additionally, the knockdown of this gene results in aberrant axon outgrowth by decreasing axon length and velocity, and increasing axon retraction. Further, we have also performed whole-mount in situ hybridization to show the localization of this gene during different stages of development, as well as whole-mount immunohistochemistry to see whether the reduction of this gene alters brain morphology. Together, our results suggest that this gene may play a significant role during early embryonic development and future studies will focus on the mechanisms by which this gene regulates different aspects of craniofacial morphology, brain morphology, and axon outgrowth patterns.

P1759
Board Number: B777
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Little is known about the maternal factors that function in body axis formation during vertebrate embryonic development. To identify these factors, a recessive maternal-effect mutagenesis screen was performed in the zebrafish Danio rerio. A number of mutants with defects in early developmental processes, including early morphogenesis and body axis formation were identified. One such mutant, split top exhibits a dorsalization of the embryonic axis. Clutches of embryos from split top mutant mothers are characterized by the five classic dorsalized phenotypic classes, as well as some additional defects. The mutant embryos show an expansion of dorsal markers and a corresponding reduction in ventral markers during gastrulation indicative of dorsalization. The dorsalization defects can be rescued by misexpression of either BMP2 or BMP7 ligands, or by derepression of BMP signaling by knockdown of BMP antagonists. The additional defects appear to be the result of altered morphogenesis, including
defects in epiboly progression, the process by which the blastoderm cells migrate over and surround the yolk. Mutant embryos display altered microtubule and actin cytoskeletal networks in the yolk cell, which can account for the epiboly defects observed. split top mutant embryos also appear to be defective in the cell movement process of convergence and extension. We mapped the split top mutation to chromosome 17, and identified cathepsin B, a through RNA-Seq and traditional positional cloning methods as the gene disrupted in split top mutants. This work was supported by NIH grant R01-GM56326, NIH training grant T32HD007516, the PENN-PORT training program, and by the Mississippi INBRE, funded by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103476.

P1760
Board Number: B778
NLRP7's Key Role in Primate Trophoblast Differentiation.
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Hydatidiform mole (HM), a gestational trophoblastic disease, can be explained as a pregnancy with no or improper embryo development and abnormal trophoblast proliferation. NLRP7, a member of NOD Like receptor family, which takes part in innate immunity, was identified as the first causative gene of recurrent HM. Very little is known about the function of NLRP7 in inflammation whereas its mechanistic contribution in HM formation is still unknown. To elucidate NLRP7's function in HM, we generated induced pluripotent stem cells (iPSC) from a patient who carries a heterozygous NLRP7 and NLRP2 deletion (NLRP7+2Δ). This strategy allows us to overcome the ethical considerations in working with human embryonic tissues and primary trophoblast cells and we also circumvent the inability to perform in vivo animal experiments since rodents lack NLRP7. Patient’s primary fibroblast cells were successfully converted to iPSCs using non-integrating episomal strategy and iPSCs were differentiated to trophoblast cells upon BMP-4 exposure together with inhibitors of ACTIVIN and FGF2 pathways. We demonstrated that under BMP-4 conditions, cells begin to express several trophoblast markers; CDX2, CGB, HLA-G, PGF whereas they lose expression of pluripotency markers; POU5F1 and NANOG. iPSCs derived from NLRP7+2Δ have a tendency to differentiate faster and more into trophoblast cells compared to wild type cells, which is possibly the root of HM. This idea is further supported by recovery experiments showing that introduction of NLRP7 decelerates this differentiation process. In conclusion, NLRP7 appears to have a critical contribution in trophoblast differentiation during early embryo development and is an essential regulator of primate embryogenesis.

P1761
Board Number: B779
microRNA cross regulation of gene regulatory network and signaling pathways.
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The microRNAs are small non-coding RNAs that repress the translation and reduce the stability of target mRNAs in animal cells. microRNA-31 (miR-31) has been found to play a role in cancer, bone formation, and lymphatic development. However, the function of miR-31 in embryogenesis is not well described. We examined the role of miR-31 in early development, using the sea urchin embryo as a model. We
found that miR-31 is expressed in all stages of development and its knockdown (KD) resulted in defects in the patterning and function of the skeletogenic primary mesenchyme cells (PMCs). Using bioinformatics and luciferase reporter constructs, we identified miR-31 to repress genes within the PMC gene regulatory network (GRN). The majority of the mislocalized PMCs in miR-31 KD embryos did not express VegfR10, indicating that miR-31 regulates the ability of PMCs to respond to positioning cues. We also found that miR-31 directly suppresses Eve and indirectly regulates Vegf3 in the ectoderm. These results indicate that miR-31 coordinate suppresses genes within the GRN of PMCs and in the ectoderm to impact PMC patterning and skeletogenesis. To understand the function of miR-31 at a systems level, we used miR-31 pull down assays to identify its direct targets. This study will reveal how miR-31 cross-regulates GRNs and signaling pathways to ensure proper development. Since miR-31, GRNs, and signaling pathways are highly conserved in animals, this study will enhance the understanding of fundamental mechanisms used by a developing embryo to build its precise organization.

P1762
Board Number: B780
MicroRNA regulation of Dishevelled in early embryonic development.
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MicroRNAs (miRNAs) are highly conserved, small non-coding RNAs that regulate gene expressions by binding to the 3’ UTR of target mRNAs and silence translation. MiRNAs are key regulators of the Wnt signaling pathway that are known to affect cell proliferation, migration, polarity and other developmental processes. This study investigates miRNA regulation of different isoforms of Dishevelled (Dsh/Dvl), an important signaling protein located upstream of β-catenin. At least three isoforms of Dvl are found in the sea urchin embryo, where they have similar spatial localization in early development but later show distinct ciliary staining in select isoforms. Using luciferase assays and site-directed mutagenesis, we demonstrated that the different isoforms of Dvl are directly regulated by miRNAs. By blocking miRNA regulation of all Dvl isoforms using miRNA target protector morpholino oligonucleotides (miRNA TP MASO), we observed dose-dependent defects of the embryonic skeleton, patterning of the skeletogenic primary mesenchyme cells, and morphological defects in the gut. We will identify the molecular mechanism of how miRNA regulation of Dvl impacts early development.

P1763
Board Number: B781
Histone Abundance Adjusts the Timing of the Zygotic Genome Activation in Drosophila.
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The mid-blastula transition (MBT) is a crucial period during embryogenesis where the embryos of many species including flies, fish, and frogs switch from fast cell cycles driven by maternal components to slower cycles dependent on zygotic transcripts. Prior to the MBT zygotic transcription is repressed and major zygotic transcription is activated during this transition. The timing of the MBT is sensitive to changes in the ratio of DNA to cytoplasm which is exponentially increasing as cells complete repeated rounds of DNA replication without growth. This lead to the hypothesis that titration of a maternally provided component against the increasing quantity of DNA may serve as a trigger to activate many downstream events. Our previous work suggests that histones may be this titrated component. Here
show that in Drosophila quantitative overexpression of the early embryonic histone pool leads to delayed cell cycle slowing and delayed gastrulation. Similarly, histone-overexpressing embryos activate transcription of many early genes more weakly and in later cell cycles than controls. Conversely, transcription is initiated more strongly and earlier in histone depleted embryos. Furthermore we explore the relationship between these changes in transcriptional levels and nucleosome occupancy on the genome. This work opens the door for more detailed molecular analysis of the interaction between histone levels, transcription factors, and the cell cycle machinery during early development.

P1764

Board Number: B782

Identifying functional domains in the histone anchor Jabba.

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Because both excess and dearth of histones result in widespread cellular defects, histone synthesis and turnover are typically tightly controlled. Early Drosophila embryos employ a unique sequestration mechanism to regulate histone availability: histones H2B, H2A, and H2Av are transiently recruited to lipid droplets (LDs), fat storage organelles in the cytoplasm. This sequestration mediates both storage of excess histones and short-term buffering of the histone supply. A key player is the protein Jabba, which anchors histones to LDs. However, its molecular mechanism remains unknown.

We have generated a genomic Jabba transgene that restores Jabba expression to wild-type levels. It rescues histone localization to LDs, short-term H2Av buffering, and the developmental defects associated with lack of Jabba. This sets the stage for a structure-function analysis of Jabba.

To identify functional domains in Jabba, we employ two strategies: characterizing Jabba isoforms in vivo and Jabba deletion constructs in cultured cells. Using RNAseq and RT-PCR, we find that in embryos Jabba expression is dominated by three of seven predicted isoforms, namely B, G, and H. We have generated embryos expressing solely mCherry-tagged JabbaB or JabbaG fusion proteins in a Jabba null background. Both constructs localize to LDs and are sufficient to recruit histones. These results suggest that LD localization and histone recruitment are mediated by Jabba’s common N-terminal 316aa.

Using imaging and a luciferase complementation assay, we have identified distinct Jabba regions that mediate targeting to LDs and interactions with histones in Kc167 cells, respectively: Jabba[1-108] localizes to LDs, but does not interact with histones; Jabba[193-320] interacts with histones, but is diffusely distributed throughout the cell. In addition, deleting amino acids 223-248 abolishes histone binding, without compromising LD targeting.

To determine in vivo relevance, we are now testing various Jabba transgenes in flies. Jabba[1-192] and Jabba(delta223-248) localize to embryonic LDs as expected. Jabba[193-320] is undetectable in embryos, but expressed in ovaries, where it is highly enriched in the nuclei of nurse cells. Similar nuclear localization of Jabba[193-320] is observed in fat body, salivary glands, and Kc167 cells. During normal development, the cytoplasm of nurse cells is transferred to the oocyte, while their nuclei degenerate via apoptosis. We propose that Jabba localization to LDs retains histones in the cytoplasm and thus promotes their transport from nurse cells to oocyte.
P1765
Board Number: B783
The functional and structural analysis of Drosophila robo2 in axon guidance.
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No matter the complexity of bilateral animals, axons are posed with the central problem of whether or not they should cross the midline. Roundabout (Robo) family proteins regulate many axon guidance decisions in the Drosophila embryonic central nervous system. Robo1 and Robo2 facilitate midline repulsion in response to Slit, while Robo2 and Robo3 define the lateral position of longitudinal axon pathways. In addition to these shared roles, Robo2 can also promote midline crossing of axons, an activity that is not shared by the other Drosophila Robos. My project will give me insight on molecular mechanisms of Robo2’s functional diversity as a transmembrane receptor and therefore also may apply to vertebrate species. Drosophila Robo2 plays at least three distinct roles in axon guidance in the fly embryo (midline repulsion, pro-midline crossing, and lateral positioning). Previous gain of function and genetic rescue studies suggest that the different roles of Robo2 are specified by individual immunoglobulin-like (Ig) domains within the receptor. Ig2 is required for Robo2’s pro-crossing function, while Ig1 and Ig3 are thought to regulate lateral positioning. It has been assumed (but not directly demonstrated) that Robo2 acts as a canonical cell-autonomous Slit receptor to signal midline repulsion; if so, this activity would likely require the Slit-binding Ig1 domain of Robo2.
We are using a CRISPR/Cas9-based gene replacement approach to investigate which domains of Robo2 (Ig & Fn) are required for each of its axon guidance activities. By replacing the robo2 coding region with epitope-tagged cDNAs, in which individual domains have been deleted, we are examining the contributions of each domain to receptor localization, regulation, and Robo2-dependent axon guidance outcomes. We observed a mislocalization of protein when looking at Robo2 without its Ig1 and Ig3 domains. We are also currently examining the roles of the cytoplasmic domains of Robo2 with a similar approach. Our results promise to increase our understanding of how individual receptors can contribute to multiple axon guidance outcomes during developmental wiring of the nervous system.

P1766
Board Number: B784
The role of MNL1 during neural crest cell developmental defects in mice.
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MNL1 is required for development in several species and associated with human diseases having development defects. Previously, we demonstrated that mice lacking MNL1 displayed several development defects during embryonic days, especially in development defects of peripheral nerve. In addition, MNL1 (conventional) deficiency mice showed hypopigmentation on their belly and limbs. This phenotype is caused by a depletion of melanocytes that are differentiated in the neural crest (NC) cells. In this connection, the transcription factor microphthalmia-associated transcription factor (MITF) occurs the primary regulator of melanocyte and regulates the development of melanocytes from the NC. Neural crest (NC) cells are transient group of cells unusual to vertebrates that occur in the embryonic ectoderm cell layer and produce a variety cell lineage including melanocytes, peripheral and intestinal neurons, and glia cells. Here, we used neural crest (NC) specific deleted MNL1 (conditional) mice to determine precise effects of MNL1 in NC cells. To produce deletion of MNL1 in NC cells, we used floxed
version of MNL1 which has subsequently been crossed with Wnt1-cre which expresses in the NC precursor cells. We confirmed the expression of MNL1 in NC cells during embryonic days to determine that deletion of MNL1 occurred in NC cells. We observed that the expression of MNL1 was disappeared. As a result, hypopigmentation observed in Neural crest-specific deletion of MNL1. Also, the hydrocephaly, postnatal lethality and abnormalities during embryonic days were observed in Neural crest-specific deletion of MNL1. In addition, we further studied Sox10 which plays an important role in NC development, MITF and its down-regulation genes in neural crest-specific deletion of MNL1. Thus, we demonstrated that decreased levels of these genes reduces melanocyte development. In conclusion, our data report that MNL1 plays an important role in hypopigmentation.

P1767
Board Number: B785
Role of Peptidylglycine α-Amidating Monooxygenase in the Adaptive Plasticity of Embryonic Hatching in Zebrafish.
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Hatching in anamniotes is a critical developmental event, but its timing is adaptively plastic, responding to environmental conditions. To elucidate the role of nutrient availability in this process, we took advantage of the well-defined phenotypes exhibited by zebrafish with genetic and nutritional deficits in the metabolism of copper, a trace element essential for development. Treatment of developing embryos with neocuproine, a membrane permeant copper chelator, resulted in a dose-dependent decrease in the rate and percent of hatching through 96 hours post fertilization. These data indicated that a copper-dependent pathway was involved in the hatching process. Peptidylglycine α-amidating monooxygenase (PAM), an evolutionarily conserved cuproenzyme, is essential for the biosynthesis of several peptides required in neuroendocrine physiology. Consistent with a direct role for this cuproenzyme in the copper-dependent hatching pathway, antisense (morpholino) abrogation of PAM expression resulted in a similar reduction in the rate and percent of hatching. Assays for PAM enzymatic activity revealed a marked decrease in PAM-morpholino injected embryos when compared to uninjected and control-morpholino injected embryos. In normal embryonic development the hatching gland, a developmentally regulated single sheet of cells, forms just over the yolk sac. As the embryo develops, hatching gland cells release a cysteine proteinase, cathepsin L, into the perivitelline fluid to degrade the chorion; hatching gland cells then undergo apoptosis. Although hatching gland morphology was normal in PAM-morpholino injected embryos, the expression and activity of cathepsin L were reduced in both PAM-morpholino injected and neocuproine-treated embryos, revealing a role for PAM in hatching gland function. To further understand the role of the hatching gland, transgenic fish expressing cytosolic EGFP under control of the cathepsin L promoter (Tg[ctslb:EGFP]) were generated. Transgenic embryos treated with neocuproine displayed more EGFP+ hatching gland cells at embryonic stages beyond when hatching should have taken place, indicating that these cells continued to express cathepsin L and failed to undergo apoptosis. Taken together, these studies reveal a critical role for copper availability in the adaptive plasticity of hatching and suggest a role for peptidergic signaling in this process. Support: DK032949 and R37DK44464
The effects of bisphenol A & alternatives, individually & in combination, on the development of *Xenopus laevis* (clawed frog).

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Bisphenol A (BPA) is used as an intermediate in the production of polycarbonate plastics and epoxy resins, which can be found in adhesives, food and drink packaging, and paper coatings. We are comparing the effects of BPA, a known environmental endocrine disruptor, to its replacements, bisphenol S (BPS) and bisphenol F (BPF), individually and in combination, on the development of *Xenopus laevis* (clawed frog). BPA is known to stimulate cellular responses by binding to estrogen receptors as well as being a thyroid hormone antagonist. The effects of BPS and BPF are not yet understood, however they have been found in urine samples. The development of *X. laevis* has been used as a model system due to the large number of offspring produced, the relatively rapid rate of development, and the organism's dependence on thyroid hormones for metamorphosis. Two trials were completed: the first trial had 11 groups starting at stages 8-9 and the second trial had 9 groups starting at stage 16. Embryos were incubated in concentrations of 5 μg/mL and 10 μg/mL of BPA, BPS, and BPF and combinations of the three at concentrations of 5 μg/mL, 3 μg/mL, and 6 μg/mL. Survivorship, length and rate of development were measured and any changes in morphology were noted. We observed a bubble malformation in the following groups: 5 μg/mL BPF, 5 μg/mL BPA, 10 μg/mL BPS, 10 μg/mL BPF, 5 μg/mL BPA: 5 μg/mL BPF, and 6 μg/mL BPA: 3 μg/mL BPS. The bubble malformations were found in the head or body region of the tadpoles. Two malformations of the tail were also found in 10 μg/mL BPS and 5 μg/mL BPA: 5 μg/mL BPS groups. Several statistically significant differences in length were found. The 5 μg/mL BPA: 5 μg/mL BPF group averaged around 2 cm smaller than the control group. The 3 μg/mL BPA: 6 μg/mL BPS and the 6 μg/mL BPA: 3 μg/mL BPS groups were 1 cm larger than the control group. These differences in length were found only in combination groups. The highest rates of mortality were seen in 10 μg/mL BPF and 10 μg/mL BPA groups. Results indicate lower doses of BPS may have greater effects on mortality than the higher doses. There is a need for further investigation due to important environmental implications. Supported by a grant from the TriBeta Research Foundation.

Physical and molecular mechanisms of cell cycle synchronization in early Drosophila embryos.

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One of the most fascinating questions in biology is how spatiotemporal coordination is achieved in cells, tissues and populations. A remarkable example is the synchronized divisions that take place in the developing *Drosophila* embryo. The Drosophila embryo develops as a multinucleated syncytium (a common cytoplasm not divided by membranes), which undergoes 13 rapid and synchronous nuclear divisions. The synchrony of these early cell cycles is essential for proper embryonic development. In my talk, I will describe the recent work of my lab to dissect the molecular and physical mechanisms that ensure the spatiotemporal coordination of the cell cycle in early *Drosophila* embryos. I will show how
waves of Cdk1 activity synchronize the cell cycle, how such waves are controlled by an active mechanism during DNA replication, while mitotic waves are a passive, kinematic consequence of S-phase waves. Molecularly, the waves do not require the positive feedbacks that regulate mitosis, but are controlled by a double negative feedback between Cdk1 and Chk1. I will also present theoretical arguments, supported by experimental observations, which indicate that the observed waves are originated by a new physical mechanism, in which waves of chemical activity arise in time-dependent bistable systems. Finally, I will discuss how tissue-wide synchronization is established, presenting a new model integrating Cdk1 oscillations, actomyosin contractility and nuclear spreading. Our experiments argue for a new mechanism by which global synchronization can arise from a spreading of local synchrony.

P1770
Board Number: B788
Sperm Aster Growth and Dynamics during Pronuclear Migration.
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The fertilizing sperm brings with it centrioles from which the sperm aster and mitotic spindle are formed. The role of the sperm aster is to capture the female pro-nucleus for pronuclear fusion and to centrally position the nucleus for the first cleavage division. Sperm aster migration was recently hypothesized to be due to microtubule (MT) length-dependent cytoplasmic pulling forces in which aster asymmetry and dynein are essential for male pronuclear centration (Tanimoto et al, 2016). We have re-examined the role of sperm astral MT growth and dynamics during pronuclear migration in the same higher invertebrate Lytechinus pictus. In contrast to the modeling of Tanimoto et al, we find that astral MTs are longer on the cortical facing side compared to those on the side facing the cell center. Furthermore, cortical facing MTs are anchored to the inner cell surface, which is also inconsistent with a MT length-dependent cytoplasmic pulling model. We next manipulated aster asymmetry in 1-cell zygotes by either increasing MT catastrophe or polymerization (Strickland et al, 2005). Neither compound affects fertilization or the length ratio of cortical vs cytoplasmic facing astral MTs. The result of suppressing sperm aster MT growth in L. pictus is arrested sperm-egg pronuclear migration, resulting in failure to center the nucleus and delayed cytokinesis despite having identical aster asymmetry when compared to controls. Conversely, increasing MT polymerization rates causes longer asters and faster sperm aster migration, without affecting female pronuclear migration. Finally, decreasing detyrosination of astral MTs prevents nuclear centration and causes defective aster morphology. Recent studies have indicated a tyrosinated tubulin preference for dynein (McKenney et al, 2016) and a detyrosinated preference for different kinesins (Sirajuddin, et al, 2014). Taken together, we predict a motor asymmetry in which the tyrosination profile of the sperm aster allows more dynein transport on the cytoplasmic astral MTs and more kinesin transport on the cortical-facing astral MTs, allowing the foce-balance required for male pronuclear centration. Such motor asymmetry coupled with potential cortical pushing forces by growing astral MTs may provide the forces necessary for sperm aster positioning.

Funded by: NSF MCB 1244425 to DB
P1771
Board Number: B789
Functional roles of hnRNPA2/B1 by RNA epigenetic modification in mammalian embryonic development.
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Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) plays an important role in influence of pre-messenger RNA (pre-mRNA) processing, mRNA metabolism and transportation in cells. Current research reveals that hnRNPA2/B1 can m6A modification on pre-mRNA or pre-miRNA and affect alternative splicing and miRNA processing. However, functional role and relationship between m6A and hnRNPA2/B1 in early embryo development are unclear. Here, we present evidence that hnRNPA2/B1 is crucial for the early embryo development by regulating of specific gene transcripts. hnRNPA2/B1 was localized in the nucleus during subsequent embryonic development since fertilization. Then, Knockdown of hnRNPA2/B1 induced by RNA interference (RNAi) was used to analyze the role of hnRNPA2/B1 during mouse preimplantation development. Knockdown of hnRNPA2/B1 delayed embryo development and blocked to further post-implantation development after 4-cell stage. Transcriptome analysis indicated that hnRNPA2/B1 KD blastocyst was changed of global gene expression patterns related with transcription, translation, cell cycle, ES cell differentiation, and RNA methylation. Interestingly, ICM marker OCT4 and Sox2 was significantly decreased in blastocyst stage. m6A RNA modification regulator methyltransferase like 3(METTL3) knock-down embryos mis-localize of hnRNPA2/B1 at the nucleus and decreased of m6A RNA methylation. On the other hand, hnRNPA2/B1 knock-down embryos increased m6A intensity. Together, our results suggested that hnRNPA2/B1 essential for early embryogenesis through the regulation of transcription related factors and the effect of cell fate transition through m6A RNA epigenetic modification.

P1772
Board Number: B790
The role of cadherin-based adhesions during trigeminal ganglia assembly.
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Cranial sensory ganglia play a vital role in the nervous system through their relay of sensory information from extrinsic and intrinsic sources to the brain. These ganglia are generated from the intermixing and coalescence of two distinct cell populations: neural crest cells (NCCs) and placode cell (PC)-derived neurons. Defects in NCC and PC development can lead to various congenital and hereditary malformations, disorders and diseases, including CHARGE and Branchio-Oto-Renal spectrum syndromes, heart-related anomalies, and cancers. Cranial ganglia assembly requires the formation of cadherin-based adherens junctions within the NCC and PC-derived neuron populations; however, the molecular composition of these adherens junctions is still unknown. To this end, we are using the assembly of the cranial trigeminal ganglia in the developing chick embryo as a model to define the molecules mediating these intercellular interactions. We hypothesize that NCCs and PCs intermingle and aggregate through the creation of specific cadherin-based adherens junctions, thereby permitting proper trigeminal gangliogenesis. Our prior work demonstrated that NCCs and PC-derived neurons express a distinct repertoire of cadherin proteins, with Cadherin-7 (Cad-7) and N-cadherin (N-cad) observed in migratory NCCs and PC-derived neurons, respectively. Using gene perturbation experiments, we now show that alterations in NCC Cad-7 influence trigeminal ganglia assembly, including the contribution of NCCs and
PC-derived neurons to the ganglion. Furthermore, in vivo biochemical studies point to a potential role for the formation of heterophilic cadherin-based adhesions between NCCs and PC-derived neurons. Taken together, our work will provide additional insight into the mechanisms that orchestrate the cellular movements essential for cranial gangliogenesis, and, collectively, will impact our understanding of the normal formation of, and diseases associated with, NCCs, PCs and the sensory nervous system. Importantly, our results may be directly translatable to other tissues generated from heterotypic cell types and will therefore have broad implications for human health.

P1773
Board Number: B791
Sonic hedgehog guides axons through release of a Dock-ELMO complex.
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In the developing spinal cord, Sonic hedgehog (Shh) attracts commissural axons toward the floorplate. How Shh regulates the cytoskeletal remodelling that underlies growth cone turning is unknown. We found that Shh activation of the Rho GTPase Rac1 requires the activity of Docks, which are unconventional GEFs. Knockdown of Dock3 and 4, or their binding partner ELMO1 and 2, abolished commissural axon attraction by Shh in vitro. Dock and ELMO were also required for correct commissural axon guidance in vivo. Moreover, we found that polarised Dock activity was sufficient to induce axon turning, indicating that Docks are instructive for axon guidance. Mechanistically, we show that Dock and ELMO interact with Boc, the Shh receptor, and that this interaction is reduced upon Shh stimulation. Furthermore, Shh stimulation translocates ELMO to the growth cone periphery. Together, we propose a model where release of the Dock/ELMO complex from Boc by Shh leads to Rac activation and growth cone turning. This identifies Dock/ELMO as new effectors of non-canonical Shh signaling in axon guidance.

P1774
Board Number: B792
Characterizing cell size dependent transcription with Xenopus embryos and cytoplasmic extracts.
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Recent work has demonstrated that cell size plays a fundamental role during early embryogenesis. After fertilization, cells within the embryo divide without growing, which results in reduced cell size. Prior to gastrulation, these divisions are driven maternally by pre-loaded mRNAs and proteins while the zygotic genome lies dormant. Upon zygotic genome activation (ZGA), genes necessary for gastrulation and the formation of differentiated germ layers are expressed and maternal products are cleared from the embryo. However, the mechanisms by which ZGA occurs are poorly understood. Previous research has suggested that timer and sizer mechanisms may control the timing of ZGA. For example as cell size reduces, an increased DNA to cytoplasm ratio (D:C) has been proposed to trigger transcriptional
activation. Studies on the effect of D:C ratio on ZGA have shown that addition of exogenous DNA in vivo or in vitro is sufficient to trigger a global onset of transcription. However, these studies have not directly tested the contribution of cell size to ZGA. A limitation of studies in embryos is that it is difficult to manipulate cell size. In order to mimic the broad range of cell sizes present in an embryo and to do so without compromising the complex processes that drive cellular function, our lab has developed a synthetic cell like system with controllable dimensions. This system allows me to investigate the relationship between cell size and transcription. To fully characterize this relationship, we require an assay sensitive enough to detect early zygotic transcripts. Our lab has developed a method for detecting nascent transcripts with 5-ethyl-uridine (5-EU) metabolic labeling in Xenopus laevis embryos and extracts. Using this method, I measure global changes in nascent transcription in Xenopus embryos, size sorted dissociated blastomeres, and embryo extracts. Using RT-PCR on biotinylated 5-EU RNA conjugated to streptavidin beads, I detect a specific subset of early RNA polymerase II dependent transcripts associated with ZGA, confirming that 5-EU labels nascent zygotic transcripts. The results of my studies support a cell size dependent model for ZGA and provide a novel system in which to characterize the mechanisms that link cell size and gene expression.

P1775
Board Number: B793

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During gastrulation prospective mesodermal cells must be brought onto the inside of the developing embryo. In the fruit fly \textit{Drosophila melanogaster} this requires precise spatial and temporal regulation of \textit{folded gastrulation (fog)} and \textit{T48} gene expression. The \textit{fog} and \textit{T48} gene products then act in parallel to activate Rho mediated cell signaling pathways. This in turn leads to constriction of the apical side of the cells, thereby initiating the internalization of the prospective mesoderm. This process of apical constriction and many of the molecular components involved are conserved in other morphogenetic events and in other species (including neural tube formation in vertebrates). However, direct homologs of \textit{fog} and \textit{T48} in vertebrates have not been identified. We are interested in understanding the evolution of this morphogenesis pathway and have begun by identifying \textit{fog} and \textit{T48} homologs in other dipterans. Sequence analysis of the identified \textit{fog} homologs shows \textit{fog} to be a rapidly evolving gene while \textit{T48} is evolving less rapidly. We are now analyzing the role of functional motifs in the Fog protein in fruit fly species. We have also identified homologs of \textit{fog}, the Fog receptor \textit{MIST}, and \textit{T48} in the mosquito \textit{Anopheles gambiae}. This insect has been reported to gastrulate differently to \textit{Drosophila melanogaster} and was previously thought to lack a \textit{fog} homolog. We are therefore examining the cell shape changes underlying gastrulation, and the expression of \textit{fog}, \textit{MIST} and \textit{T48} in \textit{A. gambiae} embryos. Ultimately we hope these studies will provide insight into the evolutionary processes that shape the developmental pathways of morphogenesis.
P1776

Board Number: B794

*Wolbachia* infection status, embryo-wide distribution and subcellular localization patterns during early embryonic development in a variety of *Drosophila* species.

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*Wolbachia* are intracellular endosymbionts found within the cells of many arthropods, including an estimated 40-60% of all insects. These endosymbiotic bacteria are known to be involved in the pathogenesis of a number of insect and filarial nematode mediated diseases in humans. In many cases *Wolbachia* also have an impact on the fertility and fecundity of the host insects in ways that maximize the maternal transmission of the bacteria from one generation to the next. These effects on the host have led to much interest in using *Wolbachia* as a biopesticide to reduce pest insect populations. However, little is known about how *Wolbachia* interact with their host at a cellular and molecular level. Fruit fly species provide a useful model system to explore such interactions. In our lab, and others, it has been shown that different fly-*Wolbachia* species combinations show varying patterns of *Wolbachia* distribution in the host embryo. In this study we have investigated the embryo-wide distribution patterns of different host-*Wolbachia* species combinations in a variety of lab cultured and locally caught fruit fly species. We have found that differences in distribution patterns persist during the period of incorporation of the *Wolbachia* into the developing germline of the host. However, our initial studies imply that on the level of individual cells the *Wolbachia* localize in a consistent sub-cellular pattern and show a likely interaction with microtubules. This is consistent with results from others that have demonstrated association between *Wolbachia* and microtubules in individual fly species during oogenesis and/or embryogenesis. Our ongoing work involves further investigation into shared aspects of *Wolbachia*-host interaction during early embryogenesis across species. We have also extended our study to include *Wolbachia* infection status and capability of locally caught *Drosophila suzukii*. This invasive fly species causes substantial damage to U.S. fruit crop production. There is therefore interest in whether *Wolbachia* could be used as an alternative population control technique. However, such approaches require a better understanding of *Wolbachia-host interactions in local fly populations.* Ultimately we hope that the discovery of conserved aspects of host-*Wolbachia* interaction will lead to a deeper understanding of the mechanisms whereby these important bacteria interact with a wide range of host species.

P1777

Board Number: B795

Spatiotemporal Regulation of Zygotic Genome Activation During Early Embryogenesis.

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Thousands of zygotic genes are activated during maternal-to-zygotic transition (MZT); however, how massive gene expression is spatially regulated within an embryo is still unknown. Using Xenopus as a model, we detected nascent transcripts by labeling RNA with 5-ethynyl uridine (5-EU). In combination with click chemistry and confocal imaging, we were able to track zygotic genome activation (ZGA) at the single-cell level in an embryo during the mid-blastula transition (MBT). As expected, we found that the
EU-RNA was not detected in embryos prior to cleavage 10 (C10), consistent with published gene profiling data derived using RNA extracted from whole embryos. Interestingly, ZGA is not uniform in space or time – high levels of zygotic transcription were evident in only a few cells at C10, approximately half the embryo at C13 and entire embryo at C16. This observation argues against a timer model, in which ZGA onset corresponds to a specific time post-fertilization. To validate our labeling approach, we co-microinjected 5EU and α-amanitin. We found that α-amanitin treatment eliminated nearly all nascent transcription, suggesting that the EU-labeled RNAs we image are predominantly RNA polymerase II dependent. The spatial patterns of ZGA prompted us characterize whether ZGA is cell size dependent. By quantifying the EU-RNA level in single cells at different stages, we found that the EU-RNA level was inversely correlated with cell size, supporting a cell-size sensing model for ZGA. To differentiate between the effects of cell size and number of cell divisions, we generated mini-embryos that are one eighth volume of the normal embryos. Large-scale transcription initiated as early as cleavage 8 in mini-embryos but was still off in wild-type embryos. These data suggest that by reducing cell size below a size threshold, genome activation is triggered. Finally, a higher percentage of cells in the presumptive ectoderm compared to the presumptive endoderm were transcriptionally active at the MBT, suggesting a potential link between spatial onset of ZGA and germ layer specification. Taken together, our results provide new insights into ZGA regulation and a spatial map of ZGA onset during early embryogenesis in vertebrates.

Tissue Development and Morphogenesis 1

P1778
Board Number: B796
Characterization of epithelial cell rearrangements during lens placode invagination.
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Development of the vertebrate eye serves as a valuable model for epithelial morphogenesis. At the onset of lens formation, the surface ectoderm thickens to form the lens placode, a structure positioned adjacent to the retinal primordium that undergoes a series of cell rearrangements and shape changes that drive invagination of the tissue to form the lens pit and subsequently the lens vesicle. Currently, the cellular mechanisms driving epithelial invagination of vertebrate structures such as the lens placode remain incompletely characterized. Although, the role of apical constriction (AC) and mechanically constrained tissue growth have previously been investigated, their contribution only partially explains this process. In order to better characterize lens placode invagination, cellular junctions of mouse and chick embryos were visualized using fluorescent live microscopy with either a transgenic fluorescent protein (E-cadherin(TG) or a fluorescent probe that binds to F-actin (SiR-F-actin). It was observed that the lens placode cells and those immediately surrounding them have distinct behaviors. The central lens placodal cells significantly reduce their apical area isotropically and maintain their cellular junctions with neighboring cells within the placode. However, the cells immediately peripheral to the lens placode are anisotropic in shape and dynamically rearrange themselves such that a net central/radial tissue movement is achieved. One of the dynamic cell behaviors observed is the formation and resolution of epithelial rosettes, a hallmark of intercalation movements that can elongate epithelial tissues. Because the Par-complex protein Par3 has previously been implicated to regulate invertebrate epithelial rosette resolution, its role during vertebrate placode invagination was determined. While Par3 protein appears to be isotropically localized in lens placode peripheral cells, it appears to be localized to the vertices of rosettes. Static analysis of fixed embryos lacking Par3 protein demonstrated that rosette structure and
anisotropic distribution depend on Par3. Further analysis of lens placode cell behavior in the absence of Par3 using live imaging and automated pattern recognition tools will shed light into the role of rosette resolution, radial cell movement, and Par3 function during vertebrate epithelial invagination.

P1779
Board Number: B797
Determining how cellular phase transitions partition the myotube cytoplasm.
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Syncytia, cells containing multiple nuclei, are found throughout the biosphere. These cells are essential for mammalian muscle and placenta development, and are a common state of fungal pathogens. Interestingly, nuclei in fungal, muscle, and placenta syncytia have different transcriptional programs despite sharing the same cytoplasmic environment. This independent nuclear behavior suggests that the cytosol is compartmentalized thereby insulating neighboring nuclei. We have found that mRNAs are non-randomly positioned near nuclei in fungal syncytia via incorporation into granule-like RNA/protein (RNP) structures. These structures are droplet-like and the core components can undergo liquid-liquid phase separation. In this system, liquid droplets act to spatially regulate mRNAs controlling the cell cycle. We hypothesize that cytosol compartmentalization via RNP droplets is a conserved mechanism for multinucleate cells to spatially organize the cytosol. To test this hypothesis, we are using mouse myotubes in which nuclei have autonomous transcriptional programs in a common cytoplasm and mRNAs appear spatially patterned. Transcripts encoding the acetylcholine receptor (AchR) show non-random subcellular localization, where AchR protein clusters also form, suggesting these transcripts are locally translated. Here, we identified FXR1, or Fragile X-Related protein, as a potential AchR binding protein through a bioinformatics screen. FXR1 is an intrinsically-disordered RNA-binding protein, and is essential for muscle development with knockout mice dying shortly after birth due to respiratory or cardiac failure. To study FXR1 in muscle syncytia we are using the C2C12 cell line, which can be easily differentiated in culture. We found that FXR1-GFP forms liquid-like droplets that fuse in the myotube cytosol. We have optimized smFISH for differentiated C2C12 myotubes to visualize AchR mRNA, and found that AchR transcripts are not homogenously distributed in the cytoplasm, consistent with previous findings. Additionally, FXR1 droplets partially colocalize with AchR mRNA. To identify regions of FXR1 required for droplet formation, we created mutations in predicted RNA binding domains and disordered regions. By imaging artificial syncytia created using U2OS human osteosarcoma fibroblasts transfected with these constructs, we identified mutations that alter the ability of FXR1 to form droplets as well as the morphology of the droplets formed. This work furthers our understanding of how cytoplasmic partitioning via phase transitions is conserved, by identifying and characterizing FXR1 RNP droplets in myotube syncytia.
P1780
Board Number: B798
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Though cell cycle regulation plays a key role in numerous developmental processes, including morphogenesis and differentiation, the regulatory systems that control the cell cycle remain poorly understood due to the inability to visualize changes in cell cycle state in vivo. We have adapted a cell cycle state biosensor originally designed for cell culture for use in zebrafish. The sensor provides a visual readout of cell cycle state based on the nuclear and cytoplasmic localization of DNA Helicase B (DHB), which is determined by CDK2 levels, in addition to a nuclear mask and a membrane marker. To test DHB, we compared it to Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI), a previously validated cell cycle sensor with limited resolution, and found that DHB accurately differentiates between G1 phase and S/G2 phases and is also able to distinguish between S/G2. Next, we induced cell cycle arrest in the zebrafish tailbud using pharmaceuticals to provide a baseline for quantifying the nuclear/cytoplasmic ratio of DHB in each phase of the cell cycle. Finally, using the newly calibrated biosensor, we explored the effects of cell cycle dysregulation on tailbud development. Through microinjection of a plasmid which ubiquitously expresses CDKN1A/p21, a cyclin-dependent kinase inhibitor associated primarily with inhibition of CDK2, we found that overexpression of CDKN1A/p21 induced G1 cell cycle arrest in tailbud cells and disrupted notochord development. Our sensor provides new insights into the relationship between cell cycle regulation and morphogenetic changes and allows for high-resolution live imaging of the zebrafish tailbud.

P1781
Board Number: B799
The role of polycomb group ring finger 5 (Pcgf5) in pressure overload hypertrophy.
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Pcgf5 is one of core components of polycomb repressive complex 1 (PRC1)-related complexes which catalyze monoubiquitination of histone H2A to repress gene expression. However, the biological function of Pcgf5 remains largely unknown. We generated Pcgf5 knockout mice with LacZ-reporter. Pcgf5 expression is restricted to the heart of embryos and adult mice. Mice homozygous for a Pcgf5 null allele survived to adulthood. To study the role of Pcgf5 in adult heart, we induced pressure overload cardiac hypertrophy in 10 - 12 week-old wild-type (WT) and Pcgf5-/- mice using surgically creating transverse aortic constriction (TAC). The cardiac functions of WT and Pcgf5-/- mice were assessed at 0 (before TAC), 1, 3, 6, 9, 12 and 15 weeks after TAC by echocardiography. Echocardiography revealed that Pcgf5-/- mice had significant increased heart weight to body weight ratio, increased left ventricular (LV) mass (1 week after TAC), increased LV anterior wall (1 week after TAC), increased diastolic LV internal diameter (12 weeks after TAC) and reduced ejection fraction (1 week after TAC), compared with WT mice. Taken together, Pcgf5-/- mice developed cardiac hypertrophy at an earlier time point after TAC, suggesting that Pcgf5 plays a role in development of cardiac hypertrophy.

Sunday-496
P1782
Board Number: B800
A fluorescence based cell cycle state biosensor in C. elegans and its use in characterizing cell cycle state during vulval morphogenesis.
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During organismal development, differential regulation of the cell cycle is critical to many cell biological processes, including differentiation and morphogenesis. While the complete cell division lineage of C. elegans is known, how the control of cell cycle is linked to fate specification and morphogenesis remains poorly understood, due to our inability to directly visualize cell cycle state. In order to visualize cell cycle state live, we have adapted a CDK2 biosensor for use in C. elegans. Our biosensor uses the dynamic nuclear/cytoplasmic localization of a portion of Human DNA Helicase B (DHB) linked to GFP to assess cell cycle state. The dynamic localization is the result of phosphorylation of the biosensor by CDKs. We have modified this sensor to allow for algorithmic assessment of cell cycle state. Similar to reported results from cell culture, we are using this biosensor to quantify lineage specific differences between cycling cells, quiescence and differentiation, provide new biological insights into the role of cell cycle during gastrulation and the control and timing of the cell cycle in specification of uterine cells and the morphogenesis of the C. elegans vulva.

P1783
Board Number: B801
Myocardial-specific functions of Jarid2 in the heart.
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Jarid2 (Jumonji A/T-rich interaction domain 2) is an essential factor for normal heart development. We have demonstrated that deletion of Jarid2 in mice results in cardiac malformations recapitulating human congenital cardiac disease, and dysregulation of gene expression during development. However, the cardiac-specific developmental function and the precise epigenetic regulation of gene expression by Jarid2 remain to be elucidated. Here we demonstrated that cardiac-specific deletion of Jarid2 in the developmental or postnatal myocardium causes cardiac malformations and functional defects. We employed three different cardiac-expressing Cre transgenic mice. Deletion of Jarid2 by Nkx2.5-Cre (Jarid2Nkx) caused cardiac malformations including ventricular septal defects, thin myocardium, hypertrabeculation, increased cardiac jelly and neonatal lethality. In contrast, later deletion of Jarid2 in mice with cTnt-Cre or aMHC-Cre transgenic lines did not cause gross abnormalities in development. By employing combinatorial genome-wide approaches and molecular analyses, we show that Jarid2 together with PRC2 regulates a subset of Jarid2 target gene expression and H3K27me3 enrichment during heart development. Specifically, Jarid2 is required for PRC2 occupancy and H3K27me3 on the Isl1 locus, leading to proper repression of the target gene expression during cardiac development. Jarid2 represses neural gene expression, cardiac jelly, and several important factors such as Isl1 and Bmp10, all of which are crucial for normal ventricular development. Thus, early deletion of Jarid2 in the myocardium results in dysregulation of gene expression and developmental defects later in development. Interestingly, deletion of Jarid2 by aMHC-Cre (Jarid2aMHC) resulted in complete lethality by 9 months of age with dilated cardiomyopathy. Jarid2aMHC mice showed an increase in fetal gene
expression, such as Tnni1 and Acta2, and a decrease in p27 levels at young ages. Therefore, Jarid2 is also required for myocardial maturation and maintaining cardiac function in adult stages. Our studies revealed epigenetic regulation of Jarid2 in association with PRC2 within the developing myocardium. We discovered that Jarid2 is necessary to establish correct epigenetics on the target genomic loci during a narrow developmental window, which is prior to differentiation of cardiac progenitors into cardiomyocytes and maturation of cardiomyocytes.

P1784
Board Number: B802
The Micropeptide Myomixer Controls Cell Fusion and Skeletal Muscle Formation.
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Skeletal muscle formation occurs through fusion of myoblasts to form multinucleated myofibers. From a genome-wide clustered regularly interspaced short palindromic repeats (CRISPR) loss-of-function screen for genes required for myoblast fusion and myogenesis, we discovered an 84-amino acid muscle-specific peptide that we call Myomixer. Myomixer expression coincides with myoblast differentiation and is essential for fusion and skeletal muscle formation during embryogenesis. Myomixer localizes to the plasma membrane, where it promotes myoblast fusion and associates with Myomaker, a fusogenic membrane protein. Myomixer together with Myomaker can also induce fibroblast-fibroblast fusion and fibroblast-myoblast fusion. We conclude that the Myomixer-Myomaker pair controls the critical step in myofiber formation during muscle development.

P1785
Board Number: B803
Tks5-Mediated Podosome Formation Governs Mammalian Myoblasts Fusion.
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The multi-nucleated skeletal muscle fibers arise from the fusion of myoblasts. While actin cytoskeleton has been known as the driving force to promote myoblast fusion, how exactly actin is reorganized in fusing myoblasts and by what means myoblast becomes fusion competent remain unclear. Here, we demonstrated that myoblast utilizes podosome, a protrusive actin-enriched structure, to propel cell-cell fusion. The ability of myoblast to form podosome is governed by the induction of a podosome scaffold protein, tyrosine kinase substrate with 5 SH3 domain (Tks5). Importantly, the expression of Tks5 is critical for myoblast fusion but not its differentiation. Furthermore, we found that Tks5 recruits another podosome component, dynamin-2, to the plasma membrane where myoblast fusion occurs. Dynamin-2 is a large GTPase functioning on membrane fission and actin reorganization. Tks5 regulates both the subcellular distribution of dynamin-2 in myoblast and its self-assembly around actin filaments. Specifically, the actin binding ability of dynamin-2, not the membrane fission activity, is required for myoblast fusion. Taken together, our results unveil that myoblast acquires fusion competency through Tks5 induction which controls the maturation of podosome thus impels myoblast fusion. Our findings also explain the paradoxical function of the membrane fission protein, dynamin-2, on intercellular membrane fusion.
Acquisition of myofibroblastic phenotype of stromal cells under pathological condition is critical process for the pathogenesis of fibrotic disease and cancer progression. Although the mechanical properties of extracellular matrices under the pathological condition is variable, most researches for the myofibroblast differentiation has been carried out under the conventional culture condition (e.g., plastic stiff culture dish). In this report, we show that the microtubule acetylation induced by TGF-β1 is a critical factor for initiation of fibroblast differentiation under the soft ECM condition resembling the early pathological condition. Inhibition of tubulin acetylation by knockout (KO) of αTAT1, a microtubule acetylation transferase 1, using CRISPR/Cas9 system in mouse embryonic fibroblast (MEF) resulted in the decrease of several myofibroblast marker genes expression and cellular contractility. Comparison of RNA-seq results obtained from WT and αTAT KO MEF under the soft ECM culture condition indicated that microtubule acetylation is responsible for bulk of gene expression corresponding to the myofibroblast differentiation. Moreover, overexpression of K40Q α-tubulin (acetyl-mimic mutant) not K40A α-tubulin (acetyl-null mutant) in αTAT KO MEFs recovered myofibroblast marker gene expression. Mechanistically, acetylated-α-tubulin is sufficient to dynein dependent nuclear translocation of YAP coactivator, via enforced acetylated-α-tubulin-dynein complex. Blockade of dynein activity with pharmacological inhibitor, EHNA and overexpression of dynamitin significantly reduced nuclear translocation of YAP and expression of myofibroblast marker genes on soft ECM condition. Collectively, these findings demonstrate that the signaling axis for TGF-β1/acetylated-MT/dynein-based nuclear translocation of YAP is critical progress for the acquisition of myofibroblastic phenotype on soft ECM environment and it rendered to advantage for development pathogenesis.

Regeneration of missing tissue requires both generation of new cells after wounding as well as information to correctly pattern the new tissue. However the sources and regulation of positional information during regeneration remain poorly understood. Planarians are flatworms with a robust ability to regenerate from nearly any injury. By one week after amputation of the head or tail, planarians regenerate all missing tissue in a newly formed outgrowth termed the blastema. Prior work has identified positional information in planarians using RNA interference phenotypes in which incorrect tissues are regenerated. For example, Wnt pathway inhibition leads to regeneration of ectopic heads instead of tails, and inhibition of the BMP pathway leads to formation of ectopic ventral tissue on the dorsal side. Remarkably, recent work has shown that members of these key developmental signaling pathways are expressed predominantly in planarian muscle cells, leading to the hypothesis that muscle cells maintain positional information during regeneration. The planarian body-wall musculature forms a
sub-epidermal network composed of three layers: circular fibers that are oriented along the medial-lateral (ML), diagonal fibers, and longitudinal fibers that are oriented along the anterior-posterior (AP) axis. We found that the planarian bHLH family transcription factor myoD, a homolog of the mammalian myogenic factor, controlled formation only of longitudinal fibers. Inhibition of myoD reduced the number of longitudinal fibers and led to regeneration failure despite normal homeostatic tissue turnover. This phenotype revealed that longitudinal fibers express key factors that are required to initiate regeneration. Conversely, the NK1 homeodomain transcription factor nkk1-1 is required only for circular fiber formation. Inhibition of nkk1-1 reduced the number of circular fibers and resulted in abnormal regeneration of a bifurcated AP axis where two fused heads form within a single anterior blastema. Planarian muscle is therefore more than a contractile and structural tissue with subsets of body-wall muscle playing distinct regulatory roles in wound signaling and patterning to enable regeneration.

P1788
Board Number: B806
Deciphering Heart Regeneration by Histone Exchange Profiling.
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Unlike humans, vertebrate species like zebrafish activate cardiomyocyte division after cardiac tissue damage, regenerating lost muscle. Regulation of chromatin organization is a principal mechanism underlying animal development, it is unknown to what extent injury-induced structural changes in chromatin underlie heart regeneration. Here, we set-out to define nucleosome changes during heart regeneration. We generated a transgenic zebrafish strain expressing a biotinylatable H3.3 histone variant in cardiomyocytes and derived a cell type-specific profile of histone exchange in heart muscle. We identified thousands of inter- and intragenic regions that revise H3.3 occupancy during regeneration, many of which were undetectable by alternative chromatin scanning technology. In transgenic reporter lines, a subset of H3.3-enriched regions directed gene expression in subpopulations of ventricular cardiomyocytes, whereas other elements showed enhancer activity preferential to injury- or Neuregulin1-elicited cardiomyocyte proliferation. Many consensus binding motifs near regeneration-associated genes were enriched among predicted enhancer sequences, suggesting a regulatory network for heart regeneration. Our cardiomyocyte H3.3 profiles provide a fertile resource to understand and manipulate the genetic program of the heart to build new muscle.

P1789
Board Number: B807
Preterm birth compromises cerebellar development: evidence from a pig model.
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A large fraction of preterm infants have compromised neurodevelopment. Motor dysfunction and cognitive impairment in preterm infants were associated with hypoplasia and cellular lesions in the cerebellum. However, the cellular and molecular mechanisms that mediate cerebellar abnormalities in preterm infants remain largely unknown. In this study we investigated the impact of preterm birth on
cerebellar development and identified specific molecular targets affected by preterm birth using the pig as a large animal model. Cerebella were collected from preterm piglets delivered at 91% term (day 105 out of 115 days of gestation) via c-section and raised for 10 days using milk replacer. Naturally born term pigs were used as controls. Specific cerebellar populations were identified by immunohistochemical staining. Cells were counted using unbiased stereological methods. For gene expression analysis, external granule cell layer was isolated by laser capture microdissection and analyzed by real-time RT-PCR. Based on Calbindin immunohistochemistry, we found that preterm birth does not affect either morphology or density of Purkinje cells at term equivalent age. Similarly, there was no difference in the number of Pax2+ molecular layer interneurons between preterm and term pigs. Ki67 and Tag1 immunohistochemical analysis revealed, however, that preterm birth specifically compromises proliferation, but not differentiation of cerebellar granule cells. Laser capture microdissection / quantitative-PCR analysis identified several key cerebellar developmental genes, including both secreted molecules and intrinsic factors, that were misregulated in preterm pigs, likely contributing to the granule cell abnormality. Thus, our analysis of a pig model argues that preterm birth compromises cerebellar development by primarily affecting proliferation of granule cells and suggests that disruption of this cellular population contributes to motor and cognitive impairment in preterm human infants.

P1790
Board Number: B808
Identification of novel transcription factor in the generation of mid-brain during embryo development: Application of alternative transcription factor binding site-prediction-method.
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Cell fate specification relies on the action of critical transcription factors (TF) that become available at distinct stages of embryonic development. Mid-brain dopamine neurons (mDA\textit{n}) play a central role in the modulation of several brain functions, including voluntary movements, emotion, and cognition. The progressive degeneration of substantia nigra mDA\textit{n} gives rise to some of the main motor features of Parkinson’s disease (PD). Current treatments for PD are symptomatic and how the phenotype of DA neurons is normally established and the ways in which pathology affects the maintenance of cell identity are, therefore, important considerations. A number of transcription factors regulate the development of this set of neurons and some remain constitutively expressed throughout life. These maintenance transcription factors are closely associated with essential neuro-physiological functions and are required ultimately for the long-term survival of the mid-brain dopamine neurons. The development of mDA\textit{n} is controlled by a combination of between cell extrinsic pathway (SHH-FGF8 or Wnt1) and intrinsic signals (FOXA2, LMX1A). Both Nurr1 and Pitx3 are required for mDA\textit{n} differentiation and survival. Here, human neural progenitor cells were more efficient differentiation into dopamine neurons in the treatment to the small chemical compound X than other conventional differentiating factors, confirmed by in vitro results. To understand the developmental patterns of gene expression in the dopamine differentiation of human neural progenitor cells, we performed transcription analysis of targeting neurons at different treatment time points by in vitro culture with or without small molecules or SHH or negative control condition using next-generation RNA sequencing (RNA-seq). We showed that distinct gene expressions shown by different factor-treated cells (negative, SHH and compound X) and most genes were down-regulated and four genes uniquely up-regulated by compound X and predicted to be related to neurogenesis by bioinformatics data analysis. Furthermore, that genes uniquely up-regulated by compound X, Nurr1 and TH act in a coordinated manner for dopamine differentiation and they have
motifs in its promoters which novel transcription factor X binds by alternative transcription factor binding site prediction method. We found that novel transcription factor X expressed mid- brain of E10 embryonic mouse but not mid-brain of over E12 embryonic mouse brain. The Transcription factor X protein merged with TH+ cells and bind to the promoter of TH gene. Our results suggest that a novel transcription factor X is one of the required factor for mDA specification during brain development

P1791
Board Number: B809
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Embryonic morphogenesis is a developmental process that constructs organs and body plans by coordinating cell shape and cell migration to establish tissue organization. The roles that the cytoskeleton and cell adhesion have during morphogenesis for cell shape changes and movement have been well documented. However, many of the signaling and biochemical pathways that underlie these dynamics remain incompletely understood.

Genes that regulate morphogenesis are likely to also have requirements earlier in development, making their identification challenging. Thus temperature-sensitive, embryonic lethal (TS-EL) alleles in C. elegans provide an excellent tool for investigating genetic pathways necessary for morphogenesis. Our lab has isolated a collection of ~1,000 TS-EL mutants. We now seek to identify and clone all of the morphogenesis-defective mutants within our collection. To date, we have identified 68 mutants with penetrant and 29 mutants with variable terminal morphogenesis-defective phenotypes (respectively defined as ≥70% or 50<70% of mutants arresting at a single embryonic stage). The majority of the highly penetrant mutants arrest without elongation. We then identify the causative genes using a combined SNP mapping and whole genome sequencing approach, along with genetic complementation tests. In a collaboration, the mutants we identify are further analyzed for cell fate patterning defects using an automated cell lineage platform developed in Zhirong Bao’s lab. We reason that mutants exhibiting normal cell fate specification but abnormal cell shape or location represent true morphogenetic mutants. So far, we have identified 19 alleles representing 13 genes, including glp-1 (3 alleles), let-19 (3 alleles), emb-5 (3 alleles), mom-4, emb-4, chaf-1, rib-1, hlih-1, sart-3, cdc-25.2, and Irr-1.

One gene, Irr-1 (Leucine rich repeat), encodes a protein that functions as a substrate recognition subunit for Cullin 2-RING ubiquitin ligase complex (CRL2\textsuperscript{LRR})\textsuperscript{1}. Introduction of C. elegans CLR2\textsuperscript{LRR} in human cell culture prevents inhibition of the Rho/ROCK/LIMK pathway and activates cofilin, an actin depolymerizer (Starostina et al., 2010). Therefore, LRR-1's role in regulating the remodeling of the actin cytoskeleton makes it particularly interesting, as let-502/ROCK is known to be important for C. elegans embryonic morphogenesis. We are currently characterizing the Irr-1 morphogenesis defects using the cell lineage platform and by using CRISPR to make an endogenous GFP::LRR-1 transgenic line to characterize its expression. Our long term goal is to advance our understanding of embryonic morphogenesis in C. elegans by identifying previously unknown players that influence this fundamentally important biological process.

Sunday-502
P1793
Board Number: B811
Cardiac transcriptome profiling during regeneration in zebrafish.
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Myocardial infarctions (MIs) are a prevalent form of cardiovascular disease (CVD) and are a leading cause of global mortality. MI is characterized as an ischemic injury that induces cardiomyocyte (CM) necrosis and subsequent fibrosis of the damaged region. The post-MI scar leaves patients at risk for recurrent MI and heart failure, and one reason for the scar's permanence is the heart's inability to induce sufficient CM proliferation. Mammalian CMs proliferate rapidly during development, but become post-mitotic shortly after birth and fail to divide after injury in adults. Unlike mammals, adult zebrafish possess robust CM proliferation and undergo complete cardiac regeneration. Using ventricular amputation in adult zebrafish as our injury model, we sought to determine which genes play a significant role in CM proliferation after injury and which mechanisms they operate through. In this study, we performed RNA-seq on uninjured and 3 days post-amputation (3dpa) AB* ventricles and validated gene up-regulation with semi-quantitative PCR and RNAseq. cenpf and foxm1 were among those genes induced after amputation. Both genes are highly expressed during mammalian cardiac development but their expression is significantly down-regulated 7 days after birth suggesting they could be important regulators of CM proliferation. Cenpf is a kinetochore-binding protein that may also play roles in vesicle trafficking, DNA-binding, and cell migration, whereas Foxm1 is a transcription factor that regulates the expression of multiple pro-mitotic genes. We hypothesize that cenpf and foxm1 are critical for CM proliferation after injury and that their loss will disrupt the regenerative capacity in adult zebrafish. Currently we are expanding our preliminary findings that cenpf−/− mutant hearts possess large scars even at 30dpa when WT hearts have completed regeneration. Moreover, Cenpf is significantly expressed proximally to the injury zone at 3dpa, which hints at potential roles in CM proliferation. Our goal is to elucidate the exact mechanisms these genes in heart regeneration in hope of providing new therapeutic avenues for mammalian heart repair.

P1794
Board Number: B812
Understanding the molecular basis of human craniofacial disorders using Caenorhabditis elegans as a model organism.
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Craniosynostosis is a birth defect in which one or more of the fibrous sutures in an infant’s skull close prematurely resulting in an abnormal head shape. Craniosynostosis occurs in about one of every 2,000 births. Mutations in TWIST1, a basic helix-loop-helix transcription factor, has been found in patients with craniofacial disorders. In this study, we aim to understand the molecular basis of craniofacial disease using Caenorhabditis elegans as a model organism. C. elegans is an excellent model genetic system to study Twist because it has a single TWIST homolog known as HLH-8. Also, HLH-8, its partner HLH-2, and downstream target genes are well conserved with hTWIST proteins. Twist-containing dimers regulate the expression of target genes by binding an enhancer element called the E-box. We study the Notch ligand gene, arg-1, as an HLH-8 target gene. The 385 bp arg-1 promoter region contains three different E-boxes that could be bound by either HLH-8/HLH-8 homodimers or HLH-8/HLH-2 heterodimers. The three E-boxes are sufficient for maintaining the full expression pattern of arg-1 in a subset of
mesodermal tissues including the head-mesodermal cell (HMC), vulval muscles (VMs), and enteric muscles (EMs). In our previous work, we generated an allelic series of different glutamic acid substitutions (E29) at a conserved residue of the HLH-8 DNA binding domain, to mimic the mutations found in craniofacial patients. We discovered that not all of the E29 homozygous mutants were equal regarding phenotypes. E29G, A, and Q were ~100% Egg-laying defective but were mildly constipated. Additionally, 74%-100% of these mutants were able to drive expression of the HLH-8 target gene arg-1 in EMs but not in VMs indicating that these alleles retain the ability of DNA binding. We propose that E29 mutants favor binding a particular E-box DNA sequence over the other. To generate reagents to test our hypothesis, we swapped the different E boxes found in the 385 bp arg-1 promoter region and studied the resulting gene expression. We generated three arg-1 promoter constructs. The first construct was mimicking the WT pattern (E1-E2-E3). In the second one, we swapped E3 with E1, and in the third construct, we swapped E1 with E3. We found that the E1 box sequence is essential for arg-1 expression in HMC but not VMs. Also in animals with swapped constructs, we noticed a decrease in the VMs arg-1::gfp expression indicating that in VMs, arg-1::gfp expression requires having both E-boxes (E1 and E3) besides E2, for maximum VMs expression levels. Next, we will be crossing the HLH-8 E29G, A, and Q mutants in these reporters to evaluate the contribution of the E-box sequences and to see if in these mutants the expression is altered.

P1795
Board Number: B813
Breaking Hertwig’s Rule in the Drosophila Follicular Epithelium.
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We investigated proliferation and tissue regularity in a simple epithelium undergoing elongation. Through live imaging and semi-automated image analysis, we found that cell division drives the early Drosophila follicular epithelium towards optimal geometric packing. This increase in regularity demands that cells break Hertwig’s rule, a long-established principle of cell biology which holds that divisions orient along the cell shape long axis. We show that planar division orientation aligns with the elongating axis of the egg chamber, and not predicted by interphase cell shape. Planar division orientation relies on the actin scaffolding protein Canoe/Afadin, but in contrast to other systems, is not achieved through the asymmetric enrichment of the Pins/Mud spindle-orienting machinery. Spindle orientation in the apical-basal and planar axes are independent. This work highlights the diversity of division control in epithelial tissues, even within the same organism, and suggests a broad means of planar division orientation in epithelia.
P1796
Board Number: B814
Wnt Signaling in Migratory Neural Crest Cells In The Chick Spinal Cord.
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Neural crest (NC) cells are a subset of migratory cells that generate a variety of different cell types during embryonic development. Neural crest cells have filopodia that have long been implicated in migration. Recently, it has been shown that neural crest cells provide important signals, such as Wnts, to tissues along the path of migration. Wnt producing cells, such as neural crest, require functional PORCN and WLS to generate and transport Wnts to target cells. PORCN, an ER transmembrane protein, adds a palmitate to Wnt that is essential for Wnt secretion and signaling. WLS regulates the sorting and transport of Wnts to the cell surface where it can then be transported out of the cell. Recent studies in Drosophila and zebrafish have shown that Wnts can be transported via actin-based filopodia. Thus, we hypothesized that filopodia in neural crest cells might also function in the delivery of signals such as Wnt. To test this hypothesis, we developed biologically active WNT1-GFP and WLS-mCherry fusion proteins. These constructs were introduced into premigratory neural crest cells via in ovo electroporation. Migratory neural crest cells were subjected to live-imaging using confocal microscopy. In the absence of exogenous WLS-mCherry, WNT1-GFP was primarily localized to the ER and Golgi. Upon co-expression of WLS-mCherry and WNT1-GFP, most WNT1-GFP was redistributed to the cell surface. In addition, we observed an apparent increase of filopodia containing both WLS-mCherry and WNT1-GFP in migratory neural crest cells. To test whether these projections were actin based, we tested for the co-localization of Utrophin-261-EGFP. We found that Utrophin-261 is indeed localized to these projections, thus indicating that the projections are actin-based. As Myosin-X (MyoX) is often found in actin-based projections, we then tested for the localization of a variant of MyoX, HmmMyoX, to these projections. HmmMyoX was found localizing at the tips of some of the projections. Thus, our results show that co-expression of WLS and WNT1 1) induced formation of new filopodia 2) promoted localization of WNT1 to these structures 3) the structures contain actin and 4) MyoX is localized to the tips of the filopodia.

P1797
Board Number: B815
Mask and Yorkie are required for cell adhesion in the Drosophila retina.
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The conserved Hippo signaling pathway is critical for the regulation of tissue growth in both Drosophila and mammals. Transcription of Hippo pathway target genes is mediated by the transcriptional coactivator Yorkie (YAP/TAZ in mammals). Active Yorkie (Yki) translocates to the nucleus where it forms complexes with transcription factors to facilitate the transcription of growth-promoting genes. Recently, the protein Mask was discovered to be a cofactor of Yki and required for the transcription of several Yki target genes. Our results show that Mask is required during development of the Drosophila retina. When Mask levels are reduced, retina display a decrease in overall cell number along with severe patterning defects. Such mispatterning is likely attributed to junctional defects observed when Mask and Yki levels are reduced. This suggests that Mask and Yki are regulators of cell adhesion in the retina. Such a requirement would implicate the Hippo signaling pathway as a novel regulator of cell adhesion during organ morphogenesis.
P1798
Board Number: B816
Quantitative analysis of the contribution of apical constriction to neural tube closure.
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Neural tube closure is a complex morphogenetic process that requires several distinct cell behaviors to be successfully executed. Apical constriction (AC) of neural plate cells has long been known to contribute to neural plate folding, but the cell-biological mechanisms of AC in the neural plate are poorly understood. Additionally, recent studies of AC in Xenopus have shown that AC itself is a multifaceted process that may be comprised of both apical surface and apical junction constrictions. To better understand how AC contributes to neural plate morphogenesis, we are combining live imaging and quantitative analysis in the Xenopus neural plate in order to understand how various forms of AC contribute to neural tube closure. Additionally, we are utilizing modern genetic manipulations such as CRISPR/Cas9-mediated gene knockouts and AC effector proteins such as Shroom3 or SH3PXD2B to study the contribution of AC to apical constriction behaviors in the neural plate at both the whole-tissue and sub-cellular levels. Together, these techniques will allow for dynamic analysis of many aspects of AC during neural tube closure while simultaneously building workflows that can be used to study many aspects of neural tube closure in a live organism.

P1799
Board Number: B817
C-cadherin is required for localization of actomyosin contractility machinery during convergent extension.
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Convergent extension (CE) is a process of collective cell migration that drives anterior-posterior (AP) axis elongation in animals ranging from Drosophila to mammals. A key step during CE is polarized junction exchange in which AP cell-cell junctions shrink and are exchanged for mediolateral cell junctions. This requires polarized actomyosin contractility at the shrinking junctions, but it is unclear how the contractility machinery interacts with the cell adhesion molecules that hold these junctions together. Here, we use in vivo subcellular time-lapse imaging of Xenopus laevis mesoderm to investigate how the cell-cell adhesion protein C-cadherin interfaces with the actomyosin contractility machinery. Our initial prediction, based on study of E-cadherin during epithelial CE, was that junction shrinking required reduction of cell adhesion through active clearing of C-cadherin. However, during mesenchymal CE, we observed the opposite and C-cadherin was enriched at AP junctions. Also, we observed concurrent pulses of C-cadherin and actin specifically at shrinking junctions, suggesting cooperative function. To directly test if C-cadherin was required for junctional enrichment of the actomyosin contractility machinery, we knocked down C-cadherin. We found that contractile myosin was depleted from all cellular junctions after disruption of C-cadherin. Interestingly, actin was still present at cell junctions in the C-cadherin depleted tissue. This result lead us to conclude that C-cadherin is required to bring contractile myosin, but not actin to cell-cell junctions and suggests that C-cadherin has a role in constructing or maintaining the actomyosin machinery. Also, these results support a novel mechanism of cadherin function during mesenchymal CE as C-cadherin is not removed from shrinking cell junctions and instead actively contributes to junction remodeling.
P1800
Board Number: B818
Hedgehog signaling constrains cell movements during early eye development.
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During development, cells and tissues must undergo dramatic movements to form 3-dimensional organs, the specific structure of which is critical for function. Precise tissue organization is required for the morphogenesis of the vertebrate eye as developmental defects in the eye structure commonly account for visual impairment. One such defect, uveal coloboma, is a significant cause of blindness worldwide and is characterized by a hole or cleft in the eye. Mutations in the Hedgehog (Hh) receptor Patched lead to hyperactive Hh signaling and have been shown to cause coloboma in both humans and zebrafish. Using a combination of 4-dimensional live imaging, computational methods, and molecular genetics, we have determined the morphogenetic events including cell movements, division and death underlying optic cup morphogenesis and how these are disrupted by hyperactive Hh signaling. At the cellular level, we used 4D cell tracking to pinpoint a population of cells whose origin and movements are disrupted when Hh signaling is overactivated. Further, at the single cell level, migration behaviors are disrupted: rather than a migratory bipolar morphology, these cells exhibit multipolar morphology and cease to move. To determine the molecular mechanism by which hyperactive Hh signaling disrupts cell movements and eye formation, we assayed both transgenic reporter and endogenous target gene expression to determine when and where hyperactive Hh signaling first alters development. Using transplantation experiments, we additionally determined that overactive Hh signaling acts non-cell autonomously to disrupt cell morphology and migration, through a Gli transcription-dependent mechanism. With these findings, we present a model in which a specific level of Hh signaling is critical to regulate cell behaviors: we hypothesize that overactive Hh signaling leads to increased production of a secreted molecule or cell-surface protein that in turn disrupts migration behaviors. We have thus identified the cellular mechanism by which overactive Hh signaling disrupts optic cup morphogenesis, and we are currently working to identify the critical Hh factor downstream of Hh signaling that directly regulates migration.

P1801
Board Number: B819
Fetal programming and induction of inflammatory response in the gerbil prostate caused by n-6 fatty-acid intake from corn oil.
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The quality of dietary lipids in the maternal diet during gestation can negatively affect organogenesis and enhance susceptibility of the offspring to diseases in adult life, a process known as fetal programming. Excessive n-6 fatty acid (FA) intake can favor the development of some diseases, especially those related to inflammatory response. The capacity of these FA to induce fetal programming of prostate has not yet been investigated. The present study evaluated whether maternal intake of n-6 FA during gestation leads to fetal programming of the gerbil prostate. Pregnant Mongolian gerbils (Meriones unguiculatus) were randomly divided into Control (C) and Oil (O) groups. O females received, by gavage, 0.1mL of corn oil from gestational day 8 to 23. Adult male offspring (16 weeks old, n=10...
animals/group) were euthanized and the ventral prostatic lobe was analyzed using stereological, histopathological, immunohistochemical [androgen receptor (AR), phospho-histone H3 and cluster of differentiation 3 (CD3)] and Western blotting [AR, phosphorylated protein kinase B (pAkt), estrogen receptor α (ERα) and liver X receptor α (LXRα)] methods. Serum levels of testosterone (T) and 17β-estradiol (E2) were measured by ELISA. Maternal intake of n-6 FA during gestation decreased the prostatic weight of offspring at adulthood. Stereological analysis showed that this reduction was due to a decrease of 22.6 and 29.7% in the volume of the epithelial and luminal compartments, respectively. Although T levels did not differ between the groups, AR-positive stromal cells and prostatic AR protein expression were enhanced in O. E2 levels and ERα protein expression increased 46 and 61% in this group, respectively. According to histopathological analysis, maternal intake of n-6 FA caused an increase in the incidence and multiplicity of inflammatory-related disorders such as reactive hyperplasia, and also of prostatic intraepithelial neoplasia. These lesions exhibited a particular aspect with inflammatory cells, including CD3 lymphocytes, located predominantly in the subepithelial layer of the prostatic acini. The stromal proliferative index and pAkt protein expression were enhanced in O. LXRα protein expression also increased in this group. This may represent a tendency to down-modulate the accumulation of inflammatory molecules and to ameliorate the inflammatory effects caused by n-6 FA intake. Together, these data indicate that the maternal intake of n-6 FA during gestation affects prostatic organogenesis and leads to perturbations in the epithelial-stromal interaction, disrupting AR, ERα and LXRα and promoting inflammatory-related disorders in Mongolian gerbils at adulthood.

Financial support: FAPESP (#2014/03300-8), CAPES, CNPq (#147426/2014-6, #308367/2014-6).

P1802
Board Number: B820
Mouse trophoblast lineage development requires Smad4-dependent signaling.
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Mouse trophoblast stem (TS) cells established from the outgrowth of the polar trophectoderm of blastocysts or the extraembryonic ectoderm of post-implantation embryos are precursors of trophoblasts and can contribute to all trophoblast lineage derivatives in vivo, providing a powerful in vitro system for studying trophoblast stem cell self-renewal and differentiation. Although it is known that Transforming Growth Factor beta (TGF-β)/Nodal related signaling together with FGF4 signaling is critical for TS cell self-renewal, the intracellular signaling transduction of TGF-β related signaling and its crosstalks with other signaling pathways in trophoblast lineage development remain poorly understood. The function of Smad4, the central mediator of TGF-β related signaling, in trophoblast lineage development has not been fully investigated, partially due to the early lethality of Smad4 null embryos. Utilizing Smad4-deficient TS cells derived from preimplantation embryos, we find that Smad4 deficiency alters the kinetics and lineage distribution during trophoblast lineage differentiation in vitro. In particular, Smad4-deficient TS cells exhibit enhanced differentiation in the spongiotrophoblast and trophoblast giant cells lineages at the expense of the syncytiotrophoblast lineage. Moreover, our studies provide evidence linking the differentiation defects seen in the Smad4 null TS cells to alterations in TGF-β, MAPK signaling pathways, as well as Wnt signaling pathway. Pharmacological manipulation studies reveal that canonical Wnt signaling is involved in the regulation of TS cell self-renewal and differentiation. Our analyses provide insights into the molecular interplays of signaling pathways that regulate trophoblast lineage development.
Prokaryotic Cell Biology

P1803
Board Number: B822
A Trimeric Transmembrane Mechanism Underlying Enterobacter cloacae Resistance to Triclosan.
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Multidrug resistant bacterial infections are rising worldwide, shifting from what once was a biological phenomenon to what is quickly becoming a global health crisis. The creation of antibiotic resistant strains has been facilitated by the overuse of antimicrobial compounds, such as triclosan. For the past several decades, triclosan was added to consumer products such as toothpaste, hand sanitizer, and plasticware. Its overuse has created an environmental crisis, as it has been found in water runoff, soil, sediment, and other substrates. Triclosan contamination provides a selective pressure that likely leads to novel strains that are resistant to this and other antimicrobial compounds. Despite these potential outcomes and limited FDA ban, triclosan continues to be included in some products (e.g. toothpaste) globally, worsening the environmental contamination that has been accumulating for over 40 years. We have previously demonstrated that repeated exposure of various bacterial species to triclosan, tetracycline, and chloramphenicol selects for strains with both resistance to that compound and cross-resistance to other antimicrobials. In addition, growth curve analysis has shown an inverse correlation between novel strain resistance and rate of growth. This study focuses on the transmembrane efflux pump of Enterobacter cloacae. This Gram-negative pathogen is prevalent in both the environment and clinical settings, and is one of the major underlying causes of severe antibiotic resistant intensive care unit infections. Previous studies have indicated that its AcrAB-ToIC efflux pump is associated with the removal of an array of antibiotics including tetracycline and chloramphenicol from the cell, thus increasing organismal resistance to these compounds; this study extends those findings to triclosan-resistant strains and further assesses the connection between efflux pump overexpression and antibiotic resistance. Study methods include real time qPCR analysis of associated genes and intracellular real-time dye efflux assays. Our expression studies show a significant increase in AcrAB transcription. Current studies are extending the scope of these findings by looking at ToIC and a number of activators and repressors involved in operon regulation (e.g. SoxS, MarA, EnvR). Future research includes an extensive study of the consequences of overexpression and repression of the AcrAB operon via cloning into a controlled expression plasmid in E. coli. Ultimately, data derived from this study could lead to a better understanding of the cellular mechanisms that underlie the multidrug resistance, provide a tool for assessing the impact of environmental triclosan on the creation of multidrug resistant species, and offer a potential target for bacterial inactivation.

P1804
Board Number: B823
An antibacterial mechanism of bac8c via apoptosis-like response on Escherichia coli.
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Bac8c (RIWVIWRR-NH₂), an 8-mer peptide modified from amino acids 4-11 of Bac2a, has been known to exert significant broad-spectrum antimicrobial activity. In the present study, the findings suggest a novel
mechanism for the antibacterial effect of bac8c on *Escherichia coli*, namely, the induction of a bacterial apoptosis-like response. We propose a possible mechanism for the bacterial apoptosis-like death that includes the following: accumulation of reactive oxygen species (ROS) (detected with H₂DCFDA staining), increased intracellular calcium levels (detected with Fura-2 AM), disruption of the membrane potential [detected with DiBAC₄(3)], activation of a bacterial caspase-like protein (detected by FITC-VAD-FMK staining) and DNA degradation (detected with TUNEL assay) which is the hallmarks of late apoptosis in bacterial cells treated with bac8c. We also performed RecA expression assay with western blotting and observed activation of SOS response to repair the damaged DNA using RecA and LexA mutant strains. To summarize, bac8c are involved in the apoptosis-like response in *E. coli* and the novel mechanisms which were identified in this study, suggest that bac8c may be an effective antimicrobial agent with far lower propensity for inducing microbial resistance than antibiotics.

**P1805**
**Board Number: B824**
**Understanding Role of VraT in Methicillin-Resistant *Staphylococcus aureus***.
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*Staphylococcus aureus* is a major causative organism of infective endocarditis and may result in mortality rates as high as 35% in isolated breakouts. Resistance to frontline antibiotics such as oxacillin and vancomycin suggests novel antibiotics or prophylactic agents are required. Recent studies have shown that deleting a gene coding for the membrane protein called VraT re-sensitizes *S. aureus* to vancomycin via disruption of bacterial signaling pathways. This confirms VraT is a biologically validated drug target. We are currently attempting to understand the structure of VraT and how it promulgates antibiotic resistance. As a first step in our project, we are deleting the VraT gene from methicillin-resistant *S. aureus* using a Group II intron-disruption cassette to generate a re-sensitized mutant strain unable to make the VraT protein. We use this mutant as a tool to design prophylactics against drug resistance or adjuvants for combination therapy with oxacillin and vancomycin.

**P1806**
**Board Number: B825**
**Apple cider vinegar changes Escherichia-coli pathogenic protein expression profiles and curbs infected leucocyte cytokine secretion.**
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Antimicrobial infections in the elderly and immunocompromised patients can often lead to complications and host resistance. The World Health Organisation predicts a global escalation in antimicrobial resistance in years to come. Extra-intestinal pathogenic *Eschericia-coli* (E-coli) are the most frequent cause of blood bourne, urinary tract and hospital acquired infections. They can be difficult to treat and can easily spread causing sepsis and even organ failure if not treated in time. Treatment options are still limited hence there is a real need for novel or alternative therapeutics which could combat these infections. Objective: The aim of this study was to investigate the antimicrobial activity of Apple cider vinegar (ACV) on E-coli growth and cellular interactions. Methods: We diluted the ACV at various concentrations and incubated these directly with E-coli at various concentrations using the microdilution technique. We also infected freshly isolated leucocytes from peripheral blood obtained by consensus from healthy human peripheral blood donors. Leucocytes were co-incubated with E-coli for
6, 12, 24 hour time periods and with ACV. Collected supernatants were analysed for cytokine content using ELISAs. We also looked at the impact of ACV directly on the structure of E-coli carrying out tandem mass spectral proteomic analysis. Results: We showed that ACV inhibited E-coli growth, the minimum inhibitory concentration was found to be 1/100 dilution of 5% concentrated ACV. We also saw a dose dependent down regulation of induced monocytes tumour necrosis factor alpha secretion from 2.3 +/- 0.3 ng/ml to 0.2 +/- 0.1 ng/ml (Mean +/- SD) with p<0.05. Proteins analyses of microbial cultures following ACV treatment for 24 hours compared to untreated showed there was an alteration in expression of key metabolic protein involved in E-coli growth cycles. Other proteins that were noticeably absent were those involved in pathogenesis, organelle structure, carbon, glucose metabolism and cellular oxidation or reduction reactions. Conclusion: This study demonstrates that ACV displays antimicrobial activity which can penetrate microbial membranes as well as organelles and destroys key metabolic proteins.

P1807
Board Number: B826
The effects of acetic acid on biofilm formation and wound healing using a zebrafish model.
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Biofilm is an accumulation of multi-species and multicellular microbial mass embedded in polysaccharide matrix with adhesive abilities. Complex biofilm has remarkable ability to impede normal immune responses and aggressive medical interventions, leading to difficulties in treating infections of wounds, heart valves, and bone tissue. We must utilize in vitro, in vivo, and ex vivo models to gain understanding of biofilm formation and eradication. We have previously established a wound model using Danio rerio (zebrafish), both in vivo, and as a novel ex-vivo tissue explant culture, to analyze the formation, growth, and treatment of biofilm. We have compared formation of biofilm within zebrafish penetrating burn wounds using three specific strains of Staphylococcus epidermidis. S. epidermidis RP62A (WT) is a robust biofilm-former, 1457 (WT) is a moderate biofilm-former, and 1457 aapΔ/icaΔ does not form detectable biofilm. Using zebrafish tissue explant cultures, we previously found evidence of bacterial growth in all three strains; however, only the RP62A strain formed robust, macroscopic biofilm and demonstrated microscopic Gram stain evidence of cell clusters held together with polysaccharide matrix. Here, we demonstrate our efforts to begin assessment of the effectiveness of acetic acid treatments on disrupting biofilm formation, both in vitro and using our zebrafish in vivo and ex vivo explant culture models. We hope to gain understanding of the role that acetic acid treatment could play in the treatment of bacterial biofilm infections of chronic wounds.

P1808
Board Number: B827
FzlA, an essential regulator of FtsZ filament curvature, controls constriction during Caulobacter division.
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During bacterial division, polymers of the conserved tubulin-like GTPase FtsZ assemble at midcell to form the cytokinetic Z-ring, which is postulated to coordinate peptidoglycan (PG) remodeling and
envelope constriction. Determining precisely how Z-ring structure and dynamics are mechanistically coupled to PG metabolism and, more broadly, to division are major goals in the field. Curvature of FtsZ filaments was previously found to promote membrane deformation in vitro, though the role that curved filaments play in facilitating division in vivo remains undefined. Our lab studies FzlA, a bacterial division protein that stabilizes highly curved FtsZ filaments, as a tool for assessing the contribution of FtsZ filament curvature to cell constriction.

Using X-ray crystallography, we determined the structure of FzlA to inform a structure-function analysis of FzlA’s interaction with FtsZ. We phenotypically characterized a panel of mutant FzlA strains, then biochemically assayed activity of the corresponding mutant proteins toward FtsZ. We found that in Caulobacter crescentus, FzlA must bind to FtsZ for division to occur and that FzlA-mediated FtsZ curvature is critical for efficient division. Using single-cell microscopy, we subsequently observed that mutation of FzlA affects cell constriction rate, which we propose corresponds with its ability to bind and curve FtsZ polymers. Further, we found that a slowly constricting fzlA mutant develops “pointy” poles, suggesting that FzlA influences the relative contributions of radial versus longitudinal PG insertion at the septum. These findings implicate FzlA as a critical coordinator of envelope constriction through its interaction with FtsZ and demonstrate a functional link between FtsZ curvature and efficient constriction in C. crescentus.

P1809
Board Number: B828
Spatial-temporal regulation of bacterial cell division machinery by FtsZ treadmilling dynamics.
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Bacteria cells recruit more than twenty proteins to assemble the cell division machinery or ‘divisome’. How these proteins are regulated and coordinated in the future division site is still not completely clear. In Escherichia coli, a tubulin homolog FtsZ could polymerize and form a ring-like structure (Z-ring) at the middle cell where the cell divides. Z-ring formation initiates the recruitment of downstream proteins of a ‘matured’ divisome. Previous studies have shown FtsZs in the ring turnover dynamically both in vivo and in vitro. By using total internal reflection microscopy (TIRF) and 3D structural illumination microscope (3D-SIM), we demonstrated the FtsZ filaments in the ring constantly treadmill around the cell circumference. The dynamic treadmilling behaviour is not caused or related to the known FtsZ regulators, such as ZapAB, MinCDE, and SlmA, but coupled with its intrinsic GTPase activity. The treadmilling speed reduces significantly in FtsZ GTPase-deficient mutant strains, and the septal morphology becomes abnormal and asymmetric. The asymmetric or incomplete septal morphology highly indicates that treadmilling dynamic could modulate the distribution and/or activity of other proteins in divisome, which are responsible for cell wall synthesis. We monitored the localization and mobility of the essential transpeptidase FtsI by 3D-single molecule tracking. These molecules showed directional movement around the future septum with the speed highly correlated with the treadmilling speed of FtsZ. In wild type cells, both FtsI treadmilling and FtsI movement could complete the whole ring circumference in ~100 second time scale which is 1/10-1/20 of the total cell division time under our experiment condition. This indicated that FtsZ ring dynamically redistribute the cell division machinery to build the septal cell wall piece by piece until a uniform septum completed. However, the defected FtsZ with slow treadmilling speed (similar to the division time) can only sample part of the division site and lead to an incomplete septum.

P1810
Board Number: B829
Dual labeling of bacterial peptidoglycan and tubulin FtsZ to study bacterial cell division.
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Peptidoglycan (PG), an essential component of bacterial cell wall, protects bacterial cells from changes in osmotic pressure and helps to maintain cell shape. PG is constructed from short peptide chains and two carbohydrates, N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc). We recently developed a method combined organic synthesis, biochemistry, and genetic engineering approaches to label Escherichia coli bacterial PG on its MurNAc sugar backbone. Unnatural PG intermediates with bioorthogonal functionalization were synthesized and incorporated into PG via a specific cell wall recycling pathway. Remodeled cells were then labeled with a bioorthogonal click reaction to link a fluorescent molecule onto the cell wall. Using this method, the bacterial cell wall including the dividing PG-ring was successfully visualized and analyzed by Structured Illumination Microscopy (SIM) and Stochastic Optical Reconstruction Microscopy (STORM). To achieve a better understanding of the dynamics and three-dimensional architecture of bacterial cell wall during cell division, we engineered the E. coli cell strain to label on the bacterial tubulin FtsZ, a protein serves as the scaffold of divisome assembly. Dual labeling on the bacterial PG and FtsZ protein provides a new approach to study the interaction between PG carbohydrate and cell divisome, and the forming of Z-ring during cell division. This study would also help to reveal new details about the assembly order during cell wall dividing and determine that whether the PG biosynthesis or FtsZ polymer constriction provides the force for cell division.

P1811
Board Number: B830
Mechanical strain sensing implicated in cell shape recovery in Escherichia coli.
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The shapes of most bacteria are imparted by the structures of their peptidoglycan cell walls, which are determined by many dynamic processes that can be described on various length scales ranging from short-range glycan insertions to cellular-scale elasticity. Understanding the mechanisms that maintain stable, rod-like morphologies in certain bacteria has proved to be challenging due to an incomplete understanding of the feedback between growth and the elastic and geometric properties of the cell wall. Here, we probe the effects of mechanical strain on cell shape by modelling the mechanical strains caused by bending and differential growth of the cell wall. We show that the spatial coupling of growth to regions of high mechanical strain can explain the plastic response of cells to bending and quantitatively predict the rate at which bent cells straighten. By growing filamentous Escherichia coli cells in doughnut-shaped microchambers, we find that the cells recovered their straight, native rod-
shaped morphologies when released from captivity at a rate consistent with the theoretical prediction. We then measure the localization of MreB, an actin homologue crucial to cell wall synthesis, inside confinement and during the straightening process, and find that it cannot explain the plastic response to bending or the observed straightening rate. Our results implicate mechanical strain sensing, implemented by components of the elongosome yet to be fully characterized, as an important component of robust shape regulation in E. coli.

P1812
Board Number: B831
Brownian ratchet mechanism for faithful segregation of low-copy-number plasmids.
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Bacterial plasmids are extrachromosomal DNA that provides selective advantages for bacterial survival. Plasmid partitioning can be remarkably robust. For high-copy-number plasmids, diffusion ensures that both daughter cells inherit plasmids after cell division. In contrast, most low-copy-number plasmids need to be actively partitioned by a conserved tripartite ParA-type system. ParA is an ATPase that binds to chromosomal DNA; ParB is the stimulator of the ParA ATPase and specifically binds to the plasmid at a centromere-like site, parS. ParB stimulation of the ParA ATPase releases ParA from the chromosome, after which it takes a long time to reset its DNA-binding affinity. We introduce a Brownian ratchet model that recapitulates the full range of actively segregated plasmid motilities observed in vivo. We demonstrate that plasmid motility is tuned as the replenishment rate of the ParA-depletion zone progressively increases relative to the cargo speed, evolving from diffusion to pole-to-pole oscillation, local excursions, and, finally, immobility. When the plasmid replicates, the daughters largely display motilities similar to that of their mother, except that when the single-focus progenitor is locally excursive, the daughter foci undergo directed segregation. We show that directed segregation maximizes the fidelity of plasmid partition.

P1813
Board Number: B832
16s RNA gene Restriction Fragment Length Polymorphism of Guánica Salterns Halobacteria.
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One of the most intriguing things in science are extremophiles, it is fascinating the different physiological characteristics that this organism poses to survive on those extreme environments. How they manage to support harsh environmental conditions, how the fight high osmotic pressures and other situations is really fascinating. Guánica salterns halobacteria is an extremophile organism that live in a saline in Guánica Puerto Rico, we have isolated this extremophiles and performing a full characterization of the halobacteria and we are pursuing to develop the full phylogenetic tree for this archaean. We have gather data to believe that we found a new species of halobacteria,. We are cloning the gene by PCR cloning using primers develop in our lab. DNA sequencing of the amplified fragments and restriction fragment length polymorphism analysis of the 16s RNA gene of the Guánica Saltern Halobacteria, a highly conservative gene in species, will be done. To further characterize it, the DNA sequencing data will be use on a homology Blast analysis to compare it with other known halobacteria.
We are doing clonal expansion of the halobacteria, DNA extractions, PCR cloning, restriction enzymes (RFLP), agarose electrophoresis, and bioinformatics tools. The knowledge of this halobacteria can enrich the scientific community, not only by the founding of a new species but the new characteristics that this organism can have hidden in their genome. Halobacteria are of great interest, not only for being extreme and have information in their physiology to survive in an extreme environment but we have data that shows that this organism produce pigments that can be used as food coloring, it has proteins that are functional in high salt environments that can be used in process of fermentation by big companies. Guánica Saltern Halobacteria can have the protein for the future great technique in biotechnology, or the future in the fermentation process to produce molecules that can impact the medical field.

P1814
Board Number: B833
Isolation and Characterization of Surface Bacteria Associated with the Sea Urchin Lytechinus variegatus.
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This study focusses on identifying and characterizing bacterial flora that coexist on the surface and test of the sea urchin Lytechinus variegatus. Several bacterial strains were isolated from different batches of wild type sea urchins by gently swabbing their test, mouth and gonadopores with a sterile tip and plating them onto marine agar plates. More than 60 different bacteria were re-streaked till single colonies were obtained. Results indicated that all isolated bacteria were gram negative rods. They also tested positive for the enzyme oxidase and did not ferment lactose. One of them showed agarolytic properties. A few of them harbor endogenous cryptic plasmids ranging in size from 5Kb to 20Kb. A total of 13 strains were selected to be investigated phylogenetically by 16S rDNA gene amplification and sequence analysis. The sequences obtained will be compared with 16S rDNA gene sequences from databases. Bacteria will be tested for antimicrobial properties and the presence of bioactive compounds.

P1815
Board Number: B834
Calcium plays a role in the survival of protist co-isolated from the eggs of sea urchin Lytechinus variegatus and Lyechinus willamsi.
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Without treatment, harvested sea urchin eggs disintegrate within 24 hours and become heavily infested with a certain species of protists. It is not clear if the protists disintegrate the eggs or simply consume the degrading eggs. Freshly spawned eggs of sea urchin Lytechinus variegatus were incubated in regular sea water at a normal calcium concentration and in calcium free sea water for four days. Calcium free sea water with natural salinity and low salinity sea water with calcium were used as controls. Results indicated that protists did not proliferate or survive in both calcium free waters. However, eggs disintegrated even in the absence of protists though at a slower rate. There is clear indication that protists are not responsible for disintegration of the eggs. Furthermore, it appears that calcium is very essential for the survival of this protist. Classification and the symbiotic role of this protist with sea urchins needs to be investigated.
P1816
Board Number: B835
Analysis of damaging effect of Betadine combined with the enzyme nuclease toward bacteria Serratia marcescens.
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Gram negative bacteria Serratia marcescens is recognized as important pathogen. We displayed the culture growth caused by addition into the broth of the filter papers that 14 years ago were soaked with various strains of bacteria S. marcescens, and then were dried, and after that were stored at 4C. As S. marcescens also demonstrates an incredible resistance to the action of antibiotics we decided to work out a combined preparation that includes Betadine, making pore in cells’ membranes, and the enzyme, nuclease, potently degrading both RNA and DNA.
We found a complete loss of viability of S. marcescens culture after 3 min incubation in the presence of 10-fold water diluted Betadine (povidone iodine - topical antiseptic). The further dilution of Betadine diminished its suppression of S. marcescens growth. Electronic microscopic examination of S. marcescens cells after 15 sec incubation with 5000-fold diluted Betadine showed both the damage of the boundary layers of the cells, and changes in chromatin, as well as migration of cytoplasmic membrane into the cytosol, which probably connected with the diminishing turgor. The Betadine effectiveness depended on both the culture density and the growth phase. The culture of the stationary phase was most sensitive to the action of the diluted Betadine and, vice versa, least sensitive, at the lag phase. The purified as had previously published [1] nuclease was found to maintain 30-40% of the residual activity after 20 min incubation with 2000-fold diluted Betadine. Combining the diluted Betadine, with the nuclease enhanced the damage to S. marcescens cells. The electronic microscopic examination of the cells after 15 sec incubation with a mixture of the diluted Betadine and the nuclease showed that the microbial material in the vast majority contained the cells with a distorted shape: curved, rounded, and also structures resembling fragments of cells. Quite often we observed structures resembling cells with a “trail” that suggested the outflow of the protoplast. In most cases, almost complete loss of the boundaries between periplasm, cytoplasm, cell wall, cytoplasmic membrane and nucleoid region was observed. As a result, the cells resembled a mixture of haphazardly located fragments of different electron density.
This study was conducted according to the Russian Government Program of Competitive Growth of Kazan Federal University. Albert A. Rizvanov was supported by state assignment 20.5175.2017/6.7 of the Ministry of Education and Science of Russian Federation.

P1817
Board Number: B836
Multidrug Resistance in Disparate Bacterial Species Following Triclosan Exposure.
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The Food and Drug Administration (FDA) considers the increased resistance of bacteria to antimicrobials to be a major public health threat. Initiatives to ensure proper use of these agents are countered by the
public demand for substances that can regularly be used to impede the growth of bacteria that cause human disease. One such agent, triclosan, has been used as a supplement in common personal hygiene products such as toothpaste, shampoo, and hand sanitizer for several decades as a way to discourage bacterial growth. Its effectiveness was first questioned by the FDA in 1974, but its use has continued. Noting a lack of evidence regarding efficacy, the FDA banned triclosan and 18 other antimicrobial ingredients from most commercially available products beginning in January 2017; however, this merely limited its use as this chemical continues to be included in such products as plastics and toothpastes. In addition, there has already been a significant ecological accumulation of triclosan in soil, sewage, water runoff, and natural bodies of water. The Florence Statement on Triclosan and Triclocarban that was introduced at the 36th International Symposium on Halogenated Persistent Organic Pollutants in 2016 summarized concerns raised by a multitude of studies demonstrating a negative impact on the environment, concerns for the health of humans and aquatic organisms, and the potential for increased microbial resistance. The study presented here directly links triclosan to derived microbial resistance in a variety of bacterial species (e.g. Alcaligenes faecalis, Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Micrococcus luteus, and Staphylococcus aureus), and further affirms that this resistance impacts the susceptibility of those species to other antibiotics. Strains repeatedly exposed to triclosan quickly became partially- or fully-resistant to that chemical, as demonstrated by Kirby-Bauer antibiotic testing and growth curve analysis of cultures grown in the presence and absence of triclosan. Furthermore, these derived strains showed altered response to other antimicrobial drugs, such as chloramphenicol and tetracycline. Given that triclosan will likely persist in the environment, this study raises concerns regarding long-term alterations to naturally occurring bacteria and their resistance to antibiotics. Further research seeks to characterize the underlying molecular changes that lead to this cross-drug resistance, identifying commonalities among the strains, and further assessing the types of compounds rendered ineffective in these novel bacterial strains with the hope of identifying potential targets for combating multidrug resistance.

**P1818**

**Board Number: B837**

**Comparison of virulence factors in clinical and environmental strains of actinobacterium* Tsukamurella tyrosinosolvens* based on whole-genome sequencing.**

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Actinobacteria are an extensive phylum of microorganisms, including both pathogenic and biotechnologically beneficial bacteria. One of such representatives is *Tsukamurella tyrosinosolvens*. Therefore, the question of the safety of using opportunistic actinobacteria in biotechnological processes and, in particular, in the processing of industrial wastes remains open. Currently three whole genome sequences for T. *tyrosinosolvens* strains are presented in the NCBI database: PS2 (test strain), CCUG 38499 (clinical strain) and JCM 15482 (soil strain). The first strain was isolated by us as an alkane-oxidising bacteria from the industrial wastewater sludge repository. *T. tyrosinosolvens* PS2, which showed no specific activity on blood agar and salt egg yolk agar, was considered as a potential non-pathogenic strain for comparative analysis. Hemolytic and lecithinase activity for another two strains is unknown. The identification of genetic determinants of pathogenicity in the bacterial genomes among the factors that determine the adhesion, invasion, secretion and resistance of microorganisms was performed using the MP3 program (“Prediction of Pathogenic/Virulent Proteins”). A total of 533 genes were found, 516 of which were shared by all strains, 7 genes were unique for the *T. tyrosinosolvens* PS2 strain, and 10 genes appeared to be specific for the clinical and soil isolates. In the genome sequence of
the hydrocarbon oxidizing strain *T. tyrosinosolvens* PS2 no invasion factors participating in the primary attack of cells and tissues of the host organism, such as trypsin, and endopeptidase enzymes genes, that cleave peptides and proteins, were detected. In addition, a lack of genes for the pseudaminic acid synthesis, which is necessary for the bacterial cell movement, colonization of mucous membranes and host tissue invasion, was demonstrated. Key genes of the type IV secretion system, which carries out the virulence proteins secretion into the eukaryotic cell, were not found. Among the unique *T. tyrosinosolvens* PS2 strain genes, bacteriophage were found, which could be considered as indirect pathogenic factors. Thus, the differences revealed by the *T. tyrosinosolvens* strains genome comparison are of interest for studying host-pathogen interactions. Lack of genes for the primary attack of host organism cells and tissues probably reduces the level of PS2 strains’ virulence. The identical profile of virulence factors in both soil and clinical strains indicates potential danger of soil bacteria as a source of opportunistic infection.

The research was performed using the equipment of the Interdisciplinary Center for Shared Use of Kazan Federal University according to the Russian Government Program of Competitive Growth of Kazan Federal University.

**P1819**

**Board Number: B838**

**Assaying the Relative Effectiveness of Biofilm Disruption by Natural Oils.**

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Biofilm formation is a survival mechanism some bacteria have evolved to adapt to host defense systems and harsh environmental conditions. Biofilms exhibit characteristics that are distinct from those expected for planktonic microorganisms including: increased antibiotic resistance, increased resistance to environmental stresses, and the expression of virulence factors. As such, biofilms contribute to bacterial pathogenicity. A crucial component of biofilms is the polysaccharide matrix, which allows adherence of bacteria to surfaces. We developed qualitative and quantitative methods to analyze biofilms formed in static cultures of *Staphylococcus aureus* and *Staphylococcus epidermidis*, two clinically important pathogens and aim to use these methods to examine the biofilm inhibiting properties of various chemicals. Under our laboratory conditions, *Staphylococci* produce a polysaccharide rich biofilm matrix that can be disrupted by diastase. In these new studies we examine the qualitative and quantitative effects of natural, easy to find oils including: olive oil, coconut oil rapeseed oil and peanut oil to disrupt biofilms. The ability to disrupt existing biofilms using oils may inform novel topical therapies. The results of our latest findings will be presented. Grant Support: NIH-NIGMS MBRS RISE: R25 GM059244-15

**P1820**

**Board Number: B839**

**Essential roles for sterols in bacterial cell biology.**

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Sterols are necessary membrane components in eukaryotic cells, and regulate membrane structure, function, and dynamics. Bacteria generally lack sterols, and contain hopanoids as functionally analogous surrogates. The planctomycete bacterium Gemmata obscuriglobus produces membrane sterols, but
their function remains enigmatic. It belongs to the greater Planctomycete-Verrucomicrobia-Chlamydiae (PVC) bacterial superphylum, where many members contain unique membrane morphological features plausibly connected to sterols. We are pursuing two aims related to sterols. Firstly, we are investigating the function of sterols in G. obscuriglobus. As genetics have only recently been developed in this bacterium, we pursued this question using a chemical depletion approach. We found G. obscuriglobus membrane sterols to be essential for survival, the first reported instance in a bacterial species. Using time-lapse light microscopy, we observed a profound defect in cellular replication associated with sterol depletion, suggesting G. obscuriglobus membrane sterols are essential for proper cell division. Secondly, we have begun to determine the prevalence and function of sterol biosynthesis across the PVC superphylum using complementary microscopy and gas chromatography mass spectrometry approaches. We found three candidate sterol-producing species within the Verrucomicrobia, though further quantitative analysis is needed. One of these bacteria exhibits a similar sterol depletion phenotype to that observed in G. obscuriglobus. Our results support novel roles for sterols in prokaryotic cell biology, particularly in regard to cell division.

P1821
Board Number: B840
Strong Antimicrobial Activity Displayed By Newly Synthesized Hydroxamic Acids And Their Derivatives.
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The increasing antibiotic resistance of pathogenic bacteria and decreasing availability of naturally produced antibiotics have made it crucial for researchers to design and synthesize new therapeutic agents with antimicrobial properties. Several chemical compounds including hydroxamic acids, and their derivatives were newly synthesized by the chemistry department and tested in our lab for antibacterial activity against eight different ATCC strains of pathogenic bacteria. The antimicrobial activity of each compound was evaluated using the disk-diffusion assay, liquid broth assay, and data derived from microtiter plate absorbance reading. While some compounds displayed a wide spectrum of antibacterial activity others did not have any effect on bacterial growth. These results enabled us to narrow down the potential active site or functional group in the molecules responsible for the activity. Future work will focus on designing and testing new derivatives with a broader spectrum of activity.

P1822
Board Number: B841
Discovery of a Marine Bacteria with a Wide Spectrum Anti-Bacterial Activity.
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Biofilms forming and antibiotic resistant bacteria are responsible for most infectious diseases and hospital related bacterial infections. The need for new antimicrobial and antibiofilm compounds is becoming vital as the incidence of these types of infection is dramatically increasing. The focus of our research is the identification and characterization of new anti-biofilm and anti-microbial substances. An unknown marine bacterium found in the Newark Bay coastal water was isolated and tested for possible anti-biofilm and anti-microbial activity. Cell free extracts of the unknown marine bacteria were made and tested against various strains of Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Erwinia carotovora, and Escherichia coli. The unknown extract
consistently exhibited strong antimicrobial activity against a wide range of gram negative and gram positive bacteria. The active compound appears to be greater than 100 kDa in size and heat sensitive. Proteinase K, DNase and RNAse treatment had no effect on the activity of the extract. Further studies will be conducted to characterize and identify the unknown bacteria and the active fraction of the extract.

**Protists and Parasites**

**P1823**

**Board Number: B842**

*Plasmodium falciparum* GPCR-like receptor SR25 mediates extracellular K+ sensing coupled to Ca2+ signaling and stress survival.

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During its development, *Plasmodium falciparum* is exposed to changes in ionic content in their surrounding environment. We have found that the *P. falciparum* serpentine-like receptor PfSR25 is involved in KCl-evoked Ca2+. In accord, switching of isolated parasites from a high KCl solution (140 mM) to a low KCl solution (5.4 mM) triggered increases [Ca2+]cyt. The KCl-elicited Ca2+ response was blocked by a phospholipase C (PLC) inhibitor, U73122 or by internal Ca2+ store depletion. This response was never observed when NaCl was used as the KCl substitute. In contrast, the calcium response in the parasite were suppressed by the knockout of the gene for the PfSR25 receptor (PfSR25-). Our results also demonstrate that PfSR25 plays important roles in adaptive response to stress: a) hyperosmotic stress resulted in a significant decrease in wild type parasites (WT) volume, but not in PfSR25- parasites; b) sodium nitropusside (SNP) exposure decreased parasitemia and upregulated metacaspase expression in PfSR25- parasites; c) wt parasites showed a significantly greater survival than PfSR25- parasites during albumax starvation; and d) PfSR25- exhibited a smaller hemozoin formation than that of WT after treatment with piperaquine (PQ). The present data showed a critical role for PfSR25 receptor in *P. falciparum* development and a better understanding of this pathway may help to clarify their role into these different functions.

**P1824**

**Board Number: B843**

*Isolation of Apicomplexan Blood Parasites from Caribbean Stegastes Damselfishes.*

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Apicomplexa are intracellular protozoan parasites that infect a wide array of host animals including humans, other mammals, birds, reptiles, fish and invertebrates. Apicomplexa are responsible for severe human disease such as malaria, but little is known about other natural apicomplexan infections. Fish infections can be used as alternate model systems to study these parasites. In the Caribbean, some of the most abundant coral reef fish, Stegastes damselfishes, are infected with Apicomplexa parasites in their red blood cells. To characterize the lifecycle, host range, and transmission route of these parasites, our goal is to develop methods to isolate and detect them in infected fish samples. **We hypothesize that we can isolate parasites from fish blood using Percoll density step gradients to fractionate cells.** To
test our hypothesis, we captured damselfish from their natural reef habitat, collect blood, and release them. Next, patterns of fractionated cells were compared from blood samples with varying degrees of infection. We also optimized various techniques for detecting parasites, including microscopy, PCR and western blot. We identified parasites in Giemsa-stained whole blood smears from 40% (4 of 10) Stegastes adustus, dusky damselfish, captured and released in Brewers Bay, St. Thomas in June 2017. We also were able to detect parasites in some fractionated blood cells using microscopy. PCR and western blot analysis will be used to confirm that some blood cell fractions are enriched with parasite DNA and proteins. PCR analysis followed by DNA purification and sequencing tested primer specificity for the parasite 18S rDNA. Sequences amplified from damselfish blood matched to other Caribbean fish apicomplexan isolates in the NCBI nucleotide database using BLAST. Our results suggest that we can fractionate fish blood, detect parasite DNA and visualize infected cells using microscopy. These techniques can be used for future experiments to identify the range of tissues and hosts infected, towards mapping the lifecycle and transmission routes of these apicomplexan parasites. The positive impact of our research will be significant, as our findings will facilitate the application of this naturally occurring infection to the study of human disease.

P1825
Board Number: B844
Unconventional trafficking of a DNA replication protein through secretory pathway in apicomplexan Plasmodium falciparum.
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Malaria, caused by Plasmodium sp., continues to be a major factor contributing to the disease burden of many countries. Amongst the various species of Plasmodium, P. falciparum is known to induce the deadliest form of malaria. It replicates at least four times during its life cycle in human and Anopheles mosquito. Additionally, it has evolved an intricate secretory system within itself as well as in the host cells for its survival. P. falciparum owes its successful survival to the efficient replication machinery as well as the ability to manoeuvre the host system according to its requirements. This study is an attempt to unravel the unusual trafficking of a Plasmodium origin recognition complex subunit homolog, PfOrC2, possibly involved in DNA replication through the endoplasmic reticulum (ER) en route to the nucleus. Some proteins follow this path from ER to nucleus under special circumstances, but such a pathway has not been reported in a pathogenic organism like P. falciparum. Based on experiments employing specific antibodies against the N-terminus and C-terminus of PfOrC2 and a series of inhibitors acting on the anterograde transport from ER (Brefeldin A) & intra-membrane proteolysis (Z-LL)₂, we found that the full-length protein is following the ER-Golgi secretory pathway and getting processed by a signal peptide peptidase. Subcellular localisation of the full-length protein and the processed fragment was established through GFP fusion parasite line, immunofluorescence assays and fractionation. The processed form is targeted to the nucleus in an-importin dependent manner (impeded by ivermectin) constitutively and might function in DNA replication and other auxiliary function. This is the first report on the ER-association and processing of a nuclear protein implicated in DNA replication in a parasite, similar to trafficking of transcription factors in higher eukaryotes.
P1826  
**Board Number: B845**  
A cdc2 homolog is required for completion of oral development during regeneration and cell division in the giant ciliate *Stentor coerules*.  
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The giant unicellular ciliate *Stentor coerules* has the ability to fully regenerate after being cut into pieces, in a way that perfectly preserves the original polarity and structure. This regeneration ability is particularly remarkable because *Stentor* has a complex cortical architecture, with longitudinal ciliary rows running between an oral apparatus at the anterior end and a holdfast at the posterior end. The molecular details behind this incredible phenomenon have remained largely unstudied, and we wish to understand how the regeneration process is coordinated at the molecular level. To identify candidates for RNAi knockdown we analyzed the kinome of *Stentor*. *Stentor* was found to encode over 2000 kinases, making up 6% of the total protein coding genes. Many of these consist of expansions in mitotic kinase families such as CDKs, PLks, Auroras, NDRs, and NEks. As some of the details of the regeneration process parallel the events of cell division, we wish to understand whether the cell co-opts cell division signaling pathways to initiate and control regeneration. To begin to investigate the role of the cell cycle in regeneration, we performed RNAi knockdown of CDK1/cdc2 homologs in *Stentor*. For some of the cdc2 genes, knockdown resulted in slowed division with daughter cells of strikingly unequal size. For other homologs, however, knockdown results in cells unable to complete division. In these cases, the cells are still able to initiate oral development but ultimately exhibit aberrant oral morphology, both prior to division and during regeneration. We conclude that the oral regeneration process employs CDK signaling pathways. In future work we aim to understand the full extent to which *Stentor* uses cell cycle signaling pathways to coordinate and control regeneration.

P1827  
**Board Number: B846**  
The localization and functional analysis of a novel centrin (TgCentrin2) in human pathogen *Toxoplasma gondii*.  
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Centrins are EF-hand containing proteins ubiquitously found in eukaryotes and are key components of centrioles/basal bodies as well as certain contractile fibers. We previously identified several centrins in the human parasite *T. gondii*, all of which are localized to the centrioles. However, one of them, TgCentrin2, is additionally targeted to ring-like structures at the apical and basal ends of the parasite, as well as a set of 5-6 annuli about ~1/4 body length from the parasite apex. Using TgCentrin2 as the probe, we seek to elucidate the composition and function of these novel centrin-containing structures, which will provide new insights into centrin biology. Because TgCentrin2 appears to be an essential gene, we designed a dual regulation method that combines transcriptional and protein stability control to achieve tight, conditional regulation of TgCentrin2 level in the cell. We discovered that the knockdown of TgCentrin2 results in severe defects in multiple aspects of *T. gondii* lytic cycle, including cell replication, cytokinesis, and cell motility. Our data also indicate that there is differential turn-over or incorporation kinetics of TgCentrin2 in its different locations. Lastly, using proteomic methods, we
have identified and are currently characterizing a group of novel TgCentrin2 interacting proteins, which may help to design new anti-parasitic measures.

P1828  
Board Number: B847  
The activity and function of TgDCX, a component of non-tubular tubulin polymers in the human parasite, Toxoplasma gondii. 
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Purified tubulin subunits spontaneously form microtubules in vitro, and thus tube formation is an intrinsic property of the tubulin subunits. However, in the human parasite, Toxoplasma gondii, these same tubulin subunits are assembled into 14 non-tubular fibers spirally arranged in a truncated cone-shaped complex (the conoid) as well as at least 5 other structures formed of canonical (tubular) microtubules. Identifying the structural determinants of the conoid and characterizing their activity in catalyzing the formation of the ribbon-like conoid fibers will therefore provide insight into how the intrinsic tendency of tubulin subunits to form a tube can be overridden by associated proteins. One candidate for this role is TgDCX, a protein bound to the conoid fiber in Toxoplasma that has a doublecortin (DCX) domain and a TPPP/P25-alpha domain, both of which are known modulators of tubulin polymer structure. Using an icosahedral virus particle containing exactly 240 copies of fluorescent proteins as the calibration standard, we estimated that approximately every 2 tubulin subunits is decorated with one TgDCX molecule in the conoid fibers. We previously showed that loss of TgDCX radically disrupts the structure of the conoid, severely impairs host-cell invasion, and slows growth. We recently found that recombinant TgDCX bundles tubulin polymers in vitro and in vivo. Furthermore, by cross-species complementation, we are able to generate "conoids" in which the TgDCX molecules are partially or fully replaced with a homolog from a photosynthetic, free-living relative of T. gondii that has a drastically different conoid structure. This makes it possible to examine the molecular basis for the evolution of this fascinating cytoskeletal complex over hundreds of millions of years. The non-tubular polymeric form of tubulin found in the conoid is not found in the host cell, suggesting that TgDCX may be an attractive target for new parasite-specific chemotherapeutic agents. To facilitate that effort, a C-terminal fragment of TgDCX that contains the DCX domain has been crystallized by the Seattle Structural Genomics Center for Infectious Disease.

P1829  
Board Number: B848  
Deciphering the role of proteasomal machinery in phagocytosis in Entamoeba histolytica.  
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Current phagosomal maturation models have mostly been derived from the endosomal maturation models in which ubiquitination/proteasomes machinery functions at multiple steps from early to late stages, including optimal ligand based internalization and endosomal maturation via the ESCRT pathway. The role of ubiquitination/proteasomes in phagosome maturation is, however, controversial due to reductionist approaches using one phagocytic receptor [1] and opsonized latex beads [2] or due to suspected contamination with non-specific proteins in purified phagosomes [3].
We have used the protozoan parasite, E. histolytica, along with red blood cells (RBCs) to understand the molecular mechanism of phagocytosis in this organism. E. histolytica is a professional phagocytic cell. This system is likely to represent the entire complexity of the phagocytic process due to use of unmodified cells and a natural ligand for phagocytosis. Biochemical assay of phagocytosis showed a significant inhibition by proteasome inhibitors. Confocal imaging after incubation with RBCs for 5 min showed 27.3% and 18% cells with phagocytic cups and 6.2% and 2% cells with phagosomes in control and proteasome inhibitor treated cells, respectively. EhCaBP1 is an early protein to be recruited to the phagocytic cups but falls-off after the cup closure and detachment from the plasma membrane. Relative fluorescence intensity of EhCaBP1 was significantly higher at the phagocytic cups in proteasome inhibitor treated cells. This could be due to a stabilization of EhCaBP1 protein by proteasome inhibitors as was observed by western blotting. Phalloidin staining of the actin polymerization at the phagocytic cup did not show any significant difference upon inhibition of proteasome activity. Live cell imaging showed a sustained interaction of RBCs with treated cells without internalization. These results suggest a slower kinetics of phagocytic cup and phagosome formation upon inhibition of proteasome activity and will be discussed in relation to the current models of phagosome maturation in E. histolytica. These studies may also have implications of ubiquitination/proteasomal involvement in phagosomal maturation process in high eukaryotes.


P1830
Board Number: B849
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Giardia lamblia, a flagellated protozoan parasite, if ingested, infects the lumen of the small intestines in humans. This parasite is found in various animals, such as dogs, cats, and birds. G. lamblia is most commonly transmitted to humans via ingestion of contaminated food and water. Its presence in aquatic environment is relatively unorthodox and its presence in public waterways is a public health concern. The goal of this study is to determine the prevalence of G. lamblia in Atlantic oysters (Crassostrea virginica), ribbed mussels (Geukensia demissa), and steamers (Mya arenaria) collected from Orchard Beach and Soundview Park, Bronx, NY. The mussels were collected on September 15, 2016 at low tide. Tissues were dissected followed by DNA extraction and PCR analysis. Thus far, we found a prevalence of 77% (21/28) and a prevalence of 65% (11/17) of G. lamblia in Soundview Park and Orchard beach, respectively. Additional experiments will be conducted to determine whether there is a difference in the prevalence and genotype of G. lamblia between the two sites. Furthermore, we will be able to assess the temporal variation in G. lamblia prevalence at Orchard beach from 2014-2016. In conclusion, Atlantic oysters and ribbed mussels can be used as a biological sentinel to detect G. lamblia in public waterways and reservoirs.
P1831

Board Number: B850
Parasites as an Alternative Model for Lipid Metabolism: Gene Expression Analysis of an Oyster Parasite Perkinsus marinus during Lipid Starvation.
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Our laboratory is interested in understanding the response of Perkinsus marinus, a protozoan parasite of oysters, to lipid starvation as a means of understanding its underlying metabolic pathways. Previous work has shown that this parasite is capable of synthesizing its own fatty acids, and as well as acquiring them from their host. As citrate can serve as a source of Acetyl CoA, we are also investigating the role of cytosolic aconitase as a potential regulator in this process, as there is evidence for phosphorylation of this enzyme to favor citrate production. No one, to our knowledge, has delineated the role of cytosolic aconitase in fatty acid biosynthesis. We have performed differential mRNA gene expression in P. marinus, in which cells were starved of lipids for 11 days, as compared to lipid replete cells. There was not a noticeable upregulation of FAII-pathway enzymes for saturated fatty acid synthesis, although citrate synthase was upregulated approximately 3-fold, consistent with allostery activation of acetyl CoA carboxylase-1. Polyketide polyunsaturated fatty acid synthase and delta 5/ delta 6 fatty acid desaturase were upregulated by approximately 2-fold. Surprisingly, a number of enzymes involved in beta-oxidation were upregulated, which contrasted with the expectation that free fatty acids from triglycerides would be directed towards salvage pathways for membrane synthesis. Consistent with this, ACC-2 was down regulated 2-fold. Interestingly, maltose acetyl transferase was also upregulated 2-fold, implying that acetyl units from beta-oxidation may be re-directed to sugar acetylation. The implications of this complex interplay will be discussed, along with future experiments to address lipid and sugar metabolites present in this parasite under lipid starvation conditions. Finally, the activity and phosphorylation status of aconitase-1 will be assessed under these conditions.

P1832

Board Number: B851
Farnesol inhibits both translation initiation and morphological differentiation in the human fungal pathogen, Candida albicans.
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Candida albicans is a polymorphic yeast that can cause life threatening systemic infections in immunocompromised individuals. One key attribute of C. albicans that enhances its pathogenicity is the ability to switch morphologies between filamentous and vegetative modes in response to specific environmental conditions. Stressful changes in such cellular conditions commonly cause a rapid inhibition of global protein synthesis leading to altered programmes of gene expression. In Saccharomyces cerevisiae, fusel alcohols signal nitrogen scarcity and induce pseudohyphal growth enabling yeast colonies to spread towards nutrient replete areas away from toxins. These alcohols also inhibit protein synthesis by targeting the translation initiation factor, eIF2B. eIF2B is the guanine nucleotide exchange factor for eIF2, which supports eIF2.GTP production and represents a key regulated step in translation initiation. In this study, a variety of cell biological and genetic assays suggest that in C. albicans, fusel alcohols and ethanol inhibit protein synthesis by targeting the translation initiation factor, eIF2B. These alcohols also lead to a morphology switch from yeast to hyphal or pseudohyphal growth.
The capacity to switch morphology is linked to pathogenesis for C. albicans. Farnesol, a quorum sensing alcohol secreted by C. albicans in its growth medium also inhibits translation initiation but does not appear to impact upon eIF2B activity. Rather, biochemical and mass spectrometric analysis suggest farnesol affects some aspect of mRNA interaction with the small ribosomal subunit during translation initiation. Intriguingly, farnesol has the opposite effect on morphological switching to hyphal-like forms, in that it prevents such transitions. These results highlight the possibility that regulation at different stages of the translation process can result in highly diverged phenotypic outputs, presumably as a consequence of differential regulation of the translation of specific mRNAs. Overall the integration of these responses may have implications in pathogenicity.

P1833
Board Number: B852
Unraveling calcium programmed hunting biodynamics of the swan-necked predatory ciliate Lacrymaria.
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Cells are remarkable machines capable of using molecular activities to perform extraordinary mechanical feats. To achieve this, signaling mechanisms must coordinate multiple active systems within the cell and regulate their actuation, but our understanding of how such complex behaviors are programmed is poorly understood for all but a handful of systems. Here, we investigate how calcium signaling controls the mechanical systems biology and biodynamics of a hunting behavior in a swan-necked ciliate Lacrymaria olor. Lacrymaria is a single-celled predator that attaches its large body to a substrate and hunts for food through repeated cycles of extension and contraction of its highly agile head and neck that can span more than seven body lengths. By tracking and quantifying the geometric features of the head and neck over time, we observe complex oscillatory dynamics indicative of stretching, relaxation, and contraction of an elastic torsional system. Analysis of the fluidic flow-fields generated around the organism implies that stretching events arise from a strong pulling force generated by the head. In contrast, the magnitude of the restoring force and contraction events appear to be under calcium control, as dramatic changes to head and neck dynamics were observed upon perturbation of calcium signaling. Live cell imaging of calcium signaling dynamics suggests mechanisms by which the organism uses both global and localized calcium signals to sculpt the physical and material properties of this elastic system. Our ongoing work aims to identify molecular mechanisms through which calcium acts and the cues that regulate its global and local release. Together, this work clarifies the important role that signaling can play in organizing the activities in complex mechanical cellular machines to perform specific complex behaviors.
P1834
Board Number: B853
Role of PLC-IP$_3$ Pathway in Intraerythrocytic Development of *Plasmodium falciparum*.
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The apicomplexan parasite *Plasmodium falciparum* is responsible for the most severe form of malaria, a disease that affects mainly affects underdeveloped and developing countries. Previous studies from our laboratory showed that Ca$^{2+}$ mobilization within the parasite is activated by a signaling pathway initiated by melatonin and this culminates in the synchronization of parasites during the intraerythrocytic phase of the life cycle. Although no (inositol 1,4,5-trisphosphate) (IP$_3$) receptor has been identified in *P. falciparum*, melatonin signal transduction pathways in the parasite seems to be in part due to phospholipase C (PLC) activation and IP$_3$ production. 2-aminoethyl diphenylborinate (2-APB) is a well-known IP$_{3}$R inhibitor in mammalian cells. In *P. falciparum* we demonstrated that 2-APB is able to inhibit melatonin-induced Ca$^{2+}$ rise. Thus, our goal in this work was to evaluate the effect of two 2-APB analogues, DPB162-AE and DPB163-AE, in melatonin-induced Ca$^{2+}$ release and parasite development. Using the fluorescent Ca$^{2+}$ indicator Fluo-4/AM, we analyzed Ca$^{2+}$ fluctuations in *P. falciparum* 3D7 strain in the presence of 2-APB, DPB162-AE and DPB163-AE. Additionally, we investigated the effect of these compounds in intraerythrocytic development by flow cytometry. Our results demonstrate that both 2-APB analogues block melatonin-generated Ca$^{2+}$ rise in the parasite and impair SOCE. The same concentration compounds were able to reduce parasitemia after 24 and 48 hours of treatment. Interestingly, melatonin did not stimulate SOCE in *P. falciparum*, but probably may be linked to depletion of Ca$^{2+}$ from intracellular stores as previously demonstrated. Finally, efforts to find new strategies to combat *Plasmodium* development are fundamental due to arising of resistant strains to the current antimalarial drugs. Therefore, melatonin signaling pathway seems to be a potential target for the development of antimalarial drugs.

P1835
Board Number: B854
Discovery of a lineage-specific mitosomal membrane protein possibly involved in vacuole-mitosome contact in *Entamoeba histolytica*.
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Entamoeba histolytica, an anaerobic intestinal parasite causing dysentery and extra-intestinal abscesses in humans, possesses a highly reduced and divergent mitochondrion-related organelle (MRO) called mitosome. The Entamoeba mitosome has been found to have a compartmentalized sulfate activation pathway, which was recently implicated to have a role in amoebic stage conversion. Mitochondria and MROs are bound by a double-membrane barrier, lined with various proteins that allow for protein and metabolite transport, lipid transfer, dynamics and organelle-organelle interaction. However, the outer
and inner membranes of the E. histolytica mitosome possess only a few homologs involved in protein or substrate transport, suggesting that the mitosomal membranes are equipped with components that may have been modified by unique and possibly lineage-specific elements. Using a specialized prediction pipeline, we collaborated to screen and discover lineage-specific mitosomal membrane proteins. We confirmed three novel Entamoeba-specific transmembrane mitosomal proteins (ETMPs). ETMP1 was predicted to have one transmembrane domain and two coiled-coil regions by in silico analyses. It was demonstrated to be integrated to the mitosomal membranes based on carbonate fractionation and immunoelectron microscopy analysis. ETMP1 forms a 180 kDa complex and immunoprecipitation analysis detected a candidate interacting partner that is localized to vacuolar and vesicular membranes. We expressed this ETMP1-binding partner and the subsequent immunofluorescence and immunoelectron microscopy data demonstrated an unprecedented vacuole-mitosome contact.

P1836
Board Number: B855
A prospective lipid import mechanism of Plasmodium falciparum in erythrocytes stages.
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Plasmodium falciparum (P. falciparum) infection is known to cause decreases in low density-lipoprotein (LDL), high density lipoprotein (HDL) and cholesterol level in blood. P. falciparum does not have de novo cholesterol synthesis pathway and it must import cholesterol from surrounding environment. However, it is not completely known how cholesterol molecules enter infected erythrocytes by crossing multiple membranes to reach parasite. To answer these questions, we used different drugs, cholesterol synthesis inhibition (statin), transport inhibitor (ezetimibe), and activating ligand of peroxisome proliferator-activated receptorsα; PPARα(fibrate), and investigated the effects of those reagents on P. falciparum growth in erythrocyte stages. We observed growth rate of P. falciparum was inhibited at the presence of ezetimibe, and other regents did not influence the parasites growth. Reported data of the reductions in LDL and HDL in malaria patients suggested us a possibility that lipoproteins were taken up as a carrier for cholesterol transport. To answer this question, we investigated an effect of hepatic cells on P. falciparum growth by setting up a co-culture system with/without drugs. Ezetimibe was not effective for P. falciparum growth in the co-culture system, but statin strongly facilitated a better parasite growth in the presence of hepatic cells. Statin is known to enhance the Niemen-Pick C (NPC1L1) expression in small intestine as mRNA level. Our data suggest a possibility that P. falciparum imported cholesterol via NPC1L1 protein in erythrocytes, or parasite protein homologous to NPC1L1.

Immune System

P1837
Board Number: B857
MicroRNA dynamics in oligodendrocytes in the context of autoimmune demyelination.
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MicroRNAs (miRNAs) belong to a class of evolutionary conserved, non-coding small RNAs which negatively affect the expression of target genes by promoting either RNA degradation or translational inhibition. In recent years, converging studies have identified miRNAs as key regulators of
oligodendrocyte (OL) functions. OLs are the cells responsible for the formation and maintenance of myelin in the central nervous system (CNS) and represent a principal target of the autoimmune injury in multiple sclerosis (MS). Thus, clarifying the possible contribution of miRNA dysregulation to OL degeneration in MS could represent a crucial strategy for the identification of still unveiled pathogenic processes as well as novel therapeutic targets. However, due to the extreme complexity of the cyto-architecture and cell heterogeneity of the CNS, the study of miRNA dynamics in specific cellular populations of the brain has been challenging. MiRAP is a novel cell-specific miRNA affinity-purification technique which relies on genetically tagging Argonaut 2 (Ago2), an enzyme involved in the physiological processing of miRNAs. In this work, we have exploited miRAP potentiality to isolate and characterize the miRNA repertoire from OLs upon induction of experimental autoimmune encephalomyelitis (EAE), a murine disease model that recapitulates several features of MS. To this extent, the first conditional mouse model expressing an epitope-tagged version of Ago2 (GFP-Myc-Ago2) was generated by means of the Cre-Lox binary system, using an Olig1-Cre driver line. Detailed immunoprecipitation experiments and confocal microscopy imaging confirmed that transgene expression in the CNS is restricted to OLs and persists in demyelinated EAE lesions. Subsequently, Taqman assays measuring the levels of miR-219, a miRNA particularly enriched in mature OLs, confirmed miRAP efficacy in isolating OL-specific miRNAs. Finally, after this thorough biochemical characterization, Next-Generation Sequencing (NGS) was employed to characterize the full repertoire of OL-specific miRNAs at different stages of the disease. Using this approach, miR-3102-5p was found significantly down-regulated at pre-symptomatic stages of EAE (5 and 10 dpi) while miR-5107-3p was up-regulated after disease symptoms manifested (15 and 20 dpi). If confirmed, these two miRNAs hold the potential to become promising candidates for future in vivo functional studies. In summary, the overarching goal of this project consists in providing the most comprehensive portray of global miRNA dynamics in the healthy and diseased CNS in a relevant model of MS. In the long term, this knowledge will serve to explore neuroprotective gene therapy approaches based on the delivery to the CNS of miRNA mimics and inhibitors, to prevent OL loss.

**P1838**

**Board Number: B858**

TRPV2 participates in T-independent B cell responses through the modulation of membrane potential.

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The ligation of B cell receptor (BCR) triggers B cell activation through the network of complexes in the immunological synapse (IS) between cell-cell interactions. As a big family of nonselective ion channel, transient receptor potential (TRP) channels show diverse and versatile biological roles, including nociception, mechanosensation, membrane potential and thermosensation. Transient receptor potential vanilloid type 2 (TRPV2) is highly expressed in B lymphocyte. However, whether TRPV2 participates in the B cell activation and modulates the humoral response is barely addressed. Here, our recent study showed that TRPV2 is of vital importance in the initiation of B cell signaling. B cells isolated from the TRPV2 deficient mice exhibited dramatically impaired capability of B cell IS formation, signaling transduction, cytoskeleton mobilization and cell proliferation in response to cognate antigens. We evaluated membrane potential through the potentiometric fluorescent dye, FLIPR Blue. After the activation, TRPV2 deficient B cell showed a much smaller depolarization peak. The capsular polysaccharides can elicit antibody responses through BCR crosslinking in a T cell independent (TI) manner. These antigens with a large polysaccharide backbone are clinically important because they exist...
in the capsules *Streptococcus pneumonia* which is a leading worldwide cause of death for children. We immunized the mice with the *Streptococcus pneumoniae* PS1 and PS3. The TRPV2 deficient mice produced diminished amounts of PS1 and PS3 specific IgM. These findings confirmed the requirement for TRPV2 in response to the clinical human pathogen. TRPV2 modulates the B cell activation through the depolarization of the plasma membrane and the orchestration of signaling transduction.

**P1839**  
**Board Number: B859**  
**A novel IgG1 variant associates with autoimmune disease and modulates autoreactive B cell fate decision.**  
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The maintenance of autoreactive B cells in an anergic state is crucial for preventing autoimmunity. Autoreactivity is generated from somatic hyper-mutation in germinal center (GC) reaction and is substantially enriched in IgG+ memory B cells, but efficiently eliminated in the plasma cell compartment in healthy individuals. However, this immune tolerance checkpoint fails in autoimmune patients. The mechanisms that regulate the differentiation and selection of autoreactive B cells to plasma cells are still elusive. Here we reveal how illegitimate IgG-BCR signaling regulates the cell fate decision of autoreactive B cells in autoimmune disease. We found that patients harboring a novel variant of human membrane bound IgG1 (hlgG1-G396R) have more severe disease-related clinical manifestations. Using inducible autoimmune mouse models, we validate the pathogenic role of this variant in vivo in newly constructed G390R knock-in mice. Due to biased differentiation from autoreactive IgG1+ GC B cells and memory B cells, the G390R variant mice generate more plasma cells with dominant autoreactive repertoires, leading to a burst of broad spectrum autoantibodies. Mechanistically, the G390R variant lowers the threshold for BCR clustering, and drastically enhances the recruitment of Grb2 and Btk signaling molecules into the immunological synapses (IS) by potentiating phosphorylation of its immunoglobulin tail via the Lyn kinase. This triggers significantly longer dwell times of Grb2 in the IS, leading to hyper-activation of the phospho-ITT-Grb2-Btk signaling module upon antigen stimulation. Thus, our studies identify a novel risk locus for SLE and shed new light on a novel regulatory mechanism of IgG-BCR signaling and its roles in autoimmune disease pathogenesis, which may provide a target for therapies of autoimmune diseases.

**P1840**  
**Board Number: B860**  
**Hepatokine induction mediates anti-inflammatory actions of Colchicine.**  
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Inflammation recognizes and eliminates danger causing by invading pathogens or endogenous signals. However, uncontrolled inflammation also damages tissues. Colchicine, an anti-inflammatory medication that binds to tubulin and blocks microtubule polymerization, is frequently prescribed for gout, Familial Mediterranean Fever, pericarditis, and is being clinically tested for a wide spectrum of diseases. Understanding how an anti-microtubule drug blocks inflammation would help us design safer future drugs. Colchicine inhibits extravasation of neutrophils to inflamed tissues in man. In culture, it directly
inhibits neutrophil chemotaxis and maturation of the pro-inflammatory cytokine, IL-1β, but the required concentrations are 30-300x higher than plasma therapeutic concentrations, which calls into question the textbook model of direct action on leukocytes. Colchicine shows rapid clearance from blood (t1/2 ~ 0.3 hour), followed by selective accumulation in the liver, and slow elimination via bile (t1/2 ~ 1 day). This pharmacology leads us to propose an alternative “Liver Selective Action” model. By measuring biomarkers of microtubule perturbation and using hepatocyte-specific siRNAs, we identified hepatocytes as the main target of colchicine at low doses. Colchicine induced secretion of a divergent member of the TGF-β family, GDF15, from hepatocytes in mice. Conditioned serum from colchicine-dosed mice contained GDF15, blocked leukocyte adhesion, and inhibited IL-1β maturation. By comparing wild-type vs GDF15/- mice, we found that GDF15 was required for colchicine treatment of crystal-induced peritonitis (a gout model). Our results identify GDF15 as a colchicine-induced hepatokine and show that colchicine treats inflammation by selectively accumulating in hepatocytes, triggering GDF15 expression, which globally inhibits neutrophil extravasation.

P1841
Board Number: B861
Haploid genetic screens identify novel regulators of cell interaction and degranulation during cytotoxic lymphocyte-mediated cell death.
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Cytotoxic Lymphocytes (CLs), which include NK cells and CD8+ T-cells, detect and eliminate transformed and pathogen-infected cells. While the physiological roles of CL-mediated apoptosis are well established, many regulators of these apoptotic pathways in target cells remain poorly understood. Using haploid genetic screens, we identified novel target cell genes that regulate cell death after treatment with TALL-104 cells, a model CL cell line. These novel genes include the glycophasphatidylinositol (GPI)-anchor synthesis pathway, chromatin structure regulators, and actin cytoskeleton components. In order to narrow down which step in CL-mediated apoptosis is regulated by each of these genes, we studied two strong hits in more detail. PIGP, which encodes an enzyme required for GPI-anchor synthesis, regulates target cell interaction with CLs. PBRM1, a member of the PBAF SWI/SNF chromatin remodeling complex, regulates the signaling between target cells and CLs required for CL degranulation and release of the toxic proteins perforin and granzymes.

P1842
Board Number: B862
Characterization of human immune cell survival and functionality in Danio rerio.
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The inflammatory response is modulated both by tissue resident macrophages and recruited monocytes from peripheral blood. It is increasingly evident that chronic inflammation plays a critical role in human diseases such as atherosclerosis, obesity, diabetes, cancer, and neurodegenerative disorders. Here we sought a model system to interrogate human immune responses in vivo with single cell resolution in multiple tissues. Elutriated monocytes from de-identified healthy human donors were introduced directly into the circulation and into the brain of 2 day old zebrafish (Danio rerio). We determined that both monocytes and macrophages (differentiated in culture) survive in innate immune competent zebrafish for up to two weeks post-injection in the non-embryonic environment despite the lower
physiological temperature of the zebrafish (28.5°C). On average, 50% of the human cells survive after 3 days in vivo. Similar results were obtained in vitro, where flow cytometry analysis revealed that human monocytes cultured at the physiological temperature of the zebrafish survive, differentiate, and show M1 and M2 polarization phenotypes in response to cytokines comparably to cohorts cultured at human physiological temperature. To assay the functionality of human immune cells in the zebrafish microenvironment, real time, high resolution imaging allowed us to quantify cell migration for each cell type. The human cells show motility throughout multiple tissues comparable to host macrophages but slower than host neutrophils. Gene expression comparisons of in vivo educated human macrophages to in vitro cytokine treated human macrophages revealed gene expression profiles associated with the M1 and M2 phenotypes. In summary, these results characterized the function of human immune cells in the in vivo environment and physiological temperature of Danio rerio. These results suggest that we will be able to define human tumor-human immune cell interactions during the establishment of a metastatic lesion in Danio rerio at single-cell resolution and in real time.

P1843
Board Number: B863
Triclosan alters the secretion of Tumor Necrosis Factor alpha from human immune cells.
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Triclosan (TCS) is an environmental contaminant added as an antibacterial agent to many products including mouthwashes, soaps, toothpastes, deodorants, and beauty products. TCS can be absorbed through skin and mouth and it has been found in human blood plasma, urine, amniotic fluid of pregnant women, and breast milk. Tumor Necrosis Factor Alpha (TNFα) is an essential pro-inflammatory cytokine that is produced by monocytes, macrophages, lymphocytes and neutrophils. TNF acts as a systemic inflammatory mediator in response to sepsis and infectious disease. Increased levels of TNFα can cause the stimulation of macrophages and helper T cells to produce additional inflammatory cytokines contributing to inflammation. While a normal inflammatory response is critical to health, high levels of inflammatory proteins such as TNFα can lead to chronic inflammation which contributes to a number of disease states including cancer. Alternatively, if immune cells are unable to secrete adequate amounts of TNFα this would leave the organism susceptible to infections. Previously, we have shown man-made environmental contaminants that gain access to the human system are able to alter the secretion of TNFα from human immune cells and we hypothesize that TCS also has the capacity to alter TNFα secretion. In the current study, peripheral blood mononuclear cells (PBMCs) were treated with TCS at concentration of 0.05-5µM for 24 h, 48h, and 6 days. TNFα level was measured by using enzyme-linked immunosorbent assay (ELISA). The result showed that 24 h exposure to TCS caused a significant increase in TNFα secretion at 5µM in cells from 4 separate donors. This increase maintained at 48 h and 6 days of exposure. Other concentrations of TCS, including the lowest of 0.05 µM, also caused significant increases in TNFα, but the specific concentrations at which the increases were seen varied among donors. These data show that exposure of (PBMCs) to TCS alters the secretion of TNFα. TCS has the capacity to increase this important master regulator of inflammation, which would have the potential to lead to development or exacerbation of several disease states. Supported by NIH grant 2U54CA163066
P1844
Board Number: B864
Exposure of human immune cells to triclosan alters the secretion of Interferon gamma.
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Triclosan (TCS) is a synthetic chemical with antibacterial function that inhibits the growth of microorganisms. TCS is used as an antifungal and antibacterial agent in many products such as: toothpaste, soaps, detergents, toys, surgical cleaning treatments, cosmetics, kitchenware, clothes, and office and school products. It is found in human blood and tissue samples. Interferon gamma (IFNg) is a cytokine that is specialized for innate and adaptive immunity against viral infections. IFNg is important for immunity against intracellular pathogens and for tumor control. As a pro-inflammatory cytokine, inappropriately elevated levels of IFNg can cause chronic inflammation, which has been shown to enhance the development and progression of certain cancers as well as other diseases. Accurate regulation of IFNg levels is important to avoid the loss of immune capability or the occurrence of chronic inflammation. The aim of this study is to investigate whether TCS alters the secretion of IFNg from human immune cells. Human peripheral blood mononuclear cells (PBMCs) were treated with 0 -5 μM TCS for 24 h, 48 h, and 6 days. IFNg levels were measured by enzyme linked immunosorbent assay (ELISA). After a 24 h exposure to TCS there were significant increases in IFNg seen at several concentrations in all donors tested (total of 4 distinct donors). The specific concentration at which increases occurred varied among cells from different donors. Increases in IFNg secretion were also seen after 48 h and 6 day exposure to TCS at certain concentrations. Thus, it appears that TCS is capable of disrupting secretion of this important immune system regulating cytokine which could have the potential to increase the potential for chronic inflammation. Supported by NIH grant 2U54CA163066

P1845
Board Number: B865
Sodium bicarbonate is the factor in culture media that potentially regulates nitric oxide production in a mouse macrophage-like cell line, J774.1 cells, treated with LPS and IFNg.
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Macrophages are known to play pivotal roles in host-defense including inflammation, the both innate and acquired immune systems, and so on. Many of these macrophage functions are exerted through macrophage activation induced by a series of PAMPs (pathogen-associated molecular patterns) like lipopolysaccharide (LPS) as well as by cytokines like interferon γ (IFNg). We showed that the activated macrophage phenotypes induced by LPS and IFNg, such as production of nitric oxide (NO), pro-inflammatory cytokines like TNFα and IL-1β, as well as induction of superoxide-generating activity in J774.1/JA-4 cells were influenced by the culture media (Dulbecco's Modified Eagle's medium (DMEM) and Ham’s F-12 Nutrient Mixture). Among these phenotypes, inducible NO synthase (iNOS) expression was shown to be of great difference between the cells cultured in DMEM and F-12. After extensive studies on the components in these media, sodium bicarbonate (NaHCO₃) concentration was critical to exert the differences in the induction of the activated macrophage phenotypes. When the concentration of NaHCO₃ in F-12 (14 mM) was adjusted to 44 mM, which is the concentration in DMEM, iNOS expression and NO production in the activated macrophages became close to that in DMEM. The same was true vice versa. Besides, change of pH by its concentrations did not result in remarkable differences
in iNOS expression or NO production. These results suggest that NaHCO₃ could be a regulatory factor of iNOS expression and NO production in macrophages, and that the its concentration has a crucial role in macrophage activation.

**P1846**  
**Board Number: B866**  
**Mild electrical stimulation with heat shock ameliorates inflammation in imiquimod-induced psoriasis model.**  
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Psoriasis is a chronic refractory disease with skin inflammation. Antibody agents or steroids have strong anti-inflammatory effects, but long-term use of these drugs is unsuitable due to their severe side effects and inter-individual differences in therapeutic responses. Therefore, development of less invasive but highly effective treatments for psoriasis is anticipated. Previously, we have comprehensively studied the effectiveness of optimized combination treatment of mild electrical stimulation (MES) and heat shock (HS). MES+HS treatment had therapeutic effects on the inflammatory symptom in mouse models of diabetes and chronic kidney disease. Furthermore, MES+HS had beneficial effect and were tolerable in the patients with type 2 diabetes or metabolic syndrome in clinical trials. To determine the effect of MES+HS on psoriasis, we used imiquimod-induced psoriasis mouse model. Imiquimod was used as an agonist of Toll-like receptor 7, which accelerates the development of psoriasis. Daily application of imiquimod on mouse ear induced skin hyperplasia by increasing cell proliferation. Moreover, the expression of inflammatory markers such as cytokines and antimicrobial proteins was increased in imiquimod-treated skin. Importantly, MES+HS (10 min, daily) significantly suppressed skin hyperplasia as well as mRNA expression of keratinocyte proliferation marker, keratin 6A. Furthermore, MES+HS also inhibited expression of both inflammatory cytokines, such as IL-17A and IL-22, and antimicrobial proteins in keratinocytes. Together, our study shows that MES+HS is an effective treatment to suppress the inflammation in imiquimod-induced psoriasis model.

**P1847**  
**Board Number: B867**  
**IMPDH filament formation in human T cell activation.**  
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**Objectives:** Inosine monophosphate dehydrogenase (IMPDH) catalyzes the conversion of IMP to xanthosine monophosphate (XMP), the rate-limiting step in de novo guanosine monophosphate (GMP) synthesis. In cultured cells, IMPDH polymerizes into rod- and ring-shaped structures (filaments) when IMP or GMP synthesis is inhibited by substrate depletion, like glutamine or serine deprivation, or by various drugs, including ribavirin, mycophenolic acid, and methotrexate. This suggests that IMPDH filaments assemble during stress related to nucleotide metabolism, although their function is still unknown. Assembly of IMPDH filaments in untreated, undifferentiated mouse embryonic stem cells hints they might function in highly proliferative cell types. Here, we investigate IMPDH polymerization in primary human T cells, which rely heavily on de novo pyrimidine synthesis to proliferate rapidly in response to antigenic challenge.
**Methods:** Peripheral blood mononuclear cells were activated ex vivo with classical T cell mitogens anti-CD3/anti-CD28, phytohemagglutinin (PHA), or concanavalin A (ConA), or with lipopolysaccharides (LPS) as a non-T cell activator control. Spleens were harvested from healthy C57BL/6 mice to measure basal IMPDH filament formation in vivo. IMPDH filaments were detected by immunofluorescence using affinity purified anti-IMPDH antibody.

**Results:** In two independent experiments, both rod- and ring-shaped IMPDH filaments were observed in ~30% of T cells after 24-hour treatment with anti-CD3/anti-CD28, PHA, or ConA, and in ~50% of cells after 72 hours. IMPDH filaments did not form in T cells left untreated or treated with LPS. Percentage of cells with filaments was consistent among anti-CD3/anti-CD28, PHA, and ConA treatments. Rods were more commonly observed than rings under all conditions (~90% rods vs. ~10% rings), and were similar in size to structures previously reported in cancer cell lines. IMPDH filaments formed in T cells did not disassemble after addition of fresh medium. Filament formation detected by anti-IMPDH antibody was correlated with positive staining of T cell marker anti-CD3. IMPDH filaments were also detected in a low percentage of cells in both the white pulp and red pulp of healthy mouse spleen.

**Conclusions:** Our data suggest that IMPDH filament formation is functionally relevant in T cell activation, and that filaments might be useful as morphological markers for T cell activation. Considering the lack of conclusive data on the function of these structures, establishing new ex vivo and in vivo models will be critical to uncovering how IMPDH polymerization affects cell function. We are currently investigating the role of IMPDH filaments in disease conditions related to T cell function.

**P1848**

**Board Number: B868**

HIV-1 gp120_{IIIB}/HIV-1 gp120_{RFL} -mediated human α7-nAChR’s up-regulation in macrophages and its implications in the cholinergic anti-inflammatory response (CAR).

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Despite the successful reduction of the development of HIV/AIDS in patients receiving therapy, this population is at a higher risk to develop non-AIDS related diseases, which are often associated with inflammatory processes. It has been shown that acetylcholine (ACh), the main parasympathetic neurotransmitter, effectively deactivates peripheral macrophages inhibiting the release of pro-inflammatory mediators, including the cytokines TNF-α, IL-6, and IL-β. The ACh-dependent macrophage deactivation is mediated by the α7-nAChR which is expressed in peripheral macrophages and has been described as essential for the so-called cholinergic anti-inflammatory response (CAR). It is known that HIV-1 gp120 proteins can cause increased cytokines released that generate sustained immune activation in macrophages, which are the principal target for HIV-1 derived R5-tropism strains. We recently demonstrated that, in human macrophages, gp120_{IIIB} (X4-tropic strains) protein induces the up-regulation of the α7 nicotinic acetylcholine receptor (α7-nAChR), a key regulator of systemic inflammation that inhibits the production of pro-inflammatory cytokines. In that study, results indicate that gp120_{IIIB} disrupts the CAR in macrophages in vitro because the activation of α7-nAChR does not inhibit the production of pro-inflammatory cytokines (interleukins and chemokines). Here, we show that M-tropic gp120_{RFL}, induces higher level of α7-nAChRs in human derived macrophages. Our results demonstrated that HIV gp120_{RFL}/CD4-CCR5 receptor complex promotes α7-nAChRs up-regulation via a mechanism that is associated with the activation of p38-MAPK signaling that has been shown to up-
regulates an early gene (Egr1), a transcription factor known to drive the expression of α7-nAChRs. These findings suggest that HIV-1 glycoproteins can alter the expression of α7-nAChRs, resulting in changes in macrophages phenotype that influence the cholinergic anti-inflammatory response. Of biological significance, the present results suggest that HIV gp120\(_{JRFL}\) interfering with the CAR could contribute to a crucial, unresolved problem of HIV infection: chronic inflammation.

**P1849**  
**Board Number: B869**  
**HLA-DR in PMNs in autologous culture of total human leukocytes with positive serology for Chagas disease stimulated with LPS.**  
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T cells only recognize antigens that appear on cell surfaces in the context of Class I and Class II Major Histocompatibility Complex (MHC) molecules. The two classes of MHC molecules are differentially expressed in cells. Activated human T lymphocytes express Class II molecules in addition professional antigen presenting cells (APCs). LPS stimulation can induce the expression of HLA-DR molecules (MHC Class II) and our aim was to observe their expression in polymorphonuclear neutrophils (PMNs) in cellular interactions in culture in blood samples for positive Chagas serology. From healthy human blood samples (n = 10) and samples with positive serology for Chagas (n = 6), anticoagulated with Heparin (in anonymity, with serology data) with ethical consent according to procedures approved by ethical committee of National Hospital Clinicas R169/13, autologous leukocyte cultures were performed in TC199 medium (SIGMA , St. Louis, MO) and in vitro stimulation with LPS (Sigma-Aldrich) 25 ng / ml at 37 ° C in a gaseous stove, 30 minutes and other controls without stimulation. Samples at 30 min and 24 hours were performed with HLA-DR Immunofluorescence (FITC; Santa Cruz Biotechnology) technique at 4 ° C overnight. Nuclear staining with DAPI (4,6'-diamidino-2-phenylindole) (Sigma, St. Louis, MO). Some samples were processed for ultrastructural study of cell interactions in culture with MET: Zeiss LEO-906E. Results: LPS stimulation induced expression of HLA-DR (MHC Class II) molecules in PMNs and samples for positive Chagas serology showed a higher number of HLA-DR + lymphocytes (p <0.001, t-test for paired samples). Interactions between PMNs and lymphocytes were observed and the latter showed abundant cytoplasm, and polarization of mitochondria. Conclusions: The ultrastructural aspects reveal signs of cellular activation in chagasic samples and interactions between PMNs and lymphocytes. Expression of HLA-DR in PMNs could involve antigen presentation via Class II

**P1850**  
**Board Number: B870**  
**Fas (CD95) signaling pathway is involved along with BAG-1M in the regulation of Hsp70-mediated chaperoning of aPKC under pro-inflammatory conditions.**  
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Atypical PKC (aPKC) is a highly conserved key regulator of asymmetry in various tissues. A conditional (epithelium-specific) knockout mouse recently revealed that aPKC also has an essential anti-
inflammatory function as NF-κB inhibitor in intestinal epithelia. In addition, aPKC controls epithelial (anti-inflammatory) IL-10 secretion. Therefore the profound loss of aPKC observed in intestinal epithelium in a mouse model of DSS-induced colitis and in biopsy samples obtained from the patients diagnosed with inflammatory bowel disease (IBD) is likely an important pathophysiological mechanism of inflammation in organs with an epithelial parenchyma. We have established that in intestinal epithelial cells aPKC is post-translationally regulated through Hsp70-mediated refolding specifically localized to keratin intermediate filaments. Activation of inflammatory signaling pathways such as TLR4 activation, results in inhibition of Hsp70 chaperoning activity leading to aPKC ubiquitination and degradation. Two cofactors which can act as inhibitors of Hsp70 chaperoning activity have been described: FAF1 (FAS-Associated Factor 1) and BAG-1, both of which bind to ATPase domain of Hsp70. We have investigated the involvement of BAG-1 previously and were able to demonstrate that under pro-inflammatory conditions there is an upregulation of medium isoform of BAG-1 (BAG-1M). In the present study we aimed to elucidate the possible role of FAF1 and the Fas (CD95) signaling pathway. Fas (CD95) is a member of the tumor necrosis factor receptor family expressed on the basolateral membrane of intestinal epithelial cells. Fas and the cognate ligand FasL have been shown to be upregulated by inflammatory signals in intestinal epithelium. We found that administration of blocking anti-FasL antibody rescued aPKC from degradation in TNF-alpha treated Caco-2 cells. In addition, an in vitro rephosphorylation assay for Hsp70 refolding of aPKC demonstrated that anti-FasL antibody incubation restored Hsp70-dependent pT555 aPKC rephosphorylation. Likewise inhibition of Fas pathway rescued chaperoning activity of Hsp70 in luciferase refolding assays. When injected intraperitoneally in mice, anti-FasL antibody delayed the progression of disease activity index (DAI) in DSS-induced colitis. Importantly, anti-FasL antibody significantly rescued 50% of the steady-state levels of pT555 aPKC signal from the effects of DSS-induced inflammation. Altogether, these results indicate that Fas-mediated signaling similar to Bag-1M affects the chaperoning function of Hsp70 and mediates the effects of pro-inflammatory signals on the stability of aPKC.

P1851
Board Number: B871
Exploring the Role of Macrophages in Pregnancy Using a Rat Model System.
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Macrophages are professional phagocytes that survey the body for pathogens and secrete biomolecules that are relevant to cellular processes involving inflammation, development, and immunogenic tolerance. Past studies to elucidate the role of macrophages during pregnancy have utilized mouse models or human clinical samples. In rats, it is still not clear whether macrophages might have a role to play in processes necessary for gestation such as arterial remodeling, clearance of apoptotic cells, or uterine tissue repair during postpartum involution. Our lab is interested in understanding the putative roles of macrophages during pregnancy in the rat uterus. Using immunohistochemistry, we identified the presence of macrophages around the uterine lumen and within the metrial gland during mid-pregnancy and postpartum (unpublished). This led us to hypothesize that macrophages might be involved in the clearance of apoptotic cells as part of their greater role in remodeling. Using quantitative PCR (qPCR), we measured the expression levels of the genes T-cell immunoglobulin and mucin domain containing-3 (TIM-3) and Mer Tyrosine Kinase (MerTK) that are expressed by macrophages and are
involved in the binding and engulfment of apoptotic cells. While continuing to assay for TIM-3 and MerTK, we also measured the expression levels of anti-inflammatory molecules such as Transforming growth factor (TGF-1) and Interleukin-10 (IL10) in order to characterize the immunomodulatory phenotype of macrophages during mid-pregnancy and postpartum. Preliminary results suggested varying spatiotemporal patterns of expression for these genes. Of note, MerTK and TGF-1 appeared to display similar expression patterns across various time points in pregnancy such as day 12, 14, and 16 and postpartum day 3. Further, the expression of these genes in the rat metrial gland tissue did not always correlate linearly with the expression of CD68, a pan-macrophage marker in rats. This suggests that immune cell populations involved in uterine remodeling during pregnancy may be heterogeneous. Proper maternal immune function is essential to a successful pregnancy. Misregulation of key immune processes can lead to pregnancy-related disorders such as preterm labor and preeclampsia. The relevance of the macrophage to both the innate and adaptive arms of the immune system make it an important player during gestation and beyond. Our research suggests an important role for these immune cells in pregnancy-associated tissue remodeling as well. Future studies focusing on the rat uterine environment will increase our understanding of how macrophages affect pregnancy in rats and whether this may have relevant implications to humans.

This work is supported by Mount Holyoke College & Microscopy Society of America.

P1852
Board Number: B872
Expansion of His48+CD11b/c+ myeloid cells in rats after vanadium and chromium salts administration.
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Exposure to various environmental pollutants is known to induce inflammatory changes, characterized by a release of cytokines and other soluble mediators. Moreover, toxic effect of various xenobiotics, including vanadium and chromium, may cause dysregulation of the immune response leading to chronic inflammation, which induces autoimmune diseases, diabetes, and malignant transformation of cells. The purpose of this research was to study the effect of intoxication with salts of heavy metals on immunosuppressive cells - MDSCs, which is shown to accelerate chronic diseases. In our study, sexually mature rats were administered ammonium vanadate (AV) and potassium dichromate (PD) at a dose of 5 mg/kg of BW for two weeks (AV/PD), after that lymphatic organs were studied on day 1, 7, and 14 after the end of AV and PD injections. Administration of AV and PD resulted in the decrease of spleen and thymus weight, also the cellularity of the thymus during the observation periods in the AV/PD group. Phenotypical analysis demonstrated that on day 1 after the beginning of the experiment freshly obtained splenocytes of AV/PD rats contained increased percentage of CD3+CD4+ T-lymphocytes comparing to control animals. Interestingly, during the next period of the experiment, a decreasing trend of CD3+CD4+ T-lymphocytes percent and significantly lower induced expression of IFNγ by these cells accompanied by increasing trend of His48+CD11b/c+ cells was noticed. Moreover, on day 14 we observed a progressive extension of His48+CD11b/c+ and His48highCD11b/c+ cells and concurrent decrease of CD3+CD4+ T-lymphocytes and their induced expression of IFNγ and IL-4 in the AV/PD group comparing to intact rats. Thus, it is possible that a reduced number of effector T cells observed in rats exposed to vanadium and chromium salts could be a result of abundant accumulation of
His48+CD11b/c+ and His48highCD11b/c+ cells that have phenotype similar to tumor-related total subset MDSC and G-MDSC, respectively, with immunosuppressive properties, in the periphery, and, in turn, it could participate in the maintaining of the immunosuppressive environment that supports persistence of chronic inflammatory conditions.

Recent Publications

P1853
Board Number: B873
NT-07-16 reduces NF-κB signaling in RAW264.7 macrophages.
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The NF-κB pathway is vital for immune system regulation and pro-inflammatory signaling. Many inflammatory disorders and diseases, including cancer, are linked to dysregulation of NF-κB signaling. When macrophages recognize the presence of a pathogen, the signaling pathway is activated resulting in the nuclear translocation of the transcription factor, NF-κB, to turn on pro-inflammatory genes. Here we are investigating the effects of a novel microtubule-depolymerizing compound, NT-07-16, on this process. Since microtubules play a critical role in cell physiology compounds that target the microtubule network have been studied for their potential use as chemotherapeutic agents. However, some of these compounds, such as colchicine, are too toxic to be used in a clinical setting. Polysubstituted pyroles, JG-03-14 (3,5-dibromo-4-(3,4-dimethoxyphenyl)-1H-pyrrole-2-carboxylic acid ethyl ester) and its refined analog, NT-07-16, which possesses an additional methoxy group at the 2-position of the phenyl ring, also bind microtubules at the “colchicine site”. These compounds have been shown to be less toxic while still inhibiting the proliferation of many cancer cell lines. Previous studies from our laboratory also found that exposure to JG-03-14 decreased pro-inflammatory signaling in activated RAW264.7 mouse macrophages. Treatment with JG-03-14 resulted in the depolymerization of microtubules and decreased the production of nitric oxide and the pro-inflammatory cytokine, tumor necrosis factor-α in the macrophages. Our present work demonstrates that exposure to NT-07-16 also decreases the production of pro-inflammatory cytokines in lipopolysaccharide (LPS)-activated RAW264.7 macrophages, and it appears that the reduction in pro-inflammatory mediators may be due, at least in part, to a decrease in the translocation of NF-κB into the nucleus. These results suggest that upon activation of RAW264.7 macrophages, NF-κB translocation into the nucleus involves the microtubule network and that disruption of this network by NT-07-16 results in less NF-κB entering the nucleus reducing the inflammatory activity of the macrophages. In addition, since the NF-κB pathway is also known to trigger the production of Bcl-2 proteins which offer protection against apoptosis and increase the survival of cancer cells, we analyzed the effect of NT-07-16 on the production of the Bcl-2 protein in RAW264.7 macrophages. Our data suggest that exposure to NT-07-16 prior to LPS activation of the macrophages reduces the upregulation of Bcl-2 triggered by NF-κB signaling. Therefore, NT-07-16, which has already shown activity as an anti-proliferative agent towards cancer cells, may also be effective as an inhibitor of the NF-κB signaling pathway further enhancing its chemotherapeutic potential.

Sunday-539
**P1854**  
**Board Number: B874**  
**Induction of M2 regulatory macrophages through β-adrenergic receptor signaling in the RAW264.7 macrophage cell line.**  
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Catecholamines alter macrophage function by binding to adrenergic receptors on the cell membrane. Stimulation through β₂-adrenergic receptors is known to down regulate the activity of classically activated M1 macrophages while stimulation through α-adrenergic receptors typically enhances their function. Our studies with the RAW264.7 murine macrophage cell line as a model for macrophage function support these findings. Macrophages were pre-treated with the selective β₂-adrenergic receptor agonist formoterol or the non-selective α-adrenergic receptor agonist clonidine prior to lipopolysaccharide (LPS) activation and analyzed for production of the pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) by ELISA. These studies showed that formoterol decreased and clonidine increased macrophage cytokine production. Furthermore, quantitative PCR (qPCR) demonstrated that LPS activation decreased the expression of β₂-adrenergic receptor mRNA and increased the expression of α₁A - and α₂-adrenergic receptor mRNA in the macrophages. Further analyses suggested that stimulation of the adrenergic receptors by norepinephrine (NE) may alter the phenotype of the macrophages. Classically activated M1 macrophages initiate the pro-inflammatory response to eliminate infection. Macrophages activated with LPS produce, along with pro-inflammatory cytokines, the enzyme inducible nitric oxide synthase (iNOS) which catalyzes the production of nitric oxide (NO) which has anti-microbial properties. The presence of the iNOS enzyme is indicative of the M1 macrophage phenotype. Alternatively activated M2 macrophages down regulate the immune response and promote tissue repair. These M2 macrophages express higher levels of the enzyme arginase-1 (Arg-1) which catalyzes the reaction of arginine to ornithine, a precursor to molecules participating in wound healing. Our qPCR results showed that expression of iNOS mRNA was reduced while expression of Arg-1 mRNA increased in macrophages pretreated with NE prior to LPS activation. Taken together these results suggest that exposure to NE may not only reduce the pro-inflammatory activity of macrophages but also induce development of the alternatively activated M2 phenotype in RAW264.7 macrophages.

**P1855**  
**Board Number: B875**  
**Asaronic acid ameliorates atherosclerotic inflammation by polarizing M1 macrophages to M2-like macrophages.**  
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Atherosclerosis is a chronic inflammatory disease and lipid metabolism disorder, and macrophage accumulation within the vascular wall is a hallmark of atherosclerosis. In atherosclerotic lesions, macrophages respond to various environmental stimuli that can modify their functional phenotypic subsets of pro-inflammatory M1 and anti-inflammatory M2 macrophages. The current study examined whether asaronic acid induced classical M1 to M2 activation of macrophage. Asaronic acid (2,4,5-Trimethoxybenzoic acid) identified as one of purple perilla constituents has been reported to exhibit antioxidant and cytostatic activity, as well as to exert anti-allergic effects. J774A1 murine macrophages
were incubated with lipopolysaccharide (LPS) or interleukin (IL)-4 in the absence and presence of 1-20μM asaronic acid. This study found that non-toxic asaronic acid at ≤20 μM dampened the secretion of the M1 cytokine IL-6 in macrophages exposed to 2 μg/ml LPS for 48 h. Moreover, asaronic acid suppressed macrophage induction of toll-like receptor 4, CD68 and CD36 enhanced by LPS. When J774A1 macrophages were treated for 48 h with 40 ng/ml IL-4, ≤20 μM asaronic acid enhanced the secretion of the M2 cytokine IL-10, evidenced by ELISA. These results demonstrated that asaronic acid inhibited M1 phenotype following exposure to TLR ligands of LPS, whereas this compound induced M2 genes by treating macrophages with IL-4 that increase IL-10 level. Therefore, asaronic acid is a therapeutic agent capable of switching M1 phenotypic macrophages to M2 phenotypes in atherosclerotic plaques.

P1856
Board Number: B876
Cell signaling involved in acute glycolytic response to immune cell activation.
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Immune cell activation is precisely regulated to respond toward a specific stimulus only and turn on a series of cellular processes required. The cellular metabolism is one of the emerging factors required for the proper immune cell activation and differentiation. Several recent reports suggested that there is an immediate glycolytic response upon activation of T cells or macrophages not only as an indicative event but also as an essential component of activation. In the real-time monitoring of glycolysis and OXPHOS using a Seahorse XF analyzer, both CD4+ and CD8+ T cells as well as JurKat immortalized T cell lines showed a rapid increase in the basal glycolytic activity within 6 min by in situ administration of anti-CD4+ and CD8+ antibody-conjugated beads. Similarly, macrophages derived from human peripheral blood monocyte as well as RAW264.7 immortalized murine macrophage cell lines showed a significant escalation in glycolytic activity within an hour by lipopolysaccharide with or without interferon γ. The increase in glycolysis was well correspond to the key cytokine production in both cell types. The further real-time cell metabolism analysis using various inhibitors for cell signaling showed that Akt activation is an essential factor for both T cell and macrophage activation. Interestingly, T cell activation also requires actin cytoskeletal polymerization which is recently suggested to support PI3K-mediated glycolysis increase through aldolase. In contrast, macrophage activation-related glycolytic response was not affected by actin polymerization inhibitors. These results suggest that Akt activation can be a core player mediating the early glycolytic response. They also imply the glycolytic response upon immune cell activation is mediated by diverse signaling pathways depending on cell types and the real-time analysis of cellular metabolism can provide useful kinetic information to understand the cell signaling of immune cell metabolism.
P1857  
**Board Number: B877**  
**Apoptotic- and Necrotic-like Ultrastructural Phenotypes of Human Monocytes in Systemic Lupus Erythematosus.**  
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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multi-organ inflammation often associated with a high titer of anti-DNA antibodies. Monocytes are involved in the pathogenesis of SLE as a source of immune modulators for T- and B-lymphocytes and a pro-thrombotic factor due to exposure of tissue factor on the cell surface. The aim of this work was to investigate the structural changes of monocytes in SLE patients in correlation with the levels of anti-DNA autoantibodies. Monocytes of six SLE patients and three healthy donors were isolated by a density gradient centrifugation of citrated blood followed by negative immune separation and analyzed using transmission electron microscopy. The purity (>96%) of the CD14-positive monocytes was confirmed using flow cytometry and cell viability (>95%) was assessed by exclusion of trypan blue. Total individual 154 monocytes from SLE patients were analyzed and compared with 22 monocytes from healthy donors. Monocytes with normal ultrastructure (ruffled cell surface, formation of filopodia, an indented nucleus, and uniform chromatin distribution) comprised 64% in the SLE patients and 95% in healthy donors. Unlike monocytes from healthy subjects, 19% of monocytes from the SLE patients had morphological apoptotic-like features: smooth cell surface, retraction of pseudopods, electron-dense cytoplasm, mitochondrial swelling, and a spherical nucleus with perinuclear accumulation of condensed chromatin or condensation of chromatin into dense spherical particles. About 17% of monocytes from the SLE patients and 5% from the healthy donors had morphological necrotic-like features: rounding-up of the cell, retraction of pseudopods, low electron-density cytoplasm, small electron-dense mitochondria, and spherical pyknotic nucleus with more diffuse euchromatin. The SLE patients with a high titer of anti-dsDNA antibodies (>200 IU/ml) had significantly more monocytes with morphological apoptotic-like (25% vs. 6%, p<0.01, χ²-test) and necrotic-like (20% vs. 10%, p<0.05, χ²-test) phenotypes compared to the SLE patients with a normal titer of anti-dsDNA antibodies (<25 IU/ml). This study affirm that a substantial fraction of monocytes in SLE undergo morphological alternations that are more pronounced in patients with an increased level of anti-dsDNA autoantibodies. The results suggest the involvement of monocytes in the pathogenesis of SLE with adverse structural alterations associated with increased production of autoantibodies. The work was supported by the Program for Competitive Growth at Kazan Federal University.

P1858  
**Board Number: B878**  
**Parasitic wasp-derived multi-strategy extracellular vesicles specifically interact with cells of the Drosophila host’s cardiovascular/hematopoietic systems.**  
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Spiked, 300-nm multi-strategy extracellular vesicles (MSEVs, previously virus-like particles) are produced by the parasitic wasp species Leptopilina heterotoma (Lh) in the venom glands of females. Along with wasp eggs and venom, MSEVs are delivered into the larval bodies of the wasps’ drosophilid hosts.
MSEVs cause apoptosis of circulating macrophages, lysis of circulating lamellocytes, and TUNEL-positive death of immature blood cells in the host larval lymph gland. This highly successful infection strategy destroys cellular immunity and thus protects developing wasp eggs that would otherwise be encapsulated by blood cells.

MSEVs possess (a) a signature of eukaryotic vesicular/transport proteins, (b) a pharmacopoeia of infection-related proteins, and (c) a wealth of novel proteins, some of which have domains similar to those found exclusively in prokaryotic proteins. A 40-kDa protein belongs to this latter class of novel proteins. Localized to MSEV spikes and surface, it is structurally similar to Type 3 secretion system tip proteins of the IpAD/SipD superfamily.

While the protein composition of MSEVs is now understood, the mechanisms by which they recognize, enter, and destroy host blood cells are not known. To begin to address these questions, we tracked the association of MSEVs in the larval hosts’ dorsal vessel and lobes of the lymph gland. We will present data that demonstrate that MSEVs enter the cardiac cells that line the dorsal vessel and interact with the extracellular matrix proteins associated with the dorsal vessel and cells of the anterior and posterior lobes. Furthermore, blood cells of the lymph gland lobes (that are targeted for destruction), as well as circulating phagocytes, phagocytose MSEVs, demonstrated by the co-localization of MSEV and endocytic pathway proteins.

MSEVs are not internalized by the normally tightly-grouped cells of the posterior signaling center (PSC) of the anterior lymph gland lobes, but instead are found in high densities in interstitial spaces between PSC cells. Additionally, PSC cells of infected lymph glands disperse into the bodies of the lobes, either singly or in smaller groups. Genetic ablation of the PSC conferred protection to the organ: PSC-less lobes exhibit low MSEV signal and reduced cellular destruction. RNAi-mediated knockdown of specific genes coding signals for hematopoietic quiescence or differentiation in the PSC did not similarly block the destructive effects of MSEVs suggesting PSC roles in parasite-activated hematopoiesis and parasite (MSEV)-mediated immune suppression.

Thus, MSEVs utilize the cardiovascular system to penetrate the host’s hematopoietic system, disintegrate the PSC, and utilize the cell’s endocytic machinery for uptake to unleash their apoptotic effects on fly macrophages.

P1859
Board Number: B879
ARTHITIS IMMUNE REPONSE MODULATION BY LOW-INTENSITY INFRARED LASER.
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Low-level light therapy (LLLT) is a phototherapy treatment that promotes cell changes by biostimulation, modulating inflammatory processes and immunological responses. The aim of this work was to investigate the effects of LLLT arthritis treatment in immune cells of near lymph node. Inflammatory process was induced in region near talocrural and subtalar joints using zymosan solution. Eighteen C57BL/6 mice were divided into three groups according to laser (830 nm, 10mW) treatment: (I) zymosan-induced, (II) zymosan-induced + laser 3 J/cm² and (III) zymosan-induced + laser 30 J/cm². Twenty-four hours after last irradiation, the flow cytometry were performed to quantify dendritic cells CD80/CD86+, macrophage CD80/CD86+, lymphocyte TCD4+, lymphocyte TCD8+ and lymphocyte Treg population in the popliteo limph nodes. The group II showed a decrease in dendritic cells population (p<0,01), while dendritic cells CD80/CD86+ (p<0,02) (potent antigen presenter) increased. More, this group showed an increase in macrophages population (p<0,01) and a decrease of macrophage CD80/CD86+ (p<0,002), when compared to untreated group. The group III showed an increase in

Sunday-543
dendritic cells CD80/CD86+ (p<0.02), macrophage (p<0.001), lymphocytes TCD8+ (p<0.01) and lymphocytes Treg (p<0.05) populations, if compared to untreated group. Our results suggest that LLLT in both fluences tested were able to modulate the immune response through alteration in the number of antigens presentation cells. The group III (treated with LLLT at 30 J/cm²) also demonstrated the elevation of effector cells, tending to an anti-inflammatory profile by elevation of Treg cells. The modulation of immune response could be a novel anti-inflammatory pathway of LLLT and opens many possibilities for new uses for the therapy.

**P1860**

**Board Number: B880**

Myeloid cells ablation attenuates Concanavalin A-induced hepatitis by suppressing immune responses.

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Both of bone marrow (BM)-derived monocytes and tissue-resident macrophages play vital role in the maintenance of tissue homeostasis. Recently, we used our intermedilysin (ILY)-human CD59 (hCD59) mediated cell ablation method (Hu, et al, Nat Med 14, 98-) and documented that the specific elimination of myeloid cells protected against Concanavalin A (ConA)-induced acute hepatitis (Feng D., et al. JCI 126, 2321-1). ILY, a toxin secreted by Streptococcus intermedius, binds exclusively to the human-specific cell membrane protein, CD59 (hCD59). Once bound, ILY rapidly lyses the cells through necrosis by forming toxin pores. We established a floxed STOP-hCD59 knock-in mouse (ihCD59) in which hCD59 expression only occurs following Cre-mediated recombination. By crossing Cre-inducible floxed STOP-hCD59 transgenic mouse line (ihCD59+) mice with myeloid cell-specific Cre transgenic line, LysmCre+ mice, we generated a double transgene positive mouse (LysMCre+ihCD59+) in which Cre expression drives expression of hCD59 in myeloid cells. The rapid and specific ablation of myeloid cells by the injection of ILY to LysMCre+ihCD59+ mice completely blocked the development of ConA-induced hepatitis. The activation of NKT and T cells have been implicated to be necessary events in ConA-induced hepatitis. To further define the pathogenic role of myeloid cells and its interaction with other immune cells, we investigated the dynamic changes of immune cells and the relevant cytokines in the ConA-induced hepatitis with or without the myeloid cell ablation. We documented that the myeloid cell ablation suppressed infiltration of T and B cells, inhibited the activation of NKT cells without affecting the total numbers of NK and NKT cells, and decreased the serum level of IFN-gamma, a cytokine mainly secreted by NKT cells in the hepatitis. These results indicate that myeloid cells play a critical role in the maintenance of T and NKT activation, which contribute to ConA-induced hepatitis.

**P1861**

**Board Number: B881**

SALTS OF HEAVY METALS CAUSE PHENOTYPIC CHANGES OF IMMUNE COMPETENT CELLS - PARTICIPANTS AND REGULATORS OF ASEPHTIC INFLAMMATION.

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Changes in the structure and functions of various components of adaptive and genetically determined immunity in the result of the development of a secondary immunodeficiency state, can be not only due to diseases but also due to the adverse effects of chemical ecotoxicants. The course of the inflammatory
process against the background of the immune system disregulation, caused by intoxication with metal compounds, can serve as a model. Rapid development of research in the field of immunology has led to many scientific breakthroughs, and however, some aspects of immune-associate diseases remain under-explored. The goal of research was studying the functioning of individual immunity units in rats with aseptic inflammation and preliminary seeded with metal compounds. The research was carried out on not pedigree male rats weighting 180-220 grams. Animals were randomly divided into 4 groups of 30 animals each: control animals; the animals with aseptic inflammation; the animals, which had been orally fed on ammonium vanadate and potassium dichromate in a dose of 5 mg / kg bwt within two weeks; the animals in which, at the end of seeding with metal compounds, aseptic inflammation was modeled. In 1, 7 and 14 days after the modeling of aseptic inflammation in animals of all groups (10 individuals for each research period), blood was sampled under chloroform anesthesia, then euthanasia was followed by extraction of thymus, bone marrow, spleen and mesenteric lymph nodes. A two-week monitoring of the immune status of the rats under inflammation, after being preliminary seeded with ammonium vanadate and potassium dichromate, displayed prolonged course of inflammation and impaired wound healing. Haematologic state displayed a sharp inhibition of immune competent cells and the development of anemia, which is inherent in immune depression. The course of inflammation was aggravated by the intervention of anti-inflammatory activity of IL-10 in the first term, and TGF-β in the research remaining periods, which significantly influenced the development and outcome of the inflammatory process in experimental rats. Expressed structural changes in lymphatic organs throughout the experiment were represented by a decrease in the cortical-medullary index, dystrophically altered cells and their scarcity. Quite the opposite, evolving increase in cellularity was observed in the spleen, an increase in the cell populations with the MDSC phenotype having expansion toward G-MDSC, a significant reduction in the Th2 immune response, and a reduction in the cytotoxic CD3 + CD8 + T-lymphocyte content by the end of the experiment.

P1862
Board Number: B882
Effect of p53 activation on target gene expression and cytokine release in peripheral blood mononuclear cells.
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Multiple sclerosis (MS) represents a common chronic autoimmune diseases with largely unknown etiology that is characterized by demyelination of axons in brain and spinal cord tissues. In the recent years it has become apparent that p53, a well-known tumor suppressor, has a primary role in regulation of innate immune responses. Data includes increased p53 expression in MS lesions, predisposition of p53-deficient animals to autoimmune diseases and high levels of inflammatory demyelination. Here we report the effect of Nutlin-3a-induced p53 activation on expression of various p53-dependent genes and release of proinflammatory cytokines. The results indicate that treatment with gradually increasing Nutlin-3a concentrations (5 - 40 uM) leads to correspondent gradual increase in expression levels of p21, Mdm2 and Puma target genes in peripheral blood mononuclear cells. In addition, upon treatment of cells with 10 uM Nutlin-3a we observed rise in IL-6, IL-10 and TNF-alpha levels. Gene expression data was obtained by TaqMan real-time PCR using CFX96 Touch Detection System (Bio-Rad). Quantitative cytokine detection and measurements were performed using Luminex 200 multiplexing system (EMD Millipore) and ELISA assay (Vector-Best). Our results could assist better understanding the p53-dependent regulation of immune cells and aid the development of novel immunotherapies. The study
was funded by research grant 16-34-60213 mol-a-dk from the Russian Foundation for Basic Research (RFBR).


P1863

Board Number: B883

Cellular events mediating extracellular trap formation in HL60-derived and mouse neutrophils.

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Extracellular trap (ET) release is a line of host defence during which activated immune cells such as granulocytes decondense and release their chromatin to the extracellular environment, leading to the formation of web-like DNA structures decorated with histones and cytotoxic proteins. This DNA “trap” participates in innate immunity by capturing and neutralizing bacteria, fungi and viruses. However, ETs also form in sterile inflammation. While a considerable amount of knowledge regarding the in vivo relevance of ET release has been cumulating, little is known about the cellular and biophysical mechanisms leading to ET formation. To determine the cellular events occurring during ET release, we performed high resolution live cell imaging of HL60-derived neutrophils stimulated to release NETs with ionomycin as well as mouse blood neutrophils (MBN) stimulated with ionomycin or LPS (bacteria component). MBN actin, microtubule and endoplasmic reticulum (ER) networks were visualized using vital dyes. HL60 cells stably expressing F-tractin-mApple were co-transfected with mEmerald-tagged ensconsin, lamin A/C, B1 and calreticulin-KDEL for visualizing respectively microtubules, the nuclear lamina and the ER. Cellular DNA was visualized with far-red Hoechst. Spinning disk confocal and DIC microscopy revealed that both ionomycin- and LPS-stimulated MBN as well as ionomycin-stimulated HL60-derived neutrophils undergo a stereotypical series of changes in cell morphology and cytoskeleton/endomembrane dynamics prior to NET release. Within minutes after stimulation, the actin cytoskeleton disassembles followed by drastic plasma membrane vesiculation and shedding coincident with ER vesiculation, but leaving the nuclear envelope (NE) intact. Subsequently, chromatin decondenses, the nucleus rounds up, and microtubules depolymerise. The NE ruptures, leading to rapid expulsion of decondensed chromatin into the cytosol. Finally, most cells rupture their cell membrane resulting in NET release. Peptidylarginine deiminase 4 (PADI4) has been shown to be required for NETs release. To determine its requirement for the cellular events leading to NETosis, we stimulated MBN from PADI4 deficient mice with ionomycin and visualized the cytoskeleton and endomembrane using vital dyes. We found that while actin and microtubule disassembly as well as ER vesiculation occur independently of PADI4, nuclear envelope rupture requires PAID4. Our data revealed that NET release proceeds via a well conserved succession of events that mediates the systematic disassembly of cytoskeletal and membranous components prior to chromatin release into the extracellular environment. The mechanism by which PADI4 mediates nuclear envelope rupture will be further studied in HL60-derived neutrophils.
Salmonella Typhimurium of macrophages leads to the production of exosomes that activate the immune response in naive cells.

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Salmonella enterica serovar Typhimurium (S. Typhimurium) is a Gram-negative bacterium responsible for infections resulting in 1.2 million illnesses annually, therefore constituting a significant economic burden. Macrophages infected with S. Typhimurium react to this pathogen by stimulation of the receptor-mediated inflammatory pathways and by the presentation of pathogen-derived antigens to other immune cells. We hypothesized that pathogen-associated molecular patterns (PAMPs) are released via exosomes, which are formed in macrophages during S. Typhimurium infection. Exocytosis relies on multi-vesicular bodies (MVB) to invaginate creating intraluminal vesicles (ILVs), thereafter MVB will fuse with the host plasma membrane and released the ILVs to the extracellular space in which the vesicles are termed exosomes. Exosomes can carry various cargoes, including proteins, metabolites, and microRNAs, which are unique to different cell types, subpopulations of exosomes and physiological conditions present. There is a precedence for using exosomes derived from the acid-fast bacterium M. tuberculosis to provide immune protection. We demonstrate that exosomes carry PAMPs from S. Typhimurium-infected macrophages, stimulating naïve macrophages to produce chemokines such as RANTES, TNF-α, IL-1ra, MIP-2, CXCL1, MCP-1, sICAM-1, GM-CSF, and G-CSF. Moreover, we show that these exosomes stimulate primary bone marrow-derived MΦ (BMDMs) and DCs to produce some of these cytokines. LPS has been previously suggested to be transported via the secretory pathway of macrophages upon S. Typhimurium infection, and we show that this exosome-triggered stimulation of proinflammatory chemokines is TLR4-dependent. Exosomes derived from macrophages infected with S. Typhimurium producing 1-monophosphorylated lipopolysaccharides (LPS) triggered significantly less TNF-α in naïve cells in comparison to exosomes derived from macrophages infected with wild-type S. Typhimurium, suggesting the involvement of LPS. We also show that the LPS or other unknown molecule stimulating naïve cells is enclosed within exosomes. However, our data suggest that apart from LPS there might be additional PAMPs contributing to the function of exosomes, which is consistent with the fact that proteomic analysis led to the identification of bacterial antigens within these host vesicles. Finally, we show that intranasal administration of exosomes from infected MΦs leads to an anti-S. Typhimurium antibody production, and to stimulation of CD4+ and CD8+ T cells. In summary, our data support the hypothesis that bacterial antigens are carried in exosomes derived from S. Typhimurium-infected antigen-presenting cells (APCs), which contribute to the stimulation of naïve immune cells.
Monday, December 4
Science Education 2

P1865
Board Number: B1
Science Communication: A new writing exercise to highlight STEM literacy.
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An important impact of our research depends on effectively communicating our discoveries. Scientific writing is a primary tool for the dissemination of research and an important skill to develop for our trainees. For many STEM trainees scientific writing represents a style of communication that is not taught during their early education. One strategy to learn scientific writing is through reading primary research literature. Often times we assign scientific articles as a reading exercise and then the trainee leads a journal club style discussion. However, this activity can result in a passive learning experience and not help the trainee develop their scientific writing skills. In an effort to promote trainee communication skills, I tested a writing exercise as a pilot project to develop a revised version of my previously published research article. Following the writing guidelines organized by the Frontiers for Young Minds journal, I worked for several months with two high school students on drawing the results and figures in an animated style. In particular, we created a novel scale to illustrate the qualitative analyses made in the research paper (“The Ouch Scale”). We also defined key concepts of the research article and wrote basic explanations of the experimental methods. Upon completion of the revised manuscript, the students gained an in-depth understanding of the research concepts. In addition, the writing of the text and illustration of the figures promotes an engaged sense of ownership for the students with the research. The final product of our writing exercise serves as a resource to share with new students as they join the lab. As a derivative of the creative writing project, we continue to develop new illustrations of our methods and results. Current work is being done to translate this writing exercise into an undergraduate level classroom environment through using small groups and discussions with invited speakers. Another application of this writing exercise is being incorporated into a graduate level seminar series in which a final report can include a section written in a Frontiers for Young Minds style. Upon reflection of this writing exercise, I encourage all research scientists to expand the impact of their discoveries and share their knowledge with society. Improving science communication will not only benefit scientific training but also promote scientific literacy within communities with limited access to STEM fields.
P1866

Board Number: B2
How Instructors Can Enhance Biology Students’ Motivation, Learning, and Grades Through Brief Relevance Writing and Worked Examples Interventions.
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The high failure rates of students in “gateway” STEM courses has been a persistent problem for biology programs nationwide. Common wisdom contends that addressing this problem requires major curricular overhauls. As large systematic changes are often impractical, we propose an alternative approach: supplementing the regular instruction with brief online modules targeting specific motivational and cognitive (learning) mechanisms. We conducted an intervention study to test the effects of different combinations of cognitive and motivational modules on undergraduate Introductory Biology students’ motivation, biology reasoning, course achievement, and intentions to remain in science. Introductory Biology students at a large urban university were randomly assigned to a no-treatment control condition or one of eight combinations of cognition-motivation modules. Overall, six out of the eight interventions led to higher course grades ($p \leq 0.05$) compared to control group participants. In this poster, we report on a subset of these data: the cognitive module of Worked Examples (brief video demonstrations of biology problem solving) and the motivation module of Relevance Writing (brief open-ended writing assignments about connections of biology concepts to one’s life). The combination of worked examples (cognitive) and relevance writing (motivational) resulted in a 6.35% improvement in course grades ($p = 0.05$). Increased student engagement in these combined modules also led to higher motivation, biology reasoning, and course grades. While research on these modules requires collaboration between biology instructors and educational science researchers, the implementation of the Worked Examples and Relevance Writing interventions is easy and integrates with existing course content. These findings support the effectiveness of delivering brief supplemental cognition-motivation modules online to promote students’ success in introductory biology courses. This easily implemented intervention can utilize online tools such as Blackboard, Canvas, or Moodle, and takes the burden off faculty to radically change their instruction when such change is not practical.

P1867

Board Number: B3
Integration of Student-Led Independent Research Experiences in a Senior-Level Biotechnology Laboratory Course.
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An opportunity of student-led independent research was incorporated as a requirement of the Biotechnology Laboratory course. This course is a combination of skills learned throughout their undergraduate studies at the university. Students use their scientific knowledge along with math and chemistry skills to prepare laboratory solutions. While in the class, they learn basic molecular biology skills that include the isolation of DNA and protein, principles of gel electrophoresis, PCR, Southern, and Western blotting. As part of the independent research activity, the students were required to prepare a detailed research proposal summarizing how they would use at least three of the techniques learned in
the class to research a scientific topic. The areas of research were optional and could be related to any area of science but approved by the instructor. Working in groups of two or three, the students researched a topic of interest and developed a hypothesis to evaluate. The students collected scientific data on their proposed laboratory experiments to support or not support their stated hypothesis. Final research proposals included background information on topic, hypothesis stated, data collection and analysis, and a discussion/conclusion of their results. Groups also suggested additional experiments in the future to address their scientific topics. At the end of the semester, all of the students completed a Qualtrics survey to evaluate the overall success of this experience. About 87% of the students strongly agreed/agreed that their knowledge about research increased by this experience and the activity stimulated more interest in doing scientific research. In summary, this opportunity allowed students to use their knowledge of science and medicine to explore a future in scientific research.

P1868
Board Number: B4
Cellular Construction Workshop – Modeling Cells as Biological Machines.
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Cellular engineering is an emerging discipline that harnesses the power of cells to solve medical and environmental problems. By focusing on the application of biological systems for problem-solving, rather than memorizing facts and details, we aimed to excite students about a career in cellular engineering. To this end, we developed a two-week workshop for high school students and teachers. The goal of this workshop was to reframe how students think about cells - as biological machines - to illustrate their complexity and their potential to solve real-world problems. We constantly used direct analogies between various biological systems and engineering models to reinforce the concept of cells as programmable systems for accomplishing various tasks.

The course was structured such that on a daily basis the learners were introduced to different biological systems, and were immediately challenged to model an observed biological behavior using Lego Mindstorms. This structure helped develop a strong understanding of DNA as a programming interface and cells as a dynamic substrate for engineering solutions. The students and teachers worked in teams as co-learners to accomplish the various biological and programming challenges.

The workshop was viewed as a pilot for curriculum modules, and we are receiving feedback from participating teachers on how these lessons can be incorporated into the classrooms. All the lesson plans and materials were made available to the teachers to facilitate an introduction to cellular engineering in biology classrooms. The workshop presented a strong model for implementing the three-dimensional teaching described in the Next Generation Science Standards to biology teachers. Special emphasis was laid on integrating engineering practices into the teaching of biology, which can be challenging for high school life science teachers who rarely have experience with engineering. Moreover, we view this type of project-based learning as having potential for broadening student participation and increasing diversity in science by engaging underrepresented students in authentic science and engineering tasks.
P1869
Board Number: B5
Broadening Interest in STEM in High School Students through Foldscope-Based Interdisciplinary Activities.
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Data from the US Bureau of Labor Statistics predicts that by 2022, occupations in science, technology, engineering, and math (STEM) are projected to grow to more than nine million. This growing workforce is expected to make up over five percent of all jobs. Indeed, many predict that the new global economy will be built on STEM careers. However, recent studies suggests that a significant number of high school students begin to lose interest in STEM fields as they get older, in part because of lack of exposure to STEM and its applications and a lack of confidence in their ability to pursue STEM-related careers. To bolster interest and confidence in self-efficacy to pursue STEM fields, we have developed a one-day outreach program that focuses on the engineering, theoretical basis, and application of the Foldscope paper microscope. Among a population of college-bound, but at-risk matriculating high school students, we found that this outreach program increased student interest in STEM in general, improved confidence in their ability to learn STEM, and increased students’ openness to careers in STEM fields. Interestingly, students most enjoyed the construction and use of the Foldscope, and least enjoyed understanding the basic optics behind the Foldscope. These results are consistent with student data that show more interest in applied than basic study of STEM fields.

P1870
Board Number: B6
Science, Biotechnology and Society, an active learning course for non-STEM undergraduates: A comparative study between first year and higher level students in term of their outcomes.
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The Department of Biological Sciences (DBS) is offering the active-learning course Science, Biotechnology and Society (CIBI 3028) to introduce non-STEM undergraduates to biotechnology and trends in this scientific field. During the semester, the students engage in the discussion of current socioeconomic, environmental and bioethical issues related to biotech. At the laboratory, the students have the opportunity of conducting microbiology and molecular biology sequential experiences resembling the methodology used in research projects. In this study, second year and higher level undergraduates (sample 1, n = 52 participants) are compared with first year students (sample 2, n = 43 participants) in term of their outcomes in knowledge and comprehension of biotechnology concepts and processes. These aspects were evaluated with a pre/post-test instrument. A t-Test was conducted to establish the data significance level. A pre/post questionnaire was used to determine if there was a transition in the students’ interest toward biosciences, post-graduate studies and research in their future careers. The questionnaire results were analyzed through mean and kurtosis. Test results show a significant gain in knowledge and comprehension for both participant samples. In term of the participant interest toward the Biological Sciences, the first year students (sample 2) show the most remarkable positive shift. This study would be the first step to evaluate the possibility to integrate an authentic research project in CIBI 3028.
P1871
Board Number: B7
Light, Imaging, Vision: An interdisciplinary undergraduate course.
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Students in physical and life science, and in engineering, need to know about the physics and biology of light. In the 21st century, it has become increasingly clear that the quantum nature of light is essential both for the latest imaging modalities and even to advance our knowledge of fundamental processes, such as photosynthesis and human vision. But many optics courses remain rooted in classical physics, with photons as an afterthought.
I describe a new undergraduate course, for students in several science and engineering majors, that takes students from the rudiments of probability theory to modern methods like fluorescence imaging and Förster resonance energy transfer. After a digression into color vision, students then see how the Feynman principle explains the apparently wavelike phenomena associated to light, including applications like diffraction limit, subdiffraction imaging, total internal reflection and TIRF microscopy.
Then we see how scientists documented the single-quantum sensitivity of the eye seven decades earlier than `ought' to have been possible, and finally close with the signaling cascade that delivers such remarkable performance.
In short, using imaging and vision as common threads helps motivate undergraduates to learn a lot of cell biology and physics. A new textbook, published in 2017, allows others to replicate this course. The book has supplemental sections that also make it suitable for graduate-level courses.
Outcomes include student reports of improved ability to gain research positions as undergraduates, and greater effectiveness in such positions, as well as students enrolling in more challenging later courses than they would otherwise have chosen. I'll show data based on a survey of students, in some cases several years after they took the course, documenting those outcomes, and connect to current ideas in education research. Finally, PhD students staffing the class gain experience with a new mode of teaching not featured in our other TA positions.

P1872
Board Number: B8
Use of community service projects in an introductory non-majors biology class.
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Clayton State University currently has a university-wide academic community engagement initiative to involve students in community service projects that also enhance learning of concepts covered in the classroom. This mechanism of learning teaches students a variety of skills that can be beneficial to them in succeeding in various aspects of their careers. This approach was used in a non-majors introductory biology class where students had the option to volunteer at either the American Cancer Society Hope Lodge facility that provides temporary housing for patients who have cancer or the Alzheimer’s Service Center which is a day-care center for patients with dementia or Alzheimer’s disease. The objective of this project was to introduce students to community service and evaluate any changes in their attitudes towards civic engagement and identity. Additionally, the study evaluated any changes in career-building skills while also having students learn content relevant to the class. The students wrote pre- and post-reflection papers. The instructor graded their responses as benchmark, milestone 1, milestone 2 or
capstone. It was found that there were significant increases in various parameters including civic engagement and identity, problem solving and critical thinking. These increases only rose to the level of milestone 2 presumably since it was performed in an introductory class. A community series attitude scale test (CSAS) was also given to the students early in the semester and then after the completion of their projects. This test asked questions based on perception of a need to respond, moral obligations to respond, reassessment of potential responses and engagement in helping behavior. There were no significant changes in any of the areas.

P1873

Board Number: B9

Using animation to improve student learning of difficult concepts in undergraduate biology classrooms.

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Active learning has dominated recent discussion on how to improve science teaching and learning on college and university classrooms. Most teaching strategies that include active learning approaches increase student engagement with course material, improve student performance, and foster classroom inclusivity. On two campuses, Bucknell University (PA) and Juniata College (PA), we examined the impact of one strategy, animation production as a mode of learning, on student performance and attitudes. Students produced an animated science video to deconstruct difficult topics like misconceptions and reading of primary literature. They wrote a script for their video, generated storyboards, and then produced a video animation using an iPad or tablet. We posited that animation production would help students learn material more accurately, exercise creativity, and improve their communication skills by telling stories. To address whether animation production improved learning of important scientific concepts, we surveyed upper level biology students at both institutions on their attitudes on using animation production to learn. At Juniata, students produced a 1-2 minute animation to communicate a main hypothesis and results of a primary paper. A survey, using a Likert scale, showed that students either strongly agreed or agreed that using animations improved their “understanding of the hypothesis” of the primary literature and caused them to “more critically analyze the research paper more than a normal reading assignment.” Moreover, students thought that opportunities for creative expression were important in biology classes. However, students also expressed that making the animations required a lot of class time. At Bucknell, students used animation to tackle misconceptions in biology. Students produced a short animation of mitosis, then watched time-lapse videos of mitosis to reveal their misconceptions about the process. Students then reanimated mitosis based on their view of the process. A quiz given two months after the animation projects were completed revealed that students created accurate drawings of chromosome distribution in mitosis, retaining what they learned from watching time-lapse videos and preparing new animations. Further application of animation production in science classrooms may lead to gains in student knowledge and communication.
P1874
Board Number: B10
Formative evaluation of active-learning activities in the cell biology classroom.
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Moving from a lecture-based classroom to an active-learning classroom is a big change for science professors who have spent their whole careers lecturing and have little experience designing and facilitating group activities for students in their classrooms. Just thinking of questions and activities or finding them from other sources is only the first step. One major challenge is to evaluate these activities as they are taking place in the classroom to effectively manage student behavior, improve student learning, and consider ways to improve the activity for a future use. In this presentation the results of using various in-class formative assessment techniques will be shared to help instructors learn to evaluate their own new or modified activity. Additionally, this project gives many examples of active-learning activities that have been classroom-tested in either an upper-level cell biology class or an introductory biology class unit focusing on cell biology topics. Both successful and not successful classroom active-learning activities as evaluated by the formative assessment techniques will be included.

P1875
Board Number: B11
Developing Future Biologists: creating and assessing a portable short course to engage underrepresented students in developmental biology.
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Many barriers prevent underrepresented students from choosing a career in science. In order for undergraduate students to decide to pursue graduate education, they must first be exposed to a particular subject, develop an interest in that topic, and accumulate related coursework and research experience. Unfortunately, many students from underrepresented groups lack exposure to the field of developmental biology due to limited course offerings and finite resources at smaller institutions. To address this disparity, graduate students at the University of Michigan created a portable short course focusing on developmental biology, titled “Developing Future Biologists” (DFB). This week-long educational initiative provides students with hands-on laboratory sessions, interactive lectures, and professional development workshops, aiming to teach students the core concepts of developmental biology and increase awareness of scientific career options. To evaluate course effectiveness, we developed a pre-post assessment, incorporating key concepts in developmental biology outlined in the BioCore Guide (Brownell et. al., 2011). Here we present results from two separate iterations of the course in Ponce, Puerto Rico (2016) and Ann Arbor, Michigan (2017). We show that student understanding of core developmental biology concepts and perceived experience in developmental biology significantly increased as a result of DFB, despite the abbreviated nature of the course. Pre-post scores improved in all five of our main content areas, including early embryonic development, gene expression, cell signaling, organogenesis, and developmental disease. Furthermore, item analysis of our assessment tool suggested that items on the post-tests were less difficult for students than pre-test items, while simultaneously being better at discriminating between high- and low-performing students in the course. The DFB portable short course model and assessment strategy could easily be adapted to

Monday-7
any number of topics and locations in order to connect undergraduate students with opportunities for advanced study and help lower the barriers that exist for underrepresented students in science, technology, engineering, and mathematics.

P1876
Board Number: B12
Development of a learning progression on cellular membranes and transport mechanisms for high school through undergraduate students.
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A learning progression (LP) is defined as a hypothetical model of student learning that contains multiple levels. This provides a framework to understand the development of students’ conceptual understanding within a domain. Cellular biology has been an area students traditionally have struggled, since they find it difficult to conceptually understand the theoretical and abstract nature of many cellular biology concepts. Therefore, a LP serves to understand student needs within this domain. To develop this LP, a cross-sectional study took place in two high schools and a public university. This included approximately forty participants who either completed a high school biology class, an undergraduate sophomore level cellular biology course or a more advanced undergraduate junior/senior level cellular biology course. Assessments were given in the form of semi-structured interviews, and responses were then audio-recorded and transcribed. In this study, we focused our attention on the development of assessments and a LP that are effective in eliciting students’ thinking and describes students’ sophisticated ways of reasoning on cell membrane structure and function and cellular transport. Our LP can be categorized into the following concepts in three levels: cell membrane structure and function, plant and animal cellular membranes, active and passive cellular membrane transport, organelle membrane transport and molecular membrane transport. Our results revealed that within cell membrane structure and function, the lowest level of the LP described how membranes keep “things” in and out, but the upper levels describe the various components and the chemical and physical properties within the bilayer. When interviewed on knowledge on plant and animal cellular membranes, analysis revealed that only the upper level of the LP describes how membranes are found in both organelles and the outer cell membrane. For active and passive transport, the upper levels describe their exact forms of energy and mechanisms, whereas the lowest level only describes whether energy is needed or not. Organelle membrane transport is relatively unknown in the lowest LP level but the upper levels describe both the mechanism and purpose of these trafficking mechanisms—such as regulation. Finally, molecular membrane transport, such as endocytosis, is superficially known in the lowest level of the LP where the upper level describes much more sophisticated reasoning behind the two processes. Collectively, this LP describes how students’ reasoning develops from high school throughout undergrad. This is an ideal tool for educators to employ in their own curriculum development to better align with the future needs of students as they progress through their biology courses.
P1877
Board Number: B13
Promoting Leadership Development Within Undergraduate STEM Curricula.
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\(^1\)Biology Department, Austin College, Sherman, TX, \(^2\)Institutional Effectiveness, Austin College, Sherman, TX

Scientists need to work together to find innovative solutions to complex problems greater in scale and magnitude than what society has faced before. In today's complex global world, where science impacts nearly every aspect of our daily lives, scientists will be expected to lead. The STEM Teaching and Research (STAR) Leadership Program at Austin College is designed to develop five behaviors necessary for effective leadership in science: communicating ideas, problem solving, collaborative work, foresight and planning, and acting responsibly. STAR achieves this goal through integrated leadership theory and activities in science courses at all levels of the curriculum that allow students to develop, practice, and reflect on these skills. The presenter will discuss specific examples of how the STAR curriculum enhances the teaching of cell biology in both introductory and advanced undergraduate courses, including a discussion of course integration informed through program assessment. Preliminary assessment data from the STAR program demonstrate significant increases in all student learning outcomes through their undergraduate experience. Additionally, no gaps in attainment of leadership behaviors by students have been observed from factors that may typically associate with achievement gaps in STEM (i.e. first generation status, race/ethnicity, or gender) Qualitative data from the cell biology courses demonstrated student learning gains in self and situational awareness and emphasized understanding the importance of communication and collaboration in science. The STAR program has led to enhanced student engagement and collaboration in cell biology coursework which may also translate to retention and success in STEM disciplines. At Austin College, we also believe that the STAR model of education enables students to be agents of change for science in the future.

P1878
Board Number: B14
Learning in Large Introductory Biology Courses Is Effectively Facilitated by Trained Undergraduate Learning Assistants.
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\(^1\)Biological Sciences, George Washington University, Washington, DC

The purpose of the GWU undergraduate Learning Assistant (LA) program (modeled after the University of Colorado program) is to facilitate learning in active classroom environments. The LA’s take a pedagogy class taught by a STEM education specialist and meet weekly with their lead instructor. The GWU LA program is associated with courses in Biology, Chemistry, Physics and Math. Here we report on teams of LA’s who participated in the daily teaching of students in Bisc 1111 Introductory Cell & Molecular Biology classes taught 2013-2016. There were 168 students in the Fall 2016 LA class and 170 in a traditional lecture section. Each LA in the Biology classrooms worked with a specified group of 10-18 students, facilitating discussions of clicker questions, worksheet problems and practice quizzes. Each group of 3 students would submit their daily worksheet to their LA for written comments and formative assessment. Students evaluated the effectiveness of the course activities for their learning (the % of students reporting a positive effect in parentheses): assigned readings (93%), weekly online homework (70%), daily in class clicker questions (89%), daily group problem solving (89%), LA facilitation (91%), brief lectures (57%), studying alone (43%), practice quizzes (82%). Most of the students (93%) reported
that they would take another course with LA’s and 77% felt that the overall course format facilitated their learning. Learning was assessed in short answer exams and by administration of the Introductory Molecular and Cell Biology Assessment concept inventory (Shi et al, 2010, CBE Life Sci. Ed). Students in the LA supported class performed better on this assessment (74%) vs. students in the traditional lecture (64%). Published reports from other universities show that the LA program increases student retention (Talbot et al, 2015, J. College Sci. Teaching).

P1879
Board Number: B15
Assessing the Effectiveness of Student Learning and Engagement in a Content Heavy Flipped Class.
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Studies have shown that greater student learning gains are achieved during active learning, when students must apply their understanding solving problems, in application exercises, in discussion-based activities, and during team-based learning. Because these active learning approaches can take up valuable class time, leaving less time for content, it is often difficult to integrate them into a content heavy lecture course. Using a “flipped classroom” approach, student-centered learning activities can be routinely integrated into the classroom while not having to sacrifice content. To test the effectiveness of the flipped classroom approach, this study assesses a 300 level Genetics class by comparing outcomes of students in a flipped class to outcomes of students in a more traditional lecture class. To control for student population differences and to ensure that student populations between the classes were comparable, portions of the flipped class experimental group (flipped group) were taught utilizing approaches similar to those in the traditional groups [Units 1 & 3 (Control Units)], whereas other portions of the course were taught utilizing the flipped classroom approach [Unit 2 (Experimental Unit)]. Exam scores during the Control Units showed no statistical difference between traditional groups and the flipped group (Unit 1, p=0.266 & Unit 3, p=0.698); whereas the flipped group performed significantly better than the traditional groups during the Experimental Unit (Unit 2, p < 0.005). Additionally, student surveys indicated that students were more engaged in the class, came to class better prepared, dedicated more time working out problems, felt more confident with the material, better paced themselves, and overall preferred the flipped class over the traditional class. This study suggests that flipping a content heavy course can improve student engagement and result in greater learning gains.

P1880
Board Number: B16
A Flipped Classroom Approach in a Sophomore-Level Cell Biology Course to Enhance Concept Integration and Critical Thinking Skills.
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¹Biological Sciences, Bridgewater State University, Bridgewater, MA

With the advent of the Internet and established online mediums through which information can be communicated to an audience, many teachers at colleges and universities nationwide are transitioning to a flipped classroom approach in their coursework. The basic structure in a flipped classroom entails students watching recorded lectures on new content before arriving to class and then spending class time engaging in interactive discussions and/or activities in the presence of the course instructor.
Several studies comparing the effectiveness of more traditionally-structured courses to courses with a flipped classroom demonstrate that this approach can yield greater student learning and success. With this in mind, this presentation will discuss a flipped classroom approach developed for an undergraduate, sophomore-level Cell Biology class. Its design was based on the following rationale: By watching lectures of new content before class, • students have more time to process the information and identify points of confusion, which they can bring to class for clarification; • class time can be more effectively spent helping students to integrate the new information with concepts already learned; • class time can be used to develop critical thinking skills through group discussions, problem sets, and/or other activities under direct guidance from the teacher. In this course, each class meeting entails the following four-step sequence: 1. Ahead of each scheduled class period, students are assigned a “Video Lecture” corresponding to the topic that will be discussed during the upcoming class meeting; 2. Students are provided a list of questions (“Guide Questions”) for each Video Lecture, which they should be able to answer after watching and studying the Video Lecture content. 3. Each scheduled class period begins with a 10-question quiz on the material covered in the corresponding Video Lecture. 4. The remainder of class time is then spent addressing questions students have from the Video Lecture, engaging in group discussions on how the content relates to previously learned information/concepts, and working on problem sets designed to reinforce and integrate course material.

**P1881**

**Board Number: B17**

**The Effects of In-Class Group Problem Solving Sessions on Student Learning and Study Behaviors and Attitudes in Biochemistry.**

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Active learning approaches including group work have been shown to be effective for student learning. We tested the effects of a specific active learning approach, in-class group work on problem sets, on student performance and student behavior and attitudes about study strategies in a large undergraduate biochemistry course. We assigned the same problem set either as an independent homework assignment or as in-class group work where students worked together in groups of five. We found that the average exam score on one exam was increased in the class with a related in-class problem session compared to the class with an independent homework assignment. Performance on specific similar questions covering content from the problem set was also increased in the class with the in-class group work. Study surveys conducted before, during, and after the class revealed self-reported student study behaviors and attitudes regarding different study methods throughout the semester. The in-class group work did not correlate with any substantial changes in student behaviors or attitudes about studying. We conclude that in-class group problem solving sessions may have short-term impact on learning compared to individual homework assignments, but did not have large effects on student behaviors and attitudes about studying.
P1882
Board Number: B18
Building a core scaffold to achieve a real understanding of the cell by medical students.
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For more than ten years now we have been using a learning activity focused on the vesicular trafficking, one of the main features of the animal cell. The task is performed at the midterm of the course by students of Medicine during their first year. Briefly the activity can be described as follows: students working in small groups make a poster of a cell showing the involvement of the internal membrane system in the biosynthetic activity of the cell. All the usual interactions between compartments have to be depicted, however each group has a different assignment and is asked to focus on a certain compartment, a specific type of cell and, at least, two specific molecules. Recently we have also added a disease or pathological condition related to the cell or to the molecules assigned. The posters will be presented and discussed in a collaborative way during the final session. Important learning objectives are achieved: 1) highlight the dynamic/fluid nature of membranes 2) discover the endosome and the relevance of this compartment and the vesicular trafficking in disease, 3) understand that common components of animal cells are being continuously synthesized and replaced. In summary, confronting the students with the task of linking the vesicular trafficking to the synthesis of specific molecules allows the student to gain insight into the complexity of the eucaryote cell and induces a clear shift in the learning process. According to this, all the student groups surveyed were highly satisfied about the design and usefulness of the activity. In addition, performing the task provides the scaffold required for adding new cellular components, as the cytoskeleton, or complex processes as signaling and intracellular regulation, the last topic in this course. We have also used an active learning approach to teach this topic: using mostly resources from the internet, students are guided through a journey of enquiry moving along different sides of the signaling process. All the answers and evidence are collected in a portfolio that will be used for the assessment. The task promotes the integration of the whole activity inside the cell and can be easily reused or adapted just choosing different signaling molecules or pathways. In fact the design of both tasks takes advantage of the increasing number of molecules and pathways being currently described in eucaryote cells, most trying for the teacher of Cell Biology when using the lecture method, but which provides endless combinations. Although different approaches can be equally useful, the design we propose is easy to perform, does not require technical resources, allows effective learning of core concepts in Cell Biology and helps to understand disease at the cellular level.

P1883
Board Number: B19
Graduate Students in a Hybrid Histology and Cell Biology Course.
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Introduction: In a world that is becoming increasingly dependent on technology, the implementation of online and computer resources has permeated graduate medical education. These technological advances have necessitated the evolution of curricula and reformatting of class structure. In particular, histology and cell biology teaching have been highly influenced and are in a state of flux in various graduate and medical curricula. A growing trend is that many institutions are now offering an online resource of digitized histological images as opposed to the traditional glass light microscope preparations. Previous studies have reported equal if not improved performance and benefits of these
online histology and cell biology resources such as slide consistency, flexibility, ease of use, and efficiency of faculty time. An online, interactive histology atlas has been developed for undergraduate, graduate, and medical students at George Washington University in Washington, D.C. This online resource offers numerous digital preparations at both light and electron microscope levels in reference to the in-class teaching syllabus of the clinically relevant organ, tissue and cell systems. The atlas teaches both structure and proper identification techniques, and also offers accompanying texts containing background information and image clarification. Furthermore, the online resource offers testing and labeling practice to enhance the learning process. The purpose of the study is to examine the efficiency, perception, and helpfulness of the online resource in a new course format. Methods: We evaluated such measures by anonymously surveying the 2016 fall cohort of graduate students in George Washington University’s Graduate Certificate of Anatomical and Translational Sciences (GCATS) and Masters of Anatomical and Translational Sciences (MATS) programs. Practical exam grades were also reviewed to assess learning of the material. Preliminary Results and Conclusions: A preliminary survey found that 66% of students thought the atlas very useful, 75% stated the online laboratory exercised to be an effective use of time, and 100% mentioned the online component enhanced their learning of the material. Students scored very well on the final practical exam in the course. Given the feedback from our survey, combined with other studies’ findings, we hope to implement changes to make the online laboratory more effective for student use and to enhance the overall learning dynamic for cell biology and histology.

P1884
Board Number: B20
Benefits of adaptive on-line learning modules.
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Dynamic on-line learning modules that accompany textbooks are designed to provide individualized feedback to students based on the accuracy of their answers to questions and their reported level of confidence in those answers. Student response and success were assessed for traditional community college Anatomy and Physiology (A&P) courses that employed adaptive on-line learning platforms. In each class, weekly homework assignments included interactive on line quizzes that were to be completed prior to a lecture. Lectures were then followed up with standard review questions to reinforce inclusive topics. All assignments were mandatory with specified due dates. In an Anatomy and Physiology (A&P) II course, data was collected for 134 students over six semesters from spring 2015 through summer 2017. Scores for on-line assignments were compared with test and quiz grades throughout the semester and with final exam scores. Results were comparable for all classes. For 71% of all students, test scores remained consistent throughout a given semester. For 18% of all students, test scores increased greater than 10 points from week 4 of the semester to the final exam in week 15. Nearly 80% of these students demonstrated continued improvement in test/quiz scores throughout the semester and completed more than 72% of the on-line assignments. For 11% of all students, test scores decreased more than 10 points from week 4 of the semester to the final exam. Nearly 40% of these students with poor performance demonstrated no notable improvement in test scores throughout the term and completed less than 32% of on-line work. Over the six semesters, the greatest benefit has consistently been observed for students who scored low on exams early in the semester but completed on-line assignments throughout with relatively high scores. Similar data compiled from A&P I classes have not been as consistent. Although assignments were also mandatory, compliance was generally lower among these students. It is possible that the more consistent results obtained in A&P II classes reflect increased motivation and maturity of students who have successfully progressed to the more
advanced level. Data will continue to be collected from A&P I classes to increase sample size and obtain more meaningful comparisons. Overall, results from A&P II classes suggest that dynamic learning modules have the potential to make a significant impact on grade improvement and academic success.

P1885
Board Number: B21
MAMS - a cell biology and interprofessional education rich bridge program to health professional school.
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The Master of Arts in Medical Sciences (MAMS) degree at Heritage University, a predominantly Native American and Hispanic-serving institution, is a one-year bridge or pipeline program to graduate health professional schools. MAMS students are typically first generation, minority, late-blooming, or disadvantaged individuals. Coursework taken alongside first-year medical students at Pacific Northwest University of Health Sciences as well as first-year pharmacy students at Washington State University coupled with standardized exam preparation, service-learning opportunities, mentorship, study strategies, and access to medical, physician assistant, and pharmacy school faculty/staff provide MAMS students with the capability to strengthen their applications as well as demonstrate their readiness for professional school. The curriculum is cellular sciences rich and focuses on the molecular basis of health and disease. Nearly one half of the coursework is cellular sciences in nature and covers topics such as histology, immunology, embryology, microbiology, biochemical metabolism, genetics, pharmacogenomics, medical physiology, and research theory. Additional instruction is provided in medical skills and the psychosocial basis of disease and treatment. Delivery of course content utilizes lectures, in-class discussion, case-based learning, small-group learning, computer-facilitated learning, and laboratory activities to maximize student comprehension of fundamental basic science principles. Since the program’s inception in 2012, 100% of students have graduated, 41% of students have been minorities, nearly 90% of graduates have gained admission into a health professional school of their choice, and 99% of MAMS students in professional schools have remained in good academic standing. MAMS students have been admitted to medical, dental, optometry, pharmacy, podiatry, and physician assistant schools. MAMS students admitted to professional schools have achieved equivalent or superior class grades and board scores compared to other admitted students. Overall student satisfaction is running approximately 88%.

P1886
Board Number: B22
Engaging community college students in an inquiry-based learning experience using NIH IRACDA postdocs expertise.
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This work highlights the positive impact of exposing community college students to inquiry-based learning activities designed by NIH IRACDA postdocs. This initiative also shares creative ways to develop postdoc teaching skills and meaningful ways to engage students in the science learning process. The postdoctoral scholars are part of the NIH IRACDA-BETTR program that Albert Einstein College of
Medicine has in partnership with Hostos Community College and Lehman College, two CUNY Hispanic-serving institutions in the South Bronx, New York. Students were exposed to state of the art technology in molecular biology and had the opportunity to gain insight into projects developed in research-intensive institutions. These activities and discussions during recitations with postdoc researchers are intended to develop student interest in science, as well as their skills in analytical and critical thinking. Specifically, we developed recitations by engaging students in case scenarios and inviting guest speakers to talk about their scientific projects. Based on this we are developing pre and post- speaker presentation questions about the research topic to generate dialogue between students and speakers, as well as discussing a relevant scientific paper using a journal-club approach. Individual surveys were used to document the impact of the initiative on the students’ interest and willingness to learn scientific content. Furthermore, lab reports and exam questions related to the above inquiry activities demonstrated the positive impact the initiative had on student skill development. Overall, this project fostered student curiosity and ability to solve problems in research settings. While we continue to develop these activities and apply them as part of the curriculum, we also aim to increase the number of visits to nearby research-intensive institutions in addition to implementing more individual undergraduate research projects and hands on activities. Together these activities will inspire the students and eventually help them to find their paths in science. Overall, this experience sheds light on the need to actively expose underserved population students to motivating STEM experiences that include young scientists with similar cultural and ethnic backgrounds.

**P1887**
**Board Number: B23**
**Bio-Bridge: A Research and Study Skills Bridge Program for Transfer Students.**
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Bio-Bridge, a bridge program targeting transfer students from local two-year institutions, has recently been implemented at Georgia Gwinnett College (GGC). The Bio-Bridge project aims to increase academic success of GGC transfer students in introductory STEM courses. To accomplish this, students participate in a semester-long research projects, under the guidance of GGC Biology seniors serving as peer mentors, with embedded lessons on study and research skills. Specifically, each Bio-Bridge participant designs and conducts an independent study investigating the antioxidant properties of a chosen herbal supplements and their effects on cultured, mammalian cells. The Bio-Bridge curriculum was piloted during the spring and fall 2016 semesters. Students gained experience in many laboratory and research techniques including literature searches, creation and analysis of graphs, experimental design, spectrophotometry, serial dilutions, redox reactions, antioxidant detection assays, and mammalian cell culture. Assessment of the curriculum was conducted using the Colorado Learning Attitudes about Science Survey (CLASS) and Introductory Molecular and Cell Biology Assessment (IMCA) surveys to gauge attitudes and understanding of introductory biology concepts, respectively. Here, we present our curricular design, analyses of the pilot study data, and future plans to grow and develop the Bio-Bridge program at GGC.
P1888
Board Number: B24
The SIE Program: Supporting Inclusive Excellence in Biology, Biochemistry, and Neuroscience.
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The Supporting Inclusive Excellence (SIE) program at Ursinus College supports student success in Biology, Neuroscience, and Biochemistry and Molecular Biology. Scholarship recipients take the same introductory sequence of Biology courses and have additional overlap in some introductory Chemistry and upper level STEM courses. The cohort nature of the program allows them to form community as they pursue challenging majors. The main goal of the scholarship program is to recruit and retain low-income and high-achieving STEM majors and prepare them for STEM careers. Specific objectives of the scholarship program include 1) Increasing the ability of low-income students to complete degrees in STEM, 2) Increasing the number of STEM graduates who are low-income and high-achieving and are also from underserved groups, 3) Providing a diversified curricular and co-curricular support structure conducive to completion of STEM degrees, and 4) Increasing the number of low-income high-achieving students who attain careers in STEM. Several support structures are already in place at Ursinus College to assist in transition to college, provide academic support through tutoring and peer-mentoring, and research opportunities. New programs have been developed including 1) A bridge program between the first and second semesters of introductory Biology during the January break, 2) A co-curricular intensive advising program to support students’ success in STEM coursework, 3) A learning circle for faculty to discuss evidence-based inclusive pedagogy in introductory biology and chemistry courses designed to improve retention of low-income students. The SIE program was founded in 2015 through an NSF STEM grant and we have matriculated two cohorts to date. The 20 scholarship students who attend Ursinus College include 80% females, 25% Pell grant recipients, 15% African Americans, 20% Latina/os, and 45% first generation college students. Four students have participated in the HHMI funded FUTURE program in the summer before or just after their first year, two students are Bonner scholars, and one student was selected to be a Fellow for the Center for Science and the Common Good (CSCG), an HHMI center at Ursinus College. CSCG Fellows explore issues at the intersection of science and society through hosting speakers, organizing discussions, doing internships, and taking courses together that explore the application of science to the common good. Thus, the SIE program is enhancing representation of students from traditionally underserved groups in research and service projects.

P1889
Board Number: B25
Yale Ciencia Academy: Leveraging a Hispanic Science Network to Enhance Graduate Biomedical Training, Career Success, and Diversity.
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Higher rates of attrition of women and minorities from scientific paths at the doctoral level are an ongoing concern. Several factors contribute to this drain of talent: (i) socio-cognitive stress (e.g. feelings of isolation); (ii) limited access to career advice networks, particularly during academic transitions; and (iii) tension between personal values and perceived culture and expectations of academic research. Studies show that minority-serving institutions and undergraduate programs that offset the sense of
isolation can increase persistence in science. Applying these interventions at later training stages and at research-intensive institutions presents obvious challenges. Through the NIH-supported Yale Ciencia Academy, we have leveraged a large and dynamic Hispanic science network, the Ciencia Puerto Rico community (www.cienciapr.org), to enhance the training and retention of minority graduate students in science. Specifically, we: 1) create online learning communities that close the geographic gap between graduate students and role models, mentors, and peers from similar cultural backgrounds; 2) provide training that addresses common gaps and provides skills for professional development; and 3) enable trainees to contribute to their community of origin through science outreach. Now in its second cohort, the Yale Ciencia Academy has recruited a total of 62 scientific role models, and 74 life and behavioral sciences graduate students from 36 universities in Puerto Rico and the U.S. to date. We are examining the impact of the program with respect to (a) enhanced feelings of competency in science and positive expectations for a scientific career; (b) larger and more focused individual professional networks; (c) competency in important academic and professional skills; and (d) awareness of social value as scientists. Preliminary results indicate high satisfaction with program components; increased science communication, scientific teaching, and mentoring and networking skills following workshop trainings; and higher confidence and clarity in career goals, higher confidence in career navigation skills, and better interactions with mentors, compared to the start of the program. Scientists participating in the program as panelists, facilitators, and instructors also rated their experience very highly.

P1890
Board Number: B26
Academic boot-camps for undergraduate anatomy and physiology and introductory biology courses: paving pathways for student success.
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During the first semester of freshmen year, biology and prehealth professional students are often enrolled in anatomy and physiology or general biology. These rigorous and fast-paced courses require students to build upon fundamental knowledge of chemistry and biology, as well as to have exceptional study science skills in order to succeed. This poses a challenge, as many students are underprepared, lacking firm foundations in science, as well as overwhelmed by the pacing and demands of college-level coursework and ownership of their own learning. As a result, these courses often result in low levels of student success with high D, F and withdraw (DFW) rates. To address these challenges and the associated impacts on student success, academic boot-camps were developed for Anatomy and Physiology and General Biology courses for students at two New York undergraduate institutions that focus on educational access to traditional and nontraditional students, including many who are racially/ethnically diverse, low income and first-generation: Mercy College and St. Francis College. The boot-camps, which took place prior to or within the first week of the semester start and were offered free of charge to the students, introduced course pacing, effective study and testing strategies, course and college resources, peer instruction and teambuilding through intensive programming. The purpose of this study was to examine the impact on and effectiveness of these academic boot-camps on student success, specifically the course DFW rates. The boot-camps resulted in improved student success. Mean exam grades were significantly higher (p<0.0001) for students who attended the bootcampus vs. those who did not attend. In addition, 88% of students who completed the full program and 72% of students who completed even a portion of the program, completed the course with a grade of B- or above vs only 30% of students who did not attend earning similar grades. 67% of the students who
While the formation of knowledge has previously drawn the interest of historians and sociologists of science, how knowledge forms remains largely unknown. This lack of understanding is perhaps best exemplified by an inability to predict “hot” research topics and the career paths of individual scientists. Here we show that biomedical research becomes predictable when one considers hundreds of properties of genes and gene products. We connect gene-specific biology with disambiguated authorship information and citation analysis for all publications since the 1970s. Using this approach, we can predict the number of publications that will appear for individual genes in MedLine in a given year, the ability of scientists to retain federal funding, and the likelihood of transitioning from first authorship positions to a role as principal investigators. We demonstrate that the number of genes contained within one publication is indicative of its probability of becoming a highly cited publication, and we explain why the majority of all human genes remains unstudied. We find that early “discover-ability” and the presence of model systems account for today’s research on individual genes and identify the gene-specific biological and chemical properties that had favored an early discovery. Finally, we demonstrate that the gene-specific historical biases of biomedical research have propagated to current computational predictions of biological function and to large, heavily used biological databases.

Although our study marks the first predictive description of any scientific discipline, we believe that its main novelty lies in the ability to equally inform policymakers and individual scientist working on specific genes.

The RNA-guided DNA endonuclease Cas9 has emerged as a powerful new tool for genome engineering. Cas9 creates targeted double-strand breaks (DSBs) in the genome. Knock-in of specific mutations (precision genome editing) requires homology-directed repair (HDR) of the DSB by synthetic donor DNAs containing the desired edits, but HDR has been reported to be variably efficient. Here, we report that linear DNAs (single and double-stranded) engage in a high-efficiency HDR mechanism that requires only ~35 nucleotides of homology with the targeted locus to introduce edits ranging from 1 to 1000 nucleotides. We demonstrate the utility of linear donors by introducing fluorescent protein tags in...
human cells and mouse embryos using PCR fragments. We find that repair is local, polarity-sensitive, and prone to template switching, characteristics that are consistent with gene conversion by synthesis-dependent strand-annealing (SDSA). Our findings enable rational design of synthetic donor DNAs for efficient genome editing.

P1893
Board Number: B30
CASTING: A rapid method to generate pooled clone libraries for functional genomics investigations.
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Yeast strain libraries have revolutionized the study of eukaryotic gene expression, protein function, localization, and protein-protein interactions. Usually, they are composed of arrayed strain collections, in which each strain has a specific modification at a different genomic locus. The creation of new genome-wide libraries, however, is prohibitively expensive, labor-intensive, and requires specialized lab-equipment to handle the strain arrays. Here, we introduce a conceptually reversed approach by using a pooled format for library construction, high-throughput functional profiling, and next-generation sequencing. By making use of low-cost pooled oligonucleotide synthesis platforms and exploiting an RNA-guided endonuclease to facilitate the precise, site-directed integration of a tagging cassette by homologous recombination, libraries can be constructed in a single reaction tube.

We applied this strategy for C-terminal tagging of open reading frames in the yeast *Saccharomyces cerevisiae*. Integration efficiency was increased several orders of magnitude compared to conventional means, allowing for a genome-wide design. An entire library could be constructed within 4 to 5 days. Quantification of expected and observed genotypes enabled us to retrieve parameters determining library heterogeneity.

Also we exemplify the analysis of such pooled clone libraries which involves their sorting (e.g. using FACS or growth competition) followed by a target enrichment next-generation sequencing protocol to characterize the identity of strains in the individual fractions.

The CASTING approach expands the functional genomics toolkit and will serve the research community as a valuable resource.

P1894
Board Number: B31
Targeted RNA Expression Profiling for Biomarker Discovery in Complex Biological Samples.
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New rapid and robust transcriptome-based methods for cellular characterization of the tumor microenvironment and biomarker discovery are required to improve prognosis and treatment of cancer and other diseases. However, challenges with current approaches for the above applications include high sample requirements, poor sensitivity, low dynamic range, and limited throughput. To address these limitations, we have developed the Driver-Map targeted RNA expression profiling assay using a genome-wide set of 19,000 validated primer pairs that leverages the sensitivity of multiplex RT-PCR with
the throughput and digital readout depth of Next-Generation Sequencing (NGS). Starting from just 10pg (single-cell) to 100ng (10,000 cells) of total RNA is sufficient to quantify over 5 orders of magnitude variation in gene expression levels with performance similar to conventional qRT-PCR. Further, the use of gene-specific primers enables direct analysis of total RNA isolate and obviates the need for globin and rRNA depletion from whole blood samples. In this study, we present the performance of the assay for immunophenotyping of immune cells in whole blood samples from sepsis patients and assess the immune responses to complex immunomodulatory stimuli in ex vivo model system. We will also present profiling results that demonstrate how this assay can be used to analyze the level of immune cell infiltration in tumor samples, and identify active pathways in tumor and xenograft samples. Preliminary studies demonstrate the assay’s unparalleled specificity and sensitivity resulting in better detection of low abundance mRNA transcripts as well as an improved cost-effectiveness for high-throughput pre-clinical applications.

P1895
Board Number: B32
Access and Discover Biological Pathway Information from Pathway Commons.
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Pathway Commons (www.pathwaycommons.org/) serves researchers by integrating data from public pathway and interaction databases and disseminating it in a uniform fashion. The knowledge base is comprised of metabolic pathways, genetic interactions, gene regulatory networks and physical interactions involving proteins, nucleic acids, small molecules and drugs. Alongside attempts to increase the scope and types of data, a major focus has been the creation of user-focused tools and resources that facilitate access, discovery and application of existing pathway information to facilitate day-to-day activities of biological researchers. For those wishing to browse and discover pathways within the collection, we offer a web-based ‘Search’ application that enables users to query by keyword and visualize ranked search results. ‘PCViz’ is a web tool that accepts gene names and returns a customizable interaction network visualization based upon pathway data resources. These complement existing desktop software add-ons linking Pathway Commons to the Cytoscape (CyPath2) network analysis tool and the R (paxtoolsr) programming language. To facilitate analysis and interpretation of experimental data - for instance, enrichment studies that distill pathway alterations from underlying gene expression changes - pathway data file downloads can be directly used in software tools such as Gene Set Enrichment Analysis. For those wishing to learn more about pathway resources and analysis, an online ‘Guide’ includes case studies and guided workflows. Ongoing development of web apps will enhance the accessibility to pathways and integrate support for visualization and interpretation of experimental data.
P1896
Board Number: B33
Single-cell protein and gene expression profiling of stem memory T cells by BD Ab-seq.
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High-throughput single-cell RNA sequencing recently emerged as a powerful tool to profile complex and heterogeneous cell populations and dynamics. However, the lack of information on protein expression can make identifying cell types that have conventionally been defined by cell-surface markers challenging, as mRNA and protein expression are often not tightly correlated. T cells in particular contain relatively low abundance of transcripts, and different T-cell subsets often exhibit highly similar transcriptional profiles.

Here we utilized BD Ab-seq, a novel protein sequencing technology enabled by oligo-conjugated antibodies, to study stem memory T cells (Tscm), a rare long-lasting memory T-cell population with stem cell-like properties. An oligo-conjugated antibody panel against immune-relevant cell-surface markers was created and used for protein profiling alongside gene expression profiling in single cells. From human PBMCs, we enriched for Tscms, naïve T cells, central memory T cells and effector memory T cells by FACS sorting. The sorted samples were labeled with antibodies barcoded with unique sample IDs, which allowed us to pool multiple samples into one single-cell experiment, and de-multiplex the samples after sequencing. The antibody-specific oligos and sample ID-specific oligos were captured, amplified and sequenced alongside mRNAs in a single workflow on BD Rhapsody™, a massively-parallel single-cell analysis system. The resulting sequencing data provided a combined output of gene expression, protein expression and sample identity.

The detection of protein expression by BD Ab-seq was highly sensitive and specific, and we observed consistent protein expression patterns when comparing BD Ab-seq data to FACS data of the same samples. The addition of protein marker expression provided more distinct and robust clustering of single-cell data compared to clustering by gene expression alone. We were also able to correlate gene expression differences between the four T-cell subsets with protein marker expression at the single-cell level to examine heterogeneity within the Tscm subset. Our study demonstrates the power of combining BD Ab-seq with RNA-seq to gain a more comprehensive understanding of cell lineage and function at the single-cell level.

P1897
Board Number: B34
Identification of Protein-Protein Interaction Hotspots in the Hippo Signaling Pathway.
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Protein-protein interactions constitute potential drug targets for cancer due to their involvement in cell signaling, but have thus far proven difficult to target for a host of reasons. One significant challenge has been identification of interaction “hotspots” due to a lack of simple, rapid methods for detection. Protein painting, a technology developed at George Mason University, is a simple, versatile tool for determining the protein-protein interactions sites of protein complexes. The solvent accessible surfaces of proteins and protein complexes can be “painted” with small, organic dyes and differential analysis used to determine the “unpainted” regions of the complex, or the interaction “hotspots”. We applied this technique to the Hippo signaling pathway, including proteins such as YAP1 (a transcriptional
regulator), TJP1 (also known as ZO-1, involved in tight junction assembly), and LATS1 (a kinase). Current research shows that inhibition of YAP can result in unintended tumor growth; therefore, disrupting inhibitory YAP complexes is a potential therapeutic strategy. We probed recombinant protein YAP complexes YAP1-TJP1 and YAP1-LATS1, and identified unique regions on each protein corresponding to interaction “hotspots”, which are evolutionarily conserved and promising sites for further inhibitor development. We also validated our method by identifying the protein interaction hotspot of the BRCT domain of BRCA1 (a DNA repair protein with mutations implicated in breast cancer development) with a peptide inhibitor, whose co-crystal structure has been solved. Future work on functional validation of identified hotspots, inhibitor design, and adaptation to whole cell models will lead to potential new therapeutic approaches to treating cancer.

**P1898**

**Board Number: B35**

**Regulation of the Mouse Alveolar Macrophage Toponome by Surfactant Protein-A.**

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We have shown that the innate host defense molecules, human (h) surfactant protein-A1 (SP-A1) & SP-A2, differentially affect the proteomic expression profile and function of alveolar macrophages (AM). Sex differences were also observed in male and female mice. In this study, we “rescued” male SP-A knockout (KO) mice with a treatment of in h the AM were harvested by bronchoalveolar lavage, plated onto slides, fixed, and frozen for later analysis. KO mice treated with vehicle alone served as controls. We analyzed the slides using the Toponome Imaging System (TIS) that enables studying the AM toponome (combination of the “proteome” and “interactome”) within intact cells. TIS is a robotically controlled serial immunostainer that enables sequential immunostaining and imaging of a single slide with multiple FITC-conjugated antibodies. The resulting images are analyzed pixel-by-pixel to identify the proteins present in each pixel. The group of proteins present in each pixel is designated as a combinatorial molecular phenotype (CMP), which represents organized protein clusters that are postulated to contribute to a specific cell function. We analyzed 19 cells from each mouse immunostained with 13 antibodies and three mice per condition. Focusing on the 20 most abundant CMPs for each cell we tabulated the incidence of each of the 13 markers studied. Phenotypes were defined as cells exhibiting similar patterns of expression of these markers. We found that the AM populations were extremely diverse and that each sample contained cells with multiple phenotypes. Of the 114 cells analyzed, no two cells were identical. However, we did find groups of cells with similar phenotypes. Some phenotypes were found predominantly in vehicle-treated KO mice, and others found mostly in SP-A1-treated mice. There were also phenotypes that were equally abundant in both groups. These patterns suggested that SP-A had both positive and negative regulatory on the incidence of some phenotypes, whereas others were unaffected by the presence or absence of SP-A. In summary, using this novel microscopic technology to analyze individual cells within populations of AM we have confirmed that AM from a given individual are very diverse, consisting of several subpopulations, and that no two cells are identical. We have also demonstrated that AM phenotype is dependent, at least in part, on the presence of SP-A in the alveolar space. These findings suggest that SP-A may be useful for the therapeutic manipulation of AM function in vivo. Supported by AHA 15IRG22890004.
P1899

Board Number: B36

Proteomic and interactome analyses of SEPT9 in MCF7 breast cancer cells reveal isoform-specific pro-oncogenic functions.
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Septins comprise a large family of GTP-binding proteins involved in a broad spectrum of cellular processes, including organization of the cytoskeleton, exocytosis, and regulation of cytokinesis. Thirteen distinct septin genes have been identified in humans, many of which exist as multiple isoform variants, that are categorized into four groups (SEPT2, SEPT6, SEPT7, and SEPT3) based on sequence similarity. Overexpression of ubiquitously expressed SEPT9, which belongs to the SEPT3 group, has been reported in various cancers, including colorectal, breast, and prostate. SEPT9 is alternatively spliced into 18 different mRNA variants, which encode protein isoforms that vary chiefly in the length and amino acid sequence of their N-terminal extension that precedes the GTP-binding domain. Differential expression of SEPT9 variants has been observed across many commonly used breast cancer cell lines, implying an isoform-specific role of SEPT9 in cancer biology. Substantial differences in the N-terminal extensions suggest unique binding partners which may provide insight into the isoform-specific roles of SEPT9. Therefore in this study, we compared the interactomes of the long-form SEPT9-i1, and short-forms-i4, and i5, in stably-transfected MCF7 human breast cancer cells using affinity purification coupled with LC-MS/MS. Our results showed isoform-specific interactomes for SEPT9i1, i4, and i5 which yielded 205, 122, and 136 unique binding partners, respectively. Isoforms 1 & 4, 1 & 5, and 4 & 5 shared 24, 30, and 86 binding partners, respectively. Notably, 94 proteins were shared among all three isoforms. Subsequent bioinformatics analyses of the isoform-specific interactomes yielded pro-oncogenic biological process enrichments for SEPT9i1, which were absent for both interactomes of SEPT9i4 and i5. Pro-oncogenic enrichments in the SEPT9i1 interactome included cholesterol/lipid biogenesis, histone methylation, centriole assembly, regulation of cell shape, nuclear envelope assembly, heat shock response, and sugar/ATP metabolism. These results point toward a biological role of the N-terminal extension of full-length SEPT9 and point to new functions and mechanisms of action for SEPT9 isoforms in cancer biology.

P1900

Board Number: B37

Novel Platform for Profiling Ubiquitin Specific Proteome.
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Ubiquitylation regulates most of cellular activities including cell cycle, differentiation, apoptosis, and stress response. The coding is encrypted by ubiquitin modification sites and chain linkages. Proteomics provides an effective approach for decoding the ubiquitin signals. Ubiquitylation is a extremely dynamic process and ubiquitylated proteins are low in abundance and stoichiometry. Enrichment of ubiquitylated proteins is essential for improve quantitative, reproducible and sensitive proteomics analysis. The common approach is to use DiGlyGly-lysine antibody to pull down ubiquitin remnant. However, ubiquitylation from ubiquitin-like proteins such as NEDD8 and ISG15 which are undistinguishable by DiGlyGly-lysine antibody. Here we demonstrate a novel ubiquitylation specific proteomics approach. This method utilizes UbiSite antibody that recognizes the ubiquitin C-terminus which creates the specificity for ubiquitin remnant. Human liver carcinoma cell line, Hep2 and T Leukemia cell line, Jurkat,
were chosen as model systems. The alteration of polyubiquitinated proteins is either induced by b-AP15, the inhibitor of a proteasome associated DUB, USP14 or bortezomib, a 26S Proteasome inhibitor. Cell lysates were digested with Lys C and immunoprecipitated with UbiSite antibody. Enriched ubiquitinated peptides were further digested with trypsin and analyzed by LC-MS/MS. The total proteome and ubiquitome were compared among these samples. In addition, the ubiquitinomes at proteins and site-specific levels were also compared with other published studies. This novel approach will facilitate discovery of pharmacological markers for drugs, and open doors for cell biologists to specifically identify patterns of poly-ubiquitylated proteins.

P1901
Board Number: B38
Development of a pharmaco-proteomics platform for monitoring changes in thrombin-mediated signaling and aggregation of human platelets treated with dabigatran.
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Dabigatran is an oral anticoagulant, and a reversible direct inhibitor of thrombin (DTI). Thrombin is known to induce the activation of platelets to aggregate by binding to and cleaving with the extracellular N-terminal domains of protease-activated receptors 1 and 4 (PAR1 and PAR4). To date many DTI have proved to be potent inhibitors of thrombin-mediated activation of platelet aggregation. Such DTI are useful drugs for managing acute coronary syndrome (ACS). Recently it was shown that dabigatran can inhibit platelet aggregation by acting directly on platelet thrombin receptors (PAR-4), by inducing a reduced expression of GPIIb/IIIa, CD63, and P-selectin on platelets after dabigatran treatment. The research presented herein further explored the molecular and cellular pathways modulated by dabigatran in the thrombin-activated human platelets from healthy donors by employing label-free proteomics approaches. We hypothesized that a global proteomics analysis of thrombin-activated human platelets treated with dabigatran would reveal the integrin-linked kinase (ILK) and integrin-mediated signaling as pathways significantly down-regulated by the DTI dabigatran. To test our hypothesis we developed a pharmaco-proteomics platform that monitors changes in protein expression profiles of platelets purified from healthy human donors, and ex-vivo activated with thrombin in the presence or absence of dabigatran. The proteomics platform employed the nanoL C-ESI MS/MS sequencing of tryptic/Glu-C/Lys-C generated peptides from platelets releasates, as well as a Q-Exactive quadrupole orbitrap mass spectrometer coupled with the label free quantification (LFQ) module provided by PEAKS 8.0 (Bioinformatics Solutions Inc.). A quantitative analysis of these biochemical pathways was accomplished with ingenuity pathway analysis (IPA; Ingenuity Systems) using the protein ratios extracted from LFQ analyses. Our hypothesis was confirmed by the bioinformatics analysis that predicted that many proteins involved in ILK and integrin signaling pathways, the actin-mediated cell signaling and cellular movement and rhoA and rhoGDI signaling were at least two-fold statistically significant down-regulated (p<0.05) in the dabigatran-treated platelets than in the control, untreated, and thrombin activated platelets. Remarkably, the LFQ analysis validated the dabigatran-mediated downregulation of some of the previously published biomarkers of the integrin mediated signaling pathways, e.g., GPIIb/IIIa, a finding which could explain the dabigatran-mediated inhibition of thrombin-activated platelets aggregation. Our results further advocate for the implementation of platelets proteomics as a reliable assay for monitoring the efficacy of selected drugs for ACS treatment.
New Technologies in Cell Biology: Education, Public Engagement and General

P1902
Board Number: B39
Quorum: Crowdsourcing image tracing through an engaging painting game.
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Recent advancements in biological research, particularly the development of high-throughput methodologies, have allowed researchers to collect huge quantities of data at prodigious rates. For data that is visual or graphical in nature, data analysis often poses a significant challenge. Segmentation or tracing of specific features within microscopic images, for example, is often difficult to completely automate due to noise and variations in sample quality and image collection. Images must often be analyzed manually, which can be a laborious and time consuming process. In order to address this issue, we have developed a game platform for crowdsourcing visual data analysis called Quorum. Quorum is an interactive, engaging painting game that allows members of the public to trace images or other visual data. As an open platform, Quorum will allow any researcher to upload images using a custom web-based interface and specify a segmentation challenge. After the images have been traced by gamers, the researchers can retrieve their analyzed data on the Quorum website. Quorum is free to use and open-source, allowing anyone to play or modify the platform.

P1903
Board Number: B40
Improving the nutritional quality of the common bean: Producing reagents for bean transformation.
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Legumes are an important source of protein for 600 million people. However, the most abundant seed proteins are low in sulfur containing amino acids. The purpose of these experiments is to improve the nutritional value of Phaseolus vulgaris, the common bean by altering the amino composition of one class of its highly abundant seed proteins, the lectin phytohaemagglutinin PHA-E and PHA-L (NCBI). We are focusing on PHA-E. Lectins are sugar-binding proteins that have antifungal and insecticidal properties, which can help the plant survive. The lectin PHA-E contains eleven isoleucine (ATC), five of which I am transforming into methionine (ATG). Negro Jamapa is one of the few common beans that can be transformed and regenerated. Negro Jamapa was germinated and DNA extracted from the leaves. PHA-E ends were modified and the gene amplified using Polymerase Chain Reactions. The product was inserted into a 2.1PCR Vector™ and modified. The modified PHA-E cassette was inserted into the binary vector with CaMV35S with mcherry or with a seed promoter with mcherry and then we transformed into Phaseolus vulgaris. Currently, we have the T0 generation for four lines with a putative modified PHA-E, and are in the process of testing T1 generation of these four lines. Future work will include verifying the expression and accumulation of the mutant PHA-E, and determining whether these modifications alter plant growth, reproduction and improve the nutritional quality of the seeds. This work was supported by a GM 063787.
**P1904**

**Board Number: B41**

The NIH Common Fund’s Extracellular RNA (exRNA) Communication Program.

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The NIH Common Fund’s Extracellular RNA (exRNA) Communication Program has been developed to address critical issues in the exRNA research. The overarching objective is to generate a multi-component community resource for sharing fundamental scientific discoveries, protocols, and innovative tools and technologies. Key components include (a) generating a reference catalogue of exRNAs present in body fluids of normal healthy individuals that would facilitate disease diagnosis and therapies, (b) defining the fundamental principles of exRNA biogenesis, distribution, uptake, and function, as well as developing molecular tools, technologies, and imaging modalities to enable these studies, (c) identifying exRNA biomarkers of diseases, (d) demonstrating clinical utility of exRNAs as therapeutic agents and developing scalable technologies required for these studies, and (e) developing a community resource, the exRNA Atlas, to provide the scientific community access to exRNA data, standardized exRNA protocols, and other useful tools and technologies.

Recent progress to be presented include showcasing new community-wide tools for researchers. ExRNA data sets are being generated and shared to be interoperable, transportable, and maximally useful to the public. Tools and technologies under development include the exRNA Atlas, RNA-seq data analysis pipelines, tools for expression quantification, and data visualization platforms. Altogether, the goal is to enable community-wide exRNA data and resource sharing.

Author list: https://commonfund.nih.gov/ExRNA/members


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**P1905**

**Board Number: B42**


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Recent technological advances in high throughput analyses of molecular, anatomic, and physiological measurements at the single cell level promise to open a new era calling for a unified brain cell census. In 2014, the NIH BRAIN Initiative awarded 10 grants to pilot classification strategies and generate data/metadata for this comprehensive brain cell census. From these pilot projects, multiple brain regions from different organisms are being studied using a variety of advanced technologies, and awardees are collaborating on defining standards to describe experiments and data sets.

In 2017, NIH expanded support for cell census data and relevant tools by launching a coordinated set of awards under the auspices of a BRAIN Initiative Cell Census Network (BICCN). The overarching goals of the consortium are to (1) create a comprehensive 3D common reference mouse brain cell atlas that integrates molecular, anatomical, and physiological annotations of brain cell types, and (2) generate reference brain cell atlases from postmortem healthy adult human and/or non-human primate brain samples.

The BICCN is composed of a group of Centers and Collaboratories supported via four companion funding opportunities: Comprehensive (U19) Center(s) that will focus on building up a comprehensive mouse brain cell atlas; A Specialized (U01) Collaboratory that will contribute cell census data for endpoints in the mouse brain not otherwise covered in the U19 Center(s); A Specialized (U01) Collaboratory that will...
begin to collect cell census data from human or non-human primate brains; and finally, the U24 BRAIN Cell Data Center (BCDC) that will integrate, visualize, and disseminate the cell census data generated by the U19 and U01 Centers and Collaboratories as well as create a brain cell knowledge base. The expected outcomes of the BICCN include: fundamental knowledge on diverse cell types and their three dimensional organizational logic in the brain; an open-access 3D digital brain cell reference atlas with molecular, anatomical, and physiological annotations of brain cell types in mouse; a comprehensive neural circuit diagram in mouse brain; reagents for cell-specific targeting; validated high throughput and low cost approaches to characterizing cell diversity in human and/or non-human primate brain samples. The BICCN will operate as a cooperative network to promote collaboration and coordination with any research entities that have similar goals. It is expected that funded projects in the BICCN will work together to achieve the overall goals. Thus, the BICCN will leverage existing atlases and common coordinate systems to facilitate collaborative efforts for the data annotation and 3D spatial mapping.

P1906
Board Number: B43
An Image-based data generation pipeline to model stem cell organization and dynamics.
.. AICS Team¹;
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We are generating tools and data to create multi-scale visual models of cell organization that capture and predict experimental observations. This relies on high replicate, dynamic image data on cell organization and activities using endogenous fluorescently tagged human induced pluripotent stem (hiPS) cells. The endogenous fluorescent tags allow us to visualize the locations and dynamics of major organelles and other cellular structures in healthy, living human cells. To capture this organization and dynamics under diverse but defined conditions, we developed a light microscopy pipeline that images each tagged hiPS cell line in a highly standardized fashion. We describe here three imaging workflows used in our imaging pipeline to study the effect of cell division, local environment and perturbations induced by pharmacologic agents with known mechanisms on the spatio-temporal localization behavior of major structures within the cell. In its current configuration, the microscopy pipeline uses Zeiss spinning disk confocal microscopy to generate 500-3000 replicate 3D images at 120x magnification for the localization of each cellular structure in relation to markers for the plasma membrane and DNA/nucleus. Lower magnification 2D time series images are used to study the dynamics of cellular structures during processes like cell division and colony formation. The microscopy pipeline applies automation and robotics for cell preparation and microscopic imaging to generate thousands of 3D image stacks or time-lapse image series every month. Image stacks or time-series are then sent to an automated image processing pipeline generating over 10,000 individually segmented 3D cells per month. To circumvent the impossible task of viewing all structures simultaneously in the same cell, we utilize computational strategies to combine image data obtained from different cell lines to produce an integrated cell model. The cell lines, data collection and integrated cell models are made available through our website (http://www.allencell.org) and can be used to visualize and understand the fundamental organization of cells and how they change in disease.
P1907
Board Number: B44
Toward the creation of the first 3D image-based data collection of drug induced signatures of endogenous fluorescently tagged human induced pluripotent stem cells lines.
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We are building predictive models of the dynamic organization and behavior of cells using, image-based 3D data sets of fluorescently tagged structures in human induced pluripotent stem cells (hiPSC). As a part of this project, we are developing a scalable and reproducible imaging pipeline that identifies signature profiles for a range of well-characterized small molecules commonly used to perturb specific cellular processes or pathways in gene-edited hiPSC from the Allen Cell Collection (see related abstract from the Institute). Our goal is to develop a systems view of the effects of these small molecules on cellular organization and dynamics. These unique data will be used to train predictive models to identify the effects of perturbing target pathways, ascertain “off-target” effects and the mode of action of unknown compounds, and identify likely pathways influenced by mutations. The pipeline was prototyped using a small suite of well-characterized compounds that include brefeldin A, paclitaxel, rapamycin, wortmannin and staurosporine. We used low-resolution imaging (24x magnification) to test a matrix of concentrations and time points for each compound of interest, allowing us to establish an initial set of conditions for each perturbation. hiPSC colonies were monitored for morphologic changes using transmitted light and an endogenously GFP-tagged structure, such as microtubules. After establishing an end point response for several compounds, high-resolution (120x magnification) imaging of multiple cell lines from the Allen Cell Collection was performed under standardized perturbation parameters, in the presence of dyes to label the nucleus and cell membrane for reference purposes. These perturbations showed alterations roughly analogous to those seen in other cell types. For example, the microtubule stabilizing agent paclitaxel increased microtubule bundle thickness and altered the shape and position of the mitotic spindle during hiPSC cell division. In addition, paclitaxel, also induced aberrant reorganization of the ER in cells undergoing mitosis, while showing minimal effects on the bulk organization of the actin bundles and cell junctions. Other drugs, such as staurosporine, a broad kinase inhibitor, had major effects on colony and cell morphology, inducing rearrangements in cell packing and shape. It also induced re-localization of desmosomes, indicating that the cell-cell junctions undergo substantial rearrangement. By building complete combinations of image-based observations of many structures/lines in the presence of a large number of standardized biochemical perturbations, we aim to generate a comprehensive database of drug signatures on hiPSC cells in their normal, pathological and regenerative (developmental) states.

P1908
Board Number: B45
Label-free ptychographic imaging reveals a role for VGSV-mediated membrane potential depolarisation in enhancing the migratory capability of breast cancer cells.
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Breast cancer is the most common cancer in women worldwide and in ~30% incidences becomes metastatic and incurable. In numerous cancer cell types, the migratory and invasive capabilities required for metastasis have been reported to be linked to the expression of functional voltage-gated sodium
channels (VGSCs). Although previous reports have highlighted that cancerous cells exhibit a more depolarised resting membrane potential (Vm) than somatic cells, no evidence exists that attributes this phenomenon to the pro-migratory action of VGSCs. Here, we utilised timelapse Ptychographic microscopy to study the role of VGVSV-mediated Vm depolarisation upon the migration of MDA-MB-321 breast cancer cells via a scratch wound assay. Ptychographic microscopy is a label-free imaging technique that retrieves the phase of light in order to yield high-contrast images of cells that are amenable to automated segmentation. The quantitative nature of the image enables changes in the cell volume of the population to be monitored, which provides an inbuilt measure of proliferation in the assay. After performing the scratch, cells were imaged for 16h in the presence of one of two drugs that hyperpolarise the Vm in distinct ways: (i) tetrodotoxin (TTX), which blocks VGSCs; (ii) NS-1619, which causes hyperpolarisation without altering the native conductance or intracellular concentration of sodium. Analysis of Ptychographic images revealed that TTX and NS-1619 treatments result in significantly longer half-times for wound closure compared to untreated control cells. No significant difference was found in the rate of change in the normalised cell volume of the population. This demonstrated that the rate of cell proliferation was unaffected by addition of the drugs; a finding that was supported by a traditional MTT assay. When considered together, these results show that TTX and NS-1619 act to reduce the migration of breast cancer cells by means of Vm hyperpolarisation. Finally, a matrigel invasion assay was performed, which showed a significant reduction in cell invasion upon treatment with TTX but not with NS-1619. This implies that Vm depolarisation is not the cause of enhanced cell invasion in vitro. Overall, cell migration and proliferation metrics extracted from images acquired by Ptychographic timelapse microscopy proved to be powerful aids in determining that the high level of motility in breast cancer cells is caused by the constitutive action of VGSCs that maintain a Vm depolarisation.

P1909
Board Number: B46
Rapid inactivation of RNaseA by high irradiance UV LEDs.
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While it has long been known that UV can inactivate enzymes (1), inactivation of RNase is an ongoing challenge for sequencing and other studies reliant upon RNA. A further challenge is the timely inactivation of RNase on laboratory equipment and surfaces. The majority of inactivation strategies require significant investment of time that could be better spent gathering data. UVLED sources can deliver higher directed radiant power than standard mercury lamps. In this study, we constructed an adjustable high intensity 275nm UVLED source and used it to expose RNaseA contaminated dry surfaces (1 µl 0.02 U/ml RNaseA) from a distance of 25 mm. The RNaseA sample was recovered from each surface in RNase free water and the suspension fluorometrically assayed for RNase activity (RNaseAlert IDT). Samples were exposed to 275 nm UVLED for various times and at different irradiances. RNaseA was totally inactivated (as compared to a negative control) with a 3 minute exposure to an irradiance of 38.5 mW/cm² at the exposed surface for a total dose of 6.94 J/cm² . Unexpectedly the same total dose at a lower irradiance 18.74 mW/cm² did not inactivate the enzyme to the same extent. Instead a total dose of 22.49 J/cm² delivered over 20 minutes was required to reach negative control levels of enzymatic activity. Further, when the irradiance of the UV LED source was varied from X mW/cm² to Y mW/cm² for a set 5 min exposure time at a fixed distance from the source, the relationship between decreasing enzyme activity and increasing irradiance was nonlinear. This is consistent with numerous studies of bio-inactivation of enzymes and microorganisms (2) that are logarithmic. We conclude that
high irradiance UVLED sources can provide faster, more complete control of enzymatic activity in such a way that is impractical with lower-intensity UV sources.
2) Chevrefils, G.; Caron, É.; Wright, H.; Sakamoto, G.; Payment, P.; Barbeau, B.; and Cairns, B. 2006. IUVA News, 8(1): 38–45.

P1910
Board Number: B47
Cold plasma eradication of Escherichia coli and Staphylococcus aureus biofilms.
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Introduction: Screws, rods and implants made of titanium are commonly used in orthopaedic surgeries and are a favorable target for bacterial colonization and biofilm formation. Bacteria in biofilms are relatively impermeable to both antibiotics and the body’s immune system. To compound the problem, the global crisis of antibiotic resistance makes infections even harder to treat; thus other solutions must be found. High-energy electrons produced within cold plasma discharges generate Reactive Oxygen and Nitrogen Species (ROS, RNS) that have antimicrobial effects by means of cell membrane leakage and DNA damage. Our goal was to test the effectiveness of cold plasma treatment on killing Gram (+) and (-) bacteria and biofilms. Methods: Escherichia coli or Staphylococcus aureus biofilms were formed on 5mm2 pieces of titanium metal before direct treatment with cold plasma of various conditions (power, gas flow, time). Indirect treatment, (saline or water treated by cold plasma then placed on the biofilm) or combinations of direct and indirect treatments were also tested. A crystal violet solution (600 ppm) was tested as a pretreatment of S. aureus. Results: Direct cold plasma treatment was applied to both E. coli and S. aureus at 24 Watts (W) and 28W of power, with a He gas flow of 5L/s for 30 sec. At 24W, E. coli showed greater susceptibility with a 103-fold decrease in the number of colony forming units per mL (CFU/mL), while S. aureus only decreased 102 fold. To determine if a crystal violet (600 ppm) solution would attract the cold plasma to the S. aureus cell wall, the titanium biofilm was applied for 1 min before the plasma treatment. This resulted in a 104 fold reduction of S. aureus, presumably due to cell wall disruption. At 28W, both Gram (+) and (-) bacteria were reduced by 104. Direct treatment of 40W at 5L/s for 10 sec, also consistently reduced both by 104 fold. Indirect treatment using plasma treated saline for 1 min had little or no effect, a 5 min treatment showed a 102 fold reduction; and 20 min session showed a 103 fold reduction. Plasma treated water for 1 min decreased bacterial levels 102 fold. Complete eradication of biofilms of both species was observed when a combination of direct and indirect treatments was tested. Conclusion: We have taken the first steps towards developing a simple and effective antibiotic-free treatment protocol to eradicate both Gram (+) and (-) bacteria in biofilms. While the cell wall provides greater protection of S. aureus from direct cold plasma treatment, higher power or the combined direct and indirect treatment eradicates both bacterial populations (at least 105 fold). Individual treatment with either cold plasma, saline, or distilled water is insufficient for complete eradication.

Monday-30
P1911

Board Number: B48
Phage assisted continuous evolution (PACE) is a powerful tool for in vivo directed evolution.
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PACE allows investigators to use directed evolution in vivo to identify new DNA sequences that encode modified proteins with a desired new function. PACE was first developed by the David Liu laboratory at Harvard University. PACE uses both positive and negative selection of filamentous phage M13 that infect \textit{E. coli}. A chemostat is used to direct mutations to phage populations instead of \textit{E. coli} host cells. Undergraduates from Davidson College and Missouri Western State University adapted PACE to evolve new riboswitches that respond to xanthine instead of theophylline. We began with two well-studied theophylline riboswitches that regulate protein production by controlling transcription or translation. We inserted each riboswitch into a modified M13 genome between the \textit{gene III} promoter and an exogenous T7 RNA polymerase (RNAP) gene that replaced the endogenous \textit{gene III}. A plasmid in \textit{E. coli} carried the essential \textit{gene III} downstream of a T7 promoter. Protein III is required for phage release from, and infection of, host cells. Students performed positive and negative selection during PACE to isolate mutated riboswitches. After PACE with the translational theophylline riboswitch, a variant that had a seven base pair deletion in the aptamer domain dominated the phage population. This variant was tested to ascertain the effect of the mutation on riboswitch function. Populations of new riboswitches taken from different M13 samples after PACE were subjected to next generation sequencing to obtain a library of variants that had evolved over the course of PACE. The populations will be tested further to determine if a xanthine specific riboswitch evolved through PACE. Our results support the claim that cell biologists can use PACE to direct the evolution of a wide variety of RNA, DNA, and protein sequences.

P1912

Board Number: B49
Nano-Channel Electroporation System for Protein Delivery into Single Cells.
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Proteins as receptors, kinases, and transcription factors are biomolecules that are particularly important for most cell signaling pathways. However, intracellular protein transfer is difficult due to its polarity and high molecular weight. We have developed a dose-controllable nanotube electroporation system consisting of PDMS (polydimethylsiloxane) and glass to accurately deliver the desired protein to mammalian single cells. A three-dimensional microfluidic channel was created using a femtosecond laser for the glass material and connected to the PDMS channel (called a Nanoinjector). We injected red fluorescent protein (RFP) into a single cell using a Nanoinjector. By controlling the duration of the electrical pulse of the Nanoinjector, we were able to control the amount of RFP injected into the cell. In addition, the protein-injected cells remained healthy. In the future, it will be possible to use the Nanoinjector to transform cells without using a viral vector or to inject macromolecular substances into cells.
P1913
Board Number: B50
Synthetic biocompatible hydrogels with user-defined spatiotemporal control to easily create structured cellular microenviroenvironments.
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Much progress has been made in the past few decades to understand how the local three-dimensional cellular microenvironment regulates cell phenotype and behavior. Recently, more complex heterogeneous hydrogel material platforms have been coupled with microfabrication tools and techniques to create structured cellular environments. These are then used to investigate cellular interactions within and between different cell populations in response to well-defined physical or chemical cues. However, these material platforms and technologies are complex, difficult to create, and limited to few research labs with the skills to develop such tools. To overcome these challenges, we have chemically modified naturally derived polysaccharides to produce a hydrogel that is shear thinning and self-healing to more easily generate structured cellular microenvironments. Alginic acid and chitosan were chemically modified using “Click” chemistry to generate copolymer hydrogel backbones with dynamic covalent cross-linking sites. The cross-linking site density was modified by adjusting the concentration of dynamic Schiff base participants to tune the stiffness and permeability of our hydrogel. To prove cytocompatibility in our hydrogel, NIH 3T3 fibroblasts were encapsulated and cultured for 24 hours within the hydrogel, and cell viability was confirmed with Live/Dead assay. We created structured microenvironments by dissecting hydrogels encapsulated with different cell populations. These dissected blocks were stacked in various geometries and the self-healing property of the hydrogel healed the cut interfaces when incubated for 40 minutes at 37°C. The result was a single hydrogel consisting of different cell populations in defined spatial orientations. With the addition of a series of orthogonal photoresponsive “Click” chemical reactions to our crosslinking moiety, we are able to create a hydrogel suitable for 3D cell culture that permits user-defined spatiotemporal control over hydrogel stiffness. This system can be integrated with microfabrication technologies to create intricate, heterogeneous hydrogel networks and complex hierarchical tissue constructs. Additionally, our biomimetic model can serve as a more sophisticated tool than current systems to elucidate the impact of various environmental cues on cellular processes, and produce more physiologically relevant \textit{in vitro} data.

P1914
Board Number: B51
Single cell RNA sequencing of zebrafish intestines reveals enhanced inflammatory signatures in chemically-induced intestinal injury.
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The current research in Inflammatory Bowel Disease (IBD) modeling has been primarily performed using the mouse system. However, mammalian systems have limited capacities to trace bacterial infection, host-cell migration, and cell biology in real-time. For these reasons, we have established a robust and cell-type specific \textit{in vivo} zebrafish model to study intestinal cellular function during inflammation in real-time. Zebrafish embryos are translucent, allowing for easy visualization of host-pathogen interactions. Additionally, they produce large clutch sizes, and many zebrafish mutant phenotypes are reminiscent of
human disease states. Specifically, utilization of the zebrafish embryo system provides the power to assess changes of the innate immune system during disease, as the zebrafish possesses exclusively innate immunity before 21 days post fertilization (dpf). It has been suggested that the interplay between host-pathogen interactions during IBD largely involves innate immune mechanisms. Using previously reported doses of dextran sodium sulfate (DSS) (Oehler et al., 2012), we established a single injury-induced model of inflammation in the zebrafish embryo. We recapitulated the shortened intestine length observed in mice in the DSS-treated fish, and also showed impaired lysosomal function in these injured embryos. However, to understand cell-type specific changes in the intestine under inflammation, in conjunction with the model established above, we show here for the first time the ability to dissect zebrafish embryonic intestines to profile baseline and inflammatory single cell transcriptomes. Embryonic intestines from wildtype and DSS-induced inflamed fish were dissected, pooled and processed to obtain single cell suspensions. We performed live and dead cell counts with Propidium Iodide, via flow cytometry analysis, which revealed 2.5 x 10^5 live single cells. Ten thousand cells per sample were processed for library preparation and single cell RNA sequencing, with an estimated targeted cell recovery of five thousand. 300 million reads were obtained from five samples, where 83% of reads were above the Q30 score. We compared our previous human single cell RNA sequencing profiles, obtained from involved and uninvolved intestinal resections, to zebrafish embryonic intestine profiles, and found gene signatures specific to the uninflamed and inflamed profiles in both organisms. We can now extend this intestine-specific single cell RNA sequencing in the zebrafish to uncover more signatures in the inflamed context, particularly those involved in innate immunity. This will aim to uncover previously unidentified cell types, molecular players and pathways that are important in understanding host-pathogen interactions during IBD-associated inflammation.

P1915
Board Number: B52
Comparison of osteogenic differentiation supported by silk fibroin films derived from various silkworms for potential bone defect treatment.
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Protein-based biomaterials are particularly useful in the biomedical field due to their unique, tunable physical properties that can be applied to numerous applications, such as in tissue regeneration and for drug delivery. Silkworm silk, which is derived from the worms’ cocoons and is composed primarily of sericin and fibroin, is an attractive biomaterial due to its versatility and mechanical stability. Specifically, we are interested in the potential of thin films composed of fibroin derived from these silks to be used as substrates for adipose-derived mesenchymal stem cells (AD-MSCs) used to treat bone defects via tissue regeneration. In many applications, these thin films are produced in water-based solutions to preserve their biocompatibility. However, the present water-based fabrication methods are time-consuming and expensive because of the silk proteins’ low solubility in water. We have developed a rapid and cost-effective new fabrication method using a formic acid-based solution to dissolve the protein. In our previous work, five silkworm cocoon-derived silks (Mori, Thai, Muga, Eri, and Tussah) and one corn protein (Zein) produced with this method were tested for cytompatibility via qualitative (Live/Dead staining) and quantitative (alamarBlue) viability assays with AD-MSCs. The Thai, Tussah, and Muga silks were found to support cell viability over 14 days and further assessed for their ability to support AD-MSC differentiation into osteocytes in order to potentially treat bone defects. AD-MSCs
were cultured both with complete osteogenic differentiation medium containing mesenchymal stem cell osteogenic differentiation supplement (MODS), to induce osteogenic differentiation, and with differentiation medium that lacked MODS, to serve as an undifferentiated control. After 20 days, osteogenic differentiation was confirmed for Tussah and Thai through positive staining with Alizarin Red S. Due to nonspecific staining with Alizarin Red S, successful differentiation for Muga could not be established conclusively. To verify that gene expression was consistent with the osteocyte phenotype, we conducted RT-PCR with ALPL, ON, Runx-2, and ZBTB16 used as gene markers for successful osteogenic differentiation. We can conclude from RT-PCR alone that the cultures induced to differentiate to osteocytes for all three silks clearly showed a much greater level of expression of ZBTB16 than the control. The levels of expression for the other markers were only slightly higher, so we are currently reconfirming our successful differentiation with RT-qPCR. Our future work includes further confirmation of successful differentiation with immunohistochemical studies targeting the products of our selected gene markers.

P1916  
**Board Number: B53**  
**3D culture of human iPSCs using Vitrogel 3D.**  
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Recently, human induced pluripotent stem cells (hiPSCs) has gained tremendous interest for its use in drug discovery, regenerative medicine, basic sciences and most importantly in disease modelling and clinical trials (like diabetes, ALS, autism, retinoblastoma, cardiomyopathies and retinal degeneration). Human iPSCs cultured in 2D has been promising, but missing a plethora of details including morphology in 3D, cell-cell contact and interaction, polarity and size. In order to better understand the disease state as is, it is of best interest to grow hiPSCs in a well-defined and simple 3D culture system. Here we show that Vitrogel 3D® provides an adequate environment to grow hiPSCs in 3D. These cells were positive for alkaline phosphatase and stem cell regulatory factors Oct4, Nanog, SOX2, SSEA3 and SSEA4. The cells differentiated into all 3 germ layers of the body (ectoderm, endoderm and mesoderm). The cells were positive for Nestin (ectoderm marker), Sox17 (endoderm marker) and Brachyury (mesoderm marker). Further studies pertaining to gene expression changes due to 3D architecture, methylation status and histone modifications of important stem cell regulatory genes will be explored. The Vitrogel 3D system can be easily utilized for 3D cultures of hiPSCs.

P1917  
**Board Number: B54**  
**Predicting the Mechanical Response of DNA Origami Structures of Different Nick Stiffness.**  
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Although the techniques of DNA origami have been applied to build nano-sized structures of a variety of geometries, ranging from rectangular boxes intended for drug delivery to robots capable of dynamic displacement, the mechanical properties of the DNA origami structures have not been carefully studied, making it difficult to predict their long-term functionality, especially in vivo. As a first step to gain insight of the mechanics of DNA origami, we aimed to understand how placement of nicks and Holliday Junctions in the DNA origami structure affect its stiffness and tensile strength in silico. Nicks, areas where one staple strand ends and another begins, and Holliday Junctions, areas on the design where a
staple strand extends from one double-helix bundle to another, introduce discontinuity thus uncertainty into origami amidst the intact double strand DNA, whose mechanical properties have been extensively examined.

CADnano software was used to designed DNA origami structures. Three sets of DNA origami structures in the shape of beams were designed in sequence. The first set of beam structures were 74 base-pairs in length and had varying cross sections of 6, 8, 10, and 12 DNA double-helix bundles. The second set of six double-helix beam structures with varying lengths (74 base pairs and 139 base pairs) were created to test for a scaling attribute. The third set of structures were created, of the same six double-helix cross section with different nick stiffness, were created, to test the effect of nick stiffness. We then simulate the process of applying stretching forces to the DNA origami structure with one end fixed using ADINA where strain responses during the pulling process were recorded. We found that DNA origami structure exhibited strain response ranging from 0.0045 to 0.022, depending on the, the number of nicks introduced, the magnitude steps and the nick stiffness, when a stretching force of 30 pN is applied. Our results departed from the conventional theoretical prediction and may explain the discrepancy between theoretical values and experimental data reported previously.

P1918
Board Number: B55
Nanoimprinted surface topology preferentially guides and enhances axon regeneration.
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Peripheral nerve injury is a common medical problem that can have devastating consequences to a patient’s life. The standard therapy for severe peripheral nerve injury is to bridge the injury site with an autograft. However, several drawbacks limit the use of nerve autografts. First, the requirement for an additional surgery to harvest nerve from the donor site may cause the loss of function at donor site. Second, the diameter or the fascicular arrangement of the nerve autograft may not match that of the injured nerve. Third, the use of autografts can sometimes lead to infection or formation of painful neuroma. For these reasons, nerve guidance conduits (NGCs) have become increasingly popular for treating patients with peripheral nerve injuries. However, most of the existing commercialized NGCs lack a scaffold to guide the outgrowth of the regenerating nerves. To address this problem, we aimed to establish a platform that can facilitate axon regeneration and guidance. We have successfully generated submicrometer-sized grooves on the surface of a biocompatible polymer using thermoplastic nanoimprint lithography (T-NIL) technology. We found that neurons from both the central nervous system (cortical neurons) and peripheral nervous system (dorsal root ganglion neurons) were able to survive on the grooved surface. Additionally, we observed that the growing direction of axons strikingly followed the submicrometer-sized grooves. We also discovered that by controlling the stiffness and the width of the grooves, this surface topology could specifically guide the axons, but not the dendrites. This specific guiding property may serve as a potential barrier to discourage the influx of unwanted non-neuronal cells into the NGC. In addition, we observed that neurons grown on the grooved surface are capable of regeneration after injury. And the grooved surface enhances the longitudinal growth of the regenerating axons compared to the flat surface. Together, our data demonstrate that the nanoimprinted submicrometer-sized grooves have a potential to guide and promote the regenerating axons.
P1919
Board Number: B56
Pharmaco-genetic toolsets for cell-specific subcellular cGMP and calcium manipulation in vivo.
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¹Vision Institute, Sorbonne Universités, CNRS, Inserm, Paris, France, ²Brain Plasticity Unit, PSL Research University, ESPCI, CNRS, Paris, France

Second messengers are mid-point relays in signaling cascades governing a wide range of cellular functions. Calcium is central in multiple cellular responses ranging from metabolism and survival to vesicle release and motility. The blockade of calcium signals currently relies on pharmacological strategies to block calcium influx into the cell or chelate extracellular or intracellular calcium. Since calcium is crucial for a wide range of signaling pathways and for the function of a wide range of components of the extracellular matrix, those strategies lack cellular specificity and are plagued with a variety of side effects when applied to patients. Similarly, cGMP is involved in a wide range of signaling pathways and cellular processes including neurotransmission, calcium homeostasis, phototransduction, lipid metabolism and cation channel activity. The diversity of these processes suggests that cGMP signals are tightly controlled in space and time to achieve specific modulation of its downstream pathways. However, manipulating cGMP is mostly achieved using pharmacological approaches either altering the synthesis or degradation of this cyclic nucleotide or manipulating downstream signaling pathways. These techniques lack both cellular and subcellular specificity.

We have developed a pair of genetically-encoded buffers that alter physiological changes in the concentration of cGMP and calcium respectively. These tools enable disrupting signaling cascades with cellular and subcellular resolution. We provide evidence that the cGMP and calcium scavengers, both in soluble form and targeted to the lipid raft or non-lipid raft compartments of the plasma membrane, are able to locally buffer changes in the respective second messenger concentration and to alter downstream cellular processes including axon pathfinding events. When in utero electroporated in the developing brain, the cGMP and calcium buffers interfere with the migration of newly generated cortical neurons in vivo and highlights a non-cell autonomous impact of second messenger manipulation. These genetically-encoded cGMP and calcium scavengers paves the way to investigate subcellularly-localized signaling in vivo, combined with cellular resolution and to directly manipulate these second messengers for therapeutic use.

Actin and Actin-Associated Proteins

P1920
Board Number: B58
Matrix remodeling and stress relaxation are impaired in vascular smooth muscle from Acta2-/- mice.
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Mutations in genes that encode the contractile proteins in vascular smooth muscle (VSM) cells causing an abnormal smooth muscle behavior have been found in thoracic aneurysm and aortic dissection.

Monday-36
(TAAD) patients. Missense mutations in ACTA2, the gene encoding smooth muscle α-actin (SMα-actin), increase susceptibility to TAAD in humans resulting from a dominant negative effect on SMα-actin filament formation. Our study investigates how dysregulation of SMα-actin affects VSM contractility in cells and aortic rings isolated from Acta2/- mice, an animal model used to study TAAD. Cells and tissue from wild-type (wt) littermates were used as control. To determine how disruption of SMα-actin affects cytoskeletal tension development, two different contractility assays in a 3D collagen matrix were performed. Acta2/- or wt cells in suspension were dispersed within rat tail collagen I gel before polymerization and attachment to 24-well plates. After 48 hours, collagen pellets were released and pellet diameter was measured over a 48-hr period, or discrete collagen fiber remodeling around the embedded cells was measured by second harmonic generation (SHG) non-linear microscopy. Results showed that collagen pellets containing Acta2/- cells contracted 2-fold less than the pellets containing the wt cells. In addition, SHG imaging revealed that Acta2/- cells remodeled the collagen matrix fibers to a lesser extent than wt cells. To determine the mechanical properties of the aorta, stress relaxation was assessed in thoracic aortic rings from Acta2/- and wt mice using a wire myograph. Aortic rings were stretched in 4 mN increments until an estimated transmural pressure of 100 mmHg was attained. Stress relaxation was measured as the magnitude of decrease in force from peak force to equilibrium after 60 sec. Piece-wise fitting of stress relaxation curves suggested two functional regimes. Significantly lower stress relaxation was recorded in aortic rings from Acta2/- mice at tensions below 10 mN compared with wt, likely driven by cytoskeletal-dependent contractility. However, no significant differences were recorded between the two animal groups above the 10mN threshold, since at higher tension the matrix-dependent contractility may be predominant. In addition, our results showed that at any given level of stretch, transmural pressure is lower in aortic rings from Acta2/- mice. Taken together, these results are consistent with Acta2/- mice having lower blood pressure compared with wt littermates and support a reduced contractile phenotype of VSM caused by the dysregulation of SMα-actin function.

P1921
Board Number: B59
“Silent code” regulation: diverse functions of closely related actin isoforms are defined by their nucleotide, rather than their amino acid sequence.
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β- and γ-cytoplasmic-actin are nearly indistinguishable in their amino acid sequence, but are encoded by different genes that play non-redundant biological roles. The key determinants that drive their functional distinction are unknown. Here we tested the hypothesis that β- and γ-actin functions in vivo are defined by their nucleotide, rather than their amino acid sequence, using targeted editing of the mouse genome. Although previous studies have shown β-actin is essential for cell migration and embryogenesis, we demonstrate here that γ-actin protein expressed by the edited β-actin gene is functionally capable of substituting for β-actin without any significant impact on organism survival. Our data show for the first time that the differences in in vivo functions of β- and γ-actin actin are determined solely by their nucleotide sequence.
P1922
Board Number: B60
Conformational landscape of actin monomers and its implications for filament assembly.
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The actin cytoskeleton a complex polymer network that gives cells structure and allows them to move and to divide. This network is constantly turning over, with the polymers assembling and disassembling on the time scale of seconds to minutes. In order to control with spatial and temporal resolution the amount of actin polymer, cells use other proteins to nucleate formation of new polymers, including the Arp2/3 complex, which forms branched actin networks, and ENA/VASP, which is involved in the formations of cell protrusions known as filopodia. Actin monomers are large globular proteins with four major domains. Monomers adopt a twisted conformation in solution, and a flat, planar conformation when in a filament. In this work, we will show how the conformational free energy landscape in terms of this macromolecular twist angle can be sampled using metadynamics simulations, and discuss our recent methodological advancements that make this computation tractable. We will then show how the conformational landscape changes when the protein is in a filament, and how this landscape changes when actin is associated with helical peptides known as WH2 domains, involved in nucleation processes such as by Arp2/3 and ENA/VASP. Finally, we will discuss how these molecular level changes might influence the thermodynamics and kinetics of filament assembly.

P1923
Board Number: B61
Characterization of CARMIL-GAP, a Dictyostelium CARMIL isoform harboring a GTPase activating domain for Rac.
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CARMILs (Capping protein Arp2/3 Myosin I Linker) are ∼1000 residue, multi-domain scaffold proteins expressed from protozoa to man that have been studied extensively with regard to their ability to bind Capping Protein (CP) and reduce its affinity for the actin filament barbed end. CARMIL proteins also appear to play important roles in signal transduction, as they exhibit genetic and physical interactions with the Rac GEF Trio (Liang et al MBoC 2009; Vanderzalm et al. Dev. 2009), and T cells lacking CARMIL-2 exhibit a profound block in signaling downstream of CD28, the major co-receptor for T cell signaling (Liang et al Nat. Immunol. 2013). In previous work (Jung et al JCB 2001), we showed that Dictyostelium CARMIL binds CP, the Arp2/3 complex and myosin I (through its SH3 domain), and it is required for actin-dependent processes such as chemotaxis and micropinocytosis to be robust. Here we describe initial studies of CARMIL-GAP, a second Dictyostelium CARMIL that contains, in addition to all the normal CARMIL domains (including the CP binding domain), a ∼130 residue insertion that, by homology, is a GTPase activating (GAP) domain for Rho-GTPases. This domain is probably functional given that full length CARMIL-GAP can only be over-expressed if it’s GAP domain contains a point mutation (R737A) that blocks GAP activity in all characterized GAP proteins. Like CARMIL, CARMIL-GAP localizes to actin-rich structures and is expressed in both vegetative and starved, developing cells. Consistently, CARMIL-GAP null cell lines created by homologous recombination exhibit pronounced defects in several actin...
based processes, including phagocytosis, motility and chemotactic aggregation. Current efforts are directed at rescuing KO cells with versions of CARMIL-GAP mutated in different domains to identify key functions, and at identifying the Rac isoform(s) regulated by CARMIL-GAP.

**P1924**
**Board Number: B62**
The Drosophila Cortactin Binding Protein 2 (CTTNBP2/CTTNBP2NL) homolog, Nausicaa, regulates actin dynamics in a Cortactin-dependent manner.
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Drosophila CG10915 is a previously uncharacterized protein coding gene with sequence similarity to human Cortactin Binding Protein 2 (CTTNBP2), a protein that interacts with both actin and microtubules for proper dendritic spine development, and Cortactin Binding Protein 2 N-terminal like (CTTNBP2NL), which redistributes Cortactin to actin stress fibers in epithelial cells. Here we have named this gene Nausicca (naus) and characterize it through a combination of quantitative live-cell TIRF microscopy, RNAi depletion, and genetics. We found that Naus co-localizes with F-actin and Cortactin in the lamellipodia of Drosophila S2R+ and D25c2 cells and this lamellipodial localization is lost following Cortactin or Arp2/3 depletion or by mutations that disrupt a conserved proline patch found in its mammalian homologs. Using Permeabilization Activated Reduction in Fluorescence (PARF) and FRAP, we find that depletion of Cortactin alters Naus dynamics leading to a decrease in the half-life of Naus in the lamellipodia by 2.1-fold and 2.2-fold, respectively. This demonstrates that appropriate localization and dynamics of Naus are regulated by interactions with Cortactin. Remarkably, we also discovered that when Naus is depleted in S2R+ cells, lamellipodial actin density increases 1.5-fold and retrograde flow speeds decrease 1.4-fold as compared to controls. In addition to these alterations to actin dynamics, Naus depletion led to a decrease in the fluorescence half-life of Cortactin by 1.3-fold, suggesting that Naus also acts to regulate Cortactin’s dynamics. Further, Naus depletion in a Drosophila imaginal disc-derived cell line, D25c2, led to decreases in migration and directionality suggesting these changes in actin dynamics also translate to changes in cell migration. Finally, we used Naus RNAi in Drosophila 3rd instar larvae neurons and observed a decreased arborization and branch length similarly to what was reported for Naus’ mammalian homolog CTTNBP2. Our results point to a possible mechanism where Naus regulates lamellipodial actin dynamics through interactions with Cortactin. We hypothesize that Naus decreases Cortactin’s off-rate possibly leading to precocious activation of the Arp2/3, increased branch nucleation, and a corresponding alteration of actin dynamics.

**P1925**
**Board Number: B63**
Mutation of arrd-15, a gene encoding an alpha-arrestin, suppresses actin disorganization phenotypes in unc-78 AIP1 mutants in C. elegans.
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Actin depolymerizing factor (ADF)/cofilin and actin-interacting protein 1 (AIP1) promote disassembly of actin filaments and play important roles in dynamic reorganization of actin cytoskeleton. AIP1
specifically targets ADF/cofilin-bound actin filaments to enhance filament severing. However, regulatory mechanisms for this reaction are poorly understood. To identify a gene that may be involved in the regulation of AIP1, we analyzed an extragenic suppressor of AIP1 mutants in the nematode C. elegans. The C. elegans unc-78 gene encodes AIP1, and unc-78 mutant worms have disorganized muscle actin filaments and move much slower than wild-type. Previously, Francis and Waterston screened for a suppressor mutant for unc-78 and isolated sup-13 (Waterston, 1988). The unc-78;sup-13 double mutants show nearly normal worm motility and have a normal appearance of sarcomeric actin filaments. The sup-13 single mutants were superficially indistinguishable from wild-type animals. Using whole genome sequencing, we identified that sup-13 is an allele of arrd-15, which encodes a previously uncharacterized arrestin-domain-containing protein (also known as a-arrestin). The arrd-15 gene contains three alternative first exons, and at least one of them was expressed in the body wall muscle as determined by a promoter-reporter analysis. In the body wall muscle, green fluorescent protein (GFP)-tagged ARRD-15 partially colocalized with vinculin and alpha-actin at the dense bodies. C. elegans has another AIP1 gene aipl-1, which is partially redundant with unc-78. Depletion of both unc-78 and aipl-1 caused embryonic lethality. However, the sup-13/arrd-15 mutation did not suppress the lethality in unc-78;aipl-1 depleted worms, suggesting that aipl-1 is required for the suppressor phenotype. The data suggest that ARRD-15/SUP-13 may be a novel regulator of AIP1-dependent actin dynamics.

P1926
Board Number: B64
The tropomyosin isoform composition of cellular actin filaments is not a simple function of relative isoform abundance.
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The actin cytoskeleton of animals and fungi contains a mixture of filaments composed of actin alone and those which are a co-polymer of actin and tropomyosin (Tpm). We have determined the absolute amounts of individual Tpm isoforms and total actin in human fibroblasts representative of 3 types of culture cells - primary, immortal and transformed. We found that all 3 cell types contain more than sufficient total Tpm to saturate every actin filament. To determine whether Tpm isoform level reflects actin filament engagement, we sub-fractionated the cells into soluble (actin filament-free) and insoluble (actin filament-associated) fractions and measured the absolute levels of Tpm isoforms and actin in each fraction. The Tpm isoforms fell into two groups in all 3 cell lines. The high molecular weight (HMW) Tpms 1.6, 1.7, 2.1 and 4.1 were primarily found in the insoluble fraction (65-85% insoluble) whereas the low molecular weight (LMW) isoforms Tpms 1.8, 1.9, 3.1/3.2 and 4.2, were primarily found in the soluble fraction (70-80% soluble). This preferential partitioning of HMW Tpms into the actin filament-containing fraction provides an explanation of how cells accommodate an excess of Tpm, viz., HMW Tpms are selectively chosen to interact with actin filaments. Furthermore, we discovered that HMW Tpms not associated with actin filaments are susceptible to degradation by the proteasome. Disruption of actin organisation with Latrunculin A led to almost complete transfer of Tpms into the soluble pool and a substantial decrease in the levels of HMW, but not LMW Tpms. The reduction in HMW Tpm levels is blocked by the proteasome inhibitor MG132. We conclude that the Tpm isoform composition of actin filaments in cells is not a simple function of relative isoform abundance and that the class of Tpm isoform, HMW vs LMW, determines its association with actin filaments as well as ability to exist in a soluble pool.
P1927
Board Number: B65
Regulatory elements within the C-terminus of human cardiac troponin T.
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Striated muscle contraction requires actin activation of myosin ATPase activity. Actin stimulates the rate of ATP hydrolysis by roughly 100-fold. Tropomyosin binding to actin lowers the ATPase activity to about 60% of the value with actin alone. Tropomyosin binds along the length of actin filaments and can occupy several positions relative to the surface of actin. One position is favorable for stimulation of ATPase activity (M state) and two others inhibit actin activation of ATPase activity (C and B states). In the absence of other effectors, tropomyosin is in rapid equilibrium between the M and C states. The addition of troponin at low calcium concentrations reduces the ATPase rate greatly as the B state is stabilized and the M state is greatly diminished. Calcium binding to troponin increases the ATPase rate but only to about the level seen with actin-tropomyosin. The addition of rigor type myosin (i.e. NEM modified myosin S1) increases the rate to about 6x that seen with actin alone. That condition gives full stabilization of the M state.

Mutations in cardiac troponin can change the calcium dependent distribution of actin states and can lead to cardiac dysfunction. Our lab previously showed that deletion of the last 14 residues of the C-terminus of troponin T (a hypertrophic cardiomyopathy mutation) eliminates the inactive B state at low calcium and increases the population of the active M state at high calcium relative to wild type troponin. The C-terminus of troponin T is therefore an important regulatory element.

We estimated changes in the distribution of actin states at low and high calcium as different segments of the C-terminus of troponin T were truncated. Those studies resolved the C-terminus of troponin T into three regions. Changes in the inactive B state at low calcium were measured using acrylodan labeled tropomyosin fluorescence. The amplitude of the signal following rapid ATP-induced dissociation of myosin S1 from regulated actin was proportional to the amount of the inactive B state. Changes in the B state were associated with truncations between residues 275-278 and 285-288 of troponin T. Changes in the active M state at saturating calcium were estimated by measuring ATPase rates relative to rates obtained with full stabilization of the M state. Residues 275-278 of Troponin T were primarily responsible for destabilizing the active state. The region 275-278 is common to both activities and the region 279-284 has little effect on either activity. The difference in residues required for each activity suggest that the C-terminal region of troponin T has different interaction partners at low and high calcium concentrations.

P1928
Board Number: B66
E-cadherin regulates cell-cell adhesion and tissue sorting by spatially regulating contractile actin filament assembly and activity.
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Recent work has identified an important function for E-cadherin as a tension-sensitive mechano-sensor in epithelial tissues. Specifically, E-cadherin directs actin filament assembly by locally activating formin, a linear actin filament assembly factor, at cell-cell contact sites in response to tension. Additionally, E-cadherin also locally activates myosin II motor protein activity at cell-cell contact sites in response to tension. Together, these activities drive the assembly and activation of contractile actin filaments.
required for adherens junction assembly. Interestingly, using E-cadherin knockdown and function blocking antibodies, we found E-cadherin expression and homophilic binding function are also required to specify cell-cell contact localized β-actin monomer synthesis. Importantly, we found the E-cadherin dose-dependent reduction in cell-cell contact localized β-actin monomer synthesis correlates with reduced adherens junction assembly. Moreover, we found the E-cadherin dose-dependent reduction in cell-cell contact localized β-actin monomer synthesis inversely correlates with tissue permeability. Mechanistically, β-actin monomer synthesis sites are specified by reversible mRNA/Zipcode Binding Protein-1 (ZBP1) interactions. The E-cadherin dose dependent reduction in cell-cell adhesion generates a tissue organization characterized by areas of high E-cadherin expressing cells and low E-cadherin expressing cells consistent with the differential adhesion hypothesis. Incredibly, perturbing the amount of localizable β-actin transcripts through genetic manipulation of the β-actin mRNA zipcode or the amount of the translational regulatory protein ZBP1 causes a similar cell sorting phenotype based on relative expression levels. Specifically, cells were likely to adhere to other cells with similar ZBP1 protein or zipcode-containing β-actin transcripts levels. In fact, ZBP1 knockdown causes cell sorting defects where cells with “high” ZBP1 expression form normal adhesions characterized by β-actin, β-catenin, and E-cadherin transcripts localization to cell-cell contact sites. By contrast, cells with “low” ZBP1 expression form disrupted adhesions characterized by delocalized β-actin, β-catenin, and E-cadherin transcripts. Together, these data demonstrate an important unappreciated function for E-cadherin in directing adherens junction formation by assembling contractile actin filaments at cell-cell contact sites by spatially localizing β-actin monomer synthesis, formin-induced linear actin filament polymerization, and myosin II motor activation in response to tissue tension to balance tissue tension with contractility.

P1929

**Board Number: B67**

Ena/VASP proteins participate in the maintenance of actin network polarity.

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Polarity in moving cells is established, in part, by the directional assembly of actin networks at the leading edge membrane. Branched actin filaments form at the leading edge due to the activity of the main actin polymerization nucleator in the lamellipodia, the Arp2/3 complex, which is activated by the WASP/WAVE family of proteins downstream of signaling in the membrane. Added to membrane-localized nucleation is the action of capping protein that limits filament growth to the vicinity of the membrane. The end result is that networks are oriented with new growth occurring predominantly beneath the leading edge membrane to push it out.

We sought to investigate how the balance between actin nucleation and capping defined actin network polarity, and how the actin polymerization enhancing protein, Ena/VASP protein, affected this balance. To do this, we used an in vitro bead system to reconstitute actin network polymerization at a surface. Beads were coated with Arp2/3 complex activators and incubated in a minimal mix of proteins for polymerization, with and without capping protein, with and without Ena/VASP. We followed the polarity of the network using a two-color actin approach. We found that Ena/VASP proteins preserved surface-directed polarity in the absence of capping protein, and increased actin network density in the presence or absence of capping protein. This mimicked what was observed when aggressive actin nucleation was produced at the bead surface by an ultra-efficient activator, suggesting that Ena/VASP indirectly increased Arp2/3 branching activity perhaps via its barbed end elongation enhancement activity. We are currently modeling our results in collaboration with theoreticians to understand how elongation enhancement by Ena/VASP could sustain actin network polarity even when capping protein concentrations are inadequate.
P1930
Board Number: B68

Characterizing the processive mechanism of Ena/VASP on diverse F-actin bundles.
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Processive Ena/VASP proteins continuously bind near actin filament barbed ends, increasing F-actin’s elongation rate and sterically inhibiting capping protein. Ena/VASP localizes to dynamic actin networks in cells, including the leading edge and tips of fingerlike projections called filopodia. Ena/VASP is known to facilitate filopodia formation and maintenance, though fundamental questions about its underlying molecular mechanism remain unanswered. We recently discovered Drosophila Ena is 2- to 3-fold more processive on F-actin bundled by the filopodial crosslinker fascin. Using multi-color TIRF microscopy, we further characterized Ena/VASP’s processivity by measuring elongation rates and residence times of Drosophila Ena, C. elegans Unc34, and human VASP on single and fascin bundled filaments. All three Ena/VASP isoforms (1) bind processively to barbed ends and increase F-actin elongation rate 2- to 3-fold, (2) have a 2- to 3-fold increase in residence time on two-filament fascin bundles, and (3) processively bind an additional 1.3- to 1.5-fold longer on fascin bundles with three or more filaments compared to two-filament bundles. Though the absolute residence times of the isoforms differ, increased processivity is conserved from worms to flies to mammals, indicating it is important for Ena/VASP’s function in cells. Strikingly, this change in processivity is not observed in the presence of α-actinin or fimbrin bundles, suggesting that enhanced processive properties are specific to bundles formed by the filopodial crosslinker fascin.

Ena/VASP is a tetramer, whose four ‘arms’ likely contribute to its increased processive run length on fascin bundles. To test the importance of DmEna’s oligomeric state on its processive properties, we designed and purified dimeric and trimeric DmEna constructs using alternative oligomerization domains. We quantified the residence time of the dimeric and trimeric DmEna constructs on single and fascin bundled filaments as well as the filopodia phenotypes of cultured Drosophila D16 cells transfected with the constructs. Both the processive run length on individual filaments and the magnitude of the increase of processivity on fascin bundles positively correlates with the number of DmEna arms. Therefore, processive behavior of Ena/VASP is dependent on the number of arms and, thus, available actin binding sites. We have discovered that Ena/VASP’s processive molecular mechanism is dependent on the crosslinker and size of actin bundles, as well as its tetrameric state.
P1931
Board Number: B69
Single Molecule Analysis of B Cell Receptor Motion during Signaling Activation.
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B cells are an essential part of the adaptive immune system. They patrol the body looking for signs of infection in the form of antigen on the surface of antigen presenting cells (APC). B cell receptor (BCR) binding to the antigen induces a signaling cascade that leads to B cell activation and spreading. During activation, B cell receptors form signaling microclusters that later coalesce as the cell contracts. We have studied the dynamics of BCRs on activated murine primary B cells using single particle tracking. The tracks obtained are analyzed using perturbation expectation-maximization (pEM), a systems-level analysis, which allows the identification of different short-time diffusive states from a set of single particle tracks. We have identified four dominant diffusive states, two of which show low diffusivities and correspond to BCRs interacting with signaling molecules. For wild-type cells, the number of BCR in signaling states increases as the cell spreads and then decreases during cell contraction. In contrast, cells lacking the actin regulatory protein, NWASP, are unable to contract and the BCR in signaling modes are sustained for longer times. PEM analysis of single molecule trajectories of CD19 indicates that this signaling coreceptor has decreased mobility and resides mostly in signaling states. Conversely, the inhibitory receptor, Fc-gamma-RIIB is found to have a higher mobility compared to BCR. These observations strengthen the notion that actin cytoskeleton dynamics modulate BCR diffusion and clustering. Our results provide novel information regarding the interactions between actin regulation, BCR motion and signaling molecules.

P1932
Board Number: B70
Twinfilin promotes Capping Protein association with actin filament barbed ends by attenuating the inhibitory effects of CARMIL.
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Nucleation and elongation of actin filaments represent the two critical control points in the cellular regulation of actin network assembly. Until recently, our view of actin elongation regulation was that the growing barbed ends of filaments were either rapidly bound by capping protein, terminating growth, or ‘captured’ by processive elongators such as formins and Ena/VASP, resulting in accelerated growth. However, work in recent years has clearly demonstrated that cells have multiple mechanisms for spatially and temporally controlling capping and elongation. CARMIL and other CPI motif-containing proteins can rapidly displace capping protein from barbed ends, and other factors can directly attenuate or enhance formin-mediated elongation. Additionally, formins and capping protein can lock horns at the barbed end, catalyzing each other’s displacement. Here, we build on this emerging view of complex molecular interplay controlling barbed end growth by addressing the functional significance of Twinfilin binding interactions with capping protein. We find that Twinfilin directly competes with CARMIL for
binding capping protein and attenuates CARMIL-mediated displacement of capping protein, enabling capping protein to remain on barbed ends. Consistent with these biochemical effects, Twinfilin silencing in B16F10 melanoma cells leads to an increase in actin assembly and in filopodia density, phenocopying the loss of capping protein. Together, these results show that Twinfilin promotes capping protein association with barbed ends by attenuating CARMIL effects, thus tuning actin network assembly at the leading edge.

**P1933**

**Board Number: B71**

**Synergy between anti-tropomyosin and anti-microtubule drugs reveals an interaction between actin and microtubule networks.**

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We have developed a new therapeutic cancer strategy based on targeting a core component of the cancer cell actin cytoskeleton, tropomyosin Tpm3.1 [1]. The first-in-class series of anti-tropomyosin (ATM) compounds prevent the cancer-associated tropomyosin Tpm3.1 from stabilizing actin filaments, leading to the collapse of the actin cytoskeleton in cancer cells [1]. We have recently demonstrated that ATM agents strongly synergize with anti-microtubule (anti-MT) chemotherapeutics that are among the most widely used front-line treatment strategies for multiple cancer types [2]. Such synergy has been observed in childhood solid tumors (e.g. neuroblastoma) and adult cancers (e.g. cervical, prostate and lung cancer) both in vitro and in vivo. Here, we examined the mechanism by which the Tpm3.1 inhibitor TR100 synergizes with the anti-MT drug vincristine (VCR) in HeLa cells, a model more suitable for imaging analysis. Isobologram analysis of the degree of synergy between TR100 and VCR in HeLa cells revealed strong or very strong synergistic combinational effects (combination index CI < 0.3 at ED50; CI < 0.02 at ED95) on inhibiting cell proliferation and inducing apoptosis. Interestingly, TR100 alone exhibited no impact on HeLa cell cycle progression, however it significantly promoted the VCR-induced cell cycle arrest of mitosis, particularly at prometaphase and metaphase. By 3D-SIM super resolution microscopy imaging and live cell imaging we observed that this drug combination disrupted spindle assembly, promoted multipolar spindle formation, and induced astral MT defects extending to the cell cortex. Given that Tpm3.1 preferentially localizes at the cell cortex during mitosis, we hypothesized that Tpm3.1 plays a role in the orientation of the mitotic spindle possibly via interacting with astral MTs or MT-associated proteins at the cell cortex. To test this hypothesis, we performed co-immunoprecipitation analysis and found that Tpm3.1 and the MT plus-end-tracking protein EB1 formed a complex in HeLa cells. Upon co-treatment with both drugs this Tpm3.1-EB1 complex was highly enriched. Such enrichment was not seen in VCR treatment at high doses, indicating that the strengthened Tpm3.1-EB1 interaction is not simply due to an increased mitotic arrest but more likely reflects a combined disruption of both the Tpm3.1-associated actin cytoskeleton and MT networks. In summary, our results highlight a novel connection between Tpm3.1-containing actin filaments and microtubules, and provides a clearer understanding of how targeting these structures may lead to improved cancer therapy.

P1934
Board Number: B73
Direct observation of actin structural deformation in response to mechanical force.
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The ability of a cell to respond to the mechanical properties of its environment (“mechanosense”) influences core cellular processes including division, migration, differentiation, and survival. Misregulation of mechanical signal transduction (“mechanotransduction”) has been implicated in malignant transformation, tumorogenesis, and metastasis, highlighting this process as a key component of cancer progression, yet the underlying molecular mechanisms remain largely unknown. Cellular mechanics are governed by an intricately coordinated contractile network consisting of the actin cytoskeleton, myosin motor proteins, and actin binding partners (ABPs). Actin filaments are flexible polymers that can adopt multiple conformational states, and recent evidence suggests that forces can regulate interactions between actin and ABPs. Here, we explore how mechanical stimuli alter the actin filament structural landscape, potentially serving as an upstream event in mechanotransduction by regulating ABP binding. We have developed a novel reconstitution system to place actin filaments under mechanical load suitable for structural studies with cryo-electron microscopy (cryo-EM). We immobilize active myosin-V (barbed-end directed motor) or myosin-VI (pointed-end directed motor) onto the film of holey-carbon cryo-EM grids, and then tether pointed-end biotinylated actin filaments onto grids such that filaments are attached to the surface through one end and free to engage with the myosin throughout the rest of the filament. Using time-lapse fluorescence microscopy, we observe morphological changes in individual actin filaments consistent with myosin V generating compression along the filament axis, while myosin VI generates tension, consistent with the directionality of the motors. Cryo-EM images reveal a novel, persistent actin structural state found in the presence of force generation that is characterized by oscillating areas of high curvature within the filament, which we term “squiggles”. Intriguingly, both compressive and tensile forces produce these “squiggle” structures, which cryo-electron tomography reveals to be 3-dimensional spirals. We hypothesize that both compression and tension introduce defects in longitudinal contacts along the filament, and that preferential propagation of defects along one strand induces squiggle formation. Resolving the structure of squiggles at high-resolution will provide insight into the molecular mechanisms of mechanically regulating actin-ABP interactions and advance the development of targeted therapeutics against specific actin conformational states.

P1935
Board Number: B72
A Novel Actin Filament Sliding and Compaction Mechanism Jointly Catalyzed by Srv2/CAP and its Interacting Partner Abp1.
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Dynamic remodeling of filamentous actin networks is a critical step in cell migration, cell adhesion, and many other actin-based processes. However, we are only beginning to understand the mechanisms used by cells to reorganize actin network architecture. Here, we describe a new remodeling activity, jointly
catalyzed by two conserved actin binding proteins that interact: Abp1 (Actin binding protein 1) and Srv2/CAP (Cyclase-associated protein). In TIRF microscopy assays, these two proteins together induce dynamic crosslinking and sliding of filaments, resulting in filament coalescence and compaction into thick bundles. This activity was observed for both the yeast and mammalian homologs of Abp1 and Srv2/CAP, and for parallel and anti-parallel filaments. Remodeling depended on direct interactions between Abp1 and Srv2/CAP, and on interactions between Abp1 and F-actin. Structurally, Srv2/CAP self-assembles into a hexameric wheel-shaped hub with six Abp1-binding sites, making the combination of the two proteins a robust actin crosslinker. Using multi-wavelength TIRF microscopy with labeled Abp1 and Srv2/CAP molecules, we directly visualized Abp1-Srv2/CAP complexes gliding diffusively along filaments and accumulating in regions of highest filament overlap, in turn leading to further coalescence. These observations define a new mode of actin network reorganization, driven by the unique molecular architecture of Srv2/CAP hexamers and their interactions with Abp1. These activities may underlie previously suggested genetic roles for yeast Abp1 and Srv2/CAP in actin organization and for mammalian Abp1 and Srv2/CAP in organizing actin filaments in sarcomeres.

P1936
Board Number: B74
Vinculin forms a directionally asymmetric catch bond with F-actin.
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Vinculin is an actin-binding protein thought to reinforce cell-cell and cell-matrix adhesions. However, how mechanical load affects the vinculin–F-actin bond is unclear. Using a single-molecule optical trap assay, we found that vinculin forms a force-dependent catch bond with F-actin through its tail domain, but with lifetimes that depend strongly on the direction of the applied force. Force toward the pointed (-) end of the actin filament resulted in a bond that was maximally stable at 8 piconewtons, with a mean lifetime (12 seconds) 10 times as long as the mean lifetime when force was applied toward the barbed (+) end. A computational model of lamellipodial actin dynamics suggested that the directionality of the vinculin–F-actin bond can potentially establish long-range order in the actin cytoskeleton. The directional and force-stabilized binding of vinculin to F-actin may be a mechanism by which adhesion complexes maintain front-rear asymmetry in migrating cells.

P1937
Board Number: B75
How do the actin- and microtubule-based transport systems communicate?
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How the actin- and microtubule-based transport systems cooperate to achieve timely and correct delivery of intracellular cargo has been subject of intense research for decades. This intricate interplay has been extensively studied using pigment organelle transport as a model. Depending on the needs of the cell, these organelles dynamically transfer between the actin and microtubule networks to achieve cell-wide redistribution, with cAMP-dependent protein kinase (PKA) acting as a key regulator of this
process. However, despite substantial efforts the molecular mechanisms governing these microtubule-and actin-based transport processes remain largely unknown. A tripartite complex comprising the membrane-bound small GTPase Rab27a, the adaptor protein melanophilin and the myosin Va motor, was previously shown to be essential for actin-dependent pigment transport in mouse melanocytes. We found that melanophilin, particularly its C-terminal actin-binding domain (ABD), is the only target of PKA in vitro. Notably, in vitro phosphorylation of the ABD closely recapitulated the previously described in vivo phosphorylation pattern. ABD’s phosphorylation, however, showed no effects on the myosin Va-mediated transport of the tripartite complex nor did it affect melanophilin’s interaction with the actin filaments. Instead, we were surprised to find that phosphorylation robustly regulated the interaction between melanophilin and microtubules. Melanophilin interacted with microtubules strictly in its dephosphorylated state via its ABD and this interaction was abolished upon PKA treatment. Remarkably, when microtubules and actin were both present, dephosphorylation was sufficient to competitively relocate melanophilin from actin to microtubules. Indeed, when actin and microtubules were present simultaneously, melanophilin’s phosphorylation state enforced track selection of the Rab27a/melanophilin/myosin Va transport complex. Collectively, our results unmasked the regulatory dominance of the melanophilin adaptor protein over its associated motor and offer an unexpected mechanism for how filaments of the cytoskeletal network compete for the moving organelles to accomplish directional transport on the cytoskeleton in vivo.

P1938

Board Number: B76

Clues to Suppressor of IKK epsilon (SIKE):cytoskeletal interactions: Binding affinities and SIKE’s dimer interface.

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Upon pathogen challenge multiple receptors both inside and on the surface of the cell, recognize pathogen associated molecular patterns (PAMPs) and initiate the production of proinflammatory, antiviral, and apoptotic responses. Pathways converge at key hubs that serve to amplify and regulate the signals, and are often responsible for determining the downstream response. TANK Binding Kinase 1 (TBK1) serves as a catalytic hub in the antiviral TLR3 mediated innate immune pathway. Suppressor of IKK epsilon (SIKE) is a recently identified high affinity alternative substrate of TBK1. It was initially found to inhibit TBK1 activation of type 1 interferon production. Upon subsequent study, it was found that SIKE was phosphorylated at six serine residues. This phosphorylation of SIKE corresponds to the activation of the antiviral response, and mediates the SIKE:TBK1 interaction. The primary function of SIKE remains unknown. Examination of SIKE’s interaction network has established direct interactions with cytoskeletal proteins including tubulin and α-actinin. These studies suggest that SIKE may play a role in cytoskeletal rearrangements associated with innate immune responses such as migration and phagocytosis. The goal of this study is to define the interaction surface of SIKE as well as the binding affinities associated with SIKE: cytoskeletal protein interactions. A quartz crystal microbalance with dissipation (QCM-D) was employed to obtain binding affinities for the SIKE:cytoskeletal protein complexes. The kon and koff values for each cytoskeletal protein were measured and KD derived from these parameters for each complex. Prior to examining SIKE:cytoskeletal protein interactions, SIKE’s oligomeric state was defined by crosslinking studies. Crosslinking with bis(sulfosuccinimidyl)suberate (BS3) has shown that SIKE is a dimer. Chemical crosslinking followed by followed by tandem mass spectrometry was employed to determine the dimeric interface of the SIKE dimer. To interpret these results, a computer model of SIKE was predicted using Modeller software. The SIKE model contains
The geometry of the tissue microenvironment determines the local mechanical stress experienced by cells. It is known that mechanical stress can regulate mechanosignaling and subsequent gene expression. For example, it has been reported that the frequency of breast cancer occurrence is higher at the mammary ducts of higher curvatures. However, the detailed mechanism by which local geometry regulates cellular behaviors is yet to be elucidated. We have thus devised a self-induced rolling membrane (SIRM) platform, a rectangular thin elastic film rolled into a cylindrical roll, to study the effect of various curvatures on cytoskeleton organization in the epithelium. The SIRM is characterized by a continuously varying curvature ranging from a radius of 50 – 500um, allowing various curvature conditions to be investigated in one experiment. First, Mouse mammary epithelial cells (EPH4-EV) were seeded and cultured for 24 hours. The SIRM was then rolled and incubated for another 24 hours to induce the curvature effects on the epithelium. We then unrolled the SIRM for high-resolution microscopy and examined the actin organization of the cells cultured at different curvatures by phalloidin staining. It was observed under fluorescence microscopy that the ratio of cytosolic actin to cortical actin difference between high and low curvature is approximately a factor of 1.2, suggesting that the curvature at the scale 50-500um radius governs the differential organization of the cytoskeleton. In summary, the SIRM is a high-throughput platform compatible with high-resolution microscopy that makes it an ideal system to study curvature effects at tissue level.

P1940

Board Number: B78
Phosphorylation of Sharpin acts as a molecular switch to control Linear Ubiquitination Assembly Complex (LUBAC) and Arp2/3 function.
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Sharpin is a multifunctional adaptor protein that, for example, inhibits integrin adhesion receptors and is part of the linear ubiquitin assembly complex (LUBAC). In addition, using the first Sharpin interactome
we showed that Sharpin promotes lamellipodium formation through direct interaction with the Arp2/3 complex (Khan et al, J. Cell Sci. 2017). Most Sharpin interactors bind the Sharpin Ubiquitin-like (UBL) domain and, therefore, Sharpin binding specificity is likely regulated by post-translational modifications. However, such modifications of Sharpin are currently unknown. Using mass spectroscopy analyses of GFP-Sharpin, pulled down from cancer cells, and recombinant GST-Sharpin, subjected to in vitro kinase assays, we identified a number of conserved residues that are phosphorylated by oncogenic kinases. Mutation of a selection of these residues identified phosphorylation sites that critically regulate Sharpin-mediated Arp2/3 activation and LUBAC function without affecting Sharpin-mediated integrin inhibition. Interestingly, one of these phosphorylation sites is located in the Sharpin UBL domain, suggesting that phosphorylation of the UBL domain acts as a molecular switch that determines which pathway is regulated by Sharpin at a given time. We now aim to develop phosphorylation-specific antibodies to further study what role these phosphorylation events play in Sharpin function and to identify the kinases that mediate these phosphorylation events. Furthermore, we will determine if Sharpin gets differentially phosphorylated under different experimental conditions. In conclusion, we identify several Sharpin phosphorylation sites in cells and show that some of these events, one of which is in the Sharpin UBL domain, regulate LUBAC and Arp2/3 function.

P1941
Board Number: B79

Molecular mechanism of LIM domain protein recruitment to stressed actin filament networks.
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The cytokinetic contractile ring and stress fibers are comprised of bundled actin filaments that often carry mechanical loads, which can cause strain events that result in bundle thinning. The stress fiber protein zyxin rapidly localizes to strain sites, and in turn recruits the F-actin crosslinker α-actinin, and elongation factor VASP, which mediate repair of the thinning actin bundle. Zyxin is a part of a large family of proteins that contain zinc finger-like LIM (Lin11, Isl-1 & Mec-3) domains, and a large fraction of this family helps regulate actin cytoskeleton network structure and dynamics, especially in relation to mechanical stresses. Zyxin LIM domains are necessary and sufficient for localization to stress fiber strain sites in mammalian cells. Similarly, the LIM domains of the fission yeast protein Pxl1 (paxillin-like) are sufficient for its localization to the contractile ring. This suggests that the mechanism used by LIM domains to recognize mechanical stress may be conserved from yeast to humans, although the underlying molecular mechanism is not understood. The LIM domains may bind F-actin directly, or additional proteins (such as myosin or α-actinin) may be required to mediate the association of LIM domains with actin filament bundles. Therefore, I have initiated in vitro reconstitution assays to elucidate the underlying molecular mechanisms by which LIM domain proteins bind to stressed actin filament bundles. High speed sedimentation assays revealed that fission yeast Pxl1 and mammalian zyxin LIM domains have only a weak affinity for binding F-actin. However, in a minimally reconstituted stressed F-actin network, the binding affinity of zyxin LIM domains increases with the addition of α-actinin and myosin-II. We therefore utilized multi-color flow TIRF microscopy to directly visualize the interaction of fluorescently labeled LIM domains of zyxin and Pxl1 with reconstituted stressed F-actin networks. With the addition of myosin to the bundled actin network, the LIM domains rapidly accumulate along the F-actin. In fact, Pxl1 LIM domains often localize to F-actin bundles immediately prior to the bundle
Beta-Actin, a 42 kDa molecular weight (MW) protein, is a housekeeping protein that is made constitutively in the cell. It is a structurally important protein playing a paramount role in the formation of actin filaments. In addition to its cellular functions, it serves as an internal control both in gene and protein expression studies and allows normalization among biological samples. That's why in numerous studies, Beta-Actin is utilized as an internal control. In this study, we reported evident production of recombinant human Beta-Actin in E. coli W3110, BL21 and M15 strains. The production of the protein involved codon optimization, gene synthesis and cloning of the optimized gene into pQE-2 expression vector for histidine tagging. Although all three strains were successfully expressed the recombinant Beta-Actin, E. coli W3110 strain produced the highest yield. The subsequent purification of the recombinant protein was performed both under native and denatured conditions. When the purification was performed under native conditions, the protein failed to bind to the column indicating that the N-terminal his tag was hidden by the native protein. Under denatured conditions, the his-tag allowed one-step purification of Beta-Actin to almost homogeneity. The chromatogram displayed a relatively sharp single peak and SDS-PAGE analysis of the peak fractions showed the presence of a major protein band with respective MW of 42 kDa. Analysis of the purified band using in-gel tryptic digestion procedure coupled with MALDI-TOF/TOF verified that the purified band indeed belonged to human Beta-Actin protein. More than half of the peptide sequence (62%) was covered by MALDI-TOF/TOF analysis. Our aim in the near future is to create sensitive and specific in-house produced monoclonal antibodies against this protein. Such antibodies will allow us to perform normalization studies in our comparative proteomic and data verification studies.
L253P ABD. This structure, along with co-sedimentation and pulsed-EPR measurements, demonstrates that high-affinity binding caused by the CH2-localized mutation is due to opening of the two CH domains. This enables CH1 to bind actin aided by an unstructured N-terminal region that becomes α-helical upon binding. This helix is required for association with actin as truncation eliminates binding. To explore the in vivo consequence of this mutation, we developed a Drosophila SCA5 model in which an equivalent mutant Drosophila β-spectrin is expressed in neurons that extend complex dendritic arborizations, like Purkinje cells targeted in SCA5 pathogenesis. The mutation causes a proximal shift in arborization, coincident with decreased β-spectrin localization in distal dendrites. We show that SCA5 β-spectrin dominantly mislocalizes components of the endogenous spectrin cytoskeleton, α-spectrin and ankyrin-2. Our data suggest that high-affinity actin binding by SCA5 β-spectrin interferes with spectrin-actin cytoskeleton dynamics, leading to a loss of a cytoskeletal mechanism in distal dendrites required for dendrite stabilization and arbor outgrowth.

P1944
Board Number: B82
Molecular mechanisms underlying formin-associated inherited deafness.
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The sensory epithelium of the inner ear contains precisely patterned cytoskeletal structures, including the paracrystalline array of actin filaments in stereocilia, the actin and microtubule meshwork of the cuticular plate that anchors stereocilia, the tight junctions that maintain ionic composition of cochlear fluids, among others. The importance of these structures for auditory function is apparent from the large number of genes encoding cytoskeletal-associated proteins that are linked to hereditary deafness. One of the first of these genes to be identified was DIAPH1, mutations in which cause autosomal dominant, non-syndromic progressive hearing loss called DFNA1. DIAPH1 is a member of the formin family of cytoskeletal regulators and, like other formins, interacts directly with the actin and microtubule cytoskeletons. Despite the great progress made in understanding the molecular basis of other forms of hereditary deafness, very little is known about the mechanisms underlying DFNA1, and more generally about the role of formins in the inner ear. We are using a biochemical approach to test various hypotheses about the etiology of hearing loss in DFNA1. There are several DIAPH1 mutations identified in affected individuals: one in the N-terminal coiled-coil domain and two in the C-terminal tail region, near the formin homology (FH) 2 domain and the diaphanous auto inhibitory domain (DAD). Our preliminary data indicate that both C-terminal mutant forms of DIAPH1 are stable in vitro and promote actin filament assembly to a similar degree as the WT protein. Further studies will focus on auto-inhibitory interactions, microtubule binding, and F-actin binding.

P1945
Board Number: B83
Actin Filament Associated Protein 1 (AFAP1) and AFAP1L1 have distinct functions in the disassembly of actin stress fibers and the formation of podosomes in response to phorbol ester stimulation.
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Podosomes are actin-rich adhesions localized on the ventral surface of several cell types, including vascular smooth muscle cells (VSMC). Phorbol esters induce podosomes in VSMC through the activation
of protein kinase C (PKC) and canonical signaling pathways. Preceding the formation of podosomes, actin stress fibers and focal adhesions disassemble with the initial dissolution of actin stress fibers occurring at their insertion into focal adhesions. Actin filament associated protein 1 (AFAP1) is localized to both actin stress fibers and podosomes and has been postulated to play a central role in both the disassembly of actin stress fibers and the formation of and lifespan of podosomes. The interaction of AFAP1 with PKCα and the subsequent activation of c-Src have been hypothesized to contribute to the function of AFAP1 in these processes. The AFAP1L1 isoform of AFAP1 shares a similar subcellular localization with AFAP1 and has also been shown to induce podosome-like structures and invadopodia. However, the contribution of AFAP1L1 in the cellular response to phorbol ester stimulation has not been determined. In this report we have examined the contribution of both proteins to the disassembly of actin stress fibers and the formation of podosomes in response to phorbol ester stimulation. By knocking down the expression of AFAP1 with siRNA and utilizing a PKCα binding deficient mutant of AFAP1, we have found that AFAP1 is not required for the dissolution of actin stress fibers in response to phorbol ester stimulation has had been previously reported, but is necessary for podosome formation. Surprisingly, we find that the knockdown of AFAP1L1 completely blocks the disassembly of actin stress fibers and the dissolution of focal adhesions and, therefore, the formation of podosomes, in response to phorbol ester stimulation. Furthermore, the cortactin-rich microdomains located at the insertion of actin stress fibers into focal adhesions, the site at which podosomes initially form in VSCM, remain intact. Overall, our findings suggest that AFAP1 and AFAP1L1 cooperate in the formation of podosomes in VSMC in response to phorbol ester stimulation and PKC activation, but have distinct roles in the process.

P1946
Board Number: B84
Investigations into the Structure and Intermolecular Interface of Human Cofilin-2 Assembled on Actin Filaments Using Magic Angle Spinning NMR.
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Actin is one of the most ubiquitous and conserved proteins and comprises one major component of the eukaryotic cytoskeleton.¹ The coflin/ADF family of proteins play an integral role as regulators of actin polymerization dynamics.² Specifically, they regulate actin filament severing and recycling of monomers in a nucleotide-dependent manner during actin treadmilling. While actin treadmilling is crucial for cell function and is responsible for up to 50% of cellular ATP consumption,³ there is currently a lack of atomic-level information regarding the structure and intermolecular interface of coflin proteins bound to actin filaments. We have previously reported atomic level insights into these assemblies in the ADP nucleotide state through the use of magic angle spinning (MAS) NMR spectroscopy.⁴ Our MAS NMR results for the F-actin binding site were in good agreement to a previous 9A cryo-EM density map,⁵ with three patches identified on coflin for F-actin binding. Other substantial structural changes that occur from coflin binding on F-actin will be clarified upon the determination of an atomic-resolution structure. Herein, we report paramagnetic NMR parameters for coflin/F-actin assemblies, which yield long-range structural information and directly report on the intermolecular interface.⁶ Specifically; we used paramagnetic relaxation enhancements (PRE’s), which we obtained by labeling F-actin with a paramagnetic spin label. In ¹³C-¹³C correlation experiments, we see several peaks corresponding to backbone and side chain atoms disappear, and decrease in intensity.
Peak intensities are correlated to proximity to the unpaired spin, and are used to derive atomic distances. We can use these in conjunction with our MAS NMR data from $^{13}$C and $^{1,6,13}$C labeled coflin as distance restraints required to derive the atomic-resolution structure of coflin/F-actin assemblies, which is currently underway.

**P1947**

**Board Number: B85**

Leiomodin-2 regulates thin filament assembly and is necessary for proper contractile force production in the hearts of adult mice.

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Leiomodin-2 (Lmod2) is a striated muscle-specific actin binding protein that has been implicated in the assembly of thin filaments. We have previously shown that Lmod2 is critical for cardiac function in neonatal mice as Lmod2 null mice die 2-3 weeks after birth with severe contractile dysfunction and enlarged ventricular chambers consistent with dilated cardiomyopathy. However, the necessity of Lmod2 in adult mice and the role it plays in the mechanics of contraction have not been studied. To answer these questions, we generated cardiac-specific conditional Lmod2 knockout mice (cKO). These mice die within a week of induction of the knockout with severe systolic dysfunction, but no change in cardiac morphology. Analysis of muscle mechanics at the fiber and single cell levels indicate a significant decrease in maximum force production and a blunting of force increase upon stretch in the cKO. Thin filament length is also substantially reduced in the cKO (~30%). Thus, Lmod2 plays an essential role in maintaining proper cardiac thin filament length in adult mice, which in turn contributes to production of contractile force.

**P1948**

**Board Number: B86**

Structural basis of actin monomer re-charging by cyclase-associated protein.

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Cyclase-associated protein (CAP) is an evolutionarily conserved actin-binding protein that is important for cell migration, morphogenesis, polarization, and endocytosis. Moreover, CAP is upregulated in many malignant cancers. CAP is a large, multi-domain protein that, in concert with coflin, promotes rapid actin dynamics by enhancing filament disassembly and monomer recycling. The most highly conserved function of CAP from Apicomplexan parasites, yeast and plants to animals is its ability to catalyze nucleotide exchange on ADP-actin monomers through its C-terminal region composed of WH2 and CARP domains. However, the structural mechanism by which CAP re-charges ADP-actin monomers with ATP, and the physiological importance of this protein-catalyzed nucleotide exchange have remained elusive. Here, by determining a 2.8 Å crystal structure of the CARP-domain of mouse CAP in complex with ADP-actin, we reveal a unique sandwich-like assembly where the CARP domain homodimer is squeezed between two actin molecules. Unlike other actin monomer binding motifs characterized so far, the CARP domain binds to the ‘back side’ of actin on subdomains 1, 2, and 3, and induces an extended D-loop conformation in actin subdomain 2. Our extensive mutagenesis and molecular dynamics simulations experiments further revealed how the adjacent WH2 domain contributes to interactions with actin.
Interestingly, the C-terminal tail of CAP is in close proximity of the sensor loop of nucleotide-binding pocket of actin, and its deletion results in severe defects nucleotide exchange activity of CAP without compromising its affinity for ADP-actin. This suggests a specific role of the CAP C-terminus in acceleration of nucleotide exchange, and allowed also to examine the physiological role of this activity. Importantly, yeast cells where the wild-type CAP was replaced by a mutant lacking the C-terminal extension displayed severe abnormalities in the organization of the actin cytoskeleton accompanied by growth and polarity defects. Collectively, our work suggests the interactions between the C-terminal tail of CAP and the nucleotide-sensor loops of actin as well as the effects of CAP on the conformation of actin monomer are important for its ability to accelerate nucleotide exchange on actin. Moreover, in vivo studies using the C-terminally deleted CAP demonstrate that proper organization of the actin cytoskeleton and cell polarity/proliferation depend on the rapid nucleotide exchange on actin, catalyzed by the cyclase-associated protein.

Regulation of Actin Dynamics 1

P1949
Board Number: B87
Bridging scales from molecules to movement: how the interplay between actin cappers, depolymerizers, fragmenters and elongators controls actin filament length.
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Actin filament barbed ends and sides are the hotspots for regulating dynamics of actin filaments. Although, most actin regulators have so far been only studied individually in vitro, it is well appreciated that complex actin dynamics seen in vivo involves a concerted interplay between the different classes of actin regulators that can simultaneously act on the same actin filament. These regulators together control the size of not only individual actin filaments but also the resulting actin networks (Carlier & Shekhar, Nat. Rev. Mol. Cell Biol. 2017).

Using a novel microfluidics-assisted single-molecule TIRF imaging technique, we can now simultaneously record the dynamics of 100’s of actin filaments and also rapidly change biochemical conditions to which the filaments are exposed to. This system has enabled us to study how multiple molecular regulators simultaneously control actin filament dynamics (Shekhar et al, Current Biology, 2017; Shekhar et al, Nat. Comms, 2015). As an example, formins, the actin filament elongators, and capping protein, the actin filament blockers, have so far been considered to bind filament barbed ends in a mutually exclusive manner. However, we have recently demonstrated that Capping protein can bind and pause a formin-bound barbed end and in the process, controls the length of individual formin-elongating filaments. These results have important implications for the control of size as well as morphology of organelles like filopodia which are formed from formin-elongating filaments (Shekhar et al, Nat. Comms, 2015).

We have also used this approach to study functional coordination between proteins that act on the filament sides and barbed ends. We have recently provided the first visual evidence that Cofilin can not only fragment actin filaments but also enhances their rate of depolymerization, thus allowing us to for the first time observe rapid treadmilling of actin filaments (Shekhar et al, Current Biology, 2017). Upon synergy with Capping protein’s long-predicted but never observed funnelling mechanism, we show that Cofilin’s severing and depolymerization can lead to sky-rocketing of actin filament growth rates. Given the technical advances in multispectral single molecule imaging as well as enabling techniques like microfluidics and micropatterning, we believe that we are now at the cusp of unravelling novel
mechanisms of how a large number of molecular machineries acting on actin filament ends and sides lead to such intricate control of cytoskeletal dynamics.

**P1950**

**Board Number: B88**

Length control of branched actin networks.

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Geometry and growth rate of branched actin networks determine cell protrusion mechanics. These highly dynamic networks maintain a steady length determined by a balance between the actin polymerization and disassembly. We study how the actin network architecture and ADF/cofilin control the interplay between the growth and disassembly rates of a reconstituted lamellipodium. We use a high-resolution surface structuration assay to reconstitute a lamellipodium in vitro, measure treadmill rates and spatial-temporal actin and cofilin distributions in the lamellipodia at varying geometry and density of the lamellipodia, and combine the in vitro analysis with mathematical modelling. We find that a significant local depletion of ADF/cofilin limits effective disassembly rates, and that the length of the actin networks is controlled by its density, width, and the ADF/cofilin-actin ratio. Based on the model, we explain how the geometry and treadmill rate of branched actin networks emerges from the complex interplay between their biochemical, structural and mechanical properties.

**P1951**

**Board Number: B89**

Distinct VASP tetramers synergize in the processive elongation of individual actin filaments from clustered arrays.

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Ena/VASP proteins are expressed in motile cells and act as actin polymerases that drive the processive elongation of filament barbed ends in membrane protrusions or at the surface of bacterial pathogens. Based on previous analyses of fast and slow elongating VASP proteins by in vitro TIRF microscopy (TIRFM), kinetic and thermodynamic measurements we established a kinetic model of Ena/VASP-mediated actin-filament elongation. At steady-state it entails that tetrameric VASP utilizes one of its arms to processively track growing filament barbed ends while three G-actin-binding sites (GABs) on other arms are available to recruit and deliver monomers to the filament tip, suggesting that VASP operates as a single tetramer in solution or when clustered on a surface, albeit processivity and resistance towards capping protein differ dramatically between both conditions. We therefore challenged the model by variation of the oligomerization state and by increase of the number of GABs in individual polypeptide chains. In excellent agreement with model predictions we show that in solution the rates of filament elongation directly correlate with the number of up to 5 free GABs in a synthetic VASP hexamer. In constructs with additional GABs filament elongation became even faster, however, the measured elongation rates increasingly deviate from the theoretical values which turned out to be caused by a new rate-limiting step in the reaction, namely the release of the end tracking arm from the growing filament. We additionally found, that release of the VASP from growing filament ends or its
processivity is dependent on the oligomerization state of the VASP as well as the affinity of its F–actin-binding site (FAB) to the filament side. Strikingly, however, irrespective of the oligomerization state or presence of additional GABs, the filament elongation on a surface invariably proceeded with the same rate as with the VASP tetramer, demonstrating that adjacent VASP molecules collectively synergize in the elongation of a single filament. Additionally, we revisited the controversial issue of profilin on VASP-mediated filament assembly and show that VASP can utilize both actin and profilin–actin. However, as opposed to formins, neither profilin I nor profilin Ila promote actin filament elongation but rather result in diminished elongation rates. Moreover, we reveal that actin ATP hydrolysis is not required for VASP-mediated filament assembly. Finally, we show evidence for the requirement of VASP to form tetramers in clusters and provide an amended model of processive VASP-mediated actin assembly in clustered arrays as found in filopodia.

P1952
Board Number: B90
Spatiotemporal Control of Actin Assembly at the Leading Edge by IQGAP.
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Cell migration requires precise spatial and temporal control of the actin cytoskeleton, which is regulated by multiple actin binding proteins. Recent work has revealed adenomatous polyposis coli (APC) is a potent actin nucleator in vitro and works collaboratively with formins to assemble actin filaments by a ‘Rocket Launcher’ mechanism (Breitsprecher et al., 2012). Further, it was found that APC-mediated actin nucleation occurs at focal adhesions, where it is critical for microtubule-induce focal adhesion turnover and directed cell migration (Juanes et al, 2017). These and other observations suggest that APC, formins, and other key binding partners such as CLIP-170 may work in concert to control actin assembly underlying cell migration. Here, we have investigated how IQGAP mechanistically influences actin assembly, alone and together with APC, formins, and CLIP-170. IQGAP is a multi-domain scaffolding protein that bundles F–actin, caps barbed ends, and binds directly to APC, the formin Dia1, and CLIP-170. Using TIRF microscopy, we have begun defining the effects of full-length IQGAP on actin assembly dynamics, alone and in the presence of these other factors. Our preliminary in vitro observations suggest that IQGAP attenuates actin assembly by APC and Dia1, and consistent with these effects, RNAi silencing of IQGAP in HeLa cells led to excessive actin accumulation at the leading edge. Thus, IQGAP may restrain unregulated actin assembly at the leading edge until specific signals are received, and in this manner spatially and temporally control cell migration.

P1953
Board Number: B91
Phase separation enhances Arp2/3 complex-dependent actin polymerization by increasing N-WASP membrane dwell time.
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Actin regulatory proteins are often organized into higher-order, micron-sized assemblies in cells. For example, the Arp2/3 nucleation promoting factor Neuronal Wiskott-Aldrich Syndrome Protein (N-WASP) is found at punctate structures such as matrix-degrading podosomes, viral induced actin comets, and the podocyte foot process. We previously developed an in vitro reconstitution system in which multivalent interactions between nephrin, Nck, and N-WASP enables their assembly and concomitant phase
separation, forming micron-scale signaling puncta on supported lipid bilayers. While we previously showed that this phase separation increased the specific activity of molecules in the pathway toward Arp2/3 complex, the molecular mechanism by which assembly and phase separation enhances activity is not known.

To understand how phase separation regulates N-WASP activity, we developed a Total Internal Reflection Fluorescence (TIRF) microscopy based assay to measure Arp2/3 complex-dependent actin polymerization on supported lipid bilayers. With 1uM Actin (5% Alexa 647), 3nM Arp2/3 complex, and 6nM CapZ, we observe rapid actin polymerization at puncta composed of nephrin, Nck, and constitutively active N-WASP. The normalized polymerization rate (polymerization rate/local N-WASP density) was significantly faster inside phase-separated membrane puncta, suggesting that the specific activity of N-WASP molecules is increased. We found that this increase in N-WASP specific activity was not due to the increased density of N-WASP molecules, but rather was due to the increased membrane dwell-time of N-WASP in puncta. We propose that N-WASP and Arp2/3 complex membrane dwell time must exceed the rate-limiting step of Arp2/3 complex activation in order for efficient actin polymerization to occur. We found that experimentally increasing N-WASP membrane dwell time through a variety of molecular perturbations was sufficient to increase its specific activity. We are currently using computational and experimental approaches to elucidate the mechanism by which N-WASP membrane dwell time is regulated in clusters. In conclusion, we’ve found that phase separation enhances Arp2/3 complex-dependent actin polymerization by increasing N-WASP membrane dwell time.

P1954
Board Number: B92
CYRIPS (Fam49) proteins are local inhibitors of Rac1 and Scar/WAVE induced lamellipodia.
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The driving forces for cell migration depend on a complex interaction between environmental cues and internally generated feedback loops. The Scar/WAVE complex provides a main control point for actin-based protrusion generation; spatiotemporal activation of this complex via activated Rac1 recruits and activates the Arp2/3 complex, triggering branched actin polymerisation. This is reinforced through a positive feedback loop, but it is unclear how the cell restricts the eventual size of a protrusion through buffering of the positive signals to cause splitting or retraction.

We have identified a highly conserved regulator of Scar/WAVE complex, CYRIPS (Cyfip related Rac interacting pseudopod splitter) from a proteomics screen. CYRIPS shows sequence similarity to CYFIP1/2 in a sequence domain of unknown function DUF1394 and myristoylation at an N-terminal conserved sequence. We demonstrate specific binding of CYRIPS DUF1394 to activated Rac1 in a region of shared homology with CYFIP proteins, establishing DUF1394 as a new Rac1 binding domain. CYRIPS-depleted cells have broad, Scar/WAVE-enriched lamellipodia and enhanced Rac1 signalling. CYRIPS-B knockout D. discoideum and mammalian cells also frequently assume a single leading pseudopod suggesting a role for CYRIPS as a local inhibitor of the positive signals that trigger actin-based motility. Conversely, CYRIPS over-expressers show fenestrated dendritic protrusions with reduced Scar/WAVE accumulation. We have identified the primary Rac1 interaction site and created mutants to demonstrate the importance of Rac1 binding in vivo.
In a 3D-cyst model, Rac1 signal is fundamental for establishment and maintenance of apical-basolateral polarity. CYRIP5-knockdown cells affect lumen formation and Scar/WAVE complex localization and these phenotypes can be partially reverted by chemical inhibition of Rac1. We conclude that CYRIP5 is a new evolutionarily conserved protein that competes with the Scar/WAVE complex for active Rac via its DUF1394 domain.

**P1955**  
**Board Number: B93**  
**The F-BAR protein Hof1 and Bud6: negative and positive regulatory components in a feedback loop controlling formin-mediated actin assembly.**  
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In S. cerevisiae, the formin Bnr1 is anchored at the bud neck, where it assembles a network of polarized actin cables that enter the mother cell compartment and direct myosin-based transport of secretory vesicles, organelles, and other cargo to the growing daughter cell (bud) to enable polarized growth. However, it is still not well understood how Bnr1 activities are controlled spatially and temporally to generate actin cables of the correct length, shape, and architecture to enable efficient trafficking and growth. We previously showed that the F-BAR protein Hof1, which colocalizes with Bnr1 at the bud neck, uses its SH3 domain to directly inhibit the FH1 domain of Bnr1 (Graziano et al, 2014). Here, we report that the F-BAR domain of Hof1 makes a distinct, complementary contribution to inhibiting Bnr1, blocking the activities of the FH2 domain. Consistent with these biochemical effects, deletion of the Hof1 F-BAR domain in vivo leads to defects in cable formation without altering Hof1 localization, as analyzed by confocal and super-resolution microscopy. In addition, purified full-length (FL) Hof1 shows substantially stronger inhibitory effects on Bnr1 ($IC_{50} < 5 \text{ nM}$) than either half of Hof1 alone. Single particle EM analysis of the Hof1-Bnr1 complex lends insights into the mechanism of inhibition. Remarkably, the potent inhibitory effects of Hof1 on Bnr1 are overcome in vitro by the addition of a second formin ligand, Bud6. Further, bud6Δ suppressed hof1Δ defects in cable morphology and vesicle transport. Super-resolution microscopy revealed that Hof1 and Bnr1 both localize in striated patterns at the bud neck, correlating with septin sub-structures. In contrast to Hof1, Bud6 localized to secretory vesicles, suggesting that it promotes the growth of the very actin cables on which it is trafficked. Taken together, we propose that Hof1 and Bud6 function as negative and positive regulatory components in a feedback loop controlling Bnr1-mediated actin cable formation.

**P1956**  
**Board Number: B94**  
**Control of yeast actin cable formation by a novel formin regulator, Bil2.**  
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Yeast actin cable formation is a tightly regulated process. Cables are continuously being polymerized at their barbed ends at $>200$ subunits per second by two formins (Bni1 and Bnr1), balanced by disassembly at their pointed ends. Further, cable length and orientation must match the cellular compartment in which they are assembled in order to optimally transport secretory vesicles to facilitate polarized growth. Previously, we characterized four different binding partners of Bni1 and/or Bnr1 (Bud6, Bud14, Smy1, Hof1), which each influence cable architecture and dynamics in unique ways. We also identified Bil1 as a new Bud6-interacting ligand required for proper cable formation (Graziano et al., 2013). Here,
we report the characterization of Bil2, a new ligand of Bud6, which has novel effects on formin regulation. In bulk and TIRF microscopy assays, purified Bil2 alone inhibited the nucleation effects of Bnr1 but not Bni1. In contrast, full-length Bud6 enhanced the nucleation activities of both formins. In the combined presence of Bil2 and Bud6, Bnr1 was inhibited, while Bni1 was stimulated. Thus, Bil2 appears to have negative regulatory effects on Bnr1, and positive regulatory effects on Bni1. In vivo, a deletion of Bil2 led to disorganized actin cable networks and defects in secretory vesicle transport. Further, we found that Bil2 and Bud6 localize to secretory vesicles. Together, these results suggest that Bil2 and Bud6 may function as components of a ‘split-feedback loop’ mechanism controlling actin cable assembly. Bni1-nucleated cables that successfully transport Bil2-containing vesicles would be reinforced by positive feedback, whereas Bnr1-nucleated cables would be dampened by negative feedback. This might explain how Bni1, despite having much weaker nucleation activity compared to Bnr1, is able to generate increasing levels of actin cables in the expanding bud compartment.

P1957
Board Number: B95
Nance Horan Syndrome-Like 1 (NHSL1) protein is a novel Ena/VASP and Scar/WAVE ligand and negatively regulates cell migration.
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Controlled cell migration is essential for development, homeostasis and wound healing, and its deregulation has detrimental consequences, which include developmental defects and cancer metastasis. Efficient cell migration requires actin polymerisation, which pushes the plasma membrane of lamellipodia, the leading edge of cells, forward. Precise fine-tuning of actin filament length and branching is important in this process and are regulated by several proteins, which include Ena/VASP for actin elongation and the Scar/WAVE-ARP2/3 complexes for actin branching (see review: Krause and Gautreau, 2014). Both Ena/VASP and the Scar/WAVE complex localise at the leading edge of cells and are key effectors of cell migration.

We have identified Nance Horan Syndrome-Like 1 (NHSL1) protein as a novel Ena/VASP ligand, which contains two Ena/VASP binding sites. NHSL1 dynamically localises to the protruding edge of lamellipodia where it co-localises with Ena/VASP proteins. We also found that NHSL1 directly interacts with the Scar/WAVE complex via the SH3 domain of Abi, a component of the Scar/WAVE complex. We mapped this interaction to three sites within NHSL1. NHSL1 co-immunoprecipitates with both Ena/VASP proteins and the Scar/WAVE complex. Interestingly, both interactions between NHSL1 and Ena/VASP or Scar/WAVE are positively regulated by active Rac, which is a key regulator of cell migration. Surprisingly, we found that NHSL1 significantly decreases 2D random cell migration speed and persistence. Using specific NHSL1 mutants, which cannot bind to Ena/VASP, Scar/WAVE or both, we revealed that on 2D substrates, NHSL1 negatively controls random cell migration speed via Scar/WAVE but not Ena/VASP proteins.

We have previously shown that Lamellipodin, another leading edge protein, which interacts with Ena/VASP and Scar/WAVE, positively regulates 2D cell migration via Scar/WAVE and 3D migration via both, Ena/VASP and Scar/WAVE (Law et al., 2013; Carmona et al., 2016). Here, NHSL1 interaction with Scar/WAVE may provide a negative feedback loop important for the temporal and spatial fine-tuning of lamellipodia protrusion for timely and controlled cell migration.
P1958
Board Number: B96
Rapid and dynamic arginylation of the leading edge β-actin is required for cell migration.
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β-actin plays key roles in cell migration and is one of the most ubiquitous, abundant, and essential proteins in eukaryotic cells. Our previous work demonstrated that β-actin in migratory non-muscle cells is N-terminally arginylated and this is required for normal lamellipodia extension. Mouse embryonic fibroblasts lacking arginyltransferase (Ate1) show impairments in migration rates and leading edge function, but the exact role of beta actin in this process is not understood. Here we examined the function of β-actin arginylation in cell migration. We found that arginylated β-actin is concentrated at the leading edge of lamellipodia and that this enrichment is abolished after serum starvation as well as in contact-inhibited cells in confluent cultures, suggesting that arginylated β-actin at the cell leading edge is coupled to active migration. Arginylated actin levels exhibit dynamic changes in response to cell stimuli, lowered after serum starvation and dramatically elevating within minutes after cell stimulation by re-addition of serum or lysophosphatidic acid (LPA). This dynamic changes are not seen in confluent contact-inhibited cell cultures and require active translation. Microinjection of arginylated actin antibodies into cells severely inhibits their migration rates. Together, these data strongly suggest that arginylation of β-actin is a tightly regulated dynamic process that occurs at the leading edge of locomoting cells in response to stimuli and is integral to the signaling network that regulates cell migration.

P1959
Board Number: B97
Ras activity stabilizes the actin fusion focus at cell contact sites prior to cell-cell fusion.
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In fission yeast Schizosaccharomyces pombe, two cells of opposite mating type fuse during the sexual life cycle to form a zygote. This process requires the activation of pheromone signaling that engages a GPCR-Ras-MAPK cascade to trigger sexual differentiation. Cell-cell fusion further necessitates local cell wall digestion, which relies on the stabilization of a dedicated actin-based aster, the fusion focus. This structure underlies the concentration of the pheromone secretion and perception machineries at a focal point. Reciprocally, local activation of the downstream MAPK signaling cascade spatially constrains the focus, leading to its immobilization at facing locations in the two partner cells, thus providing a positive feedback for cell-cell fusion. By constructing a live-reporter of active Ras1 (Ras1-GTP), we discovered that Ras1 is active at polarity sites, with activity peaking on the fusion structure just prior to fusion. Remarkably, constitutive Ras1 activation promoted fusion focus stabilization and fusion attempts irrespective of cell-cell pairing, leading to cell lysis. Ras1 activity is restricted by the GTPase activating protein (GAP) Gap1, itself recruited to sites of Ras1-GTP. While the GAP domain of Gap1 on its own does not suffice for this localization, its recruitment to Ras1-GTP sites is essential to block untimely fusion attempts. We propose that negative feedback control of Ras activity restraints the MAPK signal and couples fusion with cell-cell pairing.
P1960
Board Number: B98
Deconvolution of subcellular protrusion heterogeneity reveals the role of VASP in accelerating cell protrusion.
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Cell protrusion is morphodynamically heterogeneous at the subcellular level. However, the mechanistic understanding of protrusion activities is usually based on the ensemble average of actin regulator dynamics at the cellular or population levels. Here, we establish a machine learning-based computational framework called HACKS (deconvolution of Heterogeneous Activity Coordination in cytoskeleton at a Subcellular level) to deconvolve the subcellular heterogeneity of lamellipodial protrusion. HACKS identifies distinct subcellular protrusion phenotypes hidden in highly heterogeneous protrusion activities and reveals their underlying actin regulator dynamics. The association between each protrusion phenotype and the actin regulator dynamics is further corroborated by predicting the protrusion phenotype based on actin regulator dynamics. Using our method, we discovered the hidden rare ‘accelerating’ protrusion phenotype in addition to ‘fluctuating’ and ‘periodic’ protrusions. Intriguingly, the accelerating protrusion was driven predominantly by VASP-mediated actin elongation rather than by Arp2/3-mediated actin nucleation. Our analyses also suggested that VASP controls protrusion velocity more directly than Arp2/3 complex, thereby playing a significant role in the accelerating protrusion phenotype. Taken together, we have demonstrated that HACKS allows us to discover the fine differential coordination of molecular dynamics underlying subcellular protrusion heterogeneity via a machine learning analysis of live cell imaging data.

P1961
Board Number: B99
Coordinated formation and disassembly of a contractile actomyosin network mediates content release from large secretory vesicles.
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Coordinated formation and disassembly of contractile actin-based structures has been shown to underlie diverse settings of tissue morphogenesis. Here we propose that such a mechanism mediates the contractile activity necessary for content release from large secretory vesicles. A common feature of these systems is formation of an actin coat around each vesicle following their fusion with the apical cell membrane and recruitment of myosin, which together mediate the forces necessary for vesicle contraction. Using live imaging of cultured Drosophila larval salivary glands, an established model for such secretory systems, we have followed the dynamics of actomyosin coat formation and content release from glycoprotein (“glue”)-filled vesicles into the gland lumen. We previously demonstrated critical regulatory roles for the Rho1 GTPase in both actin coat formation (via activation of the Formin-family protein Diaphanous) and Myosin II-based contraction (via Rho kinase). Surprisingly, we have now found that disassembly of the actin coat, which accompanies vesicle content release, is necessary for contraction of the actomyosin network. This process was monitored using secretion-arrested vesicles, and found to be dependent on Rho1 inactivation, mediated by a dedicated RhoGAP and branched-actin
polymerization. The sequential temporal recruitment of active Rho and its inhibitors is evident by cycles of active Rho1 and actin coat accumulation and depletion in such vesicles, implying that a feedback-based mechanism regulates actin coat disassembly from the vesicle surface. Contraction-driven content release, the final step of this form of exocytosis, is therefore achieved by coordinating formation and disassembly of the contractile machinery.

**P1962**

**Board Number: B100**

**Simultaneous Imaging and Functional Studies Reveal a Tight Correlation Between Calcium and Actin Networks.**

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Tip growing cells elongate in a highly polarized manner via focused exocytosis. Calcium and actin have been implicated as vital components of a feedback loop regulating the deposition of cell wall material. However, establishing a mechanistic understanding of the interaction between calcium, actin and growth in vivo has remained challenging. Here, we investigated the calcium-actin interplay in the moss Physcomitrella patens, which provides a system with an abundant source of genetically identical tip-growing cells, excellent cytology, and a large molecular genetic tool kit. To visualize calcium, we used a genetically encoded cytosolic FRET probe, revealing a fluctuating tipward gradient with a complex oscillatory profile that was analyzed with wavelet analysis. Wavelet analysis enabled the quantitative comparison of the calcium behavior in cells where growth was inhibited mechanically, pharmacologically, or genetically. We found that cells with suppressed growth have calcium oscillatory profiles with longer frequencies, suggesting that there is a feedback between the calcium gradient and growth. To investigate the mechanistic basis for this feedback, we simultaneously imaged cytosolic calcium and actin. We found that high cytosolic calcium promotes disassembly of a tip-focused actin spot, while low calcium promotes assembly. In support of this, abolishing the calcium gradient resulted in dramatic actin accumulation at the tip. Together these data demonstrate that tip-ward calcium is quantitatively linked to actin accumulation in vivo and that the moss P. patens provides a powerful system to uncover mechanistic links between calcium, actin and growth.

**P1963**

**Board Number: B101**

**CAP2 loss activates MRTF/SRF signaling in cardiomyocytes.**

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Sudden cardiac death kills 180,000 to 450,000 Americans annually, predominantly males. A locus that causes developmental disorders and confers a risk for sudden cardiac death and conduction disease is located at 6p22, near the cytoskeletal gene, CAP2. CAP2 regulates actin dynamics in the cytoskeleton by binding actin monomers and assisting filament severing to modulate the balance of filamentous actin (F-actin) and globular actin (G-actin). To determine the role of CAP2 in vivo, we generated whole-body knockout (KO) mice and conditional KO models. CAP2 KO in both models show evidence of cardiac conduction disease and all the cardiomyocyte specific KO mice died suddenly due to complete heart...
block by 26 wks of age. RNA-seq analysis of CAP2 KO hearts revealed over-activation of MRTF/SRF regulated genes such as Myl-9 and Acta-2. The over-activated SRF signaling is specific for heart tissue as well as isolated cardiomyocytes and seen in mice of all ages tested. To test if we could treat CAP2 KO mice, we synthesized and tested an SRF transcription inhibitor CCG-1423-8a. CCG-1423-8a inhibited MRTF translocation in MEF cells and the signaling of an SRF luciferase reporter cells. When tested in mice, CCG-1423-8a reduced expression of Myl-9 and Acta-2 in the heart. Our study suggests that MRTF/SRF may be targeted in some forms of sudden cardiac death and cardiac conduction disease.

**Actin-Membrane Interactions**

**P1964**

**Board Number: B102**

Arp 2/3-dependent spatial organization of the B cell receptor (BCR) impacts immune synapse formation, BCR signaling output, and B cell activation.

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Contact-dependent cell:cell communication is a critical component of many biological processes from development to tissue maintenance to the immune system. The immune synapse (IS) is a contact-dependent cellular communication platform that is important for activating, amplifying and executing immune responses. A B cell IS is formed when antigens displayed on the surface of an antigen-presenting cell (APC) are recognized by the B cell receptor (BCR), triggering dynamic, multi-scale reorganization of the BCR and associated signal transduction machinery. Initially, BCRs are gathered into microclusters that are dispersed throughout the B cell:APC contact site, which are then coalesced into a supramolecular activation cluster (cSMAC) at the center of the contact site. Although the clustering of BCRs into microclusters and the recruitment of signaling enzymes into microsignalosomes increases BCR signaling efficiency, the molecular mechanisms controlling BCR organization during IS formation are not fully understood. Importantly, how the spatial patterning of the BCR promotes the translation of extracellular information (recognition of membrane-associated antigens) into functional outcomes (B cell activation) is not clear. By imaging the B cell:APC contact site in real time, we showed that inhibition of the actin-related protein 2/3 (Arp2/3) complex, which nucleates the formation of branched actin networks, had no effect of BCR microcluster formation but prevented the coalescence of dispersed BCR microclusters into an immune synapse cSMAC. Based on this finding, we tested the hypothesis that this dynamic spatial patterning of the BCR optimizes BCR signaling and the B cell response to membrane-associated antigens. Using both Arp3 siRNA and the Arp2/3 inhibitor CK-666, we found that cSMAC formation is important for amplifying initial BCR signaling reactions that are stimulated by APC-bound antigens. Additionally, Arp2/3-dependent cSMAC formation is important for APC-associated antigens to stimulate BCR-induced transcriptional responses and upregulation of the activation CD69 and CD86. In contrast, Arp2/3 function is dispensable for BCR signaling and subsequent functional response to non-polarized soluble antigens. Therefore, our data indicate that the dynamic spatial patterning of BCRs is controlled by Arp2/3-dependent branched actin networks and that this is important for B cell responses to spatially-restricted antigens that are presented to B cells by APCs. Overall, our work shows that the spatial patterning of receptors can encode information that impacts cellular decision-making.
**P1965**

**Board Number: B103**

A geodesic septin lattice is required for actomyosin contractility on micron-scale curved membranes in vivo.

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The ability of cell membranes to remodel into various curved configurations is fundamental for processes from cytokinesis to intracellular trafficking. Generation of membrane curvature by proteins is an important biological concept that is well studied in nanometer-scale curved membranes such as endocytic vesicles, where curvature-generating proteins include clathrin, and BAR-domain proteins. However, little is known about the proteins or mechanisms driving the formation and dynamics of larger, micron-scale membrane curvature within the cell such as around the cytokinetic furrow, secretory vesicles, or the ciliary base.

To address this question under physiological conditions we used intravital subcellular microscopy (ISMIC) in live mice to study regulated exocytosis in specialized secretory cells of the salivary gland. During this process large (~1.5 μm) vesicles, called secretory granules (SGs), containing proteinaceous cargo fuse with narrow, tube-like canaliculi at the apical plasma membrane (APM) after GPCR stimulation. A fusion pore then forms to allow flow of SG contents into the canaliculi, and integration of the SG membrane into the APM. The canalicular diameter (~0.3 μm) is almost an order of magnitude smaller than the SG diameter, making membrane integration energetically unfavorable. Thus, we asked how the SG membrane is forced into the APM forming the narrow canaliculi, and found that F-actin and non-muscle myosin (NMII) localize to the fused SG membrane and provide the forces needed for integration. We then asked, how these proteins are recruited to and organized on the curved membrane. Using knockin mice expressing GFP-NMIIA and superresolution microscopy, we describe for the first time that F-actin and NMII organize around SGs as a striking geodesic lattice with triskelia-like tripolar vertices, reminiscent of the clathrin cage around endocytic vesicles. NMIIA and actin within the lattice are partially offset, consistent with the lattice being contractile. We also show that septins, which recognize membrane-scale curvature, also form lattices around SGs, that are in register with NMIIA. Disruption of F-actin assembly causes expansion of SGs, but surprisingly does not impair recruitment of NMII or septins. Pharmacological inhibition of septin, however, results in a significant decrease in activated NMII and myosin light chain kinase (MLCK) on fused SGs.

Based on our data, we propose that the septin-lattice 1) acts as a scaffold to recruit the actomyosin network to the surface of SGs, and 2) is needed for NMII activation, likely through MLCK-mediated phosphorylation. This novel septin-actin-NMII lattice on the SG surface provides new structure-function insights into protein-driven remodeling of micron-scale curved membranes in vivo.
P1966
Board Number: B104
Inflammatory-sensitive Myosin-X functionally supports leukocyte extravasation by Cdc42-mediated ICAM-1-rich endothelial filopodia formation.
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Leukocyte transendothelial migration is key to inflammation. From rolling, leukocytes transfer to adhesion where they firmly bind to the inflamed endothelium followed by diapedesis through the endothelium. Upon inflammation, endothelial cells express small finger-like protrusions that stick out into the lumen. The function and regulation of these structures are unclear. We present evidence that these ICAM-1- and F-actin-rich endothelial finger-like protrusions are filopodia and function as adhesive structures for leukocytes to transit from rolling to crawling, but are dispensable for diapedesis. Mechanistically, these structures require the motor function of Myosin-X, the activity of the small GTPase Cdc42 and PAK4. Moreover, Myosin-X expression is under control of TNFα-mediated c-jun N-terminal kinase activity and upregulated in human atherosclerotic regions. Ex vivo en face and intravital imaging of GFP-positive inflamed murine vessels revealed the filopodia phenotype. This is the first study identifying the regulation of endothelial filopodia being crucial for leukocyte extravasation and could therefore play an important role in the progression of atherogenenesis.

P1967
Board Number: B105
Type I myosin-mediated coupling of actin assembly to the plasma membrane during clathrin-mediated endocytosis.
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Interaction of the actin cytoskeleton with cellular membranes is critical for a variety of essential cellular processes, from migration to membrane trafficking. Thus, understanding how actin is coupled to membranes is central to understanding many fundamental aspects of cell biology. The actin cytoskeleton exerts forces on membranes through assembly and myosin motors. Considerable effort has been made to study each of these two modes of force generation, but less research has focused on how the two are coordinated. Clathrin-mediated endocytosis in the yeast Saccharomyces cerevisiae provides a unique opportunity for studying this coordination owing to a well-documented requirement for both actin assembly and myosin activity and the unparalleled molecular and genetic tools in this organism. During S. cerevisiae clathrin-mediated endocytosis, actin assembly is required to invaginate the plasma membrane into a pit that can undergo scission. Type I myosins are also required for pit formation, conferring at a minimum membrane binding and myosin motor activities to the essential actin assembly complex. Endocytosis was shown previously to fail in the absence of type I myosins. However, how and why endocytosis fails was unknown. Here we performed live-cell two-color imaging of clathrin-mediated endocytosis in yeast cells relying on a series of engineered type I myosin mutant genes. We show that during clathrin-mediated endocytosis, the type I myosin Myo5 couples actin assembly to the plasma membrane.
membrane. Absent this crucial linkage, vesicles fail to be internalized. Actin assembly factors and assembling actin are recruited to endocytic sites but peel away from the plasma membrane before they can initiate invagination, frequently forming actin comets that rocket through the cytoplasm. Intriguingly, the endocytic WASP/Myosin module, previously thought of as a discrete complex, separates in these instances, with the yeast Wiscott-Aldrich Syndrome Protein (WASP) Las17 being left at the plasma membrane and the yeast WASP Interacting Protein (WIP) Vrp1 localizing to the tips of cytoplasmic comets. Our data demonstrate that type I myosins organize actin assembly factors and link them to the plasma membrane during clathrin-mediated endocytosis, coupling actin assembly to membrane deformation. The presence of type I myosins in many cellular actin networks involved in membrane budding leads us to suggest that organization and membrane linkage of actin assembly is a conserved function of these enigmatic motors.

P1968
Board Number: B106
Mechanistic principles underlying regulation of the actin cytoskeleton by phosphoinositides.
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The actin cytoskeleton provides forces for vital cellular processes involving membrane dynamics. Membrane phosphoinositides regulate many actin-binding proteins, including coflin, profilin, Dia2, N-WASP, ezrin and moesin, but the underlying mechanisms have remained elusive. By applying a combination of biochemical assays, novel photobleaching/activation approaches, and atomistic molecular dynamics simulations, we revealed that these proteins interact with membranes through multivalent electrostatic interactions, and hence function as phosphoinositide density sensors at the membrane. However, their membrane-binding kinetics differ drastically. Cofilin and profilin display transient, low-affinity interactions with phosphoinositide-rich membranes, whereas F-actin assembly factors Dia2 and N-WASP reside on phosphoinositide-rich membranes for longer periods to perform their functions. Ezrin and moesin, which link cytoskeleton to plasma membrane, bind membranes with very high affinities and slow dissociation dynamics, and do not require high “stimulus-responsive” phosphoinositide density for membrane-binding. Thus, membrane-interaction mechanisms of actin-binding proteins evolved to precisely fulfill their specific functions in cytoskeletal dynamics.

P1969
Board Number: B107
Ultrastructure of actin-myosin cytoskeleton during mitochondrial fission.
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Actin-myosin II cytoskeleton and endoplasmic reticulum (ER) play important roles in mitochondrial fission. However, the exact molecular mechanism of fission is not clear due to a lack of ultrastructural insights into organization of relevant molecular components. Platinum replica electron microscopy (PREM) has been very productive in revealing cytoskeleton architecture in various contexts, but visualization of the cytoskeleton together with membrane organelles, such as ER and mitochondria, by PREM is more challenging and has not been achieved before. We now have developed PREM techniques
to simultaneously reveal the cytoskeleton and membrane organelles and used them to analyze organization of ER and the actin-myosin II cytoskeleton around mitochondria in steady-state conditions and after induction of mitochondria fission. The identity of mitochondria and ER in PREM images was confirmed by correlative light and electron microscopy. Localization of important molecular players of mitochondrial fission, such as Drp1 and nonmuscle myosin II, was determined using immunogold PREM. We show that highly constricted mitochondria, which likely represent prefission intermediates, are often associated with a criss-cross array of long and mostly unbranched actin filaments that intersect each other approximately at the constriction site. These actin filaments sometimes appeared to extend from ER to the mitochondrion constriction site and further to another ER segment on the other side of the mitochondrion. The ER-associated actin filaments are likely nucleated by INF2-CAAX, because expression of a constitutively active INF2-CAAX results in the formation of many long actin filaments at the ER surface. Drp1 immunogold label was often found in association with actin filaments present around a mitochondrion or, sometimes, crossing over a mitochondrial constriction. Myosin II, however, was often enriched next to a constricted mitochondrion, but not exactly at the constriction site. We propose that myosin II constricts mitochondria in a corset-like fashion by pulling from a distance on the ER-associated long actin filaments that criss-cross around a mitochondrion

P1970

Board Number: B108

Opposing functions of F-BAR proteins CIP4 and FBP-17 in neuronal membrane protrusion, tubulovesicle formation and neurite outgrowth are largely encoded in their first linker region. K.L. Taylor1, R. Taylor1, B. Huynh2, K. Richters1, J. Carrington2, M. McDermott3, E.W. Dent1,2; 1Neuroscience Training Program, University of Wisconsin Madison, Madison, WI, 2Neuroscience, University of Wisconsin Madison, Madison, WI

In the nervous system, the dynamic processes of membrane protrusion and invagination are necessary for cell migration and differentiation, requiring the coordination of both the plasma membrane and the actin cytoskeleton. F-BAR family proteins are involved in sensing and altering membrane curvature through their F-BAR domain and function in endocytosis. In addition to the F-BAR domain the Cdc42 Interacting Protein 4 (CIP4) subfamily of proteins (CIP4, TOCA1 and FBP17) have an HR1 domain, which binds active Rho family GTPases, and an SH3 domain, that interacts with actin-associated proteins and dynamin. Previous work on CIP4 family proteins has focused on their ability to form elongated tubulovesicles when overexpressed in nonneuronal cells. Our recent work in primary neurons indicates that CIP4 localizes to the protruding edge of neurons, increases veil protrusion and negatively regulates neurite outgrowth. Here we show that expression of FBP17, in contrast, induces tubular structures in primary cortical neurons that colocalize with the endocytic marker Cholera toxin B subunit. FBP17 expression also decreases veil formation and results in precocious neurite formation. To determine how two highly homologous family members localize and function antagonistically in neurons we performed a structure-function analysis by domain and linker region swapping. Surprisingly, we discovered that swapping CIP4 and FBP17 F-BAR, HR1 and SH3 domains had relatively little effect on the localization and function of the two proteins. Rather, the first linker region (L1), between the F-BAR and HR1 domains, is capable of almost fully reversing the localization and function of CIP4 and FBP17. Together, these results suggest that CIP4 and FBP17 work as an antagonistic pair to fine tune membrane protrusion, endocytosis and neurite formation during early neuronal development.
P1971

Board Number: B109

Formin-mediated actin polymerization triggers constriction of both mitochondrial membranes during mitochondrial division.

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While the roles for actin in cell motility are well characterized, their roles in controlling organelle dynamics are less well described. We have previously shown that actin polymerization through an endoplasmic reticulum-bound formin protein, INF2, mediates mitochondrial division by stimulating recruitment and oligomerization of the dynamin GTPase Drp1. Drp1 activity then drives constriction of the outer mitochondrial membrane (OMM). Here, we show that INF2-mediated actin polymerization plays a second role in mitochondrial division, driving inner mitochondrial membrane (IMM) constriction through a calcium-dependent mechanism. Upon stimulation of U2OS cells with histamine, INF2-mediated actin polymerization is rapidly activated (T1/2 3.8 ± 0.9 sec), followed by an increase in mitochondrial calcium (T1/2 7.5 ± 1.9 sec). The mitochondrial calcium increase is abolished by LatA treatment or INF2 suppression. Electron microscopy shows that INF2 suppression eliminates an increase in stimulus-induced ER-mitochondrial contacts (contact distance <30 nm), which are necessary for efficient transfer of calcium from ER to mitochondrion through the mitochondrial calcium uniporter (MCU). Over-expression of ER-mitochondrial tethering proteins rescues the mitochondrial calcium defect in INF2-KO cells. Mitochondrial calcium entry drives IMM constrictions, and MCU suppression abolishes these constrictions without changing Drp1 recruitment. Live-cell super-resolution microscopy shows that IMM division precedes OMM division. Myosin IIA activity is also required for stimulus-induced mitochondrial calcium increase and IMM constrictions, but this process does not require Arp2/3 complex. These results demonstrate that acto-myosin contractility drives dynamics of both mitochondrial membranes independently during mitochondrial division: IMM constriction through increased mitochondrial calcium, and OMM constriction through Drp1 recruitment.

P1972

Board Number: B110

Generation of mouse models for muscular dystrophic chicken by CRISPR/Cas9-mediated genome editing.

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A missense mutation in the WWP1 gene was reported as the cause of muscular dystrophy of the NH-413 chicken by genetic linkage analysis. WWP1 is a HECT-type E3 ubiquitin protein ligase composed of three functional domain regions, i.e., an N-terminal C2 domain for calcium-dependent phospholipid binding, a central region containing four tandem WW domains recognize substrates with the proline-rich peptide motifs, and a C-terminal HECT catalytic domain for ubiquitin transfer from E2 to substrates. In chicken skeletal muscle, WWP1 is detected as a 130-kDa protein that localizes to various structures, such as the sarcolemma, sarcoplasmic reticulum, mitochondria, and the nucleus. However, in NH-413 chickens, the mutant WWP1 protein was markedly reduced and was faint or absent in the sarcolemma. The missense mutation changes arginine into glutamine in the center of the WW domain cluster, which gives rise to speculation that the amino-acid substitution would lead to critical alterations in WWP1 function by
defect of WW domains. To test the hypothesis, we generated mice with various deletion mutations in the area of WW domain cluster by CRISPR/Cas9-mediated genome editing, and examined the expression of the WWP1 variants. In the skeletal muscle of the mice lacking both the first and second WW domains (WW1-WW2) of WWP1, the remarkable protein reduction and loss of sarcolemmal localization were observed as is the case with NH-413 chicken skeletal muscle. The same result was also obtained in the mice that have a deletion of an entire WW2 domain. On the other hand, WWP1 clearly remained at sarcolemma in the deletion of a part of WW2 domain, although the protein expression was reduced to the same levels as the cases of deletion of WW1-WW2 and a whole WW2 domain. These findings suggested that the degradation and disappearance of mutated WWP1 at sarcolemma would occur independently from each other by defect of the WW domain function. Further studies on the relation between the mutation sites of WW domains and pathological symptom of the mice are needed to understand the molecular mechanism of WWP1-deficient muscular dystrophy.

P1973
Board Number: B111
The collapsin response mediator protein 4 (CRMP4) is enriched at actin structures formed by bacteria at the plasma membrane of epithelial cells.
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The actin polymerization machinery is recruited to the plasma membrane to create filamentous actin arrays and produce the force required for cell motility. To study this, actin-rich structures generated by bacterial pathogens such as enteropathogenic Escherichia coli (EPEC) pedestals, Salmonella enterica serovar Typhimurium (S. Typhimurium) membrane ruffles, and Listeria monocytogenes (L. monocytogenes) comet tails and listeriopods are often utilized as model systems. Through previous mass spectrometry analysis of EPEC pedestals, we identified a novel actin-associated protein called the collapsin response mediator protein 4 (CRMP4), in bacterially-induced actin structures. By immunolocalizing CRMP4 in infected HeLa cells, we found the protein concentrated at the tip of EPEC pedestals, within S. Typhimurium–generated membrane ruffles and at L. monocytogenes listeriopods. Interestingly, it was not present at L. monocytogenes comet tails. Moreover, CRMP4 does not accumulate around mutant bacteria that can not form actin-rich structures despite being associated with the host plasma membrane. Through this evidence, we suggest that CRMP4 is selectively recruited to membrane bound actin-rich structures made by EPEC, S. Typhimurium, and L. monocytogenes and that CRMP4 may have a novel function at the actin-plasma membrane interface.

Kinesins 1

P1974
Board Number: B112
Cryo-Electron Microscopy 3-D Analysis of heterodimeric KIF3 Motor Head Domains on Microtubules.
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In its native configuration, the kinesin-2 KIF3 forms heterodimers between KIF3A and KIF3B, or KIF3A with KIF3C. Heterodimeric motor domains are relatively rare among kinesins, and the advantages of a
hetero- versus homodimer configurations are not fully understood. Here we use cryo-electron microscopy on microtubules that are decorated with either, non-physiological KIF3AA, KIF3BB, and KIF3CC homodimers, as well as native KIF3AB and KIF3AC heterodimers. Since high-resolution data from cryo-EM images are typically a product of intense averaging over numerous, identical image elements, getting to high resolution from heterodimeric constructs pose a particular challenge. Collecting and identifying identical image elements cannot be done by helical diffraction methods since phases of layer lines will be convoluted from the two different motor domains. To this end it will be crucial to identify each head by a specific marker that will allow for a prescreening of repetitive units along microtubules. Therefore, we collect first rounds of 3-D data by cryo-tomography, the only EM-based 3-D analysis process that does not rely on averaging or diffraction. Here we show preliminary data that point to distinct conformations of the various constructs, as well as preliminary high-resolution data that we have obtained from individual head domains.

P1975
Board Number: B113
Development of improved microscopy and data analysis tools for understanding multimotor transport.
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Intracellular transport of vesicles, organelles, and other cargoes is driven by teams of kinesin, dynein, and myosin molecular motors. Frequently there are multiple motors of each type and directionality on each cargo, but how teams of motors coordinate their activity in order to give rise to the emergent “tug of war” behavior observed in cells remains poorly understood. Even less understood is the observation that knocking down specific motors diminishes transport in both directions, not only in the direction of the inhibited motor. In vitro and intracellular cargo tracking experiments seeking to understand the molecular mechanisms underlying bidirectional transport behavior have thus far been limited by two key factors: (1) the spatiotemporal resolution of existing microscopy techniques, without which short back-and-forth motions of the cargo blur together and average out, and (2) data analysis tools capable of ascribing the dynamics of position-over-time traces to the behaviors of the individual motors taking part in multi-motor transport. In the current work, we seek to address both of these limitations. We first introduce high-speed, super-resolution scattering microscopy techniques to track cargoes with 1-nm and 1-ms resolution. We next create a stochastic differential equation model to represent coupled motor and cargo positions, and apply it to our data using a combination of particle filtering and a stochastic expectation-maximization algorithm. Overall, we work towards quantitatively describing how each motor on the cargo dynamically interacts with the track and with the other motors in order to drive bidirectional transport.
P1976
Board Number: B114
Super-resolution imaging reveals differential clustering of microtubule motors on vesicle membranes.
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Motor proteins play an essential role in cellular organization by carrying organelles along microtubules and delivering them at the right place. Recently, the clustering of dynein motors has emerged as a novel mechanism of regulating retrograde trafficking of large vesicles. We aimed to determine whether similar clustering mechanisms of retrograde and anterograde motor proteins play a role in the trafficking of small vesicles in intact cells. Using multi-color, 3D super-resolution microscopy and quantitative analysis, we revealed the organization of dynein, Kif5 and Kif3 on the membrane of lysosomes at the nanoscale level. Our results show that all motor proteins are organized in small teams composed of between 1-4 motor proteins on the lysosome membrane. However, the total number of motor teams on the membrane is differentially regulated for the three motors. In addition, this number scales differentially with the size of the lysosome among the different motors. Overall, our results reveal for the first time how various motor proteins are organized on the membrane of small vesicles in intact cells, giving new insights into mechanisms of transport regulation by motor protein clustering.

P1977
Board Number: B115
Conformational switching of microtubule and cooperative binding of kinesin-1 as a base for polarized transport.
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Kinesin-1, the founding member of kinesin superfamily proteins, is known to use only a subset of microtubules for transport in living cells. This biased use of microtubules is proposed as the guidance cue for polarized transport in neurons, but the underlying mechanisms are still poorly understood. Here we report that there is a positive feedback in the binding of kinesin-1 to the GDP-microtubule, which spontaneously produces high affinity microtubules from other low-affinity microtubules. This high affinity state requires the binding of kinesin-1 in the nucleotide-free state. Microtubules return to the initial low affinity state by washing out the binding kinesin-1 or by the binding of AMPPNP to kinesin-1. X-ray fiber diffraction, fluorescence speckle microscopy and second harmonic generation microscopy, as
well as cryo-EM, collectively demonstrated that the binding of nucleotide-free kinesin to GDP-microtubule changes the conformation of GDP-microtubule to a conformation close to the GMPCPP-microtubule.

P1978
Board Number: B116
Phosphorylation of multiple sites of Alcadein α is required for kinesin-1 association and Golgi exit of Alcadein α.
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Anterograde axonal transport in neurons is mediated by kinesin superfamily proteins (KIFs) which are microtubule-associated motor proteins. Effective axonal transport is required for proper neuronal function, and impaired axonal transport is involved in neurodegenerative diseases. However, it remains controversial how the interaction between cargo receptors and kinesin is regulated during the various stages of cargo transport, including during vesicular cargo formation at the Golgi, transportation in axons, and release in the nerve terminal.

Alcadein α (Alca)/Calsyntenin 1 is a type I membrane protein found as a molecule suppressing the cleavage of amyloid precursor protein (APP) (J. Biol. Chem. [2003] 278, 49448). Alca is also a major cargo of kinesin-1 that is subjected to anterograde transport in neuronal axons (EMBO J. [2007] 26, 1475). Two tryptophan- and aspartic acid-containing (WD) motifs located in its cytoplasmic domain directly bind the tetratricopeptide repeat (TPR) motifs of kinesin light chain (KLC), which activate kinesin-1 and recruit kinesin-1 into Alca cargo (Traffic [2012] 13, 834).

We found that phosphorylation of three serine residues in the acidic region located between the two WD motifs is required for interaction with KLC. Phosphorylation of these serine residues may alter the disordered structure of the acidic region to induce the direct association with KLC. Substitution of these serines with Ala results in a mutant that is unable to bind kinesin-1, which impairs exit of Alca cargo from the Golgi. Furthermore, vesicles containing Alca harboring these Ala mutations were analyzed for axonal transport using TIRF (Total Internal Reflection Fluorescence) microscopy. Alca mutant was still transported, albeit improperly by vesicles following mis-sorting of the Alca mutant with APP containing vesicles. This suggests that APP partially compensates for defective Alca in anterograde transport by providing an alternative cargo receptor for kinesin-1. The present results reveal the functional importance of Alca phosphorylation at multiple sites in regulating the transport of Alca cargo by kinesin-1, and the proper formation of post-Golgi Alca cargo.
P1979

Board Number: B117

Enhanced fast velocity of APP transport by kinesin-1 is regulated by KLC1 phosphorylation.
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Amyloid β-protein precursor (APP) is deeply implicated in Alzheimer’s disease pathogenesis by causing amyloid β-protein (Aβ). One of the most important functions of APP is as a cargo receptor for kinesin-1, a conventional kinesin. Functional kinesin-1 forms a tetramer comprising two kinesin heavy chains (KHCs) and two kinesin light chains (KLCs), and plays an essential role in anterograde transport of many cargos, including membrane vesicles, In neurons, APP is transported by binding to kinesin-1, mediated by JNK-interacting protein 1b (JIP1b), which generates the enhanced fast velocity and efficient high frequency of APP anterograde transport (Mol. Biol. Cell [2014] 25, 3569). The enhanced fast velocity requires a conventional interaction between JIP1b C-terminal region and kinesin light chain 1 (KLC1) tetratricopeptide repeat, whereas the efficient high frequency requires a novel interaction between the central region of JIP1b and the coiled-coil domain of KLC1. However, it remains unclear how the interaction of JIP1b with kinesin-1 is selectively regulated to control APP cargo transport. We found that the conventional interaction of KLC1 with JIP1b is regulated by a phosphorylation of KLC1 but not JIP1b. Substitution of Glu for the phosphorylatable amino acid of KLC1 abolished the conventional interaction and suppressed the enhanced fast velocity of APP cargo, but did not impair the novel interaction responsible for the efficient high frequency. Phosphorylation of KLC1 increased in aged brain and JIP1 binding to kinesin-1 decreased. The elevated phosphorylation of KLC1 that accompanies aging may induce a qualitative impairment of APP axonal transport, potentially promoting Aβ generation and contributing to neurodegenerative disorders such as Alzheimer’s disease.

P1980

Board Number: B118

Kinesin-2 motors adapted their stepping behaviour for progressive transport on axonemes and microtubules.
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Kinesin-2 is employed in both cytoplasmic and intraflagellar long-range transport. It therefore employs two structurally distinct filament tracks, axonemes in cilia and microtubules in the cell body. We tracked the heads of single kinesin-2 molecules with Fluorescence Imaging with One Nanometer Accuracy (FIONA) on both axonemes and microtubules. The distribution of step sizes and the traces observed show that the motors adapt their stepping behavior to the respective track. While kinesin-2 takes directional, off-axis steps on microtubules, it resumes a straight path on axonemes. This provides a molecular explanation for the direction-dependent allocation of cargo to one side of the same doublet microtubule that has been observed previously. The intrinsic tendency of kinesin-2 to take sidesteps on the microtubule lattice restricts the motor to the B-tubule. These results offer first mechanistic insights
into why heterodimeric kinesin-2 was co-evolved with the ciliary machine to achieve effective two-way traffic on the axoneme in vivo.

**P1981**

**Board Number: B119**

**Axonal access is regulated by KIF13/KLP-4 in vivo.**

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Cells rely heavily on a polarized and dynamic microtubule system and its associated molecular motor proteins; the (mostly) plus end directed kinesins and minus end directed dyneins, for a multitude of functions. Microtubule motors not only ensure that a cell establishes its proper morphology and polarity, but also allow the cell to have efficient spatial and temporal movement of both organelles and vesicles to facilitate robust signal transduction pathways. This is particularly true in neurons, where a specialized cellular morphology, such as long axonal projections and access to this space, is inherently tied to high degree of dynamic signaling mechanisms. To establish a signaling network along an axon likely requires motors that are highly processive, that is the ability to bind microtubules with high affinity and carry cargo a distance. Motors of the Kinesin-3 superfamily, including the KIF1, KIF13, KIF14, and KIF16 subfamilies, are all expressed in neurons and exhibit highly processive activity in vitro, however questions still remain as to how these highly processive motors contribute to neuronal cell morphology and signal transduction in vivo. Due to its well-described nervous system and quantifiable behaviors, we are using C. elegans to study Kinesin-3 motors and their roles in neuronal signaling in vivo. We began by employing a reverse genetics approach using the worm homologue to KIF13A and KIF13B, klp-4 to determine in vivo function of these motors. We isolated mutant alleles of klp-4 predicted to effect protein function. We hypothesized that klp-4 mutation would effect cellular signaling and therefore animal behavior; and mutation in klp-4 does indeed result in changes in locomotive behaviors such spontaneous reversals and nose touch challenge suggesting a role for KLP-4 in neuronal signaling. Surprisingly, when we used rab-3::eGFP to visualize the presynaptic areas of klp-4 mutants, we discovered that klp-4 mutation lead synapses that were increased number, misorganized, and larger. Additionally, touch receptor neurons also displayed increased abnormal ectopic projections. When assessed for neurotransmitter specific behaviors, klp-4 mutants display significantly increased sensitivity to acetylcholinesterase inhibitors and altered sensitivity to exogenous serotonin. The above data suggests KLP-4 is a regulator of neuronal signaling by mediating synaptic organization and neurotransmitter mediated signaling by restricting cargo movement into and long the axon and that despite their highly processive nature, certain Kinesin-3 motors might function in both moving and restricting cellular cargoes during cell signaling events in vivo.
P1982

Board Number: B120


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Motor proteins, such as kinesins and dyneins, are responsible for several fundamental transport functions inside the cell. Impaired functionality of motor proteins, possibly due to mutations, have been linked to disruptions in intracellular transport and neurodegenerative diseases such as Huntington's and Alzheimer's disease, muscular disorders such as heart disease, uterine complications and high blood pressure. A known fact about intracellular transport is that the transfer of cargoes is often facilitated by teams of motor proteins, possibly of same or different types. It is particularly significant to study transport of cargo by both wild-type and mutant motor proteins. An exhaustive experimental analysis of specific mutations and its effect on multiple motor based transport is prohibitively time consuming due to combinatorial nature of the study. Thus, there is a significant need for analytical/computational methods to inform experiments. We addressed this by developing a semi-analytical computational methodology to compute average run-length and velocity when cargo is carried by multiple types of motor proteins. The simulation technique is able to correctly predict existing experimentally observed phenomena regarding the dependence of run-length and velocity on parameters such as ATP concentration and load force, indicating its reliability. Instantiation of this method for a recently reported mutation implicated in Huntington's disease, which indicated reduced single kinesin stall force, revealed surprising insights. Primarily, in cargoes carried by multiple wild-type and mutant kinesin, mutant motors determine the average velocity and run-length even when they are outnumbered by wild type motors in the ensemble when operating under certain external conditions. It is predicted that mutants gain a competitive advantage and lead to increased run-length when load on the cargo is in the vicinity of the mutant's stalling force or multiples of its stalling force. This can have several implications towards understanding the effect of mutations on coordinated transport and knowing which single motor characteristics, when altered, leads to impaired cargo transport. Furthermore, the unique nature of this method enables study of rare events and provides with results whose accuracy is independent of the number of iterations. The approach uses significantly less computational resources than other Monte Carlo based approaches, enabling reasonably-sized ensembles to be studied using standard desktop computers. The methodology can be further extended to study the effect of other single motor mutations on coordinated cargo transport as well as the study of transport by different types of motor proteins, while providing new prognoses to be tested experimentally.
**P1983**

**Board Number: B121**

**Motor reattachment kinetics play a dominant role in multimotor-driven cargo transport.**

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Kinesin-based cargo transport in cells frequently involves the coordinated activity of multiple motors, including kinesins from different families that move at different speeds. However, compared to the progress at the single-molecule level, mechanisms by which multiple kinesins coordinate their activity during cargo transport are poorly understood. To understand these multi-motor coordination mechanisms, defined pairs of kinesin-1 and kinesin-2 motors were assembled on DNA scaffolds and their motility examined in vitro. Although less processive than kinesin-1 at the single-molecule level, addition of kinesin-2 motors more effectively amplified cargo run lengths. By applying the law of total expectation to cargo binding durations in ADP, the kinesin-2 microtubule reattachment rate was shown to be 4-fold faster than that of kinesin-1. This difference in microtubule binding rates was also observed in solution by stopped-flow. High-resolution tracking of gold-nanoparticle-labeled cargo with 1 ms and 2 nm precision revealed that kinesin-2 motors detach and rebind to the microtubule much more frequently than do kinesin-1. Finally, cargo transported by kinesin-2 motors more effectively navigated roadblocks on the microtubule track. These results highlight the importance of motor reattachment kinetics during multi-motor transport and suggest a coordinated transport model in which kinesin-1 motors step effectively against loads while kinesin-2 motors rapidly unbind and rebind to the microtubule. This dynamic tethering by kinesin-2 maintains the cargo near the microtubule and enables effective navigation along crowded microtubules.

**P1984**

**Board Number: B122**

**Effect of membrane coupling on multiple-kinesin transport.**

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Molecular motor-based transport is critical for all eukaryotic cell function and health. Although traditionally examined in the context of single motor experiments, molecular motors often work in small teams together to transport the same cargo in vivo. Factors that control and regulate the group function of multiple motors has remained unclear. Here we used a simple lipid bilayer to couple kinesin motors together, and used microtubule gliding assay to examine the effect of this membrane coupling on the group function of multiple kinesin motors.
P1985

Board Number: B123

Intracellular cargo transport by single-headed kinesin monomers.
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Kinesins are motor proteins that transport cargoes along microtubules in eukaryotic cells. The canonical view is that dimerization is required for kinesin’s processive motility, as the two motor domains of a dimer step hand-over-hand in a tightly coordinated manner. However, whether dimerization is required for cargo transport in cells remains unclear. Using intracellular cargo trafficking assays, we compared dimeric motors to their monomeric forms containing only the motor domain and the neck linker. Surprisingly, we found that monomeric motors across different kinesin families are able to work in teams to drive cargo transport, despite lacking the single-motor processivity of the dimers. To explore the mechanics of this cooperative monomeric transport, we added structural elements after the neck linker, varying their length and flexibility. In general, kinesin monomers are efficient transporters if they are short and the cargo imposes little load. As the length or flexibility of the stalk increases, monomers become less efficient, and dimerization becomes necessary to pull against load. Together, these results lend insight into the minimal requirements and mechanical modulators of collective kinesin cargo transport. They may also shed light on why most kinesins evolved to function as dimers.

P1986

Board Number: B124

The cover-neck bundle is important for teams of kinesin-1 motors to transport high-load in cells.
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Cells have a diverse group of kinesin motor proteins to direct key microtubule-based processes such as cell division, cell motility, intracellular trafficking, and cilia function. Despite strong conservation of the motor domain across the kinesin superfamily, how various kinesins have tailored their motility characteristics to best meet their functional needs in cells remains unclear. Previous studies suggest cargo transport in cells is governed by the ability of kinesin motors to resist detachment from the microtubule when under load. We developed assays to study transport of low- and high-load cargo by teams of kinesin motors using an inducible, artificial cargo system in cells. We found differences across the superfamily in the ability of kinesin motors to effectively transport high-load cargo. To delineate key features of the motor domain important for transport of high-load, we used molecular modeling to predict mutations in the kinesin-1 motor domain and tested how these mutations impacted cargo transport. We found that the integrity of the cover-neck bundle, a beta-sheet between the coverstrand at the N-terminus and the first half of the necklinker, is critical for teams of kinesin-1 motors to transport high- but not low-load cargo in cells. Truncations of the coverstrand suggest that single motor motility does not predict the ability of teams of motors to transport high load in cells. Our results indicate that cover-neck bundle formation is not only important for single motors to step against load
under purified conditions, but is also critical for teams of kinesin-1 motors to transport high-load in a cellular context. We plan to extend these studies to additional kinesin families to identify whether there are distinct motility dependencies on formation of the cover-neck bundle.

P1987
Board Number: B125
A fluid membrane enhances the velocity of cargo transport by small teams of kinesin-1.
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Kinesin-1 is a major molecular motor driving the fundamental process of transport in live cells. While the single-molecule functions of kinesin are well characterized, the physiologically relevant transport of membranous cargos by small teams of kinesins remains poorly understood. A key experimental challenge remains in the quantitative control of the number of motors driving cargo transport. Here we utilized “motile fraction” to overcome this challenge, and experimentally accessed transport by single kinesins through the physiologically relevant transport by small teams of kinesins. We use a fluid lipid bilayer to model the cellular membrane in vitro, and employed optical trapping to quantify transport of membrane-enclosed cargos versus traditional membrane-free cargos at the same motile fraction (motor number). We found that coupling motors via a fluid membrane significantly enhances the velocity of cargo transport by small teams of kinesins. Importantly, enclosing a cargo in a fluid lipid membrane does not impact single-kinesin transport, indicating that membrane-dependent velocity enhancement for team-based transport resulted from altered interactions between kinesin motors. Our study demonstrates that membrane-based coupling between motors is a key determinant of kinesin-based transport. Enhanced velocity may be critical for the timely delivery of cargos in live cells.

P1988
Board Number: B126
Origins of thermal stability of kinesin activity in cells.
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Kinesin-1 is the motor which drives a significant fraction of microtubule-based transport in cells. In addition, because of its accessibility and robustness, Kinesin has been seen as a promising motility driver for nano-engineering. However, degradation of kinesin-1 sets in around 30 C for typical in vitro assays - much below ~40 C which can be sustained by the motor in vivo. Why are the in vivo and in vitro results so different? We have examined kinesin motility assays in the presence of trimethylamine N-oxide (TMAO), which is known to stabilize secondary structure and enzymatic activity of many proteins, and its mode of action in many ways mimic molecular crowding in cells. We show that the addition of TMAO results in a dramatic enhancement of kinesin thermal range (up ~50 C). Hence, intracellular crowding emerges as the likely factor which allows kinesin-1 to function efficiently in cells at mammalian body temperatures. This helps address a long standing discrepancy between in vivo and in vitro kinesin assays and furthermore positions TMAO as a key new ingredient for engineered kinesin-based nanodevices.
Tubulin and Associated Proteins

P1989
Board Number: B128
Native Kinesin-1 Does Not Bind Preferentially to GTP-Tubulin-Rich Microtubules In Vitro.
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Molecular motors such as kinesin-1 work in small teams to actively shuttle cargos in cells, for example in polarized transport in axons. Here we examined the potential regulatory role of the nucleotide state of tubulin on the run length of cargos carried by multiple kinesin motors, using an optical trapping-based in vitro assay. Based on a previous report that kinesin binds preferentially to GTP-tubulin-rich microtubules, we anticipated that multiple-kinesin cargos would run substantially greater distances along GMPCPP microtubules than along GDP microtubules. Surprisingly, we did not uncover any significant differences in run length between microtubule types. A combination of single-molecule experiments, comparison with previous theory, and classic microtubule affinity pulldown assays revealed that native kinesin-1 does not bind preferentially to GTP-tubulin-rich microtubules. The apparent discrepancy between our observations and the previous report likely reflects differences in post-translational modifications between the native motors used here and the recombinant motors examined previously. Future investigations will help shed light on the interplay between the motor’s post-translational modification and the microtubule’s nucleotide-binding state for transport regulation in vivo.

P1990
Board Number: B129
Mechanism of microtubule stabilization by kinesin-5.
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In addition to their capacity to slide apart antiparallel microtubules during spindle formation, the mitotic kinesin-5 motor Eg5 has been shown to pause at microtubule plus-ends and enhance microtubule polymerization (Chen and Hancock, Nature Comm. 2015:8160). The goal of the present work is to understand the Eg5 microtubule polymerase mechanism by studying how the motor alters the lateral and longitudinal tubulin-tubulin interactions that stabilize the microtubule lattice. Transient kinetics and single-molecule tracking experiments demonstrate that dimeric Eg5 motors reside predominantly in a two-head-bound strong-binding state while stepping along the microtubule (Chen et al., JBC 2016:291(39), 20283-94). This suggests that when Eg5 pauses at a growing microtubule plus-end, the motor acts as a two-head-bound “staple” to stabilize the longitudinal bonds of incoming tubulin dimers. The on-rate for Eg5 binding to free tubulin is slow, suggesting that end-bound Eg5 motors do not bind free tubulin in solution; rather they stabilize incoming tubulin dimers that have bound to the plus-end. Because tubulin in the microtubule lattice resides in a “straight” conformation, while free tubulin resides in a “kinked” conformation, a second (non-mutually exclusive) model is that Eg5 stabilizes the straight conformation of tubulin. Consistent with this, monomeric Eg5 motors, which bind to the microtubule lattice without crosslinking tubulin dimers, also stabilize microtubules against depolymerization. Furthermore, the affinity of Eg5 for free tubulin is reduced in the presence of “wedge inhibitor” drugs.
that stabilize the kinked conformation of tubulin; conversely Eg5 binding to tubulin diminishes drug binding. Thus, we propose a microtubule polymerase mechanism in which binding by one Eg5 motor domain at the microtubule plus-end straightens tubulin dimers and stabilizes lateral tubulin-tubulin bonds, while the second Eg5 head binds incoming tubulin and acts as a staple to stabilize longitudinal tubulin-tubulin bonds and enhance microtubule growth.

P1991

Board Number: B130

Investigating dynein function in a mouse model carrying a Charcot-Marie-Tooth type 2O linked mutation.

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Dynein is a minus-end directed microtubule motor protein that plays a crucial role in the cellular development, maintenance, and survival. A single point substitution of histidine to arginine at amino acid 306 (H306R) in the cytoplasmic dynein heavy chain (DHC) has been linked to the autosomal dominant Charcot-Marie-Tooth type 2O (CMT2O) neuropathy in humans (Weedon et al. 2011). Individuals with CMT2O exhibit peripheral moto-sensory defects such as distal limb weakness, atrophy, skeletal deformities (pes cavus), and gait abnormalities. We have developed and characterized a novel mouse model to understand the mechanism of the mutant dynein in the progression of the CMT2O disease. The knock-in mouse model was generated with the corresponding CMT2O linked mutation (H304R) located in mouse’s dynein heavy chain. With 12-month long behavioral studies on the wild-type, H304R/+ (heterozygous), and H304R/R (homozygous) mice, we established that we had a suitable mouse model to use for further characterization studies. The H304R/+ and H304R/R mice exhibit deficits in the grip strength, rotarod, and tail suspension reflex tests. Additionally, the characterization of neuromuscular junctions (NMJ) revealed structural defects in the morphology, complexity and branching of NMJs in the H304R/+ and H304R/HR mice. Immunohistochemistry characterization of the brains of wild-type, H304R/+ and H304R/R HR mice at early postnatal time points also revealed differences in the brain architecture. In order to understand the effects of the mutant DHC allele on development, mice of all three genotypes are being examined for neurodevelopmental milestones from postnatal day 1 (P1) through postnatal day 21 (P21). Finally, we are characterizing dynein-dependent intracellular motility in neurons from the wild-type, H304R/+, and H304R/R mice embryos.

P1992

Board Number: B131

CLASP1 is Required for CLASP2 Localization and Function at Microtubules in Interphase Cells.

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CLIP-associated proteins (CLASPs) are highly conserved microtubule (MT) plus-end tracking proteins (+TIPS) that are involved in regulating MT dynamics; specifically, CLASPs were shown to promote MT rescue and enhance MT stability. Human CLASPs consists of two paralogs: CLASP1 and CLASP2, which have been found associated with the cell cortex, kinetochores, the Golgi and at the ends of growing MTs. Although both CLASPs have been structurally and functionally characterized in depth, mutual regulatory interactions between these two proteins have not been explored. In the present study, we use a variety of human cell lines to show that while CLASP2 depletion does not interfere with cellular CLASP1 localization, the loss of CLASP1 dramatically influences CLASP2 localization in interphase. In
particular, CLASP2 is no longer associated with MT plus ends, while other MT +TIPs were not affected, and CLASP2 still retained at the Golgi. This effect suggests that CLASP1 specifically facilitates CLASP2 localization at the MT plus ends. Addressing potential underlying mechanisms, we tested if CLASP1 was involved in regulating CLASP2 phosphorylation by GSK3β, which is known to abolish CLASP2 binding to MTs. Here, we found that CLASP1 did not modify CLASP2 phosphorylation levels. Moreover, when GSK3β was inhibited in CLASP1-depleted cells, CLASP2 localization to MTs was restored, indicating that CLASP1 is capable to localize CLASP2 to MTs despite its physiological phosphorylation level. Moreover, our co-immunoprecipitation assays show that CLASP1 forms a complex with CLASP2 in cells. Since prior evidence indicates that CLASP variants can homodimerize via a C-terminal coiled-coil region, we propose that CLASP1 heterodimerizes with CLASP2 in order to target it to MTs. Functionally, CLASPs play a prominent role in MT dynamics and stability, and are critical for nucleation of Golgi-derived microtubules (GDMTs). In our in vitro reconstitution assays, CLASP2 by itself strongly promotes MT nucleation. However, decrease of GDMT nucleation in cells depleted of CLASP1 alone was as prominent as the effect of depletion of both proteins. We conclude that CLASP1-dependent localization of CLASP2 to MT ends is necessary for CLASP2 function in cells, and thus CLASP1 is upstream of CLASP2 in regulating MT nucleation and dynamics.

**P1993**

**Board Number: B132**

Microtubule acetylation induced by α-tubulin acetyltransferase overexpression impairs hepatic protein trafficking and lipid droplet motility.

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Tubulin post-translational modifications (PTMs) create a “code” on the microtubule cytoskeleton to differentially regulate molecular motor and microtubule associated protein (MAP) binding and activity to support innumerable cellular processes. The “tubulin code” is further complicated in the liver because alcohol metabolism produces highly reactive products (acetaldehyde and oxygen radicals) that form various protein adducts and indirectly induce acetylation. Our focus is to understand how alcohol metabolism-derived modifications contribute to liver damage in alcoholic liver disease (ALD). Our previous studies have shown that alcohol consumption leads to enhanced microtubule acetylation and stability that can explain alcohol-induced defects in motor-mediated protein trafficking (e.g., secretion, transcytosis, and nuclear translocation) and lipid droplet motility. To directly assess the relationship between microtubule acetylation and defects in hepatic microtubule motor-mediated motility, we overexpressed α-tubulin acetyltransferase (α-TAT1) to promote acetylation in the absence of alcohol. Using a recombinant adenovirus expressing α-TAT1, we were able to induce acetylation in a dose-dependent manner and replicate similar levels of acetylation (2-3 fold increase) seen during alcohol metabolism in polarized, hepatic WIF-B cells and in livers from alcohol-fed rats. To examine the effects microtubule acetylation has on motor-mediated trafficking and motility, we monitored basolateral secretion, transcytosis and lipid droplet motility. In cells overexpressing α-TAT1, albumin secretion was impaired to a similar extent as observed in alcohol-treated cells. Transcytosis of polymeric IgA receptor (“professional transcytosing protein”) and aminopeptidase N (single spanning membrane protein) from the basolateral-to-apical membrane was impaired to a lesser extent than in alcohol-treated cells. Live cell imaging of BODIPY-labeled droplets also revealed significantly decreased droplet motility. Immotile, enlarged lipid droplets colocalized with dynactin/dynein to suggest that droplet binding properties may be impaired. Together these results demonstrate that microtubule acetylation alone can explain defects...
in microtubule motor-dependent processes that can alter cellular functions. We are currently performing microtubule binding assays and isolating lipid droplets to assess defects in dynein/dynactin binding in cells with a hyperacetylated microtubule environment. Currently there are no treatments available to relieve or reverse the disease state in the progression of ALD, therefore modulating the cellular protein acetylation state could provide a novel therapeutic target.

P1994
Board Number: B133
Human γ-tubulin isotypes: differential expression during neuronal development and under oxidative stress.
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γ-Tubulins are highly conserved members of the tubulin superfamily essential for microtubule nucleation. Humans possess two γ-tubulin genes. It is thought that γ-tubulin-1 represents ubiquitous isotype, whereas γ-tubulin-2 is found predominantly in the brain, where it may be endowed with divergent functions beyond microtubule nucleation. The molecular basis of the purported functional differences between γ-tubulins is unknown. Previously we have reported that human γ-tubulin-2 nucleates microtubules and rescues mitotic progression in γ-tubulin-1 depleted cells (PLoS ONE 7: e29919, 2012). Here we report discrimination of human γ-tubulins according to their electrophoretic and immunochemical properties depending on C-terminal regions of the γ-tubulins. Using epitope mapping, we discovered mouse monoclonal antibodies that can discriminate between human γ-tubulin isotypes. Real time quantitative RT-PCR and 2D-PAGE showed that γ-tubulin-1 is the dominant isotype in fetal neurons. Although γ-tubulin-2 accumulates in the adult brain, γ-tubulin-1 remains the major isotype in various brain regions. Localization of γ-tubulin-1 in mature neurons was confirmed by immunohistochemistry and immunofluorescence microscopy on clinical samples and tissue microarrays. Differentiation of SH-SY5Y human neuroblastoma cells by all-trans retinoic acid, or oxidative stress induced by mitochondrial inhibitors, resulted in upregulation of γ-tubulin-2, whereas the expression of γ-tubulin-1 was unchanged. Fractionation experiments and immunoelectron microscopy revealed an association of γ-tubulins with mitochondrial membranes. These data indicate that in the face of predominant γ-tubulin-1 expression, the accumulation of γ-tubulin-2 in mature neurons and neuroblastoma cells during oxidative stress may denote a prosurvival role of γ-tubulin-2 in neurons.

P1995
Board Number: B134
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Microtubules, dynamic polymers of αβ-tubulin heterodimers, carry out diverse cellular functions in eukaryotes. Microtubule-associated proteins (MAPs), including microtubule polymerases/depolymerases, regulate the organization and dynamics of these filaments in cells. The α-
and β-tubulin gene families expanded substantially in higher eukaryotes. In particular, the human genome encodes at least seven α- and eight β-tubulin isoforms that differ in amino acid sequences and show cell type-specific expression. In vivo studies have suggested the non-interchangeable roles of tubulin isoforms during development. Recent experiments using recombinant human tubulins or chimeric tubulins have begun to reveal how differences in the primary sequences of human tubulin isoforms impact MAP interactions and assembly dynamics of microtubules. However, it has been difficult to analyze the interactions of MAPs with microtubules of different human tubulin isoforms as generating affinity-tag free recombinant human tubulins is challenging. Here, we develop a strategy to generate isotypically pure recombinant human tubulin heterodimers that lack affinity tags. We generate human α-tubulin isoform 1B in combination with either human β-tubulin isoform B2B (A1B/B2B) or B3 (A1B/B3). We then use total internal reflection fluorescence (TIRF)-based assays to quantitatively examine the direct binding of MAPs to human microtubules. We find that MCAK, a kinesin-13 family microtubule depolymerase, disassembles taxol-stabilized A1B/B2B and A1B/B3 microtubules at rates between 0.2 μm/min and 7.5 μm/min. Further, related to the co-existence of different tubulin isoforms in cells, we find that varying the ratio of B3 and B2B tubulin in microtubules can tune the rate of MAP-stimulated depolymerization up to 10-fold. Together, our data reveal that the compositions of human tubulin isoforms in microtubules modulate MAP-mediated polymer dynamics.

P1996

Board Number: B135

Elucidating the Role of a Human β-tubulin, TUBB4B: Functional Analysis of a Novel Patient-Derived Mutation.

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Humans specifically express at least six genes encoding α-tubulin and seven encoding β-tubulin, known as tubulin isoforms. While expression of some isoforms is developmentally regulated and cell-type specific, it is not fully understood why humans have so many tubulin genes and how isoforms might contribute to different microtubule function in different cellular contexts. Mutations in human isoforms that are associated with human diseases provide a powerful window into isoform function. Here we report a novel, heterozygous mutation in TUBB4B, the human βIVb isoform, identified by whole genome sequencing of a patient enrolled in the NIH Undiagnosed Diseases Program (UDP) identified. The mutation causes a glutamine to arginine substitution in the GTP binding domain of TUBB4B. The βIVb isoform has been implicated in axonemal function in model organisms; therefore, we hypothesize that disrupting the function of TUBB4B may ultimately be detrimental to cilia. Interestingly, we find that a higher fraction of UDP patient fibroblasts possess cilia than control cells, indicating that TUBB4B may play a specific role in ciliary function in mammalian cells. In contrast, the overall microtubule network in UDP patient fibroblasts does not exhibit a microtubule stability defect when exposed to cold treatment and does not have increased acetylated tubulin levels. To determine how the Q11R mutation impacts β-tubulin function, we generated an analogous mutation in budding yeast β-tubulin, TUB2. We find that tub2-Q11R fails to rescue a tub2Δ null mutant, indicating that the mutation is sufficient to disrupt β-tubulin function. Heterozygous diploids expressing one copy of tub2-Q11R are viable, resistant to microtubule destabilizing drugs, and exhibit hyper-stable microtubules. To further characterize this mutation, we examined alternative substitutions at Q11, including -Q11E and -Q11S, and tested how they affect GTP-dependent microtubule properties including GTP hydrolysis and its associated conformational changes. Both tub2 -Q11E or -Q11S mutants are sufficient to rescue a tub2Δ null

Monday-84
mutant. Additionally, these mutants exhibit sensitivity to microtubule depolymerizing drugs and have a microtubule instability phenotype indicating that the substitution of a large, positively charged arginine into the exchangeable GTP binding site of β-tubulin specifically impacts microtubule functions. Based on these results, we speculate that the Q11R mutation may alter cilia formation by increasing microtubule stability in the axoneme. Further work on this project will help further elucidate both the mechanism of how the Q11 residue contributes to β-tubulin function and the role of TUBB4B in ciliary function.

**P1997**

**Board Number: B136**

**Targeted localization of microtubule severing enzymes in vivo.**

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Microtubule-severing enzymes recognize defects in microtubules and are capable of remodeling microtubule networks. They are ubiquitous and function during mitosis, meiosis, and cell migration. There has been effort to control microtubule dynamics in specific cellular compartments, in order to develop therapies that help us overcome the pathologies of Alzheimer’s disease, accelerate wound healing, and regenerate spinal neurons (Matamoros and Baas, 2016; Dent, 2017; Charafeddine, 2015). Severing enzymes have proven to be challenging to study in vivo and in vitro, since they have a tendency to aggregate when overexpressed and drastically affect microtubule network homeostasis. Given that severing enzymes function at many locations in the cell, molecular tools must be built to control their activity at specific locations. Here, we describe the development of tools to target microtubule-severing enzymes to different cellular compartments. As a proof of concept, we have successfully targeted human katanin p60 to the kinetochore in Drosophila S2 cells. The human katanin p60 is active and severs interphase Drosophila microtubules when expressed ubiquitously. While at the kinetochores, human katanin p60 had a subtle effect on kinetochore fibers, chromosomal misalignment, interkinetochore distance and mitotic duration. Future directions of the work include targeting katanin to the cellular periphery and the Golgi.

**P1998**

**Board Number: B137**

**TACC3 regulates the dynamic microtubule plus-end in an in-vitro environment.**

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The Transforming Acidic Coiled-Coil (TACC) family of proteins plays an integral role in the regulation of the plus-end of microtubules. It is at this plus-end that the characteristic dynamic nature of microtubules allows for constant tubulin heterodimer addition and removal, thereby affecting the overall extent of microtubule growth. In addition to affecting the dynamicity of microtubules, the TACC family of proteins is known to interact discretely with other +TIPs (plus end tracking proteins), such as the microtubule polymerase, XMAP215. Although such protein interactions have been described before, the knowledge of the TACC family’s complete mechanism affecting the plus-end is not known. Recent in-vitro evidence as well as live cell data from our lab supports the idea that one such member, TACC3, may possess a role in directly affecting the polymerization of microtubules. In addition, data shows variation in physical binding and localization patterns of TACC members on the ends of microtubules. We have
employed high resolution time lapse imaging, in-vitro reconstitution assays, and biochemical techniques to look further into how these TACC proteins differentially regulate the overall dynamic nature of the microtubule.

**P1999**
**Board Number: B138**

Inhibitory effects of eucalyptol on diabetes-associated loss of glomerular slit junctions and podocyte foot process effacement.

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Kidney podocytes, highly differentiated epithelial cells, establish an interdigitating foot processes and participate in the assembly of the foot processes and the slit diaphragm. The maintenance of the slit diaphragm complex is critical in the sieving function of the glomerular filter. Hyperglycemia-induced damage to podocytes is thought to be a critical early event in diabetic nephropathy. Eucalyptol is a natural organic essential oil which is a monoterpenoid present in eucalyptus oil with antiinflammatory and antioxidant properties. This study investigated renoprotective effects of eucalyptol on loss of glomerular slit junctions and foot process effacement caused by glucose stimulation. Mouse podocytes were incubated in media containing 33 mM glucose for 4 d in the presence of 1-20 µM eucalyptol. The expression of the slit diaphragm proteins of nephrin and podocin were diminished in glucose-exposed podocytes, which was dampened by eucalyptol. The expression of the slit diaphragm-associated proteins of α-actinin-4 and CD2AP, and the podocyte adhesion molecule integrin β1 were reduced in podocytes damaged to glucose. In contrast, the eucalyptol treatment enhanced the expression of these proteins responsible for the maintenance of the foot process organization. The in vivo animal model employed db/db mice orally administrated with 10 mg/kg of eucalyptol. Electronic microscopical observations found that podocyte foot process effacement occurred in diabetic mouse glomeruli. On the contrary, oral administration of eucalyptol encumbered foot process effacement of diabetic podocytes through inhibiting loss of glomerular slit junctions-associated proteins of α-actinin-4 and CD2AP. These results demonstrated that eucalyptol maintained normal structures of podocyte foot process and slit diaphragm. Therefore, eucalyptol may be a potent renoprotective agent combating diabetes-associated damage to podocytes in relation to the maintenance of the glomerular filtration barrier.

**P2000**
**Board Number: B139**

SUMO interacts non-covalently with tubulin and the TOG protein Stu2/XMAP215.

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The Small Ubiquitin-like Modifier, SUMO, can interact with target proteins two ways, covalently or non-covalently. Covalent conjugation of SUMO occurs on the target protein through an isopeptide bond formed between the carboxyl group of the terminal glycine of SUMO and the epsilon amino group of the conjugated lysine residue. A non-covalent interaction occurs thorough the hydrophobic and ionic interactions of a “SUMO-Interacting Motif” within the interacting protein. Stu2 is the yeast homologue of CKAP5/XMAP215, a TOG protein that promotes microtubule dynamics by facilitating the loading of tubulin dimers onto microtubule ends. Although Stu2p plays a vital role in microtubule function, little is known about how it is regulated. We analyzed Stu2 for both covalent and
non-covalent interactions with SUMO. Two-hybrid analysis showed that Stu2 interacts with Smt3/SUMO and several key members of the sumoylation system including the E2 conjugating enzyme, Ubc9p. Domain mapping of amino- and carboxyl- terminal truncations shows that the dimerization region of Stu2 is important for its interaction with SUMO. Using an in vitro sumoylation assay, four shifted bands of Stu2 can be observed. Inactivation of the Ulp1 SUMO protease with a temperature sensitive mutant results in Stu2 displaying a higher molecular weight band in vivo. Pull-down assays in denaturing buffer containing urea demonstrate that an anti-SUMO reactive band co-migrates with Stu2. Stu2 also interacts with a sumo-targeted ubiquitin ligase, or STUbL enzyme. Together, these results indicate that Stu2 can be conjugated by SUMO. To test whether Stu2 could interact non-covalently with SUMO, we analyzed Stu2p for an interaction with a non-conjugatable form of SUMO fused to GST. Stu2p bound to the non-conjugatable SUMO but not to GST alone. Phenotypic analysis of mutants within the SUMO interacting motif in Stu2p that is responsible for the interaction is in progress. As tubulin is a major binding partner of Stu2p, we also tested tubulin. Tubulin also bound to the non-conjugatable form of SUMO, but not GST. This binding was independent of the presence of full-length Stu2p. Two-hybrid analysis suggests that the beta-tubulin Tub2p is the major SUMO-interacting tubulin. In contrast, the SUMO targeted ubiquitin ligase, Ris1p, bound to both alpha and beta tubulins. Together our work suggests that Stu2p can interact with SUMO by multiple mechanisms, covalent and non-covalent. This has implications for its interaction with its other partner-MAPs that are also sumoylated.

P2001
Board Number: B140
C. elegans microtubules are highly dynamic and have non-canonical lattices.
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Microtubules of the model organism C. elegans differ from the textbook eukaryotic microtubule both in their structure and dynamics. For example, microtubules in somatic cells do not contain the canonical 13 protofilament (pf) lattice seen in most eukaryotes; rather, most cells have 11 pfs. Furthermore, microtubules in various C. elegans tissues grow extremely fast (up to 60 um/min) - almost an order of magnitude higher than other model organisms. To determine the origin of these phenomena, we purified tubulin from whole C. elegans lysates. Interestingly, we observed a shift to smaller pf numbers for spontaneously nucleated microtubules relative to bovine microtubules. Furthermore, C. elegans microtubules grew much faster in vitro, with an apparent on-rate constant ~3-fold higher than for bovine tubulin, and underwent more frequent catastrophes, indicating that C. elegans tubulin is the most dynamic to date. To determine how C. elegans achieves these differences, we solved the structure of C. elegans microtubules at 4.8 A resolution. We found that C. elegans microtubules have retained the helical parameters of other eukaryotes, suggesting 11 pf microtubules found in vivo are super-twisted. We confirmed this result with negative stain electron tomography of the C. elegans embryo. When we mapped sequence conservation onto our structure, we found that the lateral bond interfaces has many divergent residues. Monte-carlo simulations of microtubule growth revealed that a modest increase in lateral bond energies is sufficient to recapitulate the growth rates observed in vitro. Together, these data provide insight into tubulin evolution by characterizing divergent dynamic and structural properties in a higher eukaryote.
P2002

Board Number: B141

TACC3 mitigates Nocodazole-induced growth cone effects and is required for proper embryonic neural development.
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Proper navigation of the developing axon necessitates the complex interactions between the microtubules (MTs) of the growth cone, associated proteins, and extracellular cues. Specifically, plus-end tracking proteins (+TIPs), proteins that reside at the plus-end of microtubules, play a crucial role in the development of embryonic neurites. We have previously demonstrated that TACC3 functions as one of these +TIPs and binds plus-ends of MTs in Xenopus laevis embryonic growth cones. Here, using quantitative analysis of high-resolution live imaging, we also show that TACC3 mitigates reduction in MT dynamics parameters -growth speed, length and lifetime- in response to Nocodazole exposure at low concentrations, and that TACC3 also limits neurite retraction under low concentrations of Slit2. Furthermore, we have observed that the impairment of proper axon navigation and growth may lead to neural morphological differences between the telencephalic hemispheres in Xenopus laevis embryos. In conclusion, our results illustrate that TACC3 may play a role in altering a developing axon’s response to external guidance cues and in facilitating proper neural morphological development.

P2003

Board Number: B142

Sequence-encoded charge patterning of the intrinsically disordered tail of FtsZ impacts polymerization and bacterial cell division.
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Polymerization of FtsZ, a bacterial homolog of tubulin, is an essential step in the assembly of the divisome (Z-ring) and the regulation of cell division in rod-shaped bacteria. The modular architecture of FtsZ includes a highly conserved GTPase domain and an intrinsically disordered C-terminal tail (CTT) that encompasses a poorly conserved polyampholytic linker (CTL) that is essential for Z-ring formation. Being a polyampholyte, the conformational properties of the CTL are determined by the patterning of oppositely charged residues quantified using a parameter. Although the sequences of CTLs are poorly conserved, is bounded within a narrow range of 0.2 ≤ ≤ 0.4. This suggests that sequence-encoded conformational properties and interactions mediated by the CTL must be conserved. We tested this hypothesis by scrambling the CTL of B. subtilis FtsZ to generate CTT variants with values that are within and beyond the observed bounds. FtsZ variants that lie within the bounds support ring-formation through a GTP-dependent mechanism. However, as increases beyond the bounds, ring formation is impaired and the CTTs promote an alternative tail-mediated assembly. Sequence features within CTLs modulate the subtle interplay amongst tail- and core-driven FtsZ assembly, high and low GTPase activity, and strong and weak lateral associations of FtsZ polymers. Our findings suggest that a “Goldilocks precept” i.e., the tail-mediated interactions must not be too strong or too weak, might govern the evolution of FtsZ-CTTs. Sequence selection in accord with the Goldilocks precept requires tail sequences with appropriately complex amino acid composition and optimal -values. Interestingly, the principles we have uncovered for FtsZ appear to be relevant for understanding the evolution of a larger class of tailed or “bristled” NTPases.
Microtubules and Cell Division

P2004  
Board Number: B143

UNC-45A is a novel spindle-associated microtubule destabilizing protein that antagonizes the effect of paclitaxel in cancer cells.

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UNC-45A is a highly conserved member of the UCS (UNC-45/CRO1/She4p) protein family. Genetic studies in lower organisms (S. cerevisiae, C. elegans and Drosophila) have shown an evolutionarily conserved role for UNC-45A in regulating cytoskeletal dynamics1-5. We are interested in understanding the role of UNC-45A with respect to its cytoskeletal-associated functions in mammalian cells. In our laboratory we have shown that UNC-45A is required for cytokinesis and motility in cancer cells6, for exocytosis in immune cells7 and for neurite outgrowth in cortical neurons8. Strikingly, new evidence from our laboratory shows that UNC-45A strongly co-localizes with interphase microtubules (MTs) and mitotic spindles throughout the cell cycle. Knockdown of UNC-45A in dividing cells results in multiple mitotic defects including increased mitotic index, increased spindle length, misaligned chromosomes, lagging chromosomes, and multipolar spindles. Moreover, acetylated tubulin levels in mitotic spindles are markedly increased upon loss of UNC-45A, suggesting that UNC-45A has microtubule destabilizing activity. This was confirmed in an in vitro Total Internal Reflection Fluorescent (TIRF) microscopy system, where UNC-45A was found to directly bind and destabilize MTs in absence of any other cellular factors. MT-destabilizing proteins have an important role in drug resistance to paclitaxel, a microtubule stabilizing agent commonly used in the treatment of breast and ovarian cancer. In an ovarian cancer cell system, loss of UNC-45A sensitizes cells to paclitaxel treatment. Conversely, cells that acquire paclitaxel resistance show increased expression of UNC-45A. Finally, immunohistochemical (IHC) analysis of ovarian cancer clinical specimens revealed a statistically significant correlation between UNC-45A expression and likelihood of resistance. These studies suggest UNC-45A is a novel microtubule-associated protein (MAP) with MT-destabilizing activity that antagonizes the effect of paclitaxel in cancer cells. Uncovering mechanistic details behind these novel UNC-45A functions has the potential to impact multiple fields of study, from translational research to basic cytoskeletal cell biology.

P2005

Board Number: B144

A Microtubule Organizing Center Directing Intracellular Transport in the Early Mouse Embryo.

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The centrosome is the primary microtubule organizing center (MTOC) of most animal cells. However, preimplantation mouse embryos only establish centrosomes at the late blastocyst stage. Therefore, the mechanism by which the early mammalian embryo organizes its microtubules (MTs) is unclear. By imaging fluorescently labeled MTs in live mouse embryos using four-dimensional confocal (4D) microscopy, we recently discovered a non-centrosomal form of MT organization in the early embryo. We find that contrary to most animal cells, the cytokinetic bridge does not undergo stereotypical abscission after cell division in the developing mouse embryo. Instead, it serves as a scaffold for the accumulation of proteins that stabilize MT minus ends and promote MT outgrowth throughout interphase, thereby transforming this structure into a non-centrosomal MTOC. Moreover, we show that this MTOC directs the transport of proteins such as E-cadherin to the cell membrane, which are essential to establish the first forms of tissue architecture during development. Together, our findings reveal how MTs are organized and function during the early stages of mammalian embryogenesis.

P2006

Board Number: B145

Tissue-specific degradation of essential centrosome components in vivo reveals distinct microtubule populations at noncentrosomal MTOCs.

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Microtubule organization is critical for cell function. Nearly all dividing animal cells use the centrosome as a microtubule organizing center (MTOC) to tether chromosomes to the spindle poles to facilitate the correct segregation of DNA between daughter cells. By contrast, differentiated cells organize their microtubules in a wide variety of patterns, and establish specific noncentrosomal locations as MTOCs to achieve their microtubule arrangements. While centrosomes redundantly require AIR-1/Aurora A and γ-TuRC for microtubule nucleation and organization, it is unknown how these tasks are accomplished at noncentrosomal MTOCs (ncMTOCs). A simple hypothesis is that ncMTOCs use these essential centrosomal proteins, tethering them to noncentrosomal sites with site-specific adapters. The C. elegans embryonic intestine is a simple epithelial model in which to test this hypothesis in vivo; the apical surfaces of these cells become specified as ncMTOCs during polarization and MTOC components such as γ-TuRC and AIR-1 localize to the apical membrane. Using tissue-specific degradation of endogenous AIR-1 and γ-TuRC components GIP-1/GCP3 and MZT-1/MOZART, we have tested the role of these proteins in ncMTOC function. Consistent with their known role at the centrosome, GIP-1, MZT-1, and/or AIR-1 depletion results in strong mitotic defects in intestinal cells prior to polarization and results in fewer intestinal nuclei. Futhermore, AIR-1 depletion perturbs TAC-1/TACC and γ-TuRC recruitment to intestinal centrosomes. Like at the centrosome, γ-TuRC recruitment to the apical ncMTOC requires GIP-1. Intriguingly, although localization of GIP-1 to the centrosome in dividing intestinal cells requires MZT-1, localization of GIP-1 to the apical ncMTOC does not, suggesting that MZT-1 specifically links γ-TuRCs to the centrosome and not to MTOCs in general. Further distinguishing MTOC function at the centrosome, the recruitment of γ-TuRC or TAC-1/TACC to the apical MTOC does not require AIR-1. Surprisingly,
degradation of AIR-1, GIP-1, or MZT-1, and even the depletion of both AIR-1 and GIP-1, has no overt effect on the localization of microtubules to the apical ncMTOC. GIP-1 degradation results in reduced apical localization of EBP-2/EB1 and fewer comets, though comets are still observed, indicating that only a subset of apical microtubules is dependent on γ-TuRC function. Together, these results suggest that centrosomal and noncentrosomal MTOCs require different proteins to function and that the apical ncMTOC is composed of distinct populations of microtubules.

P2007
Board Number: B146
Reconstitution of aster movement and cell division plane positioning in Xenopus egg extract.
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During early embryogenesis in *Xenopus laevis*, each cell division plane bisects the boundary between a pair of large microtubule asters that propagate from the poles of the mitotic spindle. The asters are a network of short, dynamic microtubules oriented radially outward from the microtubule organizing center (MTOC). Before cleavage, asters grow and move so that the cell divides approximately at its midplane and perpendicular to its long axis. Aster movement is thought to depend on length-dependent forces from cytoplasmic dynein opposed by hydrodynamic drag; however, it remains unclear how these forces propagate through the network of short, and short-lived, microtubules, resulting in aster movement. We reconstituted aster movement in *Xenopus* egg extract, and imaged the growth and interaction of asters under slit-like confinement. Aster boundaries stopped growing when they interacted with neighboring asters. The boundaries between asters formed dynamic, tessellated polygonal patches that resembled 2D foams. MTOCs moved relative to aster centroids and boundaries, and this movement was partially inhibited by the CC1 fragment of dynactin, which inhibits dynein-dependent forces. We measured the dynamic geometries of MTOCs and aster boundaries at different MTOC densities, and will use numerical and analytical modeling to investigate whether known forces can explain the results. Within the egg, microtubule asters must navigate abundant obstacles including lipid droplets and yolk platelets, which are removed during preparation of the extract. To simulate them, we added agarose spheres to the extract, and asters were able to grow around these obstacles. The global organization of microtubules remained radial, and the boundaries between asters remained approximately straight and equidistant between pairs of MTOCs. We also imaged the response of asters to controlled flows. We conclude that the short, dynamic nature of microtubules in large asters facilitates aster growth and movement through a cytoplasm containing abundant obstacles.

P2008
Board Number: B147
Spindle morphology tailoring through time: Interplay between spindle architecture and morphogenesis of the mammalian brain.
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Accurate chromosome segregation is essential for genome stability. Chromosome number deviations (or aneuploidy) influence cellular fitness and performance. During mitosis, a highly dynamic microtubule-
based structure, the mitotic spindle, is assembled to connect sister chromatids to the spindle poles providing the forces to segregate the chromosomes. Centrosomes, the main microtubule-organizing centres (MTOCs) of animal cells, organize the spindle poles contributing to spindle orientation and bipolarity. Interplay between centrosomes, spindle microtubules and associated proteins influence the fidelity of each cell division.

From all the organs in the human body the brain is particularly vulnerable to centrosome defects. Aneuploidy generated by centrosome dysfunction impairs embryonic neural progenitor survival, which culminates in severe brain size reduction at birth, a pathological condition known as microcephaly. Intriguingly, aneuploidy has also been reported in physiological conditions. Thus, there seems to be an intrinsic susceptibility of embryonic neural progenitors to mitotic errors. The origin of this susceptibility is not known. In order to unravel the mechanisms of error-prone mitosis in the neuroepithelium, we characterized mitotic spindle assembly during mouse neurogenesis in the WT brains.

Strikingly, we found that the morphology of the spindle changes during neurogenesis. While at early stages, the spindles of neural progenitors contain longer astral microtubules (MTs) that interact with the cell cortex, spindles at later stages gain in central spindle robustness at the expense of astral microtubule abundance. This change is followed by significant differences in the levels/distribution of key spindle associated factors along the spindle. Moreover, we identified Tpx2, a microtubule nucleating, organizing and stabilizing factor, as one of the key factors involved in the switch of spindle morphology.

Overall, our results indicate unexpected modifications in the pathways used by neural progenitors to build a bipolar spindle during neurogenesis, which confer a different chromosome segregation capacity. Indeed, by challenging the spindle formation with the presence of extra centrosomes, we observed an improvement of mitotic accuracy during the course of neurogenesis. We thus propose that during mammalian neurogenesis not all the progenitors are equally competent to segregate chromosomes correctly.

P2009
Board Number: B148
CENP-E-PRC1 interaction provides a temporal cue for central spindle assembly.
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Mitotic kinesin CENP-E plays key roles in chromosome congression and spindle checkpoint satisfaction. We have recently identified and characterized syntelin, a novel allostERIC inhibitor selective for CENP-E (Ding et al., 2010. Cell Res. 20, 1386-1389; Mo et al., 2016. Nat. Chem. Biol. 12,226-232; Liu et al., 2016. J Mol Cell Biol. 8,144-156). Cells treated with syntelin progress through interphase, enter mitosis normally with a bipolar spindle and lagging chromosomes around the poles. Our optical trap analyses show that syntelin is an allostERIC inhibitor which tightens CENP-E-microtubule interaction by slowing inorganic phosphate release. To delineate the role of CENP-E in reorganization of interpolar microtubules into an organized central spindle, metaphase synchronized cells were exposed to syntelin and other mitotic motor inhibitors. Syntelin does not perturb inter-polar microtubule assembly but abrogates the anti-parallel microtubule bundle formation. Real-time image shows that CENP-E inhibited cells undergo central spindle splitting which is confirmed by transmission electron microscopic analyses. Using a syntelin affinity matrix, we identified a novel CENP-E complex containing PRC1. Interestingly, inhibition of CENP-E did not alter the interaction between CENP-E and PRC1 but perturbed temporal assembly of PRC1 to the midzone. These findings reveal a previously uncharacterized role of CENP-E motor in temporal control of central spindle assembly. Currently, we are reconstituting the central
spindle sliding assay in vitro and delineate how CENP-E-PRC1 interactions establish the central spindle and promote spindle elongation using super-resolution imaging analyses.

**P2010**

**Board Number: B149**

Structures of growing and shortening microtubules suggest that anaphase forces act in part on bending protofilaments.

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We have used electron tomography to examine the tips of microtubules (MTs) growing or shortening in vivo and in vitro. The plus ends of MTs in the interzone of cells in anaphase B and kinetochore-associated MTs in metaphase are polymerizing in vivo. All these MTs are capped by PFs that show curvatures ranging from ~20 deg/dimer near their tips to ~10 deg/dimer near the MT wall. All PFs bend within a plane that contains the MT axis, so they are clearly separated from their neighbors. Since tubulin polymerization in vivo is facilitated by numerous tip-associated proteins, we have also examined the polymerization of pure tubulin in vitro, nucleated by axonemes from Chlamydomonas (kindness of M.E. Porter, Univ. MN). These growing MTs also display curving PFs at their tips, even when examined by rapid freezing and electron cryo-tomography. Again, PF curvature is greatest at the PF tip and decreases linearly with approach to the MT wall. The PFs of these MTs begin to curve away from the MT wall at a range of positions along the MT axis, so growing MT ends are “ragged.” The distribution of standard deviations of the mean positions of curvature onsets from many MTs peaks at 25 nm for growth at 1.2, and 4 mg/ml tubulin, but the frequency of outliers with high deviations increases with tubulin concentration. We compared these PF curvatures with those found on MTs induced to shorten in vitro by 25-fold isothermal dilution of MTs grown at 2 mg/ml and found curvatures that were slightly higher than those on growing MTs. The raggedness of ends on shortening MTs was, however, significantly less. The plus ends of kinetochore-MTs in anaphase A are shortening in vivo. Their PFs are longer than other PFs, and the linear decrease in PF curvature is not seen. These results suggest that MT growth occurs by the addition of bent tubulin to the tips of existing PFs, not to some niche where it can make bonds with multiple neighbors. Depolymerization is the reverse of the polymerization process. The fact that the PFs on anaphase kinetochore-MTs do not increase in curvature near their tips implies the action of cellular factors acting on these PFs, inducing them to straighten. This could be due to some of the force that these MTs exert on kinetochores during anaphase A.

**P2011**

**Board Number: B150**

Cdk1 phosphorylation of Nde1 and CENP-F contributes to G2-M Nuclear Envelope and Kinetochore dynein recruitment.

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Cytoplasmic dynein exhibits marked redistribution during the cell cycle, but the mechanisms responsible for this behavior remain incompletely understood. Dynein is recruited to the G2 nuclear envelope (NE) by early and late mechanisms involving, respectively, links to the nucleoporin RanBP2 through BicD2, and to the nucleoporin Nup133 through CENP-F and Nde1 (Baffet et al., 2015; Bolhy et al., 2011; Hu et al., 2013; Splinter et al., 2010). The late pathway is initiated by Cdk1-mediated exit of CENP-F from the
nucleus. Additionally, in mitosis, Nde1 and CENP-F subsequently co-localize to mitotic kinetochores from prophase until anaphase. To determine the mechanisms responsible for Nde1 and CENP-F co-redistribution and dynein recruitment we have examined their phosphorylation by Cdk1. Using a Cdk1 phosphorylation site-specific antibody and GFP-tagged wt and triple phosphomimetic and phosphomutant cDNAs, we have defined the role of Cdk1 in Nde1 behavior in HeLa cells. We find that Cdk1 phosphorylation of Nde1 is required for Nde1 recruitment to the G2 NE and mitotic kinetochore well into anaphase, a pattern comparable to the distribution of CENP-F. Phosphomutant Nde1 is very weak at the G2 NE. However, it is present at prometaphase kinetochores, but is completely lost upon chromosome alignment and MT attachment. Cdk1 phosphorylation of Nde1 increases its binding to CENP-F in vitro by ~ 2.5-fold, suggesting a mechanism for regulating the Nde1 and dynein recruitment from G2 through much of mitosis. To test the over-all importance of this mechanism in dynein recruitment to kinetochore, we rescued Nde1 knockdown with a dynein binding-deficient Nde1 mutant, which reduced the kinetochore dynein level by 50%. We also tested the role of Cdk1 phosphorylation in brain development using in utero electroporation of embryonic rat brain. We find that double phosphomutant Nde1 cDNA blocks G2-specific apical nuclear migration in radial glial progenitor cells (RGP). In contrast, a single site phosphomutant permits apical nuclear migration to the ventricular surface of the brain, but blocks mitotic entry. Finally, we have identified 9 Cdk1 sites in CENP-F, which may be responsible for its Cdk1-dependent nuclear export (Baffet et al., 2015). Together, these results indicate that Cdk1-phosphorylation of Nde1 and, possibly, CENP-F play critical roles in G2/M dynein regulation.

P2012
Board Number: B151
Loss of Fodrin Gives Mitotic Defects.
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Fodrin, a cytoskeletal protein of non-erythroid origin, plays a pivotal role in the maintenance of cytoskeletal structure integrity of plasma membrane. It is also involved in axonal transport and movement of organelles. Recent studies, however, showed its role in apoptosis and TGF-β signaling pathway. We have earlier shown that fodrin disappears from centrosomes in brain derived cells as the cells enter the prometaphase stage and comes back after cytokinesis. To check more of its functional involvement in mitosis, we have downregulated fodrin by shRNA treatment in neuronal and glial cells where it is found in large amounts. Downregulation of fodrin gave mitotic defects, like multipolar and broken spindles were observed. In these cells, chromosomes were un congressed. Further a high percentage of cells displayed mitotic delay. Compared to control cells that were showing mean mitotic duration of 30 min, fodrin downregulated cells showed mean mitotic duration of 52 min and some cells were even blocked in metaphase during a 150 min follow-up after fodrin downregulation for 96 h. The mitotic defects and mitotic delay indicate increase in the checkpoint protein functions. Among the checkpoint proteins, BubR1 was found to be increased during metaphase stage upon fodrin downregulation as compared to control downregulation. Besides, fodrin downregulation gave a smaller spindle size as compared to the control shRNA treated spindles. About 25% decrease in the pole to pole distance of spindles was observed in fodrin downregulated cells. These results show that fodrin has much more functional importance than earlier thought.
P2013

Board Number: B152

Structural Basis for Katanin Self Assembly.
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The reorganization of microtubules in mitosis, meiosis and development requires the microtubule-severing activity of katanin. Katanin is composed of a AAA ATPase subunit and a regulatory subunit. Whereas other AAA ATPases form stable hexamers, we show that wild-type katanin only forms heterodimers and heterotetramers. Heterododecamers were only observed for an ATP hydrolysis deficient mutant in the presence of ATP, suggesting an auto-inhibition mechanism that prevents oligomerization. X-ray structures of katanin’s AAA ATPase in monomeric nucleotide-free and pseudo-oligomeric ADP-bound states reveal conformational changes in AAA subdomains and N and C-terminal expansion segments that explain this auto-inhibition of assembly. These data lead to a model in which self-inhibited heterodimers bind to a microtubule, then transition into an assembly-competent conformation upon ATP binding. Microtubule-bound heterododecamers then promote tubulin extraction from the microtubule prior to oligomer dissociation.

P2014

Board Number: B153

Dynamic Instability of Microtubule Minus Ends.
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Dynamic organization of microtubule minus ends is essential for the formation and maintenance of acentrosomal microtubule arrays, such as those found in epithelial cells, neurons, or cells undergoing meiosis. In vitro, both microtubule ends switch between phases of assembly and disassembly, behavior known as microtubule dynamic instability. However, while the minus ends have characteristically slower growth rates, the frequency at which they transition from the growing to the shrinking state (known as ‘catastrophe frequency’) is similar to that observed at the plus ends. The mechanisms underlying this difference between the two ends are not known. While kinetic parameters measured at the microtubule plus ends have informed numerous mechanistic models to describe the plus end, whether these existing models can adequately explain minus end dynamics remains to be determined. Here, we use an in vitro reconstitution approach with total internal reflection fluorescence (TIRF) microscopy to determine the parameters characterizing minus-end dynamics. Using these minus end data, we evaluate several models of dynamic instability, previously developed to describe dynamics of microtubule plus ends. The present study aims to elucidate the fundamental nature of the microtubule minus end, which is imperative for understanding the minus end in the context of cellular regulation by external factors.
P2015
Board Number: B154
Autocatalytic microtubule nucleation determines the size and mass of spindles.
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The mitotic spindle is a complex self-organized structure that distributes the DNA between daughter cells during cell division. Interestingly, the main building blocks of the spindle, the microtubules, only have a lifetime of about 20 s while the entire structure remains for minutes up to hours. This requires constant creation of new microtubules, which is called nucleation. How microtubules, however, are nucleated in spindles and how spindles obtain their defined size despite the rapid turnover of their components is largely unknown. Here, we developed a new method based on laser ablation to measure the nucleation profile of transport-inhibited spindles in \textit{Xenopus laevis} egg extract and found that microtubule nucleation is spatially regulated. In combination with theory and quantitative microscopy, we show that new microtubules grow in an autocatalytic way and in direct physical contact to pre-existing microtubules. The autocatalytic activity of this nucleation system is spatially regulated by the limiting amounts of active microtubule nucleators, which decrease with distance from the chromosomes. This mechanism provides an upper limit to spindle size even when resources are not limiting.

P2016
Board Number: B155
Elucidating the mechanics of aster positioning using labile hydrogel microenvironments.
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The cytoplasm of a eukaryotic cell is a complex and dynamic microenvironment that requires proper organization to insure cell function. The microtubule (MT) cytoskeletal network plays a fundamental role in this organization. During interphase, it serves as both a supportive scaffold for organelles and as an arborized system of tracks for intracellular transport. Whereas during mitosis, the position of the interphase aster, which often coincides with the geometric center of the cell, determines the eventual location of the spindle apparatus and ultimately the cytokinetic furrow. The ability of the aster to find the cell center is particularly impressive in large blastomeres, where scale dictates aster movement over long distances. These movements require the generation of positioning forces and in this context there are thought to be three primary forces to generate this force balance: (i) pushing forces generated by the interaction between growing MT plus ends and the cell cortex, (ii) cortical pulling forces produced by MT dependent motors anchored in the cell cortex, and (iii) cytoplasmic pulling forces generated by motor mediated transport along aster MTs and interaction with organelles in the cytoplasm. How these different forces are integrated to position asters within cells of varied sizes and geometries remains an open question. To address it, we have developed the use of photolabile hydrogels to capture and release artificial microtubule organizing centers (aMTOCs) in defined geometric hydrogel microenvironments. Combined with cell-free extracts, we used this system to visualize the effects of such geometries on aster MT dynamics, MT density and aster positioning. Our current data suggests that
constraining an aster decreases the MT density without affecting nucleation rate. Using this approach, we hope to elucidate how the cellular microenvironment impacts aster growth, especially aster positioning through experimentally defined spatial patterns of MT density and extent.

Assembly and Disassembly of Cilia/Flagella 2

P2017
Board Number: B157
Architecture of mammalian centriole distal appendages supports a matrix that gates the primary cilium.
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Distal appendages (DAPs) are nanoscale, pinwheel-like structures protruding from the centriole distal end. DAPs mediate membrane docking for ciliogenesis, marking the cilia base around the ciliary gate. Here, through a superresolved multiplex of thirteen centriole-distal-end components, we surprisingly found that, instead of pinwheels, intact DAPs exhibit a “cone-shaped” architecture with components filling the space between each pinwheel blade, a new structural element we termed the distal appendage matrix (DAM). Specifically, CEP83, CEP89, SCLT1 and CEP164 form the backbone of pinwheel blades, with CEP83 confined at the root, and CEP164 extended to the tip near the membrane-docking site. In contrast, FBF1 marks the distal end of the DAM toward the ciliary membrane. Strikingly, unlike CEP164 essential for ciliogenesis, FBF1 is required for ciliary gating of transmembrane proteins, revealing DAPs as the essential part of the ciliary gate. Our results thus redefine both the structure and function of DAPs.

P2018
Board Number: B158
Myosin-Va is required for preciliary vesicle transportation to the mother centriole during ciliogenesis.
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Primary cilia are microtubule-based organelles protruding from the apical cell surface to sense different chemical signals. Defects in ciliogenesis can lead to a number of genetic disorders collectively known as ciliopathies. Pre-ciliary vesicle accumulation at the distal appendages of mother centrioles to form a larger ciliary vesicle is critical in early step of ciliogenesis. What is the marker protein for the preciliary and ciliary vesicle? Where do the ciliary vesicles come from? How do the ciliary vesicles transport to the distal appendages of centrioles? The above questions remain largely unknown. Here, using three-dimensional structured illumination microscopy and correlative light and electron microscopy we first demonstrate that the actin-based motor protein Myosin-Va localizes to pre-ciliary vesicles, ciliary
vesicles, and ciliary sheath. Moreover, the myosin-Va-associated ciliary vesicle is not only detected in the intracellular pathway-based ciliogenesis like RPE1 cells but also the extracellular pathway based ciliogenesis like IMCD3 cells. CRISPR/Cas9-mediated Myosin-Va gene knockout in cells dramatically inhibits not only the attachment of the pre-ciliary vesicles to the distal appendages of mother centriole but also cilia assembly, implying that Myosin-Va is required for regulating the ciliary vesicle trafficking. By comparing with other early ciliary membrane protein, we found that Myosin-Va functions upstream of EHD1-Smo- and Rab11-mediated ciliary vesicle formation. Importantly, through disrupting the function of microtubules and dynein, we further demonstrate that, Myosin-Va-associated ciliary vesicles are derived from the post-Golgi membrane, and transported to the pericentrosomal region by dynein-mediated transportation along microtubules. Finally, disturbing the centrosome-based branched actin filament by Arp2/3 inhibitor, CK-666 or siRNA-mediated knockdown of Arp2 dramatically inhibits the formation of large ciliary vesicle at the distal appendages but does not affect the transportation of the pre-ciliary vesicle from the post-Golgi to the pericentrosomal region. These results indicate that Myosin-Va takes over and transports pre-ciliary vesicles from pericentrosomal region to the mother centriole along the centrosomal branched-actin network. Together, our results indicate that Myosin-Va is the first marker to identify the preciliary and ciliary vesicle and Myosin-Va-regulated preciliary vesicle transportation from the post-Golgi to the distal appendages of mother centriole is the earliest step of ciliogenesis.

P2019

Board Number: B159
Exploring PACSIN membrane tubulation regulation during intracellular ciliogenesis using 3D electron microscopy.
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Defects in cilia are linked to human genetic diseases, called ciliopathies, and cancer. Ciliogenesis is thought to occur via two distinct processes. In the extracellular pathway, the mother centriole (MC) docks with the plasma membrane prior to ciliary growth. In contrast, the intracellular pathway involves the trafficking, fusion and reorganization of vesicular membranes at the MC prior to ciliary extension followed by emergence of the cilium at the cell surface. Here, we demonstrate that the F-BAR domain containing proteins PACSIN1 and -2 play an essential role in intracellular ciliogenesis in human and zebrafish cells. PACSINs are required for the shaping of smaller MC associated vesicles into the larger ciliary vesicle (CV). Membrane rearrangement at the CV step depends on the initial recruitment of EHD1/3 proteins, two known PACSIN1/2 binding partners linked to the Rab11-Rab8 pathway as well as SNAP29. Using Correlative Light and Electron microscopy (CLEM) combined with a Focused Ion Beam-Scanning Electron microscope (FIB-SEM), we observed membrane tubules connected to the developing intracellular cilia and the ciliary pocket in mature cilia in three-dimensions. Remarkably, during ciliogenesis these tubular membranes connect the developing cilium and the plasma membrane, forming a membrane channel to the outside of the cell. Using three color live cell imaging, we found that tubule channels originate from the intracellular cillum and require PACSINs. Together, our work uncovers a novel role for F-BAR containing proteins and membrane tubulation in ciliogenesis and explains how the developing intracellular cillum emerges from the cell.
P2020  
Board Number: B160  
A Bioactive Peptide Amidating Enzyme Is Required for Ciliogenesis.  
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We recently identified peptidylglycine α-amidating monooxygenase (PAM), a cuproenzyme required for generating amidated bioactive signaling peptides, in Chlamydomonas and mammalian cilia. PAM consists of two catalytic cores (monooxygenase and lyase) located in the secretory pathway lumen, followed by a transmembrane domain and small cytosolic region important for trafficking. Chlamydomonas PAM knockdown lines exhibit normal growth rates under both photoautotrophic and photoheterotrophic conditions, and their contractile vacuole cycle time is similar to controls. However, these strains fail to assemble normal cilia beyond the transition zone (TZ), which has missing Y linkers, and upregulate levels of two TZ components, CEP290 and NPHP4. In addition, singlet microtubules oriented orthogonal to the normal ciliary long axis and amorphous material including IFT proteins were present in the ciliary stubs distal to the TZ. The knockdown strains also have altered levels of IFT subcomplex A components, abnormal Golgi architecture, enlarged starch granules without a concomitant increase in total starch (likely due to defective trafficking of Golgi-derived starch metabolic enzymes), and exhibit selective defects in protein secretion. A line expressing a mutant PAM lacking most of its cytosolic C-terminal domain assembles cilia and is motile, suggesting that the PAM catalytic cores are important for ciliogenesis. Indeed, when deciliated wildtype Chlamydomonas cells were treated with either a mechanism-based monooxygenase inhibitor or a copper-specific chelator, the rate of reciliation was significantly retarded, indicating that amidating activity is a key ciliogenic parameter. RNAi-mediated reduction of PAM gene expression in planaria reduced cilia-mediated organismal gliding velocity and motile ciliary density on the ventral surface. The remaining cilia were aberrant with dyskinetic motion, decreased beat frequency and defects in axonemal architecture. In addition, numerous cytosolic axonemes lacking a ciliary membrane were present in the ventral epithelial cell cytoplasm, suggesting that lack of PAM leads to basal body docking and ciliary membrane trafficking defects, but does not affect axonemal assembly per se. Pam−/− mouse embryos die at embryonic day ~14.5 with massive edema. At this early developmental stage, the embryos lack multiciliated cells. However, primary cilia in neuroepithelial cells of the brain ventricles were significantly shorter than controls. Our data suggest that PAM activity and alterations in post-Golgi trafficking contribute to the observed ciliogenesis defects and provide an unanticipated, highly conserved link between PAM, amidation and ciliary assembly. Supported by DK032949, GM042143 and GM051293 (NIH).  

P2021  
Board Number: B161  
A mechanistic study of how O-GlcNAcylation levels regulates cilia lengths in mammalian cells.  
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Primary cilia are microtubule (MT) based hair-like structures that extend from the surface of many types of cells. Dysfunctional primary cilia is involved in a large number of human diseases and disorders, such as polycystic kidney disease (PKD) and Bardet-Biedl syndrome (BBS), obesity and diabetes, collectively termed ciliopathies. The ciliary functions rely on defined ciliary lengths. Besides the cell types, the
nutritional environment has been shown to regulate the ciliary length. However, the cellular internal mechanism of ciliary length regulation involved in responding to external environment condition is unclear. We recently uncovered a negative correlation between the nutrient sensing O-GlcNAcylation levels and cilia lengths in mammalian cells. Increased O-GlcNAcylation correlates with shorter cilia lengths, while decreased O-GlcNAcylation correlates with longer cilia. Previous studies have shown that HDAC6 (histone deacetylase 6), acting through axoneme microtubule deacetylation, governs ciliary disassembly. We showed that HDAC6 is O-GlcNAcylated and its modification related to HDAC6 protein stability. Future work is centered on addressing the hypothesis that O-GlcNAc of HDAC6 increases its deacetylase activity, thus shortening cilia lengths.

P2022
Board Number: B162
The role of IFT54 in tubulin transport and cilia stability.
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Intraflagellar transport (IFT) shuttles tubulin, the major structural protein of cilia and flagella, from the cell body into cilia to promote elongation. The IFT proteins IFT81 and IFT74 form a tubulin binding module: IFT81 binds the dimers via its calponin homology (CH) domain and the basic N-terminal domain of IFT74 interacts with the acidic C-terminus of β-tubulin.1 In vivo studies in Chlamydomonas reinhardtii revealed that strains lacking either the N-terminal domain of IFT74 (ΔN-IFT74) or carrying mutations incapacitating the CH-domain of IFT81 (CH5E-IFT81) build near full-length flagella albeit at a slower rate. ift81 CH5E-IFT81 ift74 ΔN-IFT74 double mutants, however, fail to assemble cilia with a subset of cells possessing stumpy flagella with a normal axonemal structure.2 Thus, IFT81/74 appear to form the major tubulin binding site on IFT trains. However, several other IFT proteins possess CH-domains and the N-terminal CH of IFT54 has been shown to bind tubulin in vitro.3 To address the question whether IFT54 contributes to tubulin transport, we characterized an insertional mutant in the IFT54 gene. The mutant expresses a C-terminally truncated non-functional IFT54 protein and completely lacks flagella. The mutant phenotype is rescued by both full-length and truncated CH-less IFT54 (ΔCH-IFT54). Specifically, the ift54 ΔCH-IFT54 strain assembles full-length, motile cilia at a normal rate, arguing against an essential role of IFT54 CH in tubulin transport. However, ift54 ΔCH-IFT54 interfered with cilia assembly in combination with the above described mutations in the IFT81 or the IFT74 genes: An ift54 ΔCH-IFT54 ift74 ΔN-IFT74 double mutant is entirely flagella-less similar to other IFT loss of function mutants. Only about 20% of the cells of the ift54 ΔCH-IFT54 ift54 CH5E-IFT81 double mutant possess near full-length flagella, and flagellar regeneration is slower than in the parental strains. Double mutant cells often remain entrapped in the mother cell wall despite the presence of flagella. Electron microscopy revealed that the mutant flagella are prone to abscission and fragmentation. Thus, the CH domain of IFT54 is required for normal cilia assembly when the tubulin binding sites of either IFT74 or IFT81 are impaired. To determine if this phenotype is the result of decreased tubulin transport or a more general defect in IFT, we are investigating the transport of GFP-tagged tubulin and tubulin derivates in ift54 ΔCH-IFT54 and suitable double mutants. 1. Bhogaraju, S., L. et al. 2013. Science. 2. Kubo, T., et al. 2016. J Cell Sci. 3. Taschner, M., K. et al. 2016. EMBO J.
P2023
Board Number: B163
The flagellar associating protein FAP85 of *Chlamydomonas* is one of the microtubule inner proteins.
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*Chlamydomonas* flagellar axonemes are composed of 200-600 types of proteins. Despite of extensive studies, many proteins have remained un-characterized. Among these un-characterized flagellar associating proteins (FAPs), we focused on the protein with an EF-hand motif, FAP85 which is specific for *Chlamydomonas*. We have identified the protein with molecular weight of 22kDa in the high salt extract of *Chlamydomonas* flagella with using peptide mass fingerprinting, which was previously named FAP85. We then cloned cDNA encoding FAP85 and expressed it in *E. coli* cells as a ProS2-conjugated form. The polyclonal antibody against the expressed protein was prepared and was confirmed to interact with native FAP85. The western blot analyses demonstrated that the anti-FAP85 antibody recognizes a prominent band of 20-kDa in every isolated axonemes from mutants lacking major axonemal components (the outer arm-lacking mutants, *oda1* and *oda2*; the inner arm dynein I1-lacking mutant *ida1*; inner-arm dynein (subspecies, a c d and e) lacking mutant, *ida5*; DRC-defect mutants, *pf2* and *pf3*; radial spoke-lacking mutants, *pf14*, the central pair lacking mutant, *pf18*; beak-like structure-lacking mutants, *mbo1*). N-Lauroylsarcosine (Sarkosyl) treatment solubilized the FAP85 from the axonemes, concomitantly with the solubilization of the A tubule. Immuno-gold electron microscopy on Sarkosyl-treated axonemes showed that gold-particles were observed only on the inner wall of A tubules of the doublet exposed with Sarkosyl treatment and had the structural repeat of ca. 50 nm. Furthermore, FAP85 had effects on tubulin kinetics, in which the critical concentration of tubulin decreased and the rate of tubulin polymerization slightly became faster in the presence of FAP85. According to these results, we conclude that FAP85 is a novel component of microtubule binding proteins localized on the inner wall of the A tubule.

P2024
Board Number: B164
The growing motile cilium tip undergoes a time-dependent development process.
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Eukaryotic motile cilia are microtubule-based structures responsible for cellular locomotion and movement of extracellular fluids. While motile cilium regeneration and the structure of the motile cilium axoneme have been well described, the development of the distal tip region and its structure during ciliogenesis remain poorly characterized. We studied tip region development during cilium regrowth and discovered that the tips of motile cilia display dynamic morphology as the cilium develops. In these investigations, we sought to investigate the factors that control ciliary tip region lengths, and determine whether these different morphologies are conserved across species. The motile cilia of deciliated *Chlamydomonas reinhardtii* and *Tetrahymena thermophila* were observed during their regrowth using scanning electron microscopy, negative staining transmission electron microscopy, and cryo-electron tomography. The investigation revealed that the ciliary tip region is subject to a time-dependent
development process that is independent of species. Furthermore, the cillum tip continues to develop after the regrowing cillum reach their stable, full lengths.

P2025  
Board Number: B165  
Phosphatidylinositol 4,5-bisphosphate (PIP2) is Essential for Cillum Assembly and Function in *Drosophila melanogaster*.  
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Cilia are antenna-like sensory organelles whose malfunction underlies many genetic disorders in humans. Recent studies have found that dephosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) by inositol 5-phosphatase (INPP5E) is necessary for cillum assembly and function. We present evidence against a simplistic model for the PIP2 code of cilia. Specifically, we show that PIP2 is essential for both formation and proper function of chordotonal cilia in *Drosophila melanogaster*. We report the presence of a tightly localized pool of relatively high PIP2 levels within the chordotonal cillum, suggesting that the notion of cillum as organelles devoid of PIP2 should be reconsidered. We also observe that neuronal clones mutant for the type I phosphatidylinositol 4-phosphate 5-kinase A/B ortholog Skittles (sktl) do not assemble cilia. Furthermore, in contrast to overexpression of Sktl in chordotonal neurons [1], RNAi-mediated sktl knockdown does not affect the localization of the Tubby domain protein Tulp. Instead, sktl RNAi induces defects in intraflagellar transport, which is unaffected in inpp5e mutants that contain high intraciliary levels of PIP2 [1]. We also report a novel role for PIP2 in transition zone length control – sktl RNAi induces aberrant elongation of transition zone-associated Cep290. This phenotype is mimicked by loss of PIP2 in the ciliated male germline of *Drosophila*. Our observations reveal unappreciated complexity in the functional relationship between PIP2 and cilia.  
References  

P2026  
Board Number: B166  
Identification and characterization of cillum proteins in *Caenorhabditis elegans*.  
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In addition to forming centrosomes in dividing cells, centrioles have an evolutionarily conserved role as basal bodies templating the formation of cilia. While numerous centriolar and ciliary components have been identified, key aspects of ciliogenesis, including basal body membrane docking and initiation of axoneme elongation remain poorly understood. In addition, previous studies of cilia in *Drosophila* and *Plasmodium* revealed an intraflagellar transport-independent biogenesis pathway, for which few molecular components are currently known. Finally, the genes linked to many human ciliopathy cases have not been determined. Together, these results indicate that additional cilia proteins and regulators remain to be identified.  
We have been undertaking a phylogenetic profiling approach to identify novel genes whose conservation pattern across eukaryotes suggests a potential role in cillum assembly or motility, which we are characterizing using *Drosophila* and *C. elegans* as experimental models. The nematode *C. elegans*
has proven to be a particularly powerful system to dissect the function of novel cilia genes because cilia are exclusively found in post-mitotic neurons, which are dispensable for viability and fertility. The availability of a wide range of ciliary markers, together with the stereotypical pattern of nematode development, makes it easy to characterize the role of novel proteins involved in cilia biogenesis. Of the 386 human genes whose inheritance pattern is characteristic of cilia, 135 are conserved in C. elegans including a set of 60 novel genes. Preliminary characterization of available mutants revealed a number of additional cilia genes. This poster will present the primary data from our screen, as well as more detailed characterization by light and electron microscopy of several of these novel proteins, including an apparent regulator of cilia biogenesis conserved across eukaryotes.

P2027
Board Number: B167
TrypTag.org – Genome wide protein localisation reveals organelle sub-domains, with functional consequences in the flagellum.
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Organelles have highly organised, complex internal structures that enable them to perform a diverse set of biological functions. Analysing this complex structural organisation on a genome-wide scale, using methods like mass spectrometry is typically extremely challenging and often loses fine-scale structural resolution. To address this, we are determining the localisation of every protein encoded in the Trypanosoma brucei genome using our high-throughput tagging methodology and automated quantitative image analysis of cell ultrastructure. Trypanosomes are ideal for this study as they combine an exquisitely ordered cellular architecture with conserved core eukaryotic biology. Trypanosome organelles have precisely defined positions/distributions in the cell, and inter-organelle contact occurs at specific positions; crucially they have greater internal order than yeast cells or typical human cell lines. To date we have localised over 80% of the 8129 proteins encoded in the genome and the data we are generating is made freely available on our website http://tryptag.org. We have shown many proteins localise to specific organelle sub-domains and this organelle asymmetry/inhomogeneity applies to all organelles, including the flagellum, endoplasmic reticulum and mitochondrion. Here, as one example, we concentrate on our comprehensive molecular cartography of the flagellum and the role of protein asymmetry within the flagellum. So far, we have found 43 proteins that localised to the proximal region of the flagellum only, 29 to the distal region only, 62 flagellar tip proteins and 289 proteins in the basal body or transition zone. Comparable asymmetry of protein localisation was also observed for flagellar membrane proteins. The cohort of distal flagellum proteins included orthologs of the docking complex proteins DC1 and DC2, required for attaching the outer dynein arms to the microtubule doublets of the axoneme. We showed there are also proximal flagellum DC1-like and DC2-like orthologs. This proximal/distal asymmetry is important for flagellum beat control in trypanosomes, and predicts tissue-specific control of flagellum beat by DC1 and DC2 orthologs in humans. This one example of exploiting the TrypTag dataset has identified a new candidate ciliopathy gene and produced a map at molecular detail of the entire flagellum. TrypTag is a rich dataset that will help define organelle sub-domains and therefore refine overall organelle function with implications for the entire eukaryotic cell biology field.
P2028
Board Number: B168
Mammalian primary cilia disassembly is heterogeneous but primarily occurs via rapid whole cilium shedding.
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The regulated cycle of assembly and disassembly of primary cilia is critical for correct ciliary signaling function and cell cycle control. While assembly and maintenance of the primary cilium are well studied, much less is known about the mechanism(s) of ciliary disassembly. Based on studies of the single celled biflagellate algae Chlamydomonas reinhardtii in which anterograde and retrograde intraflagellar transport (IFT) control ciliary assembly and maintenance, it is thought that a change in the balance of IFT could lead to ciliary disassembly. Here we tested this, and other hypotheses for ciliary disassembly in mouse epithelial IMCD3 cells expressing fluorescent markers of the ciliary membrane (SSTR3) and basal body (pericentrin PACT domain). Confocal time lapse imaging at high spatiotemporal resolution revealed a striking diversity of cilia disassembly behaviors that included gradual reabsorption into the cell and direct shedding from the cell surface, and ranged from seconds to hours. Inhibition of retrograde IFT with ciliobrevin D had no effect on overall loss of cilia or disassembly dynamics, indicating that IFT is not the principal regulator of cilia disassembly in these cells. The most prevalent method of cilia disassembly (~70\% of cases) was super-fast cell shedding, which occurred in seconds (2.8 ± 1.3 um cilia length/min). Immunofluorescence and Western blot analysis of material concentrated from the medium confirmed the presence of ciliary material detached from cells following induction of cilia disassembly. Ciliary shedding was preceded by a calcium influx within cilia, and was disrupted by depolymerization of actin with cytochalasin D or latrunculin A. This work reveals unexpected diversity in mechanisms of ciliary disassembly, and points to new potential regulators of this highly dynamic and heterogeneous process.

P2029
Board Number: B169
How cells control the size of their organelles: Flagellar length control in Chlamydomonas.
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How cells control the size of their organelles is an unanswered question in Biology. Flagella are an ideal organelle to study this question because of their simplistic structure; they are essentially one dimensional structures, only changing in length, which offers an advantage in simplicity over more complicated organelles like the endoplasmic reticulum. To study flagellar length control, we have built a microfluidic device capable of trapping Chlamydomonas cells for periods of time much longer than flagellar regeneration. With this device, we are able to measure length scale fluctuations predicted by theory that have not been measured before, enabling us to identify likely length control models.
P2030

Board Number: B170
THE ROLE OF SMALL GTPASE ARL3 IN THE FORMATION AND FUNCTION OF THE CHLAMYDOMONAS FLAGELLA.
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ARL3 is a cilia-related protein expanding from single-cell protozoans to mammalians. ARL3 has been shown to be involved in the ciliogenesis and serve as a displacement factor for the lipid-modified proteins. Functions of ARL3 other than ciliogenesis may be caused by the misdistribution of a subset of lipid-modified peripheral membrane proteins. These proteins depend on ARL3 in order to be properly released to the cilia. Currently, questions remain unclear about the function of ARL3 in ciliogenesis, and the relative extent that this arl3-dependent displacement pathway contributes. In this study, we characterized a Chlamydomonas arl3 mutant, which presents various subtle flagellar defects. We found that cells rescued with ARL3-GTP assemble significantly shortened flagella than others, confirming that overexpression of ARL3-GTP leads to ciliogenesis defects. Moreover, we compared the protein composition of isolated flagella between the mutant arl3 and the wild-type cc125. As we expected, the axonemal composition did not show significant change whereas the protein bands with altered intensity were detected in the detergent-rich partition, which contains membrane proteins. This study provides insights into possible mechanisms of ciliary dysfunction caused by the depletion of ARL3. Moreover, the isolated arl3 mutant can be a valuable tool in further function analysis of ARL3.

P2031

Board Number: B171
The contraction of Centrin filaments initiates assembly of the cilium by pumping IFT and flagellar precursors from the Zone of Exclusion (ZOE) into the ciliary/flagellar stubs.
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The Zone of Exclusion (ZOE) is an area of cell organelle exclusion located between the ciliary basal bodies and the nucleus, and bounded by bundles of centrin filaments connecting the basal bodies to the nucleus. We now show by TEM and fluorescence microscopy that the ZOE area is a flattened disc. Upon removal of the flagella of Chlamydomonas, calcium enters the cytosol, the centrin filaments rapidly contract, and pull the nucleus up underneath the basal bodies. This pushes the contents of the ZOE into the ciliary stubs. We now show that the ZOE contains IFT proteins and flagellar precursors. Earlier work had shown that IFT quickly appears, after flagellar detachment, in the flagellar stubs (Marshall and Rosenbaum, JCB 2001). Upon completion of flagellar assembly, the ZOE re-appears in the cytoplasm, following the kinetics of flagellar precursor utilization and re-synthesis (Lefebvre and Rosenbaum, JCB, 1978). Centrin contraction requires calcium (Salisbury et al, JCB (1987) as does the initiation of flagellar assembly (Lefebvre and Rosenbaum, JCB, 1978). The flagella will not assemble without centrin contraction. These results are consistent with the kinetics of flagellar regeneration in Chlamydomonas which have no lag phase (Rosenbaum, Moulder and Ringo, JCB, 1968) and the kinetics of flagellar precursor pool utilization during flagellar assembly in Chlamydomonas (Lefebvre and Rosenbaum, JCB, 1978). The results suggest that IFT and its associated flagellar precursors are associated with the centrin filaments in the ZOE, and are moved into the forming ciliary stubs by centrin contraction on deciliation. The IFT is initially moved into the stubs as a "bolus" which contains all the iFT the flagellum will ever
have as it elongates (Marshall and Rosenbaum, et al MBoC 2005). Future studies will probably show the molecular association of IFT, flagellar precursors, and centrin in the cytoplasm.

**P2032**

**Board Number: B172**

Biochemical and Structural Insights into the Interaction of Myristoylated Cargo with Unc119 Protein and Their Release by Arl2/3.

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Primary cilia are highly specialized small antenna-like cellular protrusions that extend from the cell surface of many eukaryotic cell types. The protein content inside cilia and cytoplasm is very different, but details of the sorting process are not understood for most ciliary proteins. Here we address the question whether the binding affinity has an impact on the transport of myristoylated cargo by the carrier proteins Unc119a and Unc119b like prenylated cago and their carried protein PDE6β. We thus analyzed the binding strength of N-terminal myristoylated cargo peptides (GNAT1, NPHP3, Cystin1, RP2, and Src) to Unc119a and Unc119b proteins. The affinity between myristoylated cargo and carrier protein, Unc119, varies between subnanomolar and micromolar. Peptides derived from ciliary localizing proteins (GNAT1, NPHP3, and Cystin1) bind with high affinity to Unc119 proteins, whereas a peptide derived from a non-ciliary localizing protein (Src) has low affinity. The peptide with intermediate affinity (RP2) is localized at the ciliary transition zone as a gate keeper. We show that the low affinity peptides are released by both Arl2-GppNHp and Arl3-GppNHP, whereas the high affinity peptides are exclusively released by only Arl3-GppNHP. Determination of the x-ray structure of myristoylated NPHP3 peptide in complex with Unc119a reveals the molecular details of high affinity binding and suggests the importance of the residues at the +2 and +3 positions relative to the myristoylated glycine for high and low affinities. The mutational analysis of swapping the residues at the +2 and +3 positions between high and low affinity peptides results in reversing their affinities for Unc119a and leads to a partial mislocalization of a low affinity mutant of NPHP3.

**Sensory and Signaling Functions of Cilia**

**P2033**

**Board Number: B173**

Loss of Arf4 causes severe degeneration of the exocrine pancreas but not cystic kidney disease or retinal degeneration.

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Photoreceptors rely on intracellular trafficking mechanisms to ensure proper delivery of specialized membrane proteins to their ciliary, light-sensitive outer segment. Unfortunately, very little is known about the mechanisms sorting cargos at the Golgi complex for transport to the ciliary membrane. Previously, the small GTPase, Arf4, emerged as a key molecule driving the selection of membrane proteins for delivery to the cilia. Specifically, Arf4 was thought to be critical for sorting the visual pigment, rhodopsin, from trans-Golgi network into transport vesicles destined for the outer

Monday-106
segment. Experiments performed in cell culture similarly suggested that Arf4 may be involved in the sorting of kidney-specific ciliary proteins as well. We now deleted Arf4 specifically in either rod photoreceptor cells, kidney, or globally during the early postnatal period. Arf4 deletion in photoreceptors did not cause rhodopsin mislocalization or retinal degeneration, as would be expected if Arf4 played a critical role in outer segment protein transport. Likewise, Arf4 deletion in kidney did not cause cystic disease, as would be expected if Arf4 were involved in general ciliary trafficking. In contrast, global Arf4 deletion in the early postnatal period resulted in growth restriction, severe pancreatic degeneration and premature death. Thus, while our data support a role for Arf4 in endomembrane trafficking and reveals its essential role in exocrine pancreatic function, it demonstrates that Arf4 is not the key molecule for sorting ciliary cargo. This work is critically important to the photoreceptor field as it will drive the search for alternative ciliary pathways and players.

P2034
Board Number: B174
An uncharacterized transport pathway for membrane proteins in primary cilia revealed by high-speed super-resolution microscopy.
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The primary cilium is a microtubule-based protrusion on the surface of many eukaryotic cells. It houses essential components of the Hedgehog, planar cell polarity, and many other signaling pathways, all of which require efficient protein trafficking between cytosol and primary cilium for transmitting signals and recycling receptors. It is believed that vesicular trafficking is employed for the intracellular protein transport from the Golgi to the primary cilia, while membrane diffusion and intraflagellar transport (IFT) are the major transport mechanisms for the intraciliary transport of transmembrane proteins. However, where post-Golgi vesicles fuse to and how membrane proteins are delivered into primary cilia remain in dispute. Using a high-speed super-resolution microscopy approach, we mapped the three-dimensional (3D) spatial locations of transport routes for transmembrane proteins translocating between the cytoplasm and primary cilia in live cells with an unprecedented spatiotemporal resolution of 2 ms and 10-20 nm. Remarkably, our high-resolution 3D data reveal that a previously uncharacterized transport pathway exists for vesicles loaded with transmembrane proteins inside the axonemal lumen through the transition zone and into the ciliary shaft. The distinct spatial locations of this new transport pathway and the IFT train route suggest a more precise and complete model for the cytoplasm-cilium transport of membrane proteins.

P2035
Board Number: B175
Ciliary GPCRs are required for adipogenesis.
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The primary cilium, a small antenna-like cellular projection, is localized on the surface of all mesenchymal progenitor cells. Moreover, the primary cilium is required for differentiation of mesenchymal cells. However, how the primary cilium functions in these processes remains poorly understood. Here, I will present a novel ciliary G protein-coupled receptor that regulates adipogenesis of both a murine cell line model of adipogenesis and primary isolated murine and human pre-adipocytes. Activation of this novel ciliary pathway by its natural ligand or a pharmacological agonist acutely raises
cyclic AMP levels in the primary cilium, resulting in entry into mitotic differentiative expansion, activation of the master adipogenic transcription factor Ppar,y and terminal differentiation. Conversely, a pharmacological antagonist inhibits adipogenesis. We will provide genetic evidence further detailing the signaling mechanisms that couple this GPCR to differentiation and ciliary signaling. Moreover, we show that this study has uncovered the physiologically relevant ligand to replace IBMX, a phosphodiesterase inhibitor commonly used to initiate adipogenesis in vitro by raising cellular cyclic AMP. This places the primary cilium at the crossroad of whether or not to initiate adipogenesis. Since adipocytes turnover regularly and excess nutrient uptake drives an increase in both the size and number of adipocytes, we strongly believe that our findings have significant impact on our understanding of what marks a pre-adipocyte and how its cell fate is regulated.

P2036  
Board Number: B176  
Isolation of ciliary proteins from the transient primary cilium of differentiating 3T3-L1 pre-adipocytes.  
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Obesity has become a leading health crisis in Western society. One biomedical approach to a cure is to target how the body manages energy storage and expenditure such that intentional pharmaceutical design can be used to manage signaling pathways. Our work focuses on the development of adipose tissue, specifically the expansion and differentiation of pre-adipocytes using murine 3T3-L1 cells as a model system. 3T3-L1 cells form a transient primary cilium during differentiation that is absolutely required for adipocyte formation. These cilia can be labeled for fluorescence microscopy or Western Blot using antibody to acetylated tubulin. Our goal is to better understand why proteins migrate to the primary cilium, the molecular interactions they participate in and the cellular processes they regulate. The specific aim of this project is to develop a ciliary protein isolation procedure for differentiating 3T3-L1 cells that would ultimately be useful for performing mass spectrometry, so that we can identify the pool of proteins in primary cilia. To date there is no established protocol for ciliary isolation from 3T3-L1 cells. Most ciliary isolation methods target model cells with lengthy cilia that aren’t dependent upon developmental stage, and initial attempts to isolate 3T3-L1 cell cilia were particularly challenging. Both shear force and calcium shock methods were attempted on large pools of ciliated pre-adipocytes. Calcium shock followed by successive discontinuous and continuous sucrose gradient fractionations was deemed most successful with acetylated tubulin detected in just 4 of 58 fractions. Furthermore, fractions are being probed via Western Blot with antibodies against other ciliary marker proteins such as IFTs and BBS proteins as well as general plasma membrane markers to confirm maximal separation of ciliary proteins prior to scale-up procedures.

P2037  
Board Number: B177  
Use of a new inducible Mchr1-CreER mouse model to study the role of primary cilia in obesity.  
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Studies of rare genetic disorders such as Bardet-Biedl Syndrome (BBS) and Alström Syndrome (ALMS) have continued to reveal the importance of genetics in the biology of appetite and satiety. Both BBS and ALMS are classified as ciliopathies because they result from dysfunctional primary cilia. Primary cilia are...
small, microtubule based cellular protrusions that function as specialized signaling centers. Several G-protein coupled receptors (GPCRs) preferentially localize to the primary cilium, including melanin concentrating hormone receptor 1 (Mchr1). While the melanin concentrating hormone (MCH) pathway plays a recognized role in feeding behavior, the importance of the localization of Mchr1 within the cilium remains unclear. To better understand the impact of ciliary Mchr1 localization on obesity phenotypes, we generated a novel transgenic mouse model (Mchr1-CreER) that uses the Mchr1 promoter to drive the expression of the inducible CreER. This model allows us to study terminally differentiated Mchr1 expressing cells. We first studied the expression pattern of Mchr1 in adult mice by crossing Mchr1-CreER mice with mice expressing a RosaLacZ Cre reporter allele. We observed robust Mchr1-CreER activity in the hypothalamus, hippocampus, and cerebellum. Two Mchr1-CreER founder lines showed similar expression patterns. We are currently comparing the expression pattern of Mchr1 in a constitutive Mchr1-Cre line with our inducible Mchr1-CreER line. Ongoing studies of the Mchr1-CreER mice include assessing the effect of conditional loss of primary cilia in terminally differentiated Mchr1 expressing cells on feeding behavior and energy homeostasis. Following cilia loss in Mchr1-expressing cells we will track body weight and feeding behavior in vivo and changes in electrical activity in vitro. These studies will further our understanding of ciliary GPCR signaling and may reveal unrecognized roles for cilia that can impact feeding behavior and obesity in adults.

P2038
Board Number: B178
Deficiency of IFT-A protein, THM1, sensitizes pre-adipocytes to insulin signaling and differentiation, and heightens adipose insulin sensitivity in a pre-obese state.
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The growing prevalence of obesity and associated insulin resistance and metabolic disease worldwide presents a significant negative impact on human health. Adipose tissue plays a critical role in insulin sensitivity. Hypertrophy or the enlargement of adipocytes is associated with metabolic dysfunction, while hyperplasia or formation of new adipocytes from pre-adipocytes may promote healthy metabolism. Ciliopathies link obesity to dysfunction of the primary cilium, an antenna-like sensory organelle that mediates signaling pathways. Mutations in THM1, an intraflagellar transport complex A (IFT-A) component which mediates ciliary protein transport, have been reported in patients with Bardet-Biedl Syndrome, which manifests obesity as a cardinal clinical feature. We have reported that global deletion of Thm1 in adult mice causes obesity. Here we examine the role of THM1 deficiency in adipogenesis. shRNA-mediated THM1 knock-down (kd) of 3T3-L1 mouse pre-adipocytes caused shortened primary cilia with bulbous distal tips. Following differentiation and Oil Red O-staining of lipid droplets, THM1 kd cells showed markedly enhanced adipogenesis, which was accompanied by earlier activation of AKT and ERK signaling and earlier nuclear expression of adipogenesis transcriptional regulators, C/EBP and PPAR. Additionally, THM1 kd pre-adipocytes showed greater AKT and ERK activation in response to insulin than control cells. White adipose tissue of pre-obese Thm1 conditional knock-out mice had higher levels of insulin sensitivity markers, PPAR and adiponectin, than control mice. Moreover, mesenteric white adipose tissue of pre-obese Thm1 conditional knock-out mice showed higher AKT activation than control littermates in response to intraperitoneal administration of insulin. Interestingly, we observed that the timing of peak insulin response was sex-dependent, and male and female mice showed maximal responses 15 and 30 minutes following insulin injection, respectively. Together, these data indicate that THM1 deficiency in pre-adipocytes promotes insulin
signaling and differentiation, and enhances adipose insulin sensitivity in a pre-obese state. This is the first demonstration of the role of an IFT-A component in regulating adipogenesis.

P2039
Board Number: B179
Primary hypothalamic neuronal culture for assessing primary cilia associated signaling.
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Primary cilia are microtubule based cellular appendages that function as sensory and signaling centers for cells in both mammalian development and adult tissue homeostasis. Interestingly, conditional loss of primary cilia from neurons in the hypothalamus leads to hyperphagia and obesity in mouse models of ciliopathies. Though \textit{in vivo} studies have helped elucidate the role of hypothalamic cilia in feeding behavior, their precise roles in signaling associated with the obesity phenotype remain unclear. Here we have developed a system for primary neuronal cultures to assess cell and neurobiology questions that affect hypothalamic neuronal populations involved in regulating feeding behavior. Specifically, we cultured neurons from wildtype C57BL/6J mice on the day of birth and evaluated the hypothalamic neuronal populations and cilia signaling proteins. Similar to our previous work with hippocampal neurons, nearly half of cultured hypothalamic neurons possess primary cilia. To determine if cilia mediated signaling can directly impact neuronal electrical activity, we cultured neurons from ciliopathy models and controls on multi-electrode arrays. Initial data show that these cultured hypothalamic neurons in our system alter their action potential activity upon addition of leptin or melanin-concentrating hormone (MCH) as predicted based on published \textit{in vivo} data. Current studies are also aimed at utilizing this system to assess the impact of specific cilia mediated signaling pathways such as Mchr1 and hedgehog on neuronal activity and morphology. This system provides a rapid and higher throughput approach to help elucidate cellular mechanisms through which cilia may influence behavior and energy homeostasis. Ultimately, a better understanding of mechanisms of centrally mediated feeding behavior will lead to new therapeutic targets for obesity.

P2040
Board Number: B180
Unusual elemental composition of the statoliths of Mnemiopsis leidyi and Beroë ovata.
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Ctenophores are the subject of much research effort in light of recent studies that have shown that the Ctenophora are the sister group to all other animals (Science 342:1242592). Tamm and coworkers (Curr Biol 24:R951; Biol Bull 227:7; Biol Bull 229:173) revealed unexpected architectural and functional details of the ctenophore statocyst, statolith and component lithocytes, which are supported on the tips of balancer cilia. Here, I describe yet another extraordinary feature of these ancient gravity sensors: namely that the lithocyte concretions of \textit{Mnemiopsis leidyi} and \textit{Beroë ovata} entirely lack calcium. In the fall of 2014, specimens of \textit{M. leidyi} and \textit{B. ovata} were collected by dipping from the docks of Woods Hole, Massachusetts. Statocysts were excised using microsurgical technique and subjected to several treatments: some were not fixed; some were fixed in 4% paraformaldehyde in sea water; some were fixed in 4% paraformaldehyde/2.5% glutaraldehyde in sea water. A total of >200 preparations were prepared. Fixation eased the fine dissection process and improved integrity of the samples but had no
impact on the composition. Individual ~10 μm diameter lithocytes were released from the preparations by further fine manual microdissection, allowed to separate from statocyst tissue by gravity and concentrated in a deep well dish. Lithocytes were refractile and easily observed in the dissection microscope. All preparations were rinsed extensively with ultrapure water. Samples for scanning electron microscopy and electron dispersion spectroscopy (SEM/EDS) were examined with a Zeiss Supra40VP equipped with an Oxford Instruments/INCA Synergy 350 Energy Dispersive X-ray and EDS microanalysis system. SEM and EDS samples were air-dried and mounted on aluminum stubs with carbon conductive tape (PELCO Tabs™), and coated with 4 nm carbon (Leica MED 020 Coating Station) to stabilize the preparations. Examination of lithocytes by differential interference microscopy revealed a lumpy botryoidal shape; many appeared to be made of several fused structures. This was confirmed by SEM. Lithocytes examined by polarization microscopy displayed no birefringence. Most of the lithocytes broke open upon contact with the carbon tabs and revealed stellate crystals. EDS analysis of the crystals and intact lithocytes, whether fixed or not, in all EDS modes, revealed the presence of only sodium, oxygen and sulfur. Known biominerals and purified reagents were used as controls; all displayed appropriate, expected composition. Acknowledgements: Louie Kerr/MLBL CMF, Drs. Oldenbourg, Tamm and Ameral-Zettler for indispensable aid and lively discussion. Funding: Sabbatical award, Office of the Vice President for Research and the Office of the Provost. NSF: EPS-0447675, MCB-0348327.

P2041
Board Number: B181
Investigating the Biological Role of the Voltage Sensitive Phosphatase.
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Our research is focused on elucidating the biological role of the voltage sensitive phosphatase (VSP). VSP is an intriguing protein in that it is one of the only examples of an enzyme regulated by the electrical state of the cell. Specifically, VSP couples a voltage sensing domain, normally found in voltage-gated ion channels, to a phosphatase capable of dephosphorylating phosphatidylinositol phosphates (PIPs). Since its discovery in Ciona intestinalis 2005, VSPs from several vertebrate model species have been characterized. Heterologously-expressed VSPs display depolarization dependent PIP phosphatase activity and endogenous VSPs are expressed in the embryos, kidneys and nervous systems of humans, mice, Xenopus frogs and chickens. Despite years of research, the biological role of VSP remains unclear. Our data suggest that VSP plays a role in cilia biogenesis or function. Primary cilia are considered sensory and signaling organelles that respond to the extracellular environment of the cell, whereas the beating of motile cilia in highly specialized cells creates extracellular fluid flow. Proper cilia function is crucial during embryonic development and during normal kidney and neuronal function. Many ciliopathic syndromes manifest with developmental defects, cognition disorders and renal cysts. Furthermore, cilia have a depolarized electrical environment distinct from the main cell body and are regulated by PIP signaling. Based on morpholino knockdown, immunocytochemistry and immunoprecipitation data, we propose that VSPs are integral ciliary proteins that mediate an unrecognized link between membrane potential and ciliary PIP signaling. Furthermore, we believe that future studies on VSP’s biological role will provide crucial insights toward the development of therapies for several poorly-understood ciliopathies.
Adenylyl cyclases (ACs) are enzymes responsible for the conversion of ATP into the second messenger cyclic adenosine monophosphate, cAMP. cAMP signaling is highly compartmentalized and differential expression and organization of the nine membrane-bound AC isoforms is important for the varied effect of cAMP signaling. ACs are involved in chemo- and mechanosensing in the brain and olfactory systems wherein the localization of key isoforms to the primary cilium, a solitary antenna-like organelle, is critical. The primary cilium is a key mechanosensor in bone and kidney tissues. We have previously shown that AC6 is critical to bone cell fluid-flow response and load-induced bone adaptation. In this work we examine the contribution of type 3 adenylyl cyclase (AC3) to osteocyte primary cilia mechanosensing.

Previously, it was only known that AC6 localized to the osteocyte primary cilium. Double immunocytochemistry for acetylated alpha-tubulin, a marker of the primary cilium, and AC3 showed that AC3 localization is enriched in the cilium of MLO-Y4 osteocyte-like cells. We assessed gene expression of Adcy3, encoding for the AC3 protein, and Adcy6, encoding for the AC6 protein, by RT-qPCR (data shown as mean ± SE, n = 8, Adcy3 and Adcy6 normalized to GAPDH). In IMCD cells, AC6 was highly expressed (3.33 ± 0.18), while AC3 was not detectable. In osteocyte-like cells, AC3 (0.92 ± 0.04) and AC6 (0.95 ± 0.13) were expressed equally, but at a significantly lower level than that of AC6 in kidney epithelial cells (p < 0.001). To investigate if AC3 is involved in mechanotransduction, we performed an siRNA-mediated knockdown of Adcy3 and applied fluid flow for one hour. We have previously shown that Cox-2 gene expression is significantly increased after fluid flow. The scramble siRNA control group had 2.3 ± 0.9 fold increase of flow/no-flow expression of COX-2 normalized to GAPDH (n = 8), while the AC3-knockdown group had a significantly (p = 0.015) greater flow/no-flow increase of 3.8 ± 1.3 (n = 12). There was no significant difference in COX-2 expression between the static control groups, indicating the knockdown alone did not alter COX-2 gene expression. Due to the differential expression pattern of adenylyl cyclases in mechanosensitive cells and the contribution of AC3 and AC6 to osteocyte mechanosensing, the adenylyl cyclase pool in the osteocyte cilium provides a novel target for new therapeutics.

P2043
Board Number: B183
Three dimensional architecture of primary cilia in kidney cells.
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Primary cilia are microtubule based structures that protrude into extracellular space as environmental sensors. In the kidney collecting duct, primary cilia are flow sensors for cell proliferation control and can deflect elastically under liquid flow. The elastic bending property relies on the framework structure of primary cilia (axoneme). It is widely accepted that the main structure of the axoneme of primary cilia has a cylindrical architecture with nine peripheral microtubule doublets (the 9+0 configuration). There are no central microtubule singlets in contrast to motile cilia. However, there are some published electron
micrographs showing structural features deviating from the 9+0 configuration. In this work, we obtained the first three dimensional (3D) architecture of a whole primary cilium from kidney cells by combining dozens of electron tomograms of serial plastic sections. In the 3D structural maps, nine microtubule doublets extend from the nine triplets of the basal body to the distal tip of the primary cilium with different lengths, and only several of them reach the ciliary tip by their A-tubes. There are electron dense networks connecting the microtubules one with another and with the ciliary membrane. The 3D architecture is anticipated to provide structural insight into the elastic bending property of primary cilia and contribute to our understanding about their mechanosensory functions.

P2044
Board Number: B184
Multiple clinical, targeted kinase inhibitors influence ciliary dynamics.
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The primary cilium is an antenna-like organelle extended from the cell surface which provides a platform for receptors for signaling pathways including PDGFα, Hedgehog, and Wnt. Mutations in genes encoding ciliary proteins can lead to the development of severe disorders called ciliopathies, such as polycystic kidney disease (PKD), and complete loss of cilia is often associated with cancerous transformation of epithelial cells. Intriguingly, in some solid tumors, the unciliated cancer cells secrete Hedgehog to activate ciliated stromal cells, allowing stromal cells to provide vital growth signals to the cancer cells. Understanding and manipulating such asymmetric ciliary signaling in tumors versus stromal cells can potentially improve therapeutic response. In previous work, we unexpectedly identified two targeted, clinical anti-cancer agents - the Aurora-A inhibitor alisertib, and HSP90 inhibitor ganetespib - as controlling ciliary dynamics. To determine if additional drugs affect cilia and cilia-dependent signaling, we analyzed the activity of 180 kinase inhibitors of known specificity for action in controlling ciliary assembly or disassembly. For this, we developed a stable hTERT-RPE1 cell line model with an integrated Arl13b-GFP reporter to visualize cilia, and performed a mid-throughput screen to identify 1) ciliary resorption in G0 phase, or 2) cilia disassembly in cells induced to cycle by serum treatment. This identified 17 compounds specifically affecting ciliary dynamics, which had never previously been linked to cilia. These include the multi-targeted receptor tyrosine kinase (RTK) inhibitor sunitinib, the EGFR inhibitor erlotinib, and additional agents targeting other RTKs and, surprisingly, specific pro-inflammatory kinases. Mechanistically, brief treatment with sunitinib and some agents inducing ciliary resorption promoted transient cell cycle independent activation of Aurora-A at the ciliary basal body to promote disassembly. Sunitinib and other pro-disassembly agents caused both rapid and durable loss of cilia in vitro and in vivo, in models for cancer and PKD, and in normal tissue. RNAi experiments assigned specific kinases as mediating ciliary effects of multi-targeted kinases: for example, sunitinib triggers cilia disassembly through inhibition of PDGFRα. In some cases, these effects on control of the primary cilium caused apparent “off-target” activity of specific drugs in modulating signaling by Hedgehog, based on control of functionality of the ciliary Hedgehog receptors. These findings suggest new mechanisms by which some cancer drugs may affect heterocellular tumor signaling, and contribute to a better understanding of mechanisms underlying the dynamics of the resorption and protrusion of the primary cilium, and cilia-related p
**P2045**

**Board Number: B185**

**Roles of Primary Cilia in the Oligodendrocyte Lineage.**

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Primary cilia are non-motile organelles that stem from the basal body and protrude from the cell surface. They are present in most vertebrate cell types. During ontogeny, cilia-associated pathways such as sonic hedgehog (Shh), Wnt/β-catenin and Notch, are vital for generating and specifying cells of the oligodendrocyte lineage. However, cilia have not been reported in mature oligodendrocytes. Thus, cilia may be lost at some point. Here we investigate the timing and function of cilia loss in vivo. In mice of postnatal ages P4 through adult, cilia immunoreactive for Arl13b or ACIII were common in oligodendrocyte progenitor cells (OPCs), but were absent in myelinating, APC/CC1(+) oligodendrocytes. Thus, primary cilia may be lost as cells give up their proliferative capacity and prepare to myelinate axons. The expression of myelin genes depends upon enhanced Wnt/β-catenin signaling. Because primary cilia sequester β-catenin, cilia loss should promote activity in this pathway. We proposed that cilia loss may lead to upregulation of Wnt/β-catenin activity, which in turn permits myelination. In support of this hypothesis, we found that only those Olig1(+) cells without cilia expressed β-catenin and TCF3. We also examined Axin2-lacz mice, in which lacz is a reporter of Wnt/β-catenin activity. In these mice, cilia were absent in Olig2(+)lacz(+) cells, but were not uncommon in the Olig2(+)lacz(-) population. We further tested the importance of cilia by examining Tg737orpk mice, which are deficient in the intraflagellar transport protein IFT88. In the mutant brain, cilia number and length decline during postnatal development. Mutant and wildtype brains were compared on P31. The Tg737orpk brain showed marked decreases in the numbers of APC/CC1(+) oligodendrocytes, and smaller declines in NG2(+) and Olig2(+) OPC populations. This result suggests that cilia loss interferes with the generation and maturation of cells in the oligodendrocyte lineage.

**P2046**

**Board Number: B186**

**A Bioactive Peptide Amidating Enzyme is Specifically Released in Ciliary Ectosomes during Mating in Chlamydomonas.**

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In both rodents and Chlamydomonas, peptidylglycine α-amidating monooxygenase (PAM), which converts C-terminal glycine residues into α-amides, is present in the secretory and endocytic pathways and in motile and non-motile cilia. A Type I integral membrane protein, the catalytic cores of PAM function in the lumen of the secretory pathway and on the external surface of cilia. PAM is required for the assembly of cilia in Chlamydomonas, planaria, zebrafish and mice. Cilia not only sense signals from the extracellular environment, but also transmit information in the form of vesicles (ectosomes) budded from the ciliary membrane (Wood et al., Curr. Biol. [2013]; Wang et al., Curr. Biol. [2014]; Cao et al., eLIFE [2015]). Topologically, ectosome formation is similar to intraluminal vesicle (ILV) formation; ILVs are released as exosomes upon multivesicular body/plasma membrane fusion. As PAM is present in ILVs in mammalian cells and in salivary and urinary exosomes, we hypothesized that CrPAM might be
released in ciliary ectosomes. *Chlamydomonas* ectosomes have a protein composition distinct from that of the ciliary membrane, implying that their content is regulated (Long et al., Curr. Biol. [2016]). Ciliary CrPAM associates with the axoneme and is only solubilized by treatment with both detergent and high salt, as are dynein arms; this axonemal attachment may be mediated directly by the cytosolic PAM C-terminal domain or via some other axoneme-tethered component. During vegetative growth, CrPAM levels are highest during the light phase, peaking towards the end of G1 just prior to mitotic entry. CrPAM protein is also increased in both gametes and early zygotes. Intriguingly, PAM mRNA levels are elevated in both resting gametes and those treated with the cell wall-degrading lysozyme, but decrease precipitously following gamete activation with dibutylryl cAMP (data from Ning et al., Genes Dev. [2013]). In ectosomes prepared from wildtype (cc124 and cc125) vegetative cells, PAM activity and PAM protein were barely detectable. In contrast, ectosomes prepared from mating gametes (cc125×Hap2 and cc124×cc125) had greatly enhanced levels of both PAM protein and activity; approximately one cilium-equivalent of PAM was released/cell/hour. Thus, the PAM-axoneme association is regulated, and PAM is specifically trafficked into ectosomes during mating. Ectosomal CrPAM may amidate secreted peptides, or ectosomal release may represent a cellular mechanism to remove CrPAM from the cilia either because it is now unnecessary or, possibly, because it is inhibitory to further progress in gamete fusion. We are currently examining the mechanisms involved in this regulated release process and testing whether ectosomal CrPAM is bioactive. Supported by DK032949 and GM051293 (NIH).

**P2047**  
**Board Number: B187**  
*Chlamydomonas* Secretes Amidated Peptides during Mating.  
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Peptidylglycine α-amidating monoxygenase (PAM) plays an essential role in the biosynthesis of many neuroendocrine peptides. We recently demonstrated that active PAM (CrPAM) is expressed in *Chlamydomonas reinhardtii*; strikingly, knockdown of CrPAM results in failure of ciliogenesis, while inhibition of its monoxygenase activity slows reciliation. Bioinformatic analysis of the *C. reinhardtii* genome identified multiple genes that encode secreted proteins whose features resemble those of neuroendocrine peptide precursors. The secretory pathway in this unicellular green alga shares much in common with other eukaryotes; orthologs of many of the enzymes associated with classical propeptide processing (endopeptidases, carboxypeptidases) have been identified in *C. reinhardtii*. In addition to the classical signal peptide pathway, bioactive ectosomes are released from *C. reinhardtii* cilia. Controlled secretion and activation of endoproteases play an essential role in the vegetative and sexual life cycles of *C. reinhardtii* and nutrient deprivation triggers the secretion of compensatory enzymes (e.g., alkaline phosphatase and arylsulfatase). Since published RNASeq studies reveal changes in CrPAM expression during sexual reproduction, we prepared cc124(-) and cc125(+) gametes. One hour after mixing the gametes, cells, debris and mating ectosomes were removed by differential centrifugation, leaving the mating secretome. PAM activity was recovered in the cell and ectosome pellets, but was undetectable in the mating secretome. Biological triplicates of the mating secretome were fractionated by SDS-PAGE; after visualization with colloidal Coomassie, proteins were separated into 10 fractions by molecular weight. Following in-gel trypsin digestion and LC-MS/MS analysis, 1232 proteins were identified. Using PredAlgo, a signal peptide was identified in 118 proteins; subcellular localization was not assigned for 77 proteins. Of the annotated signal peptide-containing proteins, 18% were pherophorins, 13% proteases and 8% Ser/Thr kinases. Protein prevalence in each gel slice was assessed using normalized spectral
counts; in this way, proteins subjected to proteolytic cleavage could be identified. Signal peptide-containing proteins and unassigned proteins were more likely to undergo proteolytic cleavage than proteins assigned to the Chloroplast, Mitochondria or Other categories. A screen for amidated products identified a signal peptide containing protein with an amidated C-terminus. Mating secretome constituents that passed through a 10 kDa cutoff filter are being analyzed to identify additional amidated products. Our data suggest a role for peptide/protein amidation in sexual reproduction in C. reinhardtii. Supported by DK032949 and GM051293 (NIH).

P2048
Board Number: B188
Characterization of the role of Diaphanous-related formin (DIAPH) in cilia.
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The primary cilium works as sensory structure by localizing receptors of various signaling pathways such as Sonic Hedgehog, Wnt and PDGF. Genetic defects that compromise cilia formation (ciliogenesis) or cilia maintenance cause a spectrum of disorders referred to as ciliopathies. Previous research had implicated RhoA signaling in ciliogenesis, but the pathways downstream of RhoA involved in promoting cilia growth are poorly understood. One class of RhoA effectors are the DIAPH (Diaphanous-related formin) family of formins that promote actin polymerization and also promote microtubule (MT) stability in an adenomatous polyposis coli (APC) and EB1-dependent manner. EB1 and EB3 have previously been shown to localize at the base of cilia and are implicated in cilia biogenesis by centrosome-related mechanisms. Intriguingly, APC mutations in Gardner’s syndrome also result in cilia defects, suggesting that in some circumstances APC might also have a role in ciliogenesis. However, the role of DIAPH proteins had not been examined. We therefore localized individual DIAPH family members by immunofluorescence microscopy and found that they localized to the base of cilia, but in non-identical patterns. Chemical inhibition of the DIAPH family, or siRNA-mediated depletion of individual family members (DIAPH1, DIAPH2 of DIAPH3) resulted in reduced cilia length and decreased ciliogenesis. In addition, we found that targeted overexpression of DIAPH proteins to the base of cilia causes cilia elongation and the formation of bulb at the distal tip of axoneme. These bulbs contain accumulations of IFT88 and membrane protein polycystin-2 (PC2), consistent with previous studies showing that an imbalance of anterograde and retrograde trafficking caused by defects in retrograde traffic causes bulbous distal ends in primary cilia. Finally, we expressed mutant forms of DIAPH that possess only the actin nucleating, or microtubule-stabilizing properties of DIAPH and found that both activities are important for efficient bulb formation. Together, our results suggest that activation of formins by RhoA promotes ciliogenesis through their actin nucleation and microtubule stabilization activities and likely do so in collaboration with +TIP proteins.
INPP5E is a ciliary phosphoinositide 5-phosphatase whose mutations are the cause of two human ciliopathies, Joubert (JBTS) and MORM syndromes, and whose activity supports Hedgehog-dependent tumor progression. We previously showed that INPP5E is a critical regulator of ciliary phosphoinositide levels, which in turn control ciliary protein composition and Hedgehog signaling (Garcia-Gonzalo et al. 2015 Dev Cell). However, how INPP5E activity and ciliary localization are controlled remains unclear. Here, we have studied whether protein kinase A (PKA) and tyrosine kinases (TKs) regulate INPP5E function. After noticing that both PKA consensus sites in INPP5E are disrupted by known JBTS mutations, we used site-directed mutagenesis to generate non-phosphorylatable and phosphomimetic mutants of the putative PKA target serines. Likewise, given that INPP5E is known to undergo tyrosine phosphorylation, we mutated several candidate tyrosines in a similar way. Our results suggest that INPP5E ciliary localization can be regulated by phosphorylation.

Cells release extracellular vesicles (EVs) that are nano-sized packages allowing for the exchange of protein and genetic content. Cilia release bioactive EVs that contain cargoes including proteases, agglutinins, and polycystins (Wood and Rosenbaum. Trends Cell Biol. 2015). Cilia may also receive EVs. Altered cilium-EV interactions are observed in ciliopathies such as polycystic kidney disease, suggesting a role for EVs in cilia function. The mechanisms that regulate ciliary EV biogenesis, release, and bioactivity, and the contributions of EVs to ciliary signaling are only beginning to be identified. In C.elegans, cilia are present at dendritic tips of sensory neurons. Only a subset of C. elegans ciliated neurons are EV releasing neurons (EVNs): 21 male-specific EVNs and six non-sex specific IL2 neurons (Wang and Barr. Cell Mol Neurobiol. 2016). The six IL2 neurons are four quadrant IL2, and two lateral IL2 neurons. Using transmission electron microscopy (TEM) and tomography, we found that all EVN cilia do not share the same axonemal architecture and that there are ultrastructural differences at the periciliary region between quadrant and lateral IL2s suggesting specialization between EVN subtypes. We hypothesize that EVN specific mechanisms impart cilia with the ability to make and release EVs. Using transcriptional profiling, we identified 335 genes that are overrepresented in the EVNs (Wang et al. Curr Biol 2015). This list includes EVN specific genes such as an alpha tubulin that establishes a specialized axonemal architecture important for releasing EVs (Silva et al. Curr Biol 2017), and pan-ciliary genes that may have specialized roles in the EVNs. rab-28 is pan-ciliary GTPase gene that is overrepresented in the EVN transcriptome and that is important for the maintenance of cilia ultrastructure, and function of amphid neurons that do not make and release EVs (Jensen, Carter et al.,
Using live imaging, we found that rab-28 mutants mislocalize EVN ciliary proteins and display hyperactive EV release of select cargo. TEM on rab-28 mutants revealed increased number of microtubule doublets in the IL2 axoneme and an accumulation of EVs in the extracellular space surrounding IL2 cilia. We are determining whether RAB-28 regulates EV release via effects on the axoneme or membrane events at the IL2 cilia base. Our identification of RAB-28 as an EV regulator is expected to identify mechanisms that generate ciliary functional diversity by modulating axonemal arrangement, ciliary membrane events, or both.

P2051
Board Number: B191
Disruption of primary cilia components leads to reduced proliferation in myoblasts.
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The primary cilia is thought to play a critical role in the regulation of myogenesis, as the presence of primary cilia in myoblasts is associated with their proliferation, and disassembly of primary cilia correlates with myogenic differentiation. In an attempt to identify proteins that may be involved with this regulation, a yeast two hybrid screen was carried out using centriolin, a known component of primary cilia, as bait. This screen revealed a member of the HECT family of E3 ligases as a binding partner to centriolin. In cultured cells, disruption of centriolin using the CRISPR/Cas9 system resulted in loss of this interaction and subsequent cell cycle arrest, followed by apoptotic death of the cells. Interestingly, there was also a marked increase in cells exhibiting morphologies consistent with a recently described cell type known as telocytes. These results suggest that centriolin and its interaction with HECT E3 ligases may play important roles in the maintenance of the proliferative state of myoblasts as disruption of this interaction leads to a cessation of the cell cycle and induction of cell death.

P2052
Board Number: B192
Loss of neuronal cilia alters olfactory bulb morphology.
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Olfactory sensory neurons (OSNs) project axons into the olfactory bulb (OB), where they form synapses with mitral cell dendrites in glomeruli. The activation pattern of glomeruli, and associated mitral cells underlie odor coding in the OB. Within this network are interneurons that shape synaptic input from OSNs as well as activity of mitral cells. Glomerular activity also depends on innervation from higher brain regions that supply modulation to the synaptic circuits. For example, neurons in the OB can be modulated by neural hormones associated with hunger and satiety. An unexplored mechanism of neural modulation involves primary cilia, evolutionarily conserved organelles, the function as signaling centers for numerous extracellular cues and several neuromodulatory G-protein coupled receptors (GPCRs) are localized to neuronal primary cilia. The role of neuronal cilia, and the GPCRs enriched in them, however remains unclear. Recent data suggests neuronal cilia regulate dendritic growth and branching patterns as well as agonist induced activity. The goal of this study is to investigate the neuromodulatory role of cilia of neurons in the OB. mRNAs for several GPCRs including GPR161, somatostatin receptor type 3 (SSTR3), and melanin-concentrating hormone receptor (MCHR1) are abundant in neurons in the OB. IHC for MCHR1 showed the receptor localized to a subset of periglomerular interneurons and granule cells, but not mitral cells. The localization of MCHR1 to largely interneurons suggests a role for their regulation.
of granules cells in response to neuromodulators in order to shape olfactory processing. Moreover, MCH positive fibers are detected in the OB, in close approximation to granule cell cilia. To investigate cilia on mature granule neurons, we used Gad2iresCre mice to selectively knockout the ciliary gene Ift88. Loss of Ift88 resulted in widespread loss of cilia on granule cells. At 6 weeks of age, the OB of knockout mice was noticeably disorganized and glomeruli were significantly smaller. Additionally, innervation by OSN axons and dopaminergic neurons was decreased. These results indicate that cilia on mature interneurons are important for their function and the maintenance of the OB.

Cytokinesis 1

P2053
Board Number: B194
Role of the Anillin Homologue in Cytokinesis of Cryptococcus neoformans.
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Cryptococcus neoformans, a basidiomycete yeast, causes lethal meningitis in immunocompromised individuals. One of the major virulence factors of C. neoformans is its ability to proliferate at host temperature of 37°C. Uniquely in C. neoformans, septin proteins are not essential for viability at 24°C but are necessary for cytokinesis and survival at 37°C. While the role of septins in cytokinesis is conserved in all eukaryotes except plants, their exact contribution to this process remains elusive. Moreover, mechanisms of cytokinesis in C. neoformans are largely unknown. Through a deletion screen we identified a homologue of anillin as essential for survival of C. neoformans at 37°C. A strain deleted for the anillin encoding gene exhibited a similar phenotype to that of the septin deletion mutants. The anillin homologue localized to the site of cell division during cytokinesis in a septin-dependent manner, and the assembly of the septin complex, specifically during cytokinesis, depended on the presence of anillin. Our data are consistent with a similar role for anillin in C. neoformans to that described for the anillin homologue, Bud4, in the ascomycete yeast, Saccharomyces cerevisiae. Interestingly, while septins are essential in S. cerevisiae under all growth conditions, presumably due to their essential role in cytokinesis, Bud4 is not essential. Thus, our data suggest that the contributions of septins to cytokinesis are not conserved between the two main fungal phyla Basidiomycota and Ascomycota.

P2054
Board Number: B195
Role of Aurora Kinases in Single-Cell Regeneration of Stentor.
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Single cells are capable of developing complex patterns and shapes, but the mechanism by which cells develop shape is largely unknown. Stentor coeruleus is a classical model system to study the development and regeneration of cell shape due to the large size (1mm cubed), the presence of distinct cortical features that define body axes, and most importantly due to the fact that we can surgically manipulate the cells and visualize their regeneration. When the oral apparatus is regenerated in Stentor, the macronucleus undergoes shape changes identical to those that occur during cell division at the point in division when a new oral apparatus is formed. This observation has suggested that the
timing of distinct steps of oral apparatus formation might be regulated by the same molecules that regulate the timing of division, possibly suggesting that mitosis mechanisms are integral to the processes of regeneration. To study whether there is a connection between regeneration and mitosis, we looked at the role of Aurora kinases in regeneration. Aurora kinase A (AurkA) is a kinase known to regulate spindle assembly. Aurora kinase B (AurkB) is known to guide kinetochore attachment to the spindle. We studied the role of AurkA and AurkB in single-cell regeneration using Aurora kinase inhibitors. We have observed that AurkA inhibitor, MLN8237, accelerates regeneration. Surprisingly, AurkA and AurkB inhibitor, PF03814735, suppresses regeneration entirely. Thus, we show that Aurora kinases are involved in single cell-regeneration and there is a link between regeneration and mitosis, which has implications in health.

P2055
Board Number: B196
The role of mitotic cell-substrate adhesion remodelling in animal cell division.
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Animal cells undergo a dramatic series of shape changes as they divide which depend on remodelling of the contractile actomyosin cortex and on release of cell-substrate adhesions. Here, we show that while focal adhesion complexes are disassembled during mitotic rounding integrins remain in place. These integrin-rich contacts connect mitotic cells to the underlying substrate throughout mitosis, guide polar cell migration following mitotic exit, and are functionally important, since adherent cells undergo division failure when removed from the substrate. Further, the ability of cells to re-spread along pre-existing adhesive contacts is essential for division in cells compromised in their ability to construct an actomyosin ring. Thus, Ect2 siRNA cells fail to divide on small adhesive islands, but successfully divide on larger patterns, as the connection between daughter cells narrows and severs as they migrate away from one another. Together these results reveal the importance of coupling adhesion remodelling to mitotic progression.

P2056
Board Number: B197
Aurora A kinase restricts contractile ring components to a narrow equatorial zone during cytokinesis in human cells.
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Cytokinesis is the final event of cell division in which the mother cell splits into two daughter cells. During cytokinesis, the contractile ring is carefully positioned between the separating chromosomes by anaphase spindle. While the spindle midzone, located between segregating chromosomes, promotes the accumulation of contractile ring components at the equator, the centrosomal microtubule asters prevent the accumulation of contractile ring proteins at the cell poles. Recently, we have identified TPXL-1, a potent Aurora A kinase activator, as the first molecular component of the aster-based inhibitory signal in C. elegans. We have shown that Aurora A kinase activation by TPXL-1 is required for...
removing the contractile ring component anillin from the polar cortex in the one-cell C. elegans embryo. To determine if Aurora A inhibits the polar accumulation of anillin in human cells, we treated HeLa cells expressing anillin::GFP with the small molecule inhibitor MK5108 for 30-60 minutes and filmed them during anaphase. We found that inhibition of Aurora A resulted in a significant increase in the amount of anillin::GFP on polar cortex and a wider anillin zone at cell equator. In addition to Aurora A, metazoans have also the closely related Aurora B kinase which localizes to the spindle midzone and was previously described to have a role in cytokinesis. We confirmed that inhibition of Aurora A by MK5108 for 30-60 minutes does not inhibit Aurora B by analyzing Aurora A and B specific phospho-epitopes. Aurora A is involved in centrosome maturation and bipolar spindle assembly. Thus we tested whether short inhibition of Aurora A with MK5108 affects astral microtubules. Our preliminary results suggest that astral microtubules are not affected by Aurora A inhibition. We conclude that Aurora A inhibits the accumulation of contractile ring components at polar cortex in human cells and thereby promotes contractile ring formation at the cell equator during cytokinesis.

P2057
Board Number: B198
Programmed variations of cytokinesis contribute to morphogenesis in the C. elegans embryo.
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The midbody forms at the end of cytokinesis and facilitates abscission, the final separation of the daughter cells. The midbody has also been implicated in regulating additional cellular processes such as cell fate specification, cilia formation and apical polarity during epithelial lumen formation. To investigate developmental roles of the midbody, we examined cytokinesis and midbody fate in the invariant lineage of the C. elegans embryo. We show that unique patterns of cytokinesis and midbody inheritance occur in different parts of the lineage. In the first mitosis, symmetric furrowing positions a central midbody that is always internalized by the P1 daughter cell. In the next AB cell division, a highly asymmetric furrow positions a midbody in contact with and engulfed by EMS instead of either AB daughter cell. A dramatic shift in midbody behavior is observed in several tissues during morphogenesis. In two lumen-forming tissues, the intestine and the pharynx, midbodies form after symmetric furrowing and migrate across the cell to the future apical midline. This midbody migration event coincides with previously characterized polarization events in intestinal epithelia. Upon reaching the apical midline, the midbody ring is internalized and disappears; however, the Aurora B kinase, AIR-2, remains on the apical surface for over an hour after polarization. A similar apical localization pattern is observed for AIR-2 in the pharyngeal primordium. Finally, in cells that form amphid sensilla, we observe symmetrical cytokinesis and a midbody migration event that leads to a focal aggregation of AIR-2. AIR-2 persists along the leading edge of developing dendrites well after cytokinesis. Other midbody markers are either internalized and degraded or maintained with AIR-2 in a tissue-specific manner. We observe PAR-6 localization at the focal aggregation and tip of the sensilla, suggesting that it is apical. Therefore, the midbody migrates from its original position at the end of furrowing to the position of the apical surface in several developing tissues. Inactivating temperature sensitive cytokinesis mutants late in embryogenesis causes defects in positioning, continuity and shaping of the intestinal and pharyngeal lumen. Many animals fail to hatch, but among those that do, a high percentage show defects in neuronal DiI staining. These data suggest that the proper execution of cytokinesis, which shows surprising flexibility during development, and specific cytokinesis regulators such as AIR-2, may regulate the final interphase architecture of a terminally dividing cell.
**P2058**

**Board Number: B199**

Systematic analysis of atx-2 suppressors reveal novel regulators of PAR-5/14-3-3sigma function during mitosis in Caenorhabditis elegans.

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RNA regulation plays a critical role in mitosis, yet the mechanisms remain unclear. Our lab recently identified that the conserved RNA-Binding Protein(RBP), ATX-2, regulates cytokinesis by regulating targeting of ZEN-4 to the spindle midzone through a conserved translation regulator, PAR-5/14-3-3sigma (Gnazzo et al., 2016). While co-depletion of ATX-2 and PAR-5 restored ZEN-4 targeting to the spindle midzone, it did not rescue cell division. To identify factors that may work in concert with ATX-2 to regulate cell division, we conducted a two-part, candidate RNAi suppressor and visual screen to identify factors that are important for cell division and also target ATX-2 to the centrosomes and spindle midzone. Using this approach, ten genes suppress both the embryonic lethality and cytokinesis defects observed in atx-2 mutant embryos and their loss leads to a failure to target ATX-2 to the centrosomes and midzone. These ten genes (*act-2, cgh-1, cki-1, hum-6, par-2, rnp-4, vab-3, vhl-1, vps-24, and wve-1*), have a role in either RNA function or cell cycle regulation or both. The strongest suppressor, CGH-1/DDX6, is a DEAD-box RNA helicase with conserved roles in cell division, RNA processing, translation, and neuron function. Loss of CGH-1 rescued the cytokinesis defect and also restored ZEN-4 localization to the spindle midzone. ATX-2 and CGH-1 are mutually required for their localization to centrosomes and the spindle midzone. Our findings provide the first functional evidence that CGH-1/DDX6 regulates ATX-2 function during mitosis to target ZEN-4 to the spindle midzone and suggest that RNA machinery is necessary for the completion of cytokinesis.

**P2059**

**Board Number: B200**

Characterization of the mammalian midbody transcriptome reveals important factors necessary for cytokinesis and cell fate determination.

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The midbody is a transient organelle derived from the spindle midzone and is essential for normal cell division. Long conceptualized as a simple structural component subject to degradation following cell division, recent studies suggest the midbody may play critical instructive roles in cell fate determination, stem cell maintenance, and oncogenesis (reviewed in Chen et al., 2013). In specific pluripotent and tumorigenic cell types, midbodies are preferentially inherited and escape autophagic degradation (Kuo et al., 2011). The correlation between midbody inheritance and cell fate specification is reminiscent of the differential inheritance of mRNAs or P granules in neurons and C. elegans embryos, respectively. Since mRNA localization to mitotic microtubules is required for cell cycle progression, we are exploring potential functional roles for specific midbody-associated mRNAs. We have identified 22 mRNAs specifically enriched in the midbodies of Chinese hamster ovary (CHO) cells, encoding proteins involved in regulating the cell cycle, cytokinesis, cell fate determination, alternative splicing, and transcription.
We are further characterizing the midbody transcriptome to determine the functional significance of these mRNAs, and will investigate the mechanisms that target them to the midbody.

P2060
Board Number: B201

Studying the role of active Ran in cytokinesis in early *C. elegans* embryos.
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Cytokinesis describes the separation of a cell into two daughters at the end of mitosis. This process is highly conserved and must be spatiotemporally coordinated to avoid aneuploidy and cell fate changes. A RhoA-dependent actomyosin contractile ring pinches in the cortex to divide the appropriate genetic and fate determinants into each daughter cell. The prevailing dogma in the field is that the mitotic spindle regulates the position of the contractile ring. However, studies have shown that microtubule-independent mechanisms also regulate cytokinesis. We found that Ran-GTP signaling from chromatin influences the localization of contractile proteins and cytokinesis in human cells. Our data supports that anillin, which is a scaffold protein that binds to and coordinates actin, myosin, RhoA and its upstream regulators, microtubules, and phospholipids, is the target of Ran-GTP signaling. Human anillin contains a C-terminal nuclear localization signal (NLS) that binds to Importin-β and is required for robust cytokinesis. The levels of free Importin-β, which are controlled by Ran-GTP, function as a molecular ruler to regulate the levels of cortical anillin. We recently discovered that the C-terminal NLS is conserved in *C. elegans* anillin, ANI-1, and binds to Importin-β. We hypothesize that Ran-GTP regulation of cytokinesis is conserved among metazoans and the strength of this pathway is proportional to ploidy, cell geometry and cell volume. To further test this, we are studying the regulation of cytokinesis by the Ran pathway in early *C. elegans* embryos. We found that Ran-GTP influences contractile proteins during early stages of cytokinesis, prior to ring ingression. Decreasing the levels of Ran-GTP by *ran-3* (RCC-1/RanGEF) RNAi decreased the time to ingression in the first cell division (P0), as well as in the next cell divisions (AB and P1). Interestingly, the decrease in rate was more dramatic in P1 vs. P0 or AB cells, and this change was independent of cell size. Imaging revealed that ANI-1 was more strongly recruited to the equatorial plane in P1 cells in comparison to P0 or AB cells, where ANI-1 levels are already quite high. Therefore, Ran signaling may be more strongly required in P1 by regulating ANI-1 localization. We are further testing this hypothesis by studying changes in cytokinesis in embryos with increased Ran-GTP levels, by *ran-2* (RanGAP) RNAi, as well as after perturbation of the importin complex.

P2061
Board Number: B202

Developing a Light-Induced Z-ring Disassembly (LiZRd) Assay to Probe FtsZ’s Role in E. coli Cell Division.
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Bacterial cell division is an essential process mediated by the prokaryotic tubulin homolog FtsZ. Early in division, FtsZ forms a cytokinetic ring (the Z-ring) at midcell and recruits all other downstream factors which coordinate constriction and nucleoid segregation. While it is clear that FtsZ is required for successful cell division, its mechanistic role(s) represent an active area of investigation. Recent work in our lab shows that, FtsZ undergoes treadmilling at the midcell and that the dynamics of this motion are highly correlated to FtsZ’s intrinsic GTPase activity. We also observed that FtsI (PBP3) undergoes
directional movement and that its speed is correlated with that of FtsZ. We therefore hypothesize that the dynamic motion of FtsZ is required to regulate the spatiotemporal distributions of cell wall remodeling enzymes during constriction. However, how imperative the requirement for FtsZ is during constriction is still not well understood.

In an attempt to better understand FtsZ’s role during constriction, we are currently developing a method to rapidly disassemble the Z-ring at different time points over the course of the cell cycle and examine the consequences in the localization, function and dynamics of other divisome components. Our method uses variants of a pair of proteins, CRY2 and CIB1, isolated from Arabidopsis thaliana. This may represent an effective method to shed light on a number of questions: Is the presence of FtsZ required to initiate constriction? To what degree is membrane attachment of FtsZ essential? How long is FtsZ required at midcell to complete cell division? How does FtsZ delocalization affect the localizations of various cell wall synthesis enzymes?

P2062
Board Number: B203
Active Ran regulates anillin function during cytokinesis.
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Several inputs spatially regulate cytokinesis to position and assemble an actomyosin contractile ring at a precise cortical location. Coupling ring positioning with the segregation of chromosomes is crucial to avoid aneuploidy, which is a precursor to genomic instability and cancer. The central dogma is that the mitotic spindle spatially controls the contractile ring, but we found that chromatin also spatially restricts cortical polarity for contractile ring positioning. A Ran-GTP gradient persists around chromosomes during mitosis and is crucial for mitotic spindle assembly. We hypothesize that this gradient also functions as a molecular ruler to regulate cortical contractility during cytokinesis. In support of this hypothesis, we found that Ran-GTP influences the localization of contractile proteins in mammalian cells. One of the molecular targets of Ran-GTP signaling is anillin, which binds to and coordinates F-actin, non-muscle myosin, active RhoA and its upstream regulators, microtubules and phospholipids. We found that a nuclear localization signal (NLS) within a C-terminal disordered loop of human anillin binds to importin-β and is required for cytokinesis. Point mutations within the NLS alter anillin’s localization, and the contractile ring oscillates and subsequently fails to ingress in a subset of cells in comparison to control cells. In addition, we found that this site is autoinhibited by the RhoA binding domain (RBD), and relieving this inhibition changes anillin’s affinity for importins and microtubules. Our current model is that the Ran pathway modulates cytokinesis by affecting anillin’s recruitment to the cortex. During mitosis, RhoA-GTP levels increase and bind to the RBD of anillin, causing a change in its conformation that makes the NLS and microtubule-binding domain more accessible. Importin-binding, which occurs in regions of the cell away from chromatin where Ran-GTP is high, may stabilize a conformation that further enhances anillin’s recruitment to the cortex. However, if importin levels are too high, this could negatively compete with anillin’s other interactions in the C-terminus. We believe that this pathway may be crucial for cells with asymmetrically displaced chromatin to help maintain polarity during division, or in cancer cells that have increased ploidy to ensure robust cytokinesis.
P2063
Board Number: B204
Cell-intrinsic and extrinsic control of cytokinetic diversity in the C. elegans embryo.
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Accurate cytokinesis, the physical division of one cell into two, is essential for the development of all multicellular organisms. In animal cells, cytokinesis is driven by constriction of an actomyosin contractile ring. It has long been known that animal cells divide using a highly conserved molecular mechanism, but growing evidence suggests that the molecular requirements for, and regulation of, cytokinesis in individual cell types has more diversity than previously realized. Thus, to fully understand cytokinesis, we need to acknowledge this diversity and compare cytokinesis between different cell types. To address this question, we studied division in the 4-to-8 cell C. elegans embryo. At this early stage of development, each individual cell already has a distinct cell identity leading to a distinct cell lineage that is controlled by inherited factors, cell-cell contacts, cell polarity, and conserved cell fate-signaling molecules (e.g. Notch, Wnt, Src). Using fast-acting (<20 sec) temperature-sensitive mutants, we determined the level of activity and time of protein function required for successful cytokinesis in each of the 4 cells. We found reproducible, cell type specific variation in the cytokinetic requirement for the filamentous-actin (f-actin) nucleator formin\(^{Ctk-1}\), but not the motor myosin-II\(^{Myr-2}\). Specifically, in two of the four cells (EMS and P2), we found cell division was more robust and able to occur successfully with greatly reduced formin activity and contractile ring f-actin levels. To determine if this cell-type specific cytokinetic robustness was dependent on cell intrinsic or extrinsic regulation, we performed blastomere isolation experiments. We found in one cell (P2), this cytokinetic robustness is dependent on cell intrinsic regulation, whereas in another cell (EMS) this cytokinetic robustness is dependent on cell-fate signaling pathways and direct cell-contact with its neighbor cell, P2. Together, our results demonstrate both cell-intrinsic and cell-extrinsic mechanisms control cytokinetic diversity in individual cell types and can protect against division failure when the contractile ring is weakened.

P2064
Board Number: B205
Integrated cytoplasmic reorganization during human iPS cell mitosis.
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The Allen Institute for Cell Science is developing high-replicate, dynamic image data on cell organization and activities using endogenous fluorescently tagged human induced pluripotent stem cells (hiPSCs). To date, we have generated a collection of genome-edited hiPS cell lines (WTC line) with ~15 key GFP-tagged structures (www.allencell.org and other Institute poster abstracts). These include major organelles, like the nucleus, mitochondria, ER, and Golgi, as well as other key organizational structures, such as microtubules, centrioles, actin bundles, and cell-cell junctions. One of our goals is to quantify the relative location and dynamics of major cellular structures and activities as the stem cells traverse the cell cycle. As an internal positional reference, we label the cells with fluorescent dyes that localize to the plasma membrane (CellMask Deep Red) and nucleus (Hoechst DNA staining) as a proxy. We image the cells in 100s-1000s replicates using 3D live-cell spinning disk microscopy. The DNA dye also allows us to identify 8 stages of the cell cycle, using DNA morphology and texture attributes. The stages are: two stages of prophase, two stages of prometaphase, metaphase, anaphase, telophase/cytokinesis, and
interphase. We manually annotated a dataset of 50-100 cells in each mitotic stage. These cells were used as expert input to train a supervised machine learning-based classifier that enabled automated cell cycle staging, performant at >90% accuracy. We used a parallel approach to integrate and analyze intracellular localization of cellular organelles and structures over the 8 stages of the cell cycle, permitting us to identify and correlate localization patterns in time for all of the structures studied. For example, we observed very similar localization for both the ER (sec61beta) and the nuclear envelope (laminB1), contrasting an anti-localization pattern for the mitotic spindle (alpha-tubulin) and mitochondria (tom20p) throughout mitosis. We validate our single timepoint-derived observations with live-cell timelapse imaging, which permits us to directly observe transitions between intracellular structure localization patterns. As part of our workflow, we are performing 3D image processing and a statistical analysis of the variation of organelle position and morphology during mitosis.

P2065
Board Number: B206
Microtubule tips act as signaling hubs for positioning the cleavage furrow.
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Successful cell division yields two daughter cells each with a complete copy of the genome. Cell division culminates with the formation of an actin-myosin containing contractile ring, positioned midway between the segregated chromosomes. Constriction of the ring generates a cleavage furrow that physically divides the cytosol. A key step in cleavage furrow formation is the localized activation of cortical RhoA, which triggers signaling cascades that drive contractile ring assembly and constriction. Microtubules (MTs) are essential for furrow positioning, but the nature and molecular composition of MT-derived positioning signals are unresolved and controversial. To examine MT-based signaling in cleavage furrow positioning, live-cell TIRF microscopy was applied to Drosophila (Dm) S2 cells to quantitatively characterize the spatio-temporal properties of lynchpin cytokinesis regulators: Pavarotti (DmMKLP1), Aurora B, and Polo kinase. Interestingly, MKLP1, Aurora B, and Polo each uniformly tip-track on astral MT tips within minutes of anaphase onset before becoming patterned onto a subset of equatorial astral MTs. We deemed these specialized MT-based signaling hubs cytokinesis signaling (CS)-TIPS. TIRF-based visualization of active RhoA revealed that CS-TIPS activate RhoA within seconds of contacting the cortex. Myosin-regulatory light chain (MRLC) then becomes locally enriched in the vicinity of CS-TIPS when cortical contacts (even outside the equatorial region) persist for minutes. Localized RhoA activation and MRLC enrichment are lost concomitant with dis-assembly of the CS-TIP complexes. Interestingly, maintenance of CS-TIPS absolutely requires Aurora B kinase activity while polo kinase is required for CS-TIP signaling, but not assembly. We propose that CS-TIPS act as critical MT-based signaling hubs that activate RhoA via physical contact with the cortex to position the cleavage furrow during cytokinesis.

P2066
Board Number: B207
Vps4 Induces a Dynamic Subunit Turnover in ESCRT-III to Mediate Membrane Remodelling During Cytokinesis.
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The Endosomal Sorting Complex Required for Transport (ESCRT)-III forms polymers that mediate membrane fission in many fundamental cellular processes, including cytokinetic abscission. To separate emerging daughter cells, ESCRT-III constricts the intercellular bridge until the membranes split. Prevailing models assume that persistent ESCRT-III filaments change their curvature to deform membranes for fission. However, it is not known whether ESCRT-III polymers exchange their subunits with soluble cytoplasmic pools, which is a common feature of many other force-generating filament systems like actin and tubulin.

Here, we investigated ESCRT-III polymer dynamics in live human cells and in vitro. Using photobleaching experiments, we found a rapid subunit turnover in growing ESCRT-III polymers at cytokinetic abscission sites. The ATPase Vps4, previously thought to primarily disassemble ESCRT-III, accumulated simultaneously with ESCRT-III, and was required for ESCRT-III dynamics and membrane constriction. To dissect the role of each component in polymer dynamics, we reconstituted polymerization of budding yeast ESCRT-III subunits on supported lipid membranes. The scaffold subunit Snf7 alone polymerized into a stable array of single-stranded filament spirals. Addition of ESCRT-III subunits Vps2 and Vps24 induced side-by-side filaments and suppressed further polymerization. Unexpectedly, this growth inhibition was alleviated upon addition of Vps4, resulting in growing ESCRT-III assemblies with turnover kinetics similar to those observed during cytokinetic abscission. High-speed atomic force microscopy revealed that Vps4 converts an immobile lattice of ESCRT-III filament spirals into a highly dynamic arrangement of growing and shrinking spirals.

Our study reveals a dynamic subunit turnover in ESCRT-III polymers, mediated by a constitutive activity of Vps4. This continuous ESCRT-III filament reorganization might increase the energy available for membrane deformation, and facilitates shape adaptation to variable membrane geometries - from nanometer-sized vesicles to micrometer-sized tubes as observed during abscission - thereby enabling ESCRT-III to function in diverse cellular processes.

P2067
Board Number: B208
Crosstalk between the Cdc42 GEFs Gef1 and Scd1 comprise a signaling network that coordinates sequential cytokinetic events.
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Cytokinesis is the process by which a cell physically separates, following mitotic chromosome segregation, to produce two daughter cells. In order for cell separation to occur, the plasma membrane must ingress and fuse to compartmentalize the daughter cells. In most eukaryotes, membrane ingression is accomplished by constriction of an actomyosin-based contractile ring. Little is known about the coordination of this multistep process, which involves ring assembly, ring constriction, and cell abscission. In the fission yeast S. pombe, cytokinesis has an additional layer of complexity due to the
presence of its cell wall. In organisms with a cell wall, the force provided by constriction of the actomyosin ring is not sufficient to promote membrane ingress due to the high turgor pressure within these cells. The cell overcomes this internal turgor pressure via the force generated by the synthesis a new cell wall at the division site, called the septum. How ring constriction and septum formation are coordinated to occur simultaneously is not understood. Our previous work demonstrates a role for the small Rho GTPase Cdc42 in this process. Cdc42 is spatiotemporally activated at the division site by its two GEFs (Guanine nucleotide Exchange Factors), Gef1 and Scd1. Gef1 localizes first and activates Cdc42 at the actomyosin ring to promote ring constriction. Scd1 then localizes to the ingressing membrane and regulates septum formation. Here we provide evidence of a novel crosstalk between these Cdc42 GEFs. Our data suggest that this novel crosstalk between the GEFs establishes a signaling network that monitors events to coordinate sequential progression through cytokinesis. We demonstrate that Gef1 localization is independent of the SIN (Septation Initiation Network) pathway kinases Plo1 and Sid2. However, disruption of the actomyosin ring results in loss of Gef1 localization at the division site. This indicates that Gef1 recruitment to the division site is independent of the SIN pathway, but requires an assembled actomyosin ring. Further, Gef1 promotes the recruitment of the other GEF Scd1 and its scaffold Scd2 to the division site. In return, Scd1 is required for Gef1 removal from the division site after completion of ring constriction. This is important because failure to remove Gef1 from the division site results in delayed cell abscission. Thus, crosstalk between the Cdc42 GEFs Gef1 and Scd1 gives rise to a gradient of active Cdc42 at the division site that coordinates the sequential events of ring constriction, septum formation, and cell abscission.

P2068
Board Number: B209
Binucleation fails to activate a tetraploidy checkpoint and instead causes chromosome segregation errors in the mouse preimplantation embryo.
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Objective: The aim of this project is to understand the consequences of binucleation in mouse preimplantation development. Methods: To induce binucleation, mouse embryos were treated with Latrunculin at the 4-cell stage. Fixed and live cell imaging was performed to evaluate embryo development, cell cycle progression and chromosome segregation errors. For cell and micronuclei counts, embryos were stained with Alexa-Phalloidin (plasma membrane) and Hoechst (DNA). For microtubule-kinetochore attachment analysis, we performed a short cold shock treatment followed by CREST (kinetochores) and a-tubulin (microtubules) staining. For live cell imaging, embryos were microinjected with fluorescently tagged mRNAs labeling the histones (H2B:RFP), an S-phase marker (PCNA:EGFP), spindle poles (CDK5RAP2:GFP), microtubules (SiR Tubulin) and centromeric regions (TALE:mClover). Live and fixed images were acquired using either confocal or structured illumination microscopy. Results: The number of cells within binucleated embryos increased normally after 12h and 24h of culture, indicating that binucleation does not prevent embryonic cell divisions. Using live imaging of PCNA:EGFP, we observed that the cell cycle occurred with normal temporal dynamics in the binucleated embryo, however the subsequent tetraploid cell cycle showed an extended G1 and a shortened S-phase as compared to the diploid control. Regarding chromosome segregation errors, we observed that embryos that had been binucleated possessed substantially greater numbers of micronuclei than the control diploid group (P<0.05). Consistent with this, with live cell imaging, we observed higher rates of abnormal divisions as compared to the control group (P<0.05), including
regulated kinase defective may be reported the separation. T265D.

During cytokinesis, a contractile ring, composed of actin filaments and myosin II filaments, is formed at the cell equator allowing the cell to divide into two daughter cells. The motor activity of myosin II, regulated by phosphorylation of its regulatory light chains (MRLCs), is a driving force of the cleavage furrow ingestion through the actomyosin contraction. Several kinases, such as Rho-associated coiled-coil kinase (ROCK) and Zipper-interacting protein kinase (ZIP kinase, also known as DAPK3), has been reported to phosphorylate MRLC at the contractile ring. However, it remains unclear whether these kinases function independently of each other. It has been reported that ROCK phosphorylates ZIP kinase at Thr265 and Thr299 in vitro. Furthermore, previous report suggests that phosphorylation of ZIP kinase at Thr265 increases its kinase activity. Therefore, we hypothesize that phosphorylation of ZIP kinase at Thr265 by ROCK contributes to control cytokinesis. To address this, we expressed ZIP kinase wild type (ZIPK-WT), a non-phosphorylatable mutant (ZIPK-T265A) or a phosphomimic mutant (ZIPK-T265D) in HeLa cells and treated cells with a ROCK inhibitor, Y-27632. The decrease of phosphorylated MRLC and the delay of furrow ingestion by a ROCK inhibitor were rescued by the expression of ZIPK-T265D, but not ZIPK-WT or -T265A. This suggests that ROCK regulates MRLC phosphorylation followed by furrow ingestion, through ZIP kinase phosphorylation.

P2070
Board Number: B211
Disruption of the division-to-growth transition in fission yeast yields a novel phenotype of precocious cell growth without cell separation.
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In most eukaryotic cells, growth and cell division do not overlap temporally. The major regulator of polarized cell growth in eukaryotes is the conserved GTPase Cdc42. Cdc42 activation at the growing cell ends terminates during the onset of cell division and resumes immediately after cell separation. How Cdc42 is regulated during this division-to-growth transition is not well understood. Here we present that a disruption of this transition in fission yeast cells results in premature cell growth before cell separation. In such cells, Cdc42 is activated at the growing cell ends at the expense of the site of cell

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separation. We achieve this disruption via Latrunculin A (LatA) treatment and subsequent recovery of wild-type cells. Quite remarkably, we find that a subset of these recovering cells resumes growth during or immediately after septum formation, and ultimately fails to separate. This subset consists of cells that are approximately 14µm and are likely in early-to-mid mitosis at the time of treatment. These PRESS cells (PRemature Elongation Sans Separation) exhibit rapid growth at a rate up to three times faster than untreated wild-type cells. To investigate why the PRESS cells fail to separate, we investigated the viability of the septum. We find that the septum forms normally, suggesting that it is competent for separation. Our previous studies in fission yeast cells show that the growing cell ends compete with each other. We propose that in PRESS cells, the growing ends out-compete the division site for cell delivery machinery. In support of this, we find that the type V myosin Myo52 localizes initially to the division site, but is recruited away to the growing cell ends. We also find that PRESS cells prematurely activate Cdc42 at the growing cell ends. To investigate the nature of Cdc42 activation at these ends, we observed mutants of Cdc42 regulators during LatA recovery. We find the PRESS phenotype is not predicated on the presence of Cdc42 activators Scd1 or Gef1. Further, loss of Cdc42 inhibitors Rga4 or Rga6 enhances the PRESS phenotype. Taken together, our data suggest that initiating cell growth after completion of division requires cell-cycle-dependent Cdc42 activation at the cell ends. Future work will investigate this cell-cycle-dependency and further characterize the higher order regulation of Cdc42 in order to define how a cell achieves its shape.

P2071
Board Number: B212
A novel role of Wee1 in regulating actomyosin ring constriction.
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Animal, fungal, and amoeboid cells assemble an actomyosin ring from conserved proteins that constricts the cleavage furrow during cytokinesis at the end of the cell cycle. Many studies have examined the physical forces during contractile ring constriction, but less is known about how cells ensure proper timing of ring assembly and constriction. Experiments on the fission yeast Schizosaccharomyces pombe have suggested that a checkpoint regulates constriction, but the molecular components and mechanisms of this checkpoint are unclear.

We hypothesize that this checkpoint includes a molecular “brake” to inhibit actomyosin ring constriction until all checkpoint requirements are satisfied. In S. pombe, complete assembly of the actomyosin ring as well as recruitment of cell wall synthesis enzymes is necessary to initiate constriction. S. pombe cells with the temperature-sensitive bgs1-191 mutation (the gene encoding one of the β-glucan synthases that synthesizes the primary septum) activate the constriction checkpoint and build rings that do not constrict at 36°C. Therefore, the brake is engaged in these cells. Using this background to activate the checkpoint reliably, we have evidence that the famous cell cycle kinase Wee1 (which inhibits CDK activity to regulate mitotic entry) has a role in this poorly understood checkpoint. In contrast with bgs1-191 alone, bgs1-191 wee1-50 double mutants successfully complete cytokinesis at 36°C. Therefore, loss of Wee1 function compromises the brake. Accordingly, Wee1 overexpression prolongs ring assembly, delays the onset of ring constriction, and slows ring constriction. These data support our hypothesis that a brake inhibits ring constriction in response to checkpoint activation, and indicate that Wee1 is a component of this brake. Additionally, ring disassembly is defective in wee1-50 cells at 36°C. These data are consistent with a model in which Wee1 inhibits myosin activity in the contractile ring, and reveal a novel role for this well-studied kinase in regulating the final steps of the cell cycle.

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P2072

**Board Number: B213**

**Cleavage-furrow formation without myosin or F-actin in Chlamydomonas.**

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Like most other eukaryotes outside the unikont group, the green alga Chlamydomonas reinhardtii has no type-II myosin yet divides by forming cleavage furrows. We asked if any of its three myosins (one type-VIII and two type-XI) and two actins (the conventional IDA5 and divergent NAP1) is involved in cytokinesis. None of the myosins localized to the cleavage furrow when tagged with Venus, indicating that none of them acts as part of a novel (no myosin-II) type of contractile-actomyosin ring (CAR). We next attempted to eliminate F-actin using latrunculin B (LatB). However, this proved challenging because of the surprising mechanisms that Chlamydomonas possesses for defense against such toxins. In particular, although LatB treatment of wild-type cells caused rapid depolymerization of F-IDA5, it also induced a strong upregulation of NAP1, which then formed LatB-resistant F-NAP1 filaments. RNA-seq analyses showed that this upregulation of NAP1 reflects a broad transcriptional response, much of which depends on three newly identified "LAT" proteins. Many of the LAT-regulated genes contain an "LRE motif", which appears to function as a cis -acting regulatory site. Among the genes induced are those encoding multiple proteins of the ubiquitin-proteasome system, and this upregulation promotes rapid degradation of LatB-bound IDA5. This degradation appears to be functionally important, because these nonpolymerizable monomers can apparently interfere with the formation or function of F-NAP1. Also induced are the genes for the actin-interacting proteins coflin and profilin. Cofilin may further the clearance of IDA5 by assisting with F-IDA5 depolymerization, whereas profilin appears to function both in protecting monomeric IDA5 from degradation and in promoting the formin-mediated assembly of F-IDA5 and F-NAP1.

These analyses of the actin-homeostasis system also provided the means to examine the effects of a complete loss of F-actin, which could be achieved by LatB treatment of a nap1 null mutant. Surprisingly, such cells formed normal-looking cleavage furrows, although they failed in the final abscission step; these results indicate both that a basal, F-actin-independent mechanism drives furrow formation and that F-actin has a CAR-independent role in abscission. Further study of the mechanisms of cleavage-furrow formation and abscission in Chlamydomonas should bring new insights into the basal mechanisms and evolution of cytokinesis and a deeper and better understanding of the true role of the CAR that evolved in the unikont lineage.

P2073

**Board Number: B214**

**Anillin and Septin Colocalize with Myosin II in Nodes and Filamentous Assemblages within the Forming Cytokinetic Contractile Ring.**

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The sea urchin embryo has been a model system for studying cell division for over a century, and it was in sea urchin zygotes that the contractile ring (CR) was first described. However, despite nearly fifty years of research since that first discovery, our understanding of the precise ultrastructure of the CR...
remains elusive. We are using a combination of confocal, super resolution and electron microscopy to define the ultrastructural organization of CR cytokinetic factors by taking advantage of the ability to analyze the structure of the large scale CRs present in cortices isolated from dividing first division sea urchin embryos. By TEM and super resolution microscopy, we recently reported that in established CRs, myosin II minifilaments are organized into concatenated polymers that run parallel to the long axis of the ring. However, in early furrows, myosin II is organized into discrete nodes that appear to undergo a process of interconnection and coalescence coincident with the formation of the CR. Actin localizes to these nodes that initially define a broad stripe at the cell equator, within the zone of active Rho GTPase. In the present study we show that the nodes also contain anillin and septin2, two components thought to play central roles in linking actin, myosin II, RhoA and the membrane during cytokinesis. 3D SIM imaging suggests that within each node, anillin and septin2 both occupy a more central position relative to the myosin II regulatory light chain. As CR formation progresses, the septin2 and anillin staining focuses into a narrower, denser and faintly linear pattern coincident with a similar change in activated myosin II distribution. This alteration in septin2 pattern may represent the ability of short septin filaments to diffuse in the membrane and elongate into longer filaments via end-to-end associations. In late stage CRs the arrangement of septin2 staining appears similar to the gauze-like filamentous patterns reported for septin assemblages in other cell types and staining was strong in the midbody. In addition, the close association of septin2 with myosin II within the CR was preliminarily demonstrated by positive staining using the Proximity Ligation Assay. Furthermore the localization of septin2 to the CR region was not dependent on actin filaments given that it occurred in embryos in which actin filaments were depolymerized prior to cell division by treatment with Latrunculin B. Taken together these results underscore the critical scaffolding roles that anillin and septin play in the formation of the CR and highlight the usefulness of the sea urchin embryo isolated cortex model for analyzing CR structure.

P2074
Board Number: B215
Imaging of bacterial cell division components reveals subcomplexes with distinct dynamics.
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The mechanisms underlying cytokinesis in bacteria remain a mystery. Remodelling of cell wall at the division site creates a peptidoglycan septum separating daughter cells. Division protein dynamics are critical for directing this cell wall remodelling: treadmilling of tubulin-homologue FtsZ limits the rates of cell wall insertion at the division site and cytokinesis overall. However, FtsZ is only one member of a complex of at least a dozen proteins implicated in bacterial cytokinesis: the divisome. While protein dynamics in the divisome are crucial for division, they remain unelucidated for most complex members. We have obtained movies of single molecules of divisome components, including cytoplasmic proteins that directly bind FtsZ and periplasmic proteins that are recruited late in the cell cycle. Cytoplasmic components, that regulate FtsZ polymerization, are stationary, like FtsZ monomers within treadmilling filaments. Periplasmic proteins move directionally, similar to FtsZ filaments, while supporting cell wall remodelling. These results provide a model of a divisome consisting of multiple subcomplexes. Continuing this work, we will investigate the effect that these proteins have on FtsZ dynamics and cytokinesis rate. Furthermore, specific perturbations will elucidate the mechanism underlying motion for each component.
FLIRT: Fast local infrared thermoptogenetics for spatiotemporal control of ts protein function during cytokinesis.

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We developed FLIRT (Fast Local InfraRed Thermoptogenetics) to manipulate fast-acting temperature-sensitive (ts) protein function at precise times and locations during complex cellular processes. Molecular control of complex and transient cellular behavior such as cell motility or cell division involves a high degree of spatiotemporal regulation, which is difficult to study using traditional genetic approaches. Fast-acting (≤20 sec) ts mutants permit studies of the temporal regulation of protein function by simply shifting to the restrictive temperature to conditionally inactivate protein function during the complex cellular behavior of interest. To harness the power of fast-acting ts mutants for spatiotemporal studies, FLIRT works by focusing an infrared laser at distinct sub-cellular structure(s) or on specific cells to locally heat and inactivate ts proteins at precisely defined moments during a complex cellular behavior while simultaneously monitoring the kinetic effects on that process in vivo. Here we use FLIRT to probe the spatiotemporal regulation of the core cytokinesis machinery in worms; however, FLIRT is applicable to any cellular or developmental process in any model system accessible by light microscopy. Cytokinesis is the physical division of one cell into two that occurs at the end of the cell cycle. We use FLIRT in C. elegans to study the spatiotemporal regulation of cytokinesis using our growing collection of fast-acting and reversible ts cytokinesis-defective mutants. Cytokinesis is driven by constriction of a contractile ring composed of diaphanous formin-nucleated f-actin and the motor myosin-II. We found that FLIRT irradiation of half of the contractile ring in formin(ts) or myosin-II(ts) mutant embryos halts ring constriction on the irradiated side of the cell, but not on the non-irradiated side. This suggests the contractile ring is made up of individual contractile units that can function independently of a full contractile ring. FLIRT irradiation does not inhibit cytokinesis in control embryos lacking ts mutations, or in formin(ts) and myosin-II(ts) embryos irradiated outside of the division plane (in the polar cell cortex). Further, FLIRT is reversible and cytokinesis can complete when the IR laser is turned off mid-way through contractile ring constriction in myosin-II(ts) mutants. These results highlight the high degree of rapid and reversible spatial control that can be achieved with FLIRT. We are currently using FLIRT to test the contributions of subcellular regions of actomyosin contractility in cytokinesis and expanding our analysis to other fast-acting ts cytokinesis-defective mutants.
Kinetochore Assembly and Functions 2

P2076
Board Number: B217
Human Replication Licensing Factor Cdt1 Serves as an Essential Link for Stabilizing Kinetochore Microtubule Attachments.
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Abstract: Robust kinetochore-microtubule (kMT) attachment is critical for accurate chromosome segregation. G2/M-specific depletion of human Cdt1, a protein that localizes to kinetochores in an Ndc80 complex-dependent manner, leads to abnormal kMT attachments and mitotic arrest indicating an independent mitotic role for Cdt1 in addition to its prototypic DNA replication origin licensing function. Our previous work demonstrates that the internal loop domain of the Hec1 subunit of the Ndc80 complex is required to recruit Cdt1 to kinetochores. Here, we show that Cdt1 directly binds to microtubules (MTs) and localizes to mitotic spindle MTs. Deletion mapping revealed that the C-terminus of Cdt1 carrying a winged-turn-helix domain is necessary but not sufficient for MT-binding. We find that Mitotic kinase Aurora B interacts with Cdt1 in mitotic extracts and phosphorylates Cdt1 in vitro. Aurora B-phosphomimetic Cdt1 exhibited attenuated MT-binding and its cellular expression induced defective kMT attachments and mitotic progression. Thus we provide evidence for a Cdt1-mediated MT-attachment site for the Ndc80 complex at kinetochores in addition to the well-established MT-binding sites at the N-terminal domain of the Hec1 subunit. We propose that Cdt1 links the loop domain of Ndc80 to MTs and enables an optimal MT-binding state for the complex.

P2077
Board Number: B218
Role of the human RZZ complex in coordinating the formation of stable kinetochore-microtubule attachments and proper chromosome alignment.
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Chromosome alignment and segregation during mitosis depends critically on stable kinetochore-microtubule (kMT) attachment formation, mediated by the function of dynein and Ndc80 kinetochore modules. However, the mechanism by which kMT attachments are controlled by the coordinated function of both these modules to drive chromosome motility during early mitosis is still unclear. The RZZ (Rod-ZW10-Zwilch) complex is central to this coordination as it has an important role in dynein recruitment and has been shown to have a key function in the regulation of the Ndc80 complex for kMT attachments. Here, we show that severe chromosome alignment defects induced by dynein or Ndc80 depletion were rescued by codepletion of Rod, a component of the RZZ complex, independent of its function in the spindle assembly checkpoint. Interestingly, in the case of dynein, the rescue was accompanied by a remarkable restoration of stable kMT attachments. Moreover, the rescue of chromosome alignment was critically dependent on the plus-end-directed force mediated by CENP-E, as cells codepleted of Rod and CENP-E along with either dynein or Ndc80 were not able to maintain the
stability of kMT attachments. Our data suggests that the components of the dynein module directly controls the function of the Ndc80 module for stabilizing kMT attachments during early mitosis in human cells. The RZZ complex exerts an inhibitory effect on the function of the Ndc80 complex while dynein and its adaptor, Spindly, serve to modulate this effect and control kMT attachment strength to facilitate proper chromosome motility and alignment at the metaphase plate.

P2078
Board Number: B219
Nonsense-mediated mRNA decay regulates mRNA levels for kinetochore proteins in Saccharomyces cerevisiae.
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Nonsense-mediated mRNA decay (NMD) is best known as a eukaryotic cell surveillance pathway that recognizes and targets aberrant mRNAs for accelerated decay. In a number of model systems, NMD also targets numerous wild-type mRNAs, thereby contributing to the regulation of gene expression. In Saccharomyces cerevisiae, we have identified three wild-type mRNAs seemingly affected by NMD. The CTF13, SKP1, and CEPI3 mRNAs encode proteins that bind centromere DNA to assemble the centromere-binding factor 3 (CBF3) complex of the kinetochore, which is essential for chromosome segregation during mitosis. Our lab has shown that the stability of CTF13 mRNA is directly affected by NMD. We aimed to determine if NMD similarly affects the stability of SKP1 and CEPI3 mRNAs. We engineered SKP1 and CEPI3 genes to express their wild-type mRNAs under the control of the carbon-source dependent regulator, Gal4p. As expected, both showed dextrose-dependent suppression and galactose-dependent induction. CEPI3 mRNA, but not SKP1 mRNA, accumulates in cells lacking NMD. Using carbon-source control, we are measuring rates of decay from steady-state for these mRNAs in yeast with and without NMD. Slower decay of either mRNA in yeast lacking NMD will indicate it is an NMD substrate. In future work, we aim to elucidate how any confirmed wild-type mRNA substrates of NMD are recognized for accelerated decay.

P2079
Board Number: B220
Regulation of PKA activity during Mitosis.
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Accurate chromosome segregation is essential for normal function of cells and organisms. Disruptions in many of the cell cycle regulatory mechanisms ensuring proper chromosomal segregation lead to aneuploidy, an incorrect number of chromosomes, which causes congenital disorders, including Down’s Syndrome, and is pervasive in cancer. Previous work in multicellular and yeast systems have shown that Protein Kinase A (PKA) serves a critical function in glucose response and regulation of the cell cycle. PKA is a heterotetrameric enzyme, composed of regulatory and catalytic dimers. We have observed that PKA activity fluctuates at different points within the cell cycle, peaking during S Phase. We have previously reported that deregulated PKA can cause chromosomal instability, yet the mechanism remains unknown. Experiments measuring total PKA activity and split DHFR assays indicate that PKA is down-regulated when spindle-chromosome attachments are compromised. We hypothesize that PKA activity plays an important role in the spindle assembly checkpoint to minimize chromosome missegregation. Experiments will be presented that directly test this hypothesis. This work helps to elucidate a
mechanism that links PKA to chromosome stability, which has been elusive in the past, and suggests that nutrient signaling may directly impact chromosome segregation.

P2080
Board Number: B221
The molecular requirements for epigenetic establishment of centromeres depend on the type of underlying DNA.
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Human artificial chromosomes (HACs) are exciting tools for current and planned synthetic biology efforts and could have important future clinical applications. A key element in a HAC is the centromere because it directs the faithful segregation of the HAC to daughter cells in mitosis. Functional centromeres are defined by the presence of nucleosomes containing the histone variant, CENP-A. The acquisition of CENP-A nucleosomes on naked DNA HAC templates is the critical step for HAC formation because it initiates the self-propagating epigenetic pathway for centromere inheritance. We sought to artificially promote centromere formation by locally seeding CENP-A nucleosome assembly via its specific histone chaperone, HJURP. We reasoned that this would allow us to more efficiently form HACs to test whether or not it would relax the DNA sequence requirements reported for previous versions (i.e. a reported strict requirement for highly repetitive centromeric α-satellite DNA). Our epigenetic seeding approach successfully stimulated HAC formation on α-satellite DNA, providing an example in a mammalian system for epigenetic spreading of CENP-A-containing chromatin. Earlier studies had reported a strict requirement in HAC formation for the DNA binding protein, CENP-B, and the 17 bp element within α-satellite monomers to which it binds, the CENP-B box (Ohzeki et al., 2002. J. Cell Biol. 159, 765-775; and Okada et al., 2007. Cell 131, 1287-1300). We found that artificial epigenetic centromere seeding completely bypasses the requirement for CENP-B. We also found that HACs can form on complex DNA that lacks any detectable α-satellite DNA or CENP-B protein, both findings in stark contrast to the longstanding models of HAC formation. Surprisingly, targeting local HUURP recruitment did not stimulate HAC formation on complex DNA. Moreover, unlike their earlier α-satellite counterparts, complex DNA HACs allowed us to define the sequence composition, copy number, and precise location of CENP-A nucleosome assembly, providing important insight into HAC formation and stability. Complex DNA HACs also circumvent the cloning difficulties caused by highly repetitive α-satellite DNA that has limited the broader utility of the earlier generation of HACs. Together, our study innovates the HAC “toolbox” and reveals molecular requirements for centromere formation that unexpectedly vary depending on DNA sequence.

P2081
Board Number: B222
RbAp46/48 (Lin-53) - Hat-1 complex is crucial for de novo centromere formation in C. elegans.
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After naked foreign DNA is injected into the C. elegans germline, artificial chromosomes (ACs) can be formed in its embryos and acquire segregation competency rapidly within a few cell cycles. The mechanisms underlying ACs segregation and de novo centromere formation are unknown. Here we show that de novo centromere formation occurs after oocyte fertilization. Immunofluorescent (IF)
staining following foreign DNA injection shows that centromeric protein CENP-A (Hcp-3) and Mis18BP1 (Knl-2) are both present on newly formed ACs in fertilized oocytes, although these ACs either do not segregate or lag at anaphase in the first round of mitosis. Furthermore, IF screening of histone modification marks on ACs in 1-cell embryos shows enrichment of H3K9ac, H4K5ac, H4K12ac, H4K20me, but lack H3K4me2, H3K4me3 and heterochromatin marks H3K9me2, H3K9me3 and H3K27me3. The H4K5 and H4K12 can be acetylated by Hat-1 in RbAp46/48-Hat-1 complex which is conserved in different species for depositing newly synthesized histone H3, H4 and CENP-A to the chromatin. The enriched H4K5ac and H4K12ac suggest that RbAp46/48-Hat-1 complex could be involved in the process of de novo centromere formation. We further show that inhibition of RbAp46/48 (Lin-53) and Hat-1 can totally abolish the H4K5ac, H4K12ac and CENP-A signal on newly formed ACs and disable the ACs’ segregation ability. These findings reveal that RbAp46/48(Lin-53)-Hat-1 complex is essential for initiating CENP-A deposition on naked DNA and facilitating de novo centromere formation in C. elegans.

P2082
Board Number: B223
A potential new error correction mechanism for chromosome segregation in anaphase.
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The effective segregation of chromosomes is essential for genome integrity. Errors can lead to birth defects and may contribute to carcinogenesis. To prevent mis-segregation, the sister kinetochores must be attached to microtubules emanating from opposite spindle poles, and anaphase onset must be delayed until all chromosomes are correctly attached in this bi-oriented state. Even in normal cells, numerous attachment errors occur prior to bi-orientation. These errors are generally detected during prometaphase by surveillance mechanisms that allow their release and correction. To do this, the centromeric kinase Aurora B is required. However, some errors, such as merotelic attachments, can persist and lead to the formation of lagging chromosomes (LCs). These are observed at the midzone during anaphase and are a potential source of aneuploidy. To date, only formation of LCs has been well studied. It remains unclear how the cell deals with LCs once they are formed, and whether they are subject to any type of error correction that operates after anaphase onset to prevent aneuploidy.

Aurora B is the major regulator of error correction in early mitosis and destabilises incorrect microtubule attachments prior chromatin separation. At anaphase onset, Aurora B transfers from chromosomes to the central spindle, resulting in an activity gradient across the dividing cell, centred on the midzone. Because Aurora B is removed from centromeres in anaphase, it is commonly assumed that its role in error correction ends at this time.

Our recent data show that Aurora B substrates involved in error correction during early mitosis are also phosphorylated on lagging chromosomes in anaphase. We are therefore testing the hypothesis that the Aurora B gradient functions as a “measuring ruler”, allowing the cell to identify LCs and to correct their kinetochore-microtubule attachments after anaphase onset. Specifically, we are investigating whether Aurora B actively regulates chromosome attachment status by phosphorylation of kinetochore proteins on LCs and, in so doing, reduces the incidence of chromosome mis-segregation. This work may reveal a new error correction mechanism for chromosome segregation in anaphase.
P2083
Board Number: B224
Structural studies of the budding yeast kinetochore using single-molecule localization microscopy.
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When a chromosome is erroneously attached during mitosis, the kinetochore undergoes structural changes which lead to checkpoint signalling, cell cycle arrest and chromosome reattachment. Thus, studies on the structural organization of the kinetochore is crucial to understand the function of this complex machinery. To understand the structural relation between the components at individual kinetochores we employed dual-color superresolution microscopy, specifically single-molecule localization microscopy, of the metaphase. We showed that the C-terminus of Cnn1 protein lies on a centromeric site of the metaphase kinetochore close to another centromere-binding protein Mif2. All C-termini of the MTW1 complex highly colocalize with each other as well as with C-terminus of Spc105 and Spc25. We developed a method for sub-unit counting based on the nuclear pore complex as a counting reference to measure the protein copy numbers of the kinetochore in metaphase and anaphase. In summary, we developed an experimental handle that allows structural studies of individual yeast kinetochores in situ at unprecedented detail, which will greatly further understanding of the kinetochore structure-function relationship.

P2084
Board Number: B225
Investigating the role of kinetochore dynein-dynactin in Spindle Assembly Checkpoint function.
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Eukaryotic cells employ a fail-safe mechanism – the Spindle Assembly Checkpoint, or SAC – to ensure that mitotic cells do not enter anaphase until all chromosomes are properly attached to spindle microtubules. SAC proteins accumulate on unattached mitotic kinetochores and generate a “wait anaphase” signal to prevent activation of the Anaphase Promoting Complex/Cyclosome (APC/C) by inhibiting its activator, Cdc20. This “wait anaphase” signal is the MCC, or Mitotic Checkpoint Complex, and is comprised of Mad2, BubR1, Bub3, and Cdc20 itself. Bub3 and BubR1 are recruited to kinetochores through KNL1 “MELT” motifs, whose phosphorylation by Mps1 generates direct binding sites for Bub3 and its binding partners Bub1 and BubR1. Mad2 is thought to be recruited to kinetochores via two mechanisms: (1) direct binding to Bub1, and (2) a poorly-understood linkage to the RZZ complex (Rod, Zwilch, and ZW10), which also recruits the dynein adaptor, Spindly, which in turn recruits dynein and dynactin to kinetochores. Upon stable attachment to microtubules, SAC proteins are evicted from kinetochores, which results in reduced MCC complex assembly and activity, and the consequent activation of APC/C to initiate anaphase onset and mitotic exit. The molecular signal that results in SAC protein eviction from kinetochores upon stable attachment remains unclear; however, it is likely that two mechanism are involved: (1) a shift in the balance between kinetochore kinases and phosphatases to favor phosphatase activity, which reverses many of the SAC protein recruitment events, and (2) activation of kinetochore dynein, which results in the “stripping” of SAC proteins from kinetochores. The precise contributions of the dynein pathway to checkpoint function have been difficult to assess given its many functions in animal cells (and thus the pleotropic effects following cellular dynein disruption), and the current limitations in studying dynein function in vitro. Recent innovations have allowed for a
more precise dissection of kinetochore dynein function, specifically: development of a one-step procedure for isolation of motility-competent kinetochore dynein-dynactin complexes from cultured human cells, and the development of reagents that allow for specific disruption of kinetochore dynein function in cells. We have used these tools to determine the network of interactions that link SAC proteins to dynein and to investigate the molecular signals that initiate dynein-mediated SAC protein eviction from kinetochores upon stable microtubule attachment.

**P2085**

**Board Number: B226**

**Regulation of kinetochore plasticity during anaphase.**

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The kinetochore is a large evolutionarily conserved protein structure that connects chromosomes with microtubules. During chromosome segregation, outer kinetochore components track the depolymerizing end of a microtubule to facilitate separation of chromosomes into two cells. In budding yeast, each chromosome has a point centromere upon which a single kinetochore is built which attaches to a single microtubule. Studies from our lab have shown that the kinetochore structure is plastic during the cell cycle. The kinetochore submodule Dam1 is unchanged during anaphase whereas MIND and Ndc80 submodules add copies to form an 'anaphase-configuration' kinetochore. Using calibrated imaging we found that microtubule depolymerization and kinesin related motors contribute to copy number addition. Also, using temperature sensitive mutants we found that the Anaphase Promoting Complex (APC) is required for the Ndc80 complex addition during anaphase and Mitotic exit pathway mutants arrest the cell with the anaphase-configuration kinetochore. We speculate that the APC promotes the addition of Ndc80 complex subunits during anaphase and the Mitotic Exit Pathway partially disassembles the kinetochore in the next cell cycle. Our study provides insight into the regulation of kinetochore structural dynamics during chromosome segregation in living cells.

**P2086**

**Board Number: B227**

**Determining the Molecular Basis for Differences in Binding Affinity Between Human and Mouse Aurora Kinase B.**

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The Aurora kinases (AURK) are a family of serine/threonine kinases tasked with preventing aneuploidies from occurring in mitosis and meiosis, by regulating multiple processes critical for cellular division. Though the role of AURKB is well defined in somatic cells, where it acts as the catalytic subunit of the chromosomal passenger complex (CPC), work in mouse oocytes has identified that the germ-line specific paralog, AURKC, assumes many of AURKB's functions from mitosis in meiosis. These results leave the requirement for AURKB in mouse oocytes unknown. Work in the Schindler lab has demonstrated that while mouse AURKB is inefficient at competing with AURKC to bind the CPC even when overexpressed, ectopically expressed human AURKB robustly localizes to chromosome arms, efficiently out-competing AURKC for CPC binding. Surprisingly, human and mouse AURKB are 90% identical at the amino acid level. Here, we sought to determine what sequence differences between these two AURKs are important in CPC binding affinity, driving the disparity in competition between the homologs. By expressing deletion
and chimeric mutant versions of the kinases in mouse oocytes, we show that amino-terminal regions that differ do not dictate the localization differences between mouse and human AURKB. Understanding the way in which the localization of the Aurora kinases is regulated will be essential to uncovering the unique and complex functions regulated by these proteins in germ cells.

P2087

Board Number: B228


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To ensure the correct segregation of sister chromatids during mitosis, the spindle assembly checkpoint (SAC) is a surveillance system that delays anaphase onset if one or more kinetochores are not properly attached to the spindle. Several kinetochore proteins, including MAD-1, MAD-2 and PCH-2, are necessary for generating the SAC response. MAD-2 is recruited at unattached kinetochore by MAD-1 and adopts two conformations: an open, inactive form, O-MAD-2, and a closed version, C-MAD-2, which acts as a SAC effector. MAD-1/MAD-2 dimers bind additional O-MAD-2, converting it to C-MAD-2 and facilitating the production of a soluble inhibitory signal. The concentration of this signal effectively tunes the SAC: the length of the cell cycle delay imposed by the SAC is governed by the ratio of the soluble checkpoint signal to cytoplasmic volume.

PCH-2 is required to localize MAD-2, but not MAD-1 at unattached kinetochore². We proposed that PCH-2’s role is to convert C-MAD-2 into O-MAD-2 to locally increase the concentration of O-MAD-2 at unattached kinetochores so that it can be converted to C-MAD-2 and generate the inhibitory signal. We hypothesize that this function ensures SAC strength, since mutants that abolish PCH-2’s localization at the kinetochores exhibit reduced SAC robustness.

To test this hypothesis, we investigated the SAC response in two scenarios in which SAC strength has been shown to increase: when cell volume decreases and in cells that take on specific developmental fates, specifically germline tissue. We perform these experiments in C. elegans, which gives us the opportunity to generate embryos of varying size in a context where cells take on specific developmental fates.

Our experiments indicate that PCH-2 function in the SAC becomes dispensable as the cell volume decreases: smaller pch-2Δ cells localize Mad-2 at unattached kinetochores and exhibit a functional SAC response. This role in SAC strength, however, is not limited to smaller cells, since we also found that PCH-2 is responsible for the stronger SAC in germline cells. These results suggest that the primary role of PCH-2 during the SAC response is to locally increase the concentration of O-MAD-2 to make the SAC stronger, a requirement that can be overcome in somatic tissue by decreasing cell volume. This may explain the requirement for PCH-2 in the SAC in some systems in which a large cell volume can present a challenge to a robust SAC response, such as mammalian cells and C. elegans embryos, as well as its dispensability in other systems characterized by small cell size, such as fission and budding yeast.

P2088
Board Number: B229
MOLECULAR REQUIREMENTS FOR THE TRANSITION FROM LATERAL TO END-ON MICROTUBULE BINDING AND DYNAMIC COUPLING.
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Accurate chromosome segregation relies on the ill-understood ability of kinetochores to convert their lateral microtubule attachment into the microtubule plus-end association, capable of the processive motion with tubulin dynamics. Here we report that this transition can be recapitulated in vitro using only two components: the plus-end-directed kinesin CENP-E and the microtubule wall-binding Ndc80 protein. CENP-E’s primary role is to establish the end configurations for Ndc80, while Ndc80 mediates the maintenance of end attachment. To gain insights into the molecular requirements for end-conversion, we paired CENP-E with other microtubule-binding proteins. Ska1, CENP-E Tail, EB1 and CLASP2 differed in their ability to retain the microtubule ends, and none performed as robustly as Ndc80. Likewise, a non-mitotic transporter Kinesin-1 failed to support the Ndc80-mediated end-conversion, implying that the pair of CENP-E kinesin and Ndc80 is optimally suited for this activity. To investigate the underlying mechanistic differences between these motors and MAPs, we developed a Brownian dynamics model for the molecular ensembles of proteins engaged stochastically in walking and diffusion on the microtubule wall. Modeling demonstrates that the observed differences in the end-retention by different MAPs can be largely attributed to their different residency times and rates of diffusion on the microtubule wall. The model also recapitulates the strikingly different behavior of Kinesin-1 and CENP-E, suggesting that it is rooted in their distinct force-detachment characteristics. Following a model prediction, we were able to achieve robust end-conversion with Kinesin-1 by amending its dynamic response via the reduced ATP concentration. Together, our results strongly argue that microtubule end-conversion is an emergent property of the ensemble of transporting motors and diffusing MAPs, limited by the microtubule end boundary. We propose that similar mechanism ensures microtubule end conversion at mitotic kinetochores.

P2089
Board Number: B230
Molecular mechanisms that prevent mislocalization of centromeric histone H3 variant CENP-A and chromosomal instability (CIN) in human cells.
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Faithful chromosome segregation is a critical event for every cell. Defects in chromosome segregation contribute to chromosomal instability (CIN) and aneuploidy. Aneuploidy is a hallmark of cancers and may contribute to tumor heterogeneity and cancer progression. The centromeric histone H3 variant (CENP-A in humans, Cse4 in budding yeast) is evolutionarily conserved and essential for faithful chromosomal segregation. CENP-A overexpression has been reported in numerous types of cancer and correlates with poor prognosis. Recently, we showed that CENP-A overexpression in human cells (HeLa and RPE1) results in mislocalization to non-centromeric regions and contributes to CIN (Shrestha et al.,
Oncotarget 2017). However, the pathways that regulate CENP-A expression to prevent its mislocalization are not fully understood. Our research objective is to identify pathways that regulate cellular levels of CENP-A and prevent its mislocalization for faithful chromosome segregation. Using genome-wide screens with budding yeast we showed that mutants for the replication independent histone chaperones (HIR complex) and E3 ubiquitin ligase (SCF-Met30) are sensitive to overexpression of Cse4 and defined roles for the Hir2 and SCF-Met30 in preventing mislocalization of Cse4. In this study, we investigated whether HIRA and -TrCP, human homologues of HIR2 and SCF-Met30, respectively, have conserved roles in regulating cellular levels and localization of CENP-A. Here we show that similar to our findings in yeast, depletion of HIRA or -TrCP results in mislocalization of CENP-A and CIN phenotypes in human cells. Moreover, we observed reduced proliferation of CENP-A overexpressing cells and increased DNA damage upon HIRA depletion. In a second approach, we are performing high-throughput RNAi screens to identify regulators of CENP-A expression and localization. In summary, our results provide insights into pathways that regulate cellular levels of CENP-A and prevent its mislocalization for faithful chromosome segregation.


P2090
Board Number: B231
Centromere dysfunction impacts the centrosome causing PCM dispersion during mitosis and centriole loss in the following interphase.
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A balanced set of chromosomes is necessary to maintain genome stability. Defects in DNA repair, chromosome segregation or in the final step of cell division – cytokinesis – can all generate unbalanced genomes. In order to maintain a correct karyotype, DNA integrity and correct repartition of chromosomes are ensured by the activity of cell cycle checkpoints. These respond to various stimuli, like DNA damage or incorrect microtubule (MT)-kinetochore attachment, blocking cell cycle progression to allow repair or correction. Accurate chromosome segregation requires two important players: the centrosomes (1) and the centromeres (2). (1) Centrosomes are composed by two centrioles surrounded by PeriCentriolar Material (PCM) and act as dominant sites of MT nucleation in mitosis. Centrosomes are duplicated only once per cell cycle, so that two centrosomes contribute to the assembly of the bipolar mitotic spindle that segregates chromosomes to the two daughter cells during mitosis. (2) MTs from the mitotic spindle contact kinetochores, which are multiprotein complexes that assemble on a specialized region of each chromosome, the centromere. Centromere specification depends on recruitment and loading of centromere protein A (CENP-A), a centromere-specific histone H3 variant. We found that depletion of any of the centromere components CENP-A, CENP-C or CENP-N, compromises centromere integrity. The centrosome defects include (1) PCM dispersion during mitosis, (2) centriole mis-position at mitotic spindle poles and (3) centrosome loss in the following interphase. Interestingly, we observed that the loss of centrosome integrity in response to centromere dysfunction is MT-dependent and that abnormal MT dynamics contribute to any of the phenotypes mentioned above. Using several approaches that include drug treatment, siRNA, live imaging and super resolution microscopy we proposed a model in which centromere dysfunction leads to abnormal MT dynamics impacting in mitotic lengthening and culminating with PCM dispersion. Consequently, centrioles are mis-positioned at the spindle poles, leading to their subsequent loss in interphase. These results reveal an unexpected
functional relationship between centromeres and centrosomes during mitosis. Moreover, they suggest that defects in maintaining an optimal centromeric structure impacts the integrity of the mitotic spindle in more ways than initially predicted, not only affecting chromosome segregation, but also compromising centosome integrity.

P2091
Board Number: B232
Defining The Physical and Spatial Properties of the Spindle Assembly Checkpoint Across Species.
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Accurate chromosome inheritance is necessary for the development and maintenance of all organisms. Thus, cells have developed a surveillance system called the Spindle Assembly Checkpoint (SAC), which suspends the onset of anaphase until all the chromosomes are positioned to be equally inherited. The SAC monitors attachment of spindle microtubules (MTs) to large protein complexes called kinetochores (KTs), which reside at the centromere of each chromosome. Unbound KTs facilitate the generation of a molecular ‘wait anaphase’ signal that manifests in the inhibition of anaphase onset. Recent phylogenetic analyses have revealed that the SAC is highly conserved across Eukarya. Intriguingly, the mechanisms by which cells carry out mitosis are quite variant across the kingdom (e.g., open vs. closed mitosis). How has the SAC adapted to function in these diverse mitotic contexts in order to achieve high-fidelity chromosome segregation? We are interested in understanding how the SAC functions in cells that undergo closed mitosis, in which the nuclear envelope (NE) remains intact during mitosis. We previously found that in cells which undergo open mitosis, ‘wait anaphase’ signals diffuse from the spindle into the cytoplasm. We hypothesized that in cells in which closed mitosis occurs, ‘wait anaphase’ signals could be confined to the nucleus. In an approach similar to our previous study, we have used binucleate budding yeast cells to define how SAC activation or anaphase onset in one nucleus impacts the mitotic progression of the other nucleus. We have found that unlike mammalian cells, anaphase onset is not coordinated between nuclei. Instead, nuclei progress into anaphase autonomously suggesting that ‘wait anaphase’ signals are not shared. We predicted that the restricted localization of SAC proteins could explain the nuclear autonomy we observe. We found that the proteins Mad1 and Bub1 remain localized within their parent nucleus whereas Mad2, Mad3 and Cdc20 localize to both nuclei. We investigated the possibility that once SAC proteins enter a nucleus, they are restricted from exiting back into the cytoplasm. This would provide individual nuclei discrete environments for SAC signaling and mitotic progression despite residing in a shared cytoplasm. Indeed, we have found that Cdc20, a primary component of the ‘wait anaphase’ signal, can enter both nuclei in the binucleate but then becomes ‘trapped’ within until mitotic exit, at which point it is degraded. Thus, these studies have revealed that in an organism which conducts closed mitosis, the NE restricts diffusion of ‘wait anaphase’ signals to the nucleus. The mechanism by which this restriction occurs and whether this is a conserved quality of all organisms which undergo closed mitosis remains unknown.
P2092
Board Number: B233
Kinetochore recruitment of the Ska complex is regulated by RSA-1 in C. elegans.
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During mitosis, the microtubule cytoskeleton is restructured to form the bipolar mitotic spindle. Dynamic microtubules interact with chromosomes via kinetochores to move and orient the chromosomes between the centrosomes. During anaphase, the sister chromatids are separated to ensure each daughter cell receives precisely one complement of the genome. Errors in chromosome segregation can cause aneuploidy, which is a driving force in tumour formation. RSA-1, a regulatory subunit for PP2A phosphatase in C. elegans, targets the enzyme to centrosomes and regulates microtubule outgrowth. We observed a genetic interaction between RSA-1 and the Ska complex. For example, increased levels of the Ska complex enhance the embryonic lethality associated with loss of rsa-1 function, and reduced levels of Ska restore embryonic viability. Furthermore, in rsa-1(or598) embryos, SKA-1 is prematurely and excessively recruited to kinetochores during spindle assembly. RSA-1 also affects the localization of Aurora A kinase on centrosomal microtubules, suggesting a functional link between RSA-1 activity at the centrosome and recruitment of Ska to the kinetochores. Additionally, using an RNAi-based screen, we identified several other components required for appropriate Ska localization in the embryo.

P2093
Board Number: B234
Outer kinetochore phosphatases - PP2A-B56 and PP1 – differently contribute to the establishment of chromosome-microtubule attachments.
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Chromosome instability is a hallmark of malignant cancers but the underlying lesions are poorly understood. Chromosomal stability requires faithful chromosome segregation during cell division. It is ensured by a stable connection between the chromosome and microtubules (MTs) via a macromolecular structure called the kinetochore (KT). Immature attachment of kinetochores along the lateral walls of microtubules is converted into mature attachment to the ends of microtubules (end-on) through a multi-step end-on conversion process. Only end-on attachments generate the mechanical forces that pull and segregate sister chromatids. In human cells, the Aurora B kinase is a master regulator of the end-on conversion process and is important for the stabilisation of lateral attachments, an early event needed for rapid chromosome capture. However, Aurora-B activity should be counteracted to allow stable end-on attachments; phosphatases that counteract Aurora-B and the end-on conversion process were not known. To address this, we used high-resolution microscopy techniques to dissect the role of BubR1-recruited PP2A-B56 and KNL1-recruited PP1 in the establishment of mature kinetochore-microtubule attachments. Although the two phosphatases are involved in a negative-feedback loop for spindle assembly checkpoint signalling, our findings show that PP2A-B56 and PP1 cover separable roles in controlling the plane of kinetochore-microtubule attachment. Next, in an effort to identify downstream targets of Aurora-B that promote kinetochore-microtubule attachments, we explored the significance of Ndc80-tail phosphorylation events. Our work reveals the presence of an Aurora-B dependent and Ndc80-tail independent pathway that controls the plane of kinetochore-microtubule
attachment. Finally, we present a mechanistic model of how chromosome attachment to spindle microtubules in human cells is finely regulated by Aurora-B kinase and a counteracting phosphatase at the outer-kinetochore. These findings shed light on how chromosome stability relies on a careful balance between phospho-networks that operate in a spatially, temporally and mechanically regulated manner.

P2094
Board Number: B235
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Two-color fluorescence co-localization in 3D (three-dimension) has the potential to achieve accurate measurements at the nanometer length scale. Here we optimized a 3D fluorescence co-localization method that uses mean values for chromatic aberration correction to yield the mean separation with ~10 nm accuracy between green and red fluorescently labeled protein epitopes within single human kinetochores. Accuracy depended critically on achieving small variances in fluorescence centroid determination, chromatic aberration across the measurement field, and coverslip thickness. Computer simulations showed that large variances in these parameters significantly increase 3D measurements from their true values. Our 3D results show that the protein linkage between CENP-A within the inner kinetochore and the microtubule binding domain of Ndc80 complex within the outer kinetochore is on average ~90 nm. The Ndc80 complex appears fully extended at metaphase and exhibits the same subunit structure in vivo as found in vitro by crystallography.

Spindle Assembly 2

P2095
Board Number: B236
MAARS: A novel high content acquisition software for the analysis of mitotic defects in fission yeast.
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Faithful segregation of chromosomes during cell division relies on multiple processes such as chromosome attachment or correct spindle positioning. Yet, mitotic progression is defined by multiple parameters, which need to be quantitatively evaluated. To study the spatio-temporal control of mitotic progression, we developed a High-Content Analysis (HCA) approach, which combines automated fluorescence microscopy with real time quantitative image analysis and allows the unbiased acquisition of multi-parametric data at the single cell level for hundreds of cells simultaneously. MAARS (Mitotic Analysis And Recording System) provides automatic and quantitative single cell analysis of mitotic progression on an open source platform. It can be used to analyze specific characteristics like cell shape, cell size, metaphase/anaphase delays as well as mitotic abnormalities including spindle mis-positioning, spindle elongation defects or chromosome segregation defects. Using this HCA approach, we were able to visualize rare and unexpected events of error correction during anaphase in wild type or mutant cells. Our study illustrates that such an expert system of mitotic progression is able to highlight the complexity of the mechanisms required to prevent chromosome loss during cell division.
P2096
Board Number: B237
Microtubules push chromosomes apart in anaphase.
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The spindle partitions chromosomes into the two daughter cells during cell division. The manner by
which the spindle generates and exerts the forces that segregate chromosomes in anaphase remain
poorly understood. We used genetic perturbations, electron tomography, laser ablation, and
quantitative optical microscopy to study chromosome segregation in Caenorhabditis elegans mitotic and
meiotic spindles, and human mitotic spindles. All of these spindles contain a population of microtubules
between chromosomes in anaphase. Perturbing these inter-chromosomal microtubules greatly
influences chromosome motion, while interfering with microtubules between chromosomes and spindle
poles has a minimal impact. In addition, these inter-chromosomal microtubules slide apart in the same
speed as sister chromosomes separate, but in a faster speed than the spindle poles move apart,
suggesting that their sliding is coupled to chromosome motion. Our results argue that inter-
chromosomal microtubules push chromosomes apart by a combination of polymerization and sliding.
Based on these findings, we propose that pushing from inter-chromosomal microtubules is the primary
driver of chromosome motion in anaphase.

P2097
Board Number: B238
The mitotic spindle is chiral due to torques generated by motor proteins.
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Mitosis relies on forces generated in the spindle, a micro-machine composed of microtubules and
associated proteins. Forces are required for the congression of chromosomes to the metaphase plate
and separation of chromatids in anaphase. However, torques may also exist in the spindle, yet they have
not been investigated. Here we show that the spindle is chiral. Chirality is evident from the finding that
microtubule bundles follow a left-handed helical path, which cannot be explained by forces but rather
by torques acting in the bundles. STED super-resolution microscopy, as well as confocal microscopy, of
human spindles shows that the bundles have complex curved shapes. The average helicity of the
bundles with respect to the spindle axis is 1.5 degrees/µm. Inactivation of kinesin-5 (Eg5/Kif11)
abolished the chirality of the spindle, suggesting that this motor generates the helical shape of
microtubule bundles. To explain the observed shapes, we introduce a theoretical model for the balance
of forces and torques acting in the spindle, and show that torque is required to generate the helical
shapes. We conclude that torques generated by motor proteins, in addition to forces, exist in the
spindle and determine its architecture.
P2098
Board Number: B239

NuMA Targets Dynein to Microtubule Minus-Ends at Mitosis.

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To build the spindle at mitosis, motors exert spatially regulated forces on microtubules. We know that dynein pulls on mammalian spindle microtubule minus-ends, and this localized activity at ends is predicted to allow dynein to cluster microtubules into poles. How dynein becomes enriched at minus-ends is not known. Here, we use quantitative imaging and laser ablation to show that NuMA targets dynactin to minus-ends, localizing dynein activity there. NuMA is recruited to new minus-ends independently of dynein and more quickly than dynactin, and both NuMA and dynactin display specific, steady-state binding at minus-ends. NuMA localization to minus-ends requires a C-terminal region outside NuMA’s canonical microtubule binding domain, and it is independent of direct minus-end binders gamma-TuRC, CAMSAP1, and KANSL1/3. Both NuMA’s minus-end-binding and dynein-dynactin-binding modules are required to rescue focused, bipolar spindle organization. Thus, NuMA may serve as a mitosis-specific minus-end cargo adaptor, targeting dynein activity to minus-ends to cluster spindle microtubules into poles.

P2099
Board Number: B240

Sensing of the Magnitude of Centromeric Tension at Metaphase Elicits a Graded Cellular Response.

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During mitosis, motors associate with microtubules to exert forces that push spindle poles apart, thus establishing a mitotic spindle. These pushing forces in turn cause tension in the chromatin that connects oppositely attached sister chromatids. This tension has been hypothesized to act as a mechanical signal that allows the cell to detect chromosome attachment errors during mitosis. However, the magnitude of any changes in tension that could be detected by the cell to initiate an error correction response during mitosis have not been measured, and so the underlying mechanics of a tension-based error detection pathway remains unknown. In this study, we generated and measured a gradient in tension over multiple isogenic budding yeast cell lines by genetically altering the magnitude of motor-based spindle forces. This allowed us, for the first time, to quantitatively elucidate the mechanics of a tension-based error detection pathway in mitosis. We found that a decreasing gradient in tension led to an exponentially increasing gradient in rates of kinetochore detachment and anaphase chromosome mis-segregation, with a corresponding gradient in metaphase times. Further, these tension-based cellular response gradients were abrogated in the absence of key error-correction pathway proteins. We conclude that the cell is exquisitely tuned to the magnitude of tension as a signal to detect potential chromosome segregation errors during mitosis.
P2100
Board Number: B241
Mechanical maturation of the mammalian centromere regulates force signaling at metaphase.
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During mitosis, the centromere acts as a dynamic linkage between sister chromatids and can be stretched in a spring-like fashion by mitotic spindle forces. This mechanical action generates spatial spreading between the sister chromatids and, correspondingly, force across the sister-centromeres. Importantly, centromere force acts as a mechanical signal during mitosis that contributes to proper chromosome segregation. However, the contribution of chromosome mechanical properties to mitotic force signaling in mammalian cells remains unknown. Further, these mechanics have yet to be quantitatively characterized in unperturbed, mammalian cells undergoing cell division. In this work, we combined quantitative, biophysical microscopy with computational analysis in order to elucidate the mechanical properties of the centromere during mitosis in mammalian cells. We find that the mechanical properties of the human centromere evolve during mitotic progression following a distinct and reproducible pattern, a process which we refer to as “centromere mechanical maturation”. Importantly, our findings suggest that this mechanical maturation leads to amplified force signaling at the centromere, specifically during metaphase. Strikingly, while we found that centromere mechanical maturation was conserved in a non-primate mammalian species, it was altered in four different cancer cell lines. Centromere mechanical maturation was also impaired in a dose-dependent manner by aneuploidy. These results provide new insights into the mechanical properties of the mammalian centromere during mitosis, their impact on force signaling during mitotic progression, and their relationship with genome stability.

P2101
Board Number: B242
Microtubule destabilizing activity of selfish centromeres drives non-Mendelian segregation.
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One of the very few “laws” in biology is Mendel’s Law of Segregation, which states that each allele has an equal chance to transmit to the gametes. However, it is increasingly clear that this law can be violated by selfish genetic elements, which manipulate the production of gametes to increase their own rate of transmission. This genetic cheating in meiosis, meiotic drive, potentially occurs whenever haploid gametes are produced from diploid parents. In female meiosis, selfish elements drive by preferentially segregating to the egg. Our previous results in a mouse model show that selfish centromeres have higher levels of inner and outer kinetochore proteins compared to centromeres of the homologous chromosomes, and that drive of these selfish centromeres depends on asymmetric microtubule (MT) tyrosination within the spindle. These findings raise the question of how selfish centromeres interact with the asymmetric spindle to bias their orientation towards the egg side. Here we show that selfish centromeres preferentially destabilize interactions with MTs on the cortical side of the spindle, thus promoting re-orientation from the cortical side to the egg side. Several lines of evidence support this conclusion. First, we observe re-orientation events initiating with selfish centromeres losing MT attachments. Second, selfish centromeres have higher levels of MT destabilizing factors, including the depolymerizing kinesin-13 MCAK and Aurora B kinase, compared to their homologous partners,
suggestions that they drive the re-orientation process. Third, tyrosinated MTs enriched on the cortical side of the spindle are less stable, which gives directionality to the re-orientation. Fourth, selfish centromeres frequently lose cold-stable MT attachments only when facing the cortical side. Together, these findings provide the first mechanistic insights into how selfish elements exploit meiotic spindle asymmetry to bias their transmission.

**P2102**

**Board Number: B243**

*Determining the molecular requirements for taxol-induced spindle multipolarity.*

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Recently we measured the intratumoral taxol concentration in breast cancer patients and showed that clinically relevant (low nanomolar) concentrations of taxol cause multipolar divisions rather than mitotic arrest. We have now extended this to demonstrate that low nanomolar concentrations of other microtubule poisons (including docetaxel, epothilone b, ixabepilone, vinblastine, vinorelbine, and eribulin) also induce multipolarity in breast cancer cells, suggesting that multipolar mitotic divisions may be a common mechanism of cytotoxicity for this class of drugs. Importantly, supernumerary centrosomes are not required for microtubule poison-induced multipolar spindles. To gain mechanistic insight into the formation of taxol-induced multipolar spindles, a variety of mitotic regulators were tested for their ability to affect spindle multipolarity in breast cancer cells treated with clinically relevant concentrations of taxol. Our results demonstrate that the mitotic kinesin Eg5 and the kinase Plk1 promote both the formation and the maintenance of taxol-induced spindle multipolarity, while the mitotic kinesin CENP-E and the kinase Mps1 do not. Interestingly, we found that decreasing the time to anaphase onset decreases the number of multipolar divisions in taxol, resulting in reduced cytotoxicity. Previous studies have shown that Mps1 inhibition sensitizes cells to taxol, and this combination therapy has entered clinical trials (NCT02138812 and NCT02366949). However, our evidence suggests that Mps1 inhibition reduces the efficacy of taxol as a result of decreased time to anaphase onset and fewer multipolar divisions. Additionally, our results suggest that inhibitors of spindle pole clustering increase the number of taxol-induced multipolar divisions and therefore have the potential to increase its efficacy. Identification of the cellular requirements for multipolarity induced by taxol, and of agents that increase execution of the metaphase-to-anaphase transition in the presence of multipolar spindles, may improve the clinical utility of taxol and other microtubule poisons.

**P2103**

**Board Number: B244**

*The Role of Actin-Microtubule Crosslinker Shortstop in Cell Division.*

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Properly executed cell division is crucial to development, maintenance, and longevity of multicellular organisms. Defects in both symmetric and asymmetric divisions can lead to improper developmental patterning, as well as genomic instability, disruption of tissue homeostasis, and cancer. Our research focuses on understanding how regulators of the actin and microtubule (MT) cellular cytoskeleton communicate to orchestrate the orientation and stability of the mitotic spindle, a critical component to
proper cell division and key to maintaining tissue homeostasis. Shortstop (Shot) is a member of the spectraplakin protein family found previously to crosslink actin and microtubule filaments, playing a vital role in stabilizing interphase microtubules in both Drosophila and human cell models. We describe a role for Shot in oriented cell divisions, with both tissue culture and in vivo Drosophila epithelial models showing spindle misalignment in Shot knockdowns (KDs). Further, we show a role for Shot in spindle assembly in these contexts, demonstrating that spindles do not contain tightly focused poles. Shot KDs also produce defects in chromosomal migration to spindle equator (congression) and chromosomal segregation. We show these activities are mediated not only through traditional Shot roles in stabilization of spindle MTs through crosslinks to actin, but also through a direct interaction of Shot actin binding domain to dynein activator subunit actin-related protein 1 (Arp1) filaments. In line with this hypothesis, only the Shot isoform possessing both MT and actin binding activities is capable of rescue of Shot KD phenotypes. We hypothesize Shot interaction with Arp1 functions to crosslink it to spindle MTs, facilitating activation and stabilization of the MT motor protein Dynein, and promoting its activity in spindle assembly, alignment, chromosomal congression, and chromosomal segregation. In support of this model, live cell imaging experiments show defects in cell division timing under Shot conditions. These timing faults implicate involvement of the spindle assembly checkpoint (SAC), with inhibition of SAC components under Shot KD conditions leading to timing rescue. Further, Shot loss in epithelial tissue in vivo leads to an increase in apoptosis, in line with previous findings linking spindle regulators to cell death. Interestingly however, while previous studies have implicated induction of the Jun kinase (Jnk) apoptotic pathway under spindle regulator KD, Shot apoptosis does not implicate Jnk in this activity. Finally, when Shot KD-induced apoptosis is inhibited, tumorigenic-like conditions result, underscoring the importance of Shot as a key component in development and maintenance of multicellular organisms.

**P2104**

**Board Number: B245**

**Most kinetochore fibers in human cells form via mechanisms intrinsic to the kinetochores and not by capture of astral microtubules.**

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The basic principle of mitotic spindle assembly is known as ‘search and capture’ (S&C). In the classic formulation of S&C hypothesis, plus ends of ‘astral’ MTs (MTs) that emanate from the centrosomes (spindle poles) are captured by the kinetochores that reside at the chromosome’s centromere. Repetitive captures lead to formation of MT bundles, termed ‘K-fibers’, that connect kinetochores with spindle poles. Live-cell microscopy proves that direct capture of astral MT by kinetochores does occur occasionally, which supports the S&C hypothesis. However, an alternative mechanism for K-fiber formation has also been observed. In this mechanism, MT bundles grow outwards from the kinetochores. Minus ends of these growing fibers are eventually captured by astral MTs and transported to the spindle poles by dynein. We seek to establish which of these alternative mechanisms dominates mitotic spindle assembly in human cells. Array tomography (fluorescence collected from serial 70-nm sections of the entire cell) reconstructions of the MT density in early prometaphase Rpe1 cells demonstrate that the density of MTs near kinetochores is higher than in those parts of the spindle where kinetochores are absent. This observation suggests that a significant number of MTs form directly at the kinetochores. Correlative light/electron microscopy analyses reveal that <2 min after nuclear envelope breakdown >75% of kinetochores are already in contact with 25-30 MTs residing <250 nm from the kinetochore. These MTs appear to emanate from the kinetochore’s outer plate and they tend
to orient orthogonally to the plate as expected for end-on attachment. Thus, shortly after NEB nascent K-fibers are already present at most kinetochores. Interestingly, we find no correlation between the presence or the number of MTs within the nascent K-fiber and the amount of the checkpoint protein Mad2 at the kinetochore. Further, inhibition of Aurora B activity does not affect the number or orientation of the MTs present at the early prometaphase kinetochores. In contrast, inhibition of the kinesin CenpE known to reside at the kinetochore increases the number of MTs at the kinetochores by ~25% and changes the MT orientation. Upon CenpE inhibition, MTs orient randomly to the outer plate with both their ends residing outside of the kinetochore. Further, live-cell recordings demonstrate that inhibition of CenpE alters the pattern of chromosome movements during early prometaphase. Together, our results suggest that most kinetochores attach to short MTs that are nucleated in their spatial proximity. Motor activity of CenpE brings the plus ends of these initially disoriented MTs into the kinetochore, converting lateral interactions into end-on attachment to a K-fiber with the appropriate MT polarity.

P2105
Board Number: B246
Physical Confinement Impairs Chromosome Segregation during Cell Division.
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Microenvironment plays critical roles in many biological processes including development and cancer metastasis. Physical confinement from the local microenvironment is considered to influence the tissue development. Studies have also shown that physical confinement can alter the migration of tumor cells. Examination of cellular dynamics in 3D confined microenvironments has far-reaching implications in regenerative medicine, drug efficacy and toxicity assays, and cancer treatment. In vivo studies suggested that intravascular tumor cell division is confined by the vascular wall. Such confined division may be linked to cancer metastasis. But exactly how physical confinement affects the progress of cell division is largely unknown. Currently cell division studies have been mostly conducted on flat surfaces that pose little to no confinement to the cells. Such flat surfaces do not accurately replicate the physiological environment in which cells grow and spread. By using nanotechnology, we have engineered microchannel devices to simulate the confined microenvironment. With these novel microchannel devices, we can study cell division in varying levels of confinement that more accurately represent physiological spaces. Our study from the microchannel devices shows that physical confinement causes delay and arrest in mitosis and such effects become more pronounced as the dimensions of microchannel decrease. Abnormal mitotic spindle also occurs in microchannel. Our study further shows that physical confinement can introduce aneuploidy. This result provides a new mechanism on the origin of chromosomal instability. The novel findings implicate that the mechanisms of cell division are different in confined microenvironment compared to unconfined condition.

P2106
Board Number: B247
Regulation of microtubule crosslinks during spindle assembly.
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Mitotic spindle assembly is a dynamic process where side-by-side microtubule organizing centers (MTOCs) are driven into a stable, bipolar configuration; a process necessary for proper partitioning of
duplicated chromosomes during cell division. This dramatic rearrangement requires that dynamic microtubules (MTs) from opposing MTOCs interact and form antiparallel crosslinks. MTs are organized in the spindle by Ase1/PRC1, which acts as a homodimer to crosslink antiparallel MTs and stabilize the bipolar configuration. How the crosslinking activity of Ase1/PRC1 is regulated to achieve these states of spindle assembly is not well understood. Here we test the hypothesis that regulation is achieved through Ase1/PRC1 interactions with the MT surface. MTs are made of αβ-tubulin heterodimers, which contain a highly conserved globular structure, and disordered carboxy-terminal tail (CTT) domains that decorate the surface of the MT. CTTs exhibit sequence variation across species and across tubulin isotypes within a species. In higher eukaryotes, the CTTs are hotspots for post-translational modifications, and are thought to regulate interactions with MT associating proteins at the surface of the MT. The Ase1/PRC1 binding site sits at the junction between the αβ-tubulin heterodimer, where the β-tubulin tail resides. Here we show that the β-tubulin CTT is important for proper regulation of Ase1 binding and spindle assembly dynamics. Mutants lacking the β-tubulin tail have increased Ase1 spindle MT localization, delays in spindle assembly, and short bipolar spindles. Furthermore, moving the β-tubulin tail to α-tubulin is not sufficient to rescue the delay in spindle assembly or the short bipolar spindles. Despite the increased recruitment of Ase1, β-tubulin mutants have disorganized, short bipolar spindles with fewer crosslinked antiparallel MTs. Taken together, these results suggest that the β-tubulin tail regulates Ase1 recruitment to spindle MTs to promote proper crosslinking during spindle assembly.

P2107
Board Number: B248

Minimal ingredients for coupled spindle assembly and chromosome bi-orientation in a computational model of fission yeast mitosis.
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Mitosis ensures the proper segregation of chromosomes into daughter cells, which is accomplished by the mitotic spindle. During fission-yeast mitosis, chromosomes establish bi-orientation as the bipolar spindle assembles, meaning that sister kinetochores become attached to microtubules whose growth was initiated by sister poles. This process must also correct erroneous attachments made by the kinetochores during the attachment process. Our goal is to build a 3D computational model of spindle assembly based on a realistic description of microtubule, kinetochore, and chromosome dynamics, in order to interrogate the role of specific mechanisms in these chromosome bi-orientation and error correction processes. We have added chromosomes to our previous computational model of spindle assembly [1], which included microtubules, a spherical nuclear envelope, motor proteins, crosslinking proteins, and spindle pole bodies (centrosomes). In the new model, each chromosome is represented by a pair of sister chromatids, and sister kinetochores are represented as chromatid-associated discs. In preliminary work, we have explored the mechanical properties of kinetochores and their interactions with microtubules that achieve amphitelic spindle attachments at high frequency. A plausible set of minimal assumptions yields simulations that generate chromosome attachment errors, but resolve them, much as normal chromosomes do.

P2108
Board Number: B249
Mechanisms of spindle assembly and scaling across Pipid frogs.
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The molecular mechanisms by which the mitotic spindle scales in size, as well as how it adapts its morphology to a wide range of genome sizes across different cell types and organisms, is poorly understood. In recent years, our laboratory has utilized two model frog species, the larger Xenopus laevis and the smaller Xenopus tropicalis, which have correspondingly larger and smaller cells, organelles, and genomes, to identify spindle scaling factors. However, it remains unclear whether these mechanisms are conserved across different species, or whether novel ones exist. To this end, we have developed an egg extract system using the tiny related Pipid frog Hymenochirus Boettgeri which recapitulates spindle assembly in vitro and enables identification and functional testing of potential scaling factors. Interestingly, mixing egg extracts of X. laevis with those from H. boettgeri results in spindles of intermediate size in a dose-dependent manner, as it does for mixing extracts of X. laevis and X. tropicalis, indicating that cytoplasmic factors are responsible for regulating spindle size. However, although spindles formed in H. boettgeri extracts are statistically similar in length to those of X. tropicalis, mechanisms of spindle scaling appear to be different. Unlike X. tropicalis, H. boettgeri egg extracts have low microtubule severing activity and spindle microtubules recruit reduced amounts of previously identified scaling factors, such as TPX2 and Eg5. Interestingly, the microtubule depolymerizing kinesin Kif2a is enriched on H. boettgeri egg extract spindles compared to those in Xenopus, highlighting a novel mechanism for spindle scaling across species. Additionally, we are starting to examine mechanisms of spindle scaling in response to very large genome size using the rare dodecaploid frog Xenopus longipes.

P2109
Board Number: B250
The Effects of Microtubule Length, Dynamics and Bundling on Spindle Assembly.
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The features of mitotic microtubules important for mitotic spindle assembly are not fully known, and descriptive parameters have proved challenging to measure directly. We are studying the dynamic instability of single and bundled mitotic microtubules (MTs) that originate from the spindle pole bodies of fission yeasts. We have labeled cellular MTs with an m-Cherry-α-tubulin chimera expressed at low levels to perturb MT dynamics as little as possible while viewing polymer dynamics with a spinning disk confocal microscope equipped with a sensitive camera. Using a temperature sensitive kinesin-5 allele (cut7-24) at restrictive temperature (37°C), in which bipolar spindle assembly is blocked, we observe monopolar spindles whose dynamic MTs can be imaged directly. This persistent monopolar spindle allows the measurement of growth, shrinkage, catastrophe, and rescue rates for both single and bundled MTs. For comparison we have made monopolar spindles with various average lengths: 2-3 times longer and more bundled MTs in a klp6 (Kinesin-8) deletion mutant, and significantly shorter MTs in an alp14 (XMAP215) deletion mutant. We have also made monopolar spindles with less bundling using a cls1 (CLASP) temperature sensitive mutant and an ase1 (Prc1) deletion mutant. We detect
image features, track fluorescent MTs, and compare MT dynamics and bundling, using four-dimensional data from each of these strains, observing the effects of each mutation on spindle assembly. To quantify relevant parameters, we have written software to automate detection and measurement of single MT and spindle dynamics as well as their changes over time in both monopolar and bipolar spindles, and in the transition from one spindle form to the other. We find that the highly bundled MTs do not play a role in the establishment of an initial bipolar spindle, while short, unbundled and highly dynamic MTs readily form an initial bipolar spindle despite the temperature block. To quantify the rate of tubulin turnover in this system, we have examined the rates of fluorescence redistribution after photobleaching (FRAP) on monopolar and bipolar spindles in which we quantify the substitution of unbleached tubulin dimers for bleached ones.

P2110
Board Number: B251
Adaptor binding sites in the clathrin terminal domain directly recruit the microtubule stabilizing protein GTSE1 to the mitotic spindle.
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The precise regulation of microtubule dynamics and stability ensures accurate chromosome segregation during cell division. Clathrin has recently emerged as an unexpected player controlling mitotic microtubule stability. Clathrin heavy chain (CHC) interacts with TACC3 in mitosis to form a complex that binds to microtubules and localizes to spindles. The CHC-TACC3 complex is important for microtubule stability and chromosome alignment, but the mechanisms by which this complex affects microtubules stability are still being unraveled. We previously identified GTSE1 as a novel microtubule-stabilizing protein that inhibits MCAK activity during mitosis. How GTSE1 is recruited to spindles for this function has remained unclear. Here we show that GTSE1 is recruited to the spindle via direct interaction with CHC, analogous to clathrin-adaptor protein interactions that recruit clathrin to sites of endocytosis. Either mutating conserved adaptor-interaction sites in the CHC N-terminal domain (NTD), or inhibiting interactions with the CHC clathrin-box binding site via the Pitstop-2 compound, dramatically reduce the interaction with GTSE1 in vitro. We also identified multiple LID (Leu-Ile-Asp) motifs in GTSE1, which are putative binding-sequences of the CHC NTD. Mutation of these motifs abolishes GTSE1 interaction with CHC both in vitro and in cells, and delocalizes GTSE1 from spindles, leading to mitotic defects. Thus, one mechanism by which the CHC-TACC3 complex promotes MT stability in mitosis is by directly recruiting GTSE1 to inhibit MCAK.

P2111
Board Number: B252
Clathrin promotes mitotic spindle assembly via interaction with the microtubule depolymerase inhibitor GTSE1.
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Genome integrity relies on the faithful segregation of chromosomes, a process driven by the dynamic microtubules of the mitotic spindle. Clathrin, a major component of the endocytosis machinery in interphase, locates to the spindle in mitosis and promotes both spindle assembly and chromosome alignment. Clathrin is recruited to the spindle via its interaction with TACC3, and the clathrin-TACC3 complex contributes to an inter-microtubule “mesh” that bridges microtubules together. The clathrin
heavy chain amino-terminal beta propeller is an interaction-hub for clathrin adaptor proteins that regulate clathrin functions in interphase. Interfering with the function of the clathrin beta-propeller in mitosis, either chemically or by mutagenesis, induces strong mitotic defects despite clathrin retaining its ability to interact with TACC3 and thereby localising to the spindle. It is therefore likely that clathrin also promotes timely mitosis independently of its bridging function. Here, we show that GTSE1, a protein that stabilizes mitotic microtubules by inhibiting the microtubule-depolymerase MCAK, associates with the clathrin-TACC3 complex in mitosis. In vitro analysis of the GTSE1-clathrin interaction (Lin Y.-C. et al. poster abstract) identified the binding site of GTSE1 on the clathrin heavy chain N-terminal beta propeller and the corresponding clathrin-binding motifs on GTSE1. GTSE1 mutated at the clathrin-binding motifs failed to bind to the clathrin-TACC3 complex and localize to the spindle, resulting in a strong mitotic delay due to a chromosome congression defect. Depleting MCAK in the mutant background restored a timely mitosis, suggesting that the observed mitotic delay results from excessive depolymerisation of spindle microtubules by MCAK. Consistent with this idea, we observed mitotic microtubule stability defects and aberrant spindle morphology in the GTSE1 mutant. We thus propose that the clathrin-TACC3 complex not only bridges microtubules but also promotes microtubule stability by recruiting GTSE1, an inhibitor of the microtubule depolymerase MCAK. Interestingly, both GTSE1 and the binding site for clathrin on TACC proteins are conserved only in vertebrates, raising the possibility that the mitotic function of clathrin may only be observed in vertebrates and might be substantially linked to GTSE1.

G1, G1-S, and S Phase Regulation

P2112
Board Number: B253
Assessing Reproductive Toxicity of Halogenated Flame Retardants Using a Novel in vitro Human Spermatogenesis Model.
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Studies have implicated the halogenated flame retardants TDCPP, TDBPP, HBCDD, and TBBPA as potential male reproductive toxicants; however, these flame retardants have not been assessed for their direct effects on human spermatogenesis. We have previously shown that our human stem cell-based *in vitro* model of spermatogenesis recapitulates human exposure phenotypes to known reproductive toxicants. We utilized this innovative human model to evaluate the effects of the flame retardants TDCPP, TDBPP, HBCDD, and TBBPA on spermatogenic cell lineages under acute conditions. Using flow cytometry to analyze markers of apoptosis, mitochondrial membrane potential, reactive oxygen species generation, cell cycle progression, and haploid cell production as well as high-content imaging of the spermatogenic markers PLZF and HILI, we showed that TDCPP, TDBPP, HBCDD, and TBBPA are human male reproductive toxicants *in vitro*. While not specifically impacting the survival of haploid spermatids, these toxicants affect viability of spermatagonia and primary spermatocytes during *in vitro* spermatogenesis through mitochondrial membrane potential perturbation, ROS generation, and by disrupting the cell cycle. Taken together, these results show that TDCPP, TDBPP, HBCDD, and TBBPA
all affect human spermatogenesis and highlight the need for increased surveillance of these chemicals in the human population.

P2113
Board Number: B254
Regulation of cell cycle progression by cell-cell and cell-matrix forces.
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It has long been proposed that the cell cycle is regulated by physical forces at the cell-cell and cell-extracellular matrix (ECM) interfaces. The evolution of these forces during the cycle has never been measured, however, and whether this evolution impacts cell cycle progression is unknown. We quantified cell-cell tension and cell-ECM traction throughout the complete cycle of a large cell population in a growing epithelium. These measurements unveil temporal mechanical patterns that span the entire cell cycle and regulate its duration, the G1-S transition, and mitotic rounding. We show that cells subjected to higher intercellular tension exhibit a higher probability to transition from G1 to S, as well as shorter G1 and S-G2-M phases. Moreover, we show that tension and surface energy are better predictors for the duration of G1 than any geometric property, including cell and nuclear area and their growth rates. Tension increases smoothly during the cell cycle but decreases three hours before mitosis. Using optogenetic control of contractility, we show that this tension drop favors mitotic rounding. Finally, we show that traction forces peak during mitosis due to the protrusion of neighboring cells under the dividing cell. Together, our results establish that cell cycle progression in a monolayer is regulated cooperatively by forces between the dividing cell and its neighbors.

P2114
Board Number: B255
Temperature-induced uncoupling of cell cycle regulatory mechanisms.
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Cell cycle progresses through a specific sequence of events, the timely order of which is pivotal to successful mitosis. Several regulatory mechanisms are in place to ensure that cell cycle events follow the right sequence. The main regulator of cell cycle is cyclin-dependent kinase 1 (CDK1), the activity of which drives entry into mitosis. In addition, other kinases such as Aurora’s and Polo-like kinases also regulate specific mitotic events. However, the overlapping function of these kinases does not allow us to determine how the exact sequence of cell cycle events are reached. We utilized a temperature-based approach to disentangle the regulatory mechanisms that control different steps of the cell cycle. If different steps of the cell cycle are controlled by distinct regulatory mechanisms, changing temperature will likely uncouple them, as such distinct processes will scale differently with temperature. We used a microfluidic device to control the temperature in the minimal cycles of Drosophila syncytial blastoderm, and employed biosensors of cell cycle regulators to monitor their activity in the living embryos. Using
this assay, we are able to show that an increase in CDK1 activity is necessary for entry into prometaphase, but not prophase. Aurora B drives entry into prophase in a CDK1-independent manner.

P2115  
Board Number: B256  
Elucidating the Role of Securin in Regulating Separse during Cortical Granule Exocytosis.  
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Meiosis involves a tightly regulated series of events leading to the production of haploid gametes. A key player during meiosis is the cysteine protease separase (SEP-1), which is well known for its canonical role in chromosome segregation. SEP-1 has an additional function during anaphase to promote cortical granule exocytosis, which blocks polyspermy. SEP-1 localizes to cortical granules and regulates their exocytosis during anaphase I. Before it appears on cortical granules, SEP-1 localizes to poorly characterized cytosolic filaments with other kinetochore proteins. We hypothesize that the cell cycle machinery known to control SEP-1 during chromosome segregation also controls its localization and function during exocytosis. Following chromosome alignment during metaphase, proper attachment of microtubules to kinetochores inactivates a signaling pathway known as the spindle assembly checkpoint, which allows cell cycle progression. This involves activation of the anaphase promoting complex/cyclosome (APC/C) to promote entry into anaphase I by targeting the SEP-1 inhibitory chaperone, securin (IFY-1), for degradation. We have shown that SEP-1 colocalizes with IFY-1 on filaments beginning in prometaphase, and both disassociate from these structures after the onset of anaphase I. Inhibition of APC/C activity prevents SEP-1 and IFY-1 from leaving the filaments and inhibits cortical granule exocytosis. Depletion of IFY-1 allows SEP-1 to localize to cortical granules, but prevents their exocytosis. This suggests that APC/C degradation of IFY-1 may regulate timely localization and proper activity of SEP-1 at vesicles. To further address this, we generated a non-degradable IFY-1 (IFY-1DM::GFP). Consistent with enhanced IFY-1 stability, IFY-1DM::GFP is not degraded following anaphase I onset, persisting on chromosomes and in the cytoplasm. As expected, expression of IFY-1DM::GFP causes embryonic lethality. IFY-1DM::GFP localizes to filaments normally and, in contrast to wild-type, can be observed on vesicle-like structures at the cortex during anaphase I. Interestingly, IFY-1DM::GFP causes polar body extrusion failure, which could be related to defects in cortical granule exocytosis. In the future we will investigate whether IFY-1DM::GFP affects SEP-1 localization to cortical granules. This will provide insight into how key regulatory components of the cell cycle control SEP-1 localization and activity to promote timely cortical granule exocytosis during anaphase I.

P2116  
Board Number: B257  
Spatiotemporal characterization of the human proteome.  
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The Cell Atlas, a subgroup of the Human Protein Atlas HPA, focuses on localizing the human proteins on a subcellular level using immunofluorescence and confocal microscopy. Using the image collection in the Cell Atlas as a starting point, my research aims to characterize variability of protein expression at a single cell level, both in terms of abundance and spatial distribution. The aim of my research project is to investigate the cell-cycle dependency of the human proteome and quantify changes both in expression
levels and spatial distribution in non-perturbed asynchronous cells with single cell resolution, using affinity proteomics, microscopy and image analysis.

Cells derived from the same clonal population may display variability in protein expression patterns. This variability in protein expression is easily noted within immunofluorescence images and we denote it single cell variation (SCV). This project aims to identify the human proteins that show such a dynamic property both in terms of expression levels and spatial distribution. By re-analyzing all images in the Cell Atlas we have classified around 1,500, corresponding to 13% of all studied proteins, to display such variability. We hypothesize this variation to mainly be linked to parameters such as cell cycle position, stress and cell confluence. It is reasonable to expect a big fraction of these variations to be related to cell cycle progression, as cells are grown under asynchronous conditions. To investigate the correlation to cell cycle, the SCV classified proteins were stained in the U-2 OS FUCCI cell line. The FUCCI cells are tagged with two different fluorescent proteins fused to cell cycle regulators that allow cell cycle monitoring; Cdt1, expressed in G1 phase, and Geminin, expressed in S and G2 phases.

In this manner we could identify 600 proteins with expression variation correlated to cell cycle progression, corresponding to 44% of the tested proteins.

**P2117**

**Board Number: B258**

**Cell fate decision via p53 status in response to nucleoside analog-inducing DNA replication stress.**

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DNA replication stress is induced by divergent causes, such as replication fork retardation, its collapse, or DNA damages. We have reported that trifluridine (FTD), a tri-fluorinated thymidine analog, was incorporated into DNA, and exhibited S phase delay without DNA damages-including single- or double-strand breaks (Matsuoka et al. Mol Cancer Ther 2015). Here we show that, in vitro, FTD interrupts DNA synthesis by the replicative DNA polymerases, Pol and Pol, at different types of behavior, that is, the inefficient incorporation of FTD triphosphate (FTD-TP) into the nascent DNA strand and the inefficient DNA synthesis at repetitive FTD sequence in the template DNA strand. Furthermore, we found that FTD led to a dramatic decrease in cellular dTTP, which could act as a dominant competitor of incorporation of FTD-TP into DNA, and hence this phenomenon might be a cause of incorporation of FTD-TP, even though FTD interrupted DNA synthesis. Together, these data suggest that FTD is a DNA replication stress-inducible reagent through interrupting DNA polymerase progression by incorporating FTD per se into DNA. We found that treatment of HCT116 cells, which have wild-type p53, with FTD exhibited the phenotypes such as cyclin B1 degradation and mitosis skip, resulting in cell cycle arrest at G1 phase and induction of senescence-associated beta-galactosidase activity. In contrast, treatment of HCT116 p53 knock-out cells, generated using CRISPR/Cas9 genome editing system, with FTD did not exhibit these phenotypes. Conversely, such cells entered mitosis, resulting in a remarkable abnormality of chromosome segregation accompanying chromosome bridges during anaphase and subsequent apoptotic cell death. Our findings strongly suggest that FTD-induced DNA replication stress results in an induction of cellular senescence in p53 wild-type cells and marked chromosomal instability in p53 knock-out cells. We propose the critical role of p53 in the cell fate decision in response to DNA replication stress induced by FTD, that is, whether cells undergo cellular senescence or apoptotic cell death.
P2118
Board Number: B259
Phosphorylation regulates protein-RNA phase separation.
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How cells control the position, timing and lifetime of phase-separated compartments in cells is not understood. The RNA-binding protein Whi3 regulates (or contributes to) the cell cycle, cell polarity and stress responses in the multinucleate, filamentous fungus Ashbya gossypii. When Whi3 is deleted or mutated, the nuclear division cycle in the continuous cytoplasm becomes more synchronous, the cells have difficulty establishing and maintaining polarized growth, and the cells show heightened sensitivity to certain environmental stresses. Whi3 contributes to these disparate cell processes by its ability to form different complexes with distinct spatial localizations and mRNA targets. For example, CLN3 (a G1 cyclin) transcripts are heterogeneously positioned near nuclei in a Whi3-dependent manner. BNI1 (a formin) transcripts are positioned at growing tips and nascent polarity sites in a Whi3-dependent manner and colocalize with Puf2, a pumilio family protein that also binds BNI1 mRNA. Additionally, the Whi3 protein can undergo a liquid-liquid phase separation in the presence of target mRNAs in vitro. We are using this system where distinct Whi3 complexes coexist in a cell to address how, when and where specific identities are established for phase-separated compartments. We hypothesized that local post-translational regulation of Whi3 protein confers specificity to establish these distinct complexes and encodes their function. We found by mass spectrometry that certain sites on Whi3 are phosphorylated specifically in response to perturbations in the cell cycle or environmental stresses. Creating genetic backgrounds in which individual sites have been mutated to be phosphomimetic or unphosphorylatable has biological consequences for the Whi3 target pathways and where and when Whi3 forms phase-separated compartments. Understanding how post-translational modification can be used to regulate different aspects of phase separated complexes is an important part of understanding the physiological role and regulation of these compartments in all cells.

P2119
Board Number: B260
The p38 MAP kinase pathway promotes cell size uniformity by linking cell cycle progression to cell size.
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Regularity in cell size is a characteristic feature of healthy tissues in multicellular organisms. We previously showed that mammalian cells autonomously correct deviations from target size on a cell-cycle-dependent manner. To date, the molecular mechanisms that control the regularity of cell size remain unidentified.

To investigate the signaling underlying size determination, we performed a drug screen searching for perturbations that disturb size regularity. Using fluorescent microscopy, each screen condition yields readout of a cell population with single-cell measurements of both cell size and cell cycle state. The data consistently identified a negative correlation between cell size and the fraction of G1 cells – suggesting
that small cells have a longer G1 – which we confirmed by live-cell imaging. This result suggests that animal cells regulate G1-length to buffer deviations in size, as has been previously suggested for yeast. To identify the pathways linking cell size to G1 length, we discriminated two types of compound hits. One: conditions that perturb either size or cell cycle but retain the coordination between the two, producing reciprocally correlated influences on cell size and G1 length (on-axis hits). Two: compounds that disproportionally affect size and cell cycle progression, falling off-axis to the negative correlation defined by the majority of screened conditions (off-axis hits). The off-axis hits may disrupt the coordination between cell size and G1 length.

Interestingly, mTOR pathway is enriched as the top scored targets for the on-axis compounds, suggesting that mTOR pathway functions to regulate cellular growth, but not the coordination between size and cell cycle. Supporting the conclusion, mTOR inhibition produces a significant decrease in cellular growth rate which is partially compensated by a prolonged cell cycle, limiting the effect on cell size. In contrast, the p38 pathway is highly enriched for the off-axis compounds, implicating its role in coordinating cell size and G1 progression. Additionally, we identified the p38 pathway significantly associated with increase in cell size variability, further suggesting its role in controlling size uniformity. Follow-up experiments with western blot and a p38 live-cell reporter have demonstrated that p38 activity upregulates in small-sized cells to retain the cells in G1 for a longer period of growth. Inhibition of p38 MAPKs, by either specific inhibitors or siRNA, leads to loss of the compensatory G1 length extension in small cells, resulting in faster proliferation, smaller cell size and increased size heterogeneity. Together, we propose a model wherein the p38 pathway responds to changes in cell size and regulates G1 exit accordingly, promoting cell size uniformity.

P2120
Board Number: B261
LKB1 links metabolic homeostasis to accurate chromosome segregation in mitosis.
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LKB1-MST4 kinase signaling plays an array of functional importance in cell fate determination. It has been shown that LKB1 exhibits a context dependent signaling in T cells to orchestrate metabolic and immunological homeostasis. Our recent studies show that MST4 cooperates with cAMP-dependent kinase to orchestrate polarized vesicular trafficking and exocytosis (Jiang et al., 2015. J. Biol. Chem. 290, 28272-85; Yuan et al., 2017. J. Biol. Chem.). However, the molecular mechanisms underlying metabolic homeostasis and accurate chromosome segregation were poorly understood. Here, we identified a novel LKB1 signaling axis independent of MST4 and AMPK which orchestrates metabolic homeostasis and accurate chromosome segregation during cell division. LKB1 activity is elicited at mitotic entry via a combination of CDK1 and metabolic homeostasis. Surprisingly, an activation of LKB1 activates SIRT1 via a phosphorylation-dependent conformational switch which promotes the deacetylation activity of SIRT1 and accurate segregation of chromosome in mitosis. Currently, we are measuring spatiotemporal gradient of LKB1 during chromosome segregation and assess how LKB1 dynamics is regulated during cell division cycle.
P2121
Board Number: B262
Deletion of Cul3 in the mouse mammary gland.
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Many proteins are involved in the process of cell division. Precise control of mitotic factors is crucial for the proper regulation of cell division. A major means of regulating proteins, including those involved in cell division, is by ubiquitin-mediated protein degradation, where a specific protein is targeted for destruction. The small molecule, ubiquitin, is attached to a protein, marking it for destruction by the proteasome. In the process of ubiquitination, an E3 ligase is used to determine which substrate protein will be targeted. Cul3 is an E3 ligase which has been shown to target many different substrates for ubiquitination, by pairing up with different substrate adaptor proteins. One substrate of interest for Cul3 is cyclin E, which regulates the G1/S transition of the cell cycle. When cyclin E is overexpressed, cells remain for prolonged periods in S-phase and are unable to proceed through mitosis normally. Previous studies indicate that when there is decreased Cul3 activity, large amounts of cyclin E protein accumulate in the cell.

Some breast cancers and cell lines derived from breast cancers are shown to have overexpressed cyclin E. We hypothesized that this may be due to reduced Cul3 activity. Therefore, we created a conditional knock-out of Cul3 in the mouse mammary gland to assess the effects of reduced Cul3 expression in this tissue. In the absence of Cul3, we observed hyperplasia in the mammary tissue, an increase in BrdU staining, and alterations to cyclin E expression. We also detected abnormalities in development in mammary glands deficient for Cul3. We conclude that Cul3 may play a role in development of the mouse mammary gland, and that in its absence, misregulation of proteins involved in cell division ensues.

P2122
Board Number: B263
Protein kinase C delta drives HGF-induced proliferation of equine satellite cells.
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Horses are athletic creatures with the vast majority engaged in physical exercise at either the amateur or professional level. Despite a large proportion of exercise-related activities, little is known about equine satellite cells (eqSC), the resident skeletal muscle stem cell and a contributor to exercise adaptation. Satellite cells exist in a state of quiescence expressing the transcription factor Pax7, and reside between the basal lamina and sarcolemma of the myofiber. Exercise prompts SCs to become alert, enter the cell cycle, proliferate, and differentiate before fusing to the myofiber. The objective of this study was to determine the role hepatocyte growth factor (HGF), a growth factor expressed in equine skeletal muscle following exercise, plays in time to first division, proliferation, and differentiation of eqSC in vitro. Equine SCs were derived from the gluteal muscle of previously raced thoroughbred geldings (n > 4) by a novel isolation protocol resulting in exceptional myogenicity as confirmed by Pax7 and Myogenin immunocytocchemistry. To determine whether HGF accelerates the time to first division, fresh isolates of eqSC were incubated with varying doses of HGF and briefly pulsed with EdU to mark cells in S-phase of the cell cycle. Though time in culture increased the percentage of EdU+ cells, HGF did not promote EdU incorporation until 96 h following isolation. Hepatocyte growth factor is likely not accelerating entry into the cell cycle, but rather, serving as a mitogen at these later time points. In a
subsequent study, serially passaged eqSC were supplemented with HGF and proliferation rate was measured. HGF stimulated proliferation of eqSC in a dose-dependent manner. Using chemical inhibitors to intracellular kinases, it was discovered that PKC was involved in HGF-mediated mitosis. Specifically, RT-PCR identified PKC alpha, delta, and epsilon expression in eqSCs. Lipid-mediated siRNA knockdown was utilized to determine which of the identified isoforms promoted HGF-induced proliferation. Only knockdown of PKC delta abrogated HGF-induced proliferation. The same dose of HGF that promoted proliferation slightly reduced differentiation as determined by differentiation index. These results indicate that, following exhaustive exercise in horses, HGF likely serves to promote proliferation of eqSC that have already entered the cell cycle.

P2123
Board Number: B264
Proximity based proteomic screen identifies novel associations of the polo like kinase 1 (Plk1).
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Human cell division is a dynamic set of events that promotes accurate transmission of genetic material. This conserved and sequential process is time-dependent and rigorously verified. The mitotic spindle is a critical component of this process, which ensures the safe transmission of chromosomes from a mother cell to a daughter cell with high fidelity. Disruption of the mitotic spindle can lead to aneuploidy, an atypical chromosome number, which is one of the hallmarks of cancer that is proposed to drive tumorigenesis. The polo like kinase 1 (Plk1) is a master regulatory kinase with important roles in mitotic spindle assembly and chromosome segregation. To better understand the functions of Plk1 during cell division we employed a proximity-based association mapping approach that utilized biotinylation of Plk1 associated proteins followed by biochemical purification of biotinylated proteins and liquid chromatography tandem mass spectrometry (LC-MS/MS). Among the associated proteins, we identified proteins known to be involved in the cell cycle and novel associations such as a kinesin-like protein. To further validate the associations identified in the proteomic screen, we performed in vitro co-immunoprecipitations with in vitro transcribed and translated S35 labeled proteins. The co-IP’s confirmed the interaction between Plk1 and the Kinesin-like protein. We further demonstrated that this interaction occurs not only in mitosis, but also in interphase. Additionally, in vitro experiments indicated that Plk1 was able to phosphorylate the kinesin-like protein at several serine residues. Our current and future work includes mapping out the determinants needed for binding and/or phosphorylation that may be critical for this interaction and the significance of this interaction for cell division.

P2124
Board Number: B266
Details Matter: Noise and Model Structure Set the Relationship between Cell Size and Cell Cycle Timing.
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Organisms across all domains of life regulate the size of their cells. However, the means by which this is done is poorly understood. We study two abstracted "molecular" models for size regulation: inhibitor dilution and initiator accumulation. We apply the models to two settings: bacteria like *Escherichia coli,*
that grow fully before they set a division plane and divide into two equally sized cells, and cells that form a bud early in the cell division cycle, confine new growth to that bud, and divide at the connection between that bud and the mother cell, like the budding yeast *Saccharomyces cerevisiae*. In budding cells, delaying cell division until buds reach the same size as their mother leads to very weak size control, with average cell size and standard deviation of cell size increasing over time and saturating up to 100-fold higher than those values for cells that divide when the bud is still substantially smaller than its mother. In budding yeast, both inhibitor dilution or initiator accumulation models are consistent with the observation that the daughters of diploid cells add a constant volume before they divide. This "adder" behavior has also been observed in bacteria. We find that in bacteria an inhibitor dilution model produces adder correlations that are not robust to noise in the timing of DNA replication initiation or in the timing from initiation of DNA replication to cell division (the C+D period). In contrast, in bacteria an initiator accumulation model yields robust adder correlations in the regime where noise in the timing of DNA replication initiation is much greater than noise in the C+D period, as reported previously. In bacteria, division into two equally sized cells does not broaden the size distribution.

**P2125**  
**Board Number: B267**  
**Functional characterization of the ORFs YJR141W & YJL055W in Saccharomyces cerevisiae.**  
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Although the sequence the genome of *Saccharomyces cerevisiae* (baker’s yeast) has been known for several decades, approximately 10% of the genes still have unknown functions for the gene ontology terms (GO terms) cellular component, molecular function and biological process. These uncharacterized open reading frames (ORFans) are the focus of the Yeast Gene ORFan project, which seeks to understand the function of these genes by utilizing bioinformatic methods to characterize previously unannotated stretches of genome. Two such examples of these ORFans are YJR141W (IPA1) and YJL055W (LOG1). IPA1 is an essential protein with an unknown function that contains a highly conserved HECT2 ubiquitin-conjugating domain and is predicted to be a E3 ubiquitin-protein ligase. Combined with transcriptional data, we hypothesized that IPA1 is a ubiquitin ligase involved in cell cycle regulation. Initial attempts to localize IPA1 within the cell via GFP-tagging were unsuccessful and current efforts are underway to identify specific substrates of IPA1 as well as the biological role of IPA1. LOG1 is a hypothesized to contribute to the degradation of mRNA via metabolism of purine and pyrimidine bases. Domain homology predicts that LOG1 to have cytokinin riboside 5'-monophosphate phosphoribohydrolase activity as well as places it in the pfam domain family 03641 which have conserved PGGXGTXXE motif presumed to be vital for enzymatic activity. Additionally, LOG1 is found to interact with several nuclear RNA-binding proteins and to localize within the nucleus and the cytosol. Initial localization studies found increased localization of LOG1 to the nucleus under nutrient limiting conditions. Currently functional assays are underway to characterize the conserved PGGXGTXXE motif found as well as phenotypic assays to determine the biological role of LOG1.
Tumor Suppressors and Regulation of Oncogenes

P2126
Board Number: B269
Is the Eicosanoid Producing Enzyme 12-Lipoxygenase (ALOX12) a Tumor Suppressor?
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Epithelial barrier disruption causes inflammation, which if unchecked over time can cause malignant transformation. The pathways that link chronic epithelial barrier disruption to transformation remain largely unknown. Emerging evidence suggests arachidonic acid (AA) metabolites, collectively termed eicosanoids, may play a central role in this process. Cyclooxygenases convert AA into proliferative and trophic eicosanoids, the prostaglandins, which are mediators of tumorigenesis. Lipoxygenases, on the other hand, convert AA into pro- and anti-inflammatory eicosanoids, such as leukotrienes and lipoxins. One of the least studied, yet most highly expressed epidermal lipoxygenases, ALOX12B, is essential for epithelial barrier integrity throughout phyla. We hypothesize that this enzyme acts as a tumor-suppressor in two ways: first, through its known function in epithelial barrier maintenance, preventing epithelial inflammation. Second, by shunting AA away from the pro-tumorigenic prostaglandin metabolism. Intriguingly, ALOX12B is co-deleted with p53 in many human tumors. Thus, understanding its cancer-related functions is highly relevant. Transparent zebrafish larvae are especially well suited to test the role of eicosanoid-mediated epithelial barrier maintenance, inflammatory, and malignant transformation by live imaging. Here we show loss, by CRISPR gene editing and morpholino mediated knockdown, of zebrafish alox12, the functional ortholog of ALOX12B, generates hyperplastic lesions in zebrafish epithelium. This phenotype is associated with leukocyte infiltration, and up regulation of inflammatory genes, indicative of a tumor-promoting tissue microenvironment. Through live imaging and lipidomic analysis in zebrafish and cell culture models, we are dissecting the fundamental mechanisms of eicosanoid-induced epithelial barrier homeostasis, inflammation, and malignant transformation that may be exploited for therapeutic benefit in the future.

P2127
Board Number: B270
Tumor suppressive role of Sestrin2 during colitis and colon carcinogenesis.
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The mTOR complex 1 (mTORC1) and endoplasmic reticulum (ER) stress pathways are critical regulators of intestinal inflammation and colon cancer growth. Sestrins are stress-inducible proteins, which suppress both mTORC1 and ER stress; however, the role of Sestrins in colon physiology and tumorigenesis has been elusive due to the lack of studies in human tissues or in appropriate animal models. In this study, we show that human SESN2 expression is elevated in the colon of ulcerative colitis patients but is lost upon p53 inactivation during colon carcinogenesis. In mouse colon, Sestrin2 was critical for limiting ER stress and promoting the recovery of epithelial cells after inflammatory injury. During colitis-promoted tumorigenesis, Sestrin2 was shown to be an important mediator of p53’s control over mTORC1 signaling and tumor cell growth. These results highlight Sestrin2 as a novel tumor suppressor, whose downregulation can accelerate both colitis and colon carcinogenesis.
P2128
Board Number: B271
PLK1-mediated RIP3 phosphorylation promotes G2/M abundance of RIP3.
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RIP3-mediated necroptosis is a form of programmed cell-death that occurs when caspase 8 is absent or inhibited, promoting the formation of the RIP1-RIP3 complex, which causes MLKL phosphorylation and rupture of the plasma membrane. Since necroptosis does not share proteins with the apoptosis pathway, it has been proposed as an alternate way of eliminating cancer cells which frequently inactivate caspases. To systematically address the effect of mitotic poisons on necroptosis, we treated mouse fibrosarcoma cell line L929 with various commonly used DNA-damaging and cytoskeletal-inhibiting drugs and discovered that instead of enhancing cell-death, many of these drugs in fact reduced necroptosis via the reduction in the expression of RIP3. Although the tumor suppressor p53 is activated, the expression of RIP3 was found to be independent of p53. We asked whether this reduction was dependent on the DNA-damage signaling and cell-cycle changes and found that the regulation by either of these impinged on the cell-cycle protein Polo-like kinase 1, which is both necessary and sufficient for RIP3 expression. Mechanistically, we discover that RIP3 protein levels are dependent on the cell-cycle with lowest abundance in the G1 and S phases whereas high levels are present in the G2/M phase, which also relate with necrosome formation and necroptosis. Further, we observe that RIP3-levels are diminished in the G2/M phase upon inhibition of polo-like kinase 1, indicating that PLK1 could facilitate in stabilizing RIP3. Finally, we identify a conserved cluster of residues in the 366-380 amino acid region of mouse RIP3 that are putative PLK1 phosphorylation and SCF-dependent degradation sites. Our work describes a novel regulatory network where PLK1 ensures abundant levels of RIP3 in the G2/M-phase. Our ongoing work explores whether high G2/M levels of RIP3 are linked to the elimination of aneuploidy cells via mitotic-catastrophe.

P2129
Board Number: B272
Novel Biogenic Role for the Retinoblastoma Protein Rb.
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We have identified a novel role for the retinoblastoma protein Rb in regulating cell biogenesis. Whereas oncogenic Ras signaling is sufficient to drive weak and sustained growth via the canonical growth pathway, PI3K-AKT-TOR and is able to preserve the uptake of extracellular nutrients required for biogenesis, we find that loss of Rb acts via signaling pathways distinct from PI3K-AKT-TOR and via an E2F-independent mechanism to drive cell growth, but the mechanisms remain unknown (Collins et al., 2012). We have generated an inducible system to rapidly remove Rb family activity from primary Schwann cells. Using this model, we are investigating the early biosynthetic and degradative changes following Rb family loss. We find that the Rb family acts via a distinct mechanism from mTOR signaling to regulate ribosome biogenic pathways. Additionally we have found that regulation of Pol I transcription is exquisitely sensitive to growth signaling pathways. Understanding how ribosomal biogenesis is linked to cell growth controls and cancer development may result in novel therapeutics that selectively target the growth of tumour cells.
P2130
Board Number: B273
Loss of SPINT2 promotes YAP activation and tolerance to aneuploidy.
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Functional inactivation of the Hippo tumour suppressor pathway is common in solid tumours, yet mutations in key components of the pathway are rare. Consequently, the underlying mechanisms that disable Hippo signalling in cancer cells remain elusive. Using a functional loss-of-function RNAi screen, we identified the protein SPINT2 as a novel activator of the Hippo pathway. SPINT2 is a single-pass transmembrane protein that functions as an extracellular serine protease inhibitor. We used multiple approaches to demonstrate that depletion of SPINT2 silences the Hippo pathway and thus activates the oncogenic transcriptional co-factor YAP. Mechanistically, we found that depletion of SPINT2 increases activation of the protease-activated receptors PAR1 and PAR2, which are known to suppress Hippo signalling. By contrast, overexpression of SPINT2 limits PAR activation, activates the Hippo pathway, and restrains cell proliferation. SPINT2 is a putative tumour suppressor gene that is commonly epigenetically silenced in a broad range of tumours. Interestingly, we discovered that SPINT2 is also epigenetically silenced in several aneuploid, but not diploid, primary human cell lines. While aneuploidy is a hallmark of tumour cells, it paradoxically limits the proliferation of normal cells. Thus, we hypothesized that epigenetic silencing of SPINT2 may represent an adaptation employed by tumour cells to better tolerate aneuploidy. Supporting this hypothesis, we found that depletion of SPINT2 imparts chromosomally stable cells with an increased tolerance for aneuploidy. Collectively, our work has identified SPINT2 as a novel regulator of Hippo signalling, whose loss both activates YAP and promotes tolerance to aneuploidy, two features of cancer cells.

P2131
Board Number: B274
Tumor suppressor cyclin C activates Bax in a redox-sensitive manner.
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In response to oxidative stress, mitochondria undergo extensive fragmentation, exhibit outer membrane permeabilization (MOMP) and release pro-apoptotic factors. Although the mitochondrial fission machinery is required for stress-induced hyper-fission, the molecular switch that induces this process remains elusive. In response to oxidative stress, cyclin C translocates from the nucleus to the outer mitochondrial membrane where it activates the fission GTPase Drp1. Previous studies revealed that cyclin C is both necessary and sufficient to induce extensive mitochondrial fragmentation. Moreover, cyclin C is required for stress-induced Bax activation, MOMP and ensuing apoptosis. Extending these studies in vivo, we show that cyclin C suppresses hyperplasia and adenoma formation in PTEN thyroid and activated KRAS pancreatic tumor mouse models, respectively. These results indicate that cyclin C is a previously underscribed solid tumor suppressor that forms a bridge between the fission and apoptotic machinery. Given that mitochondrial fission is the first step in apoptosis, we tested whether forced
cyclin C cytoplasmic localization could sensitize cancer cells to conventional chemotherapeutics. To do this, we designed a stapled peptide mimic (S-HAD) that interferes with cyclin C binding to its nuclear anchor Med13. In the absence of additional stress, S-HAD induced cyclin C release, mitochondrial fragmentation and mitochondrial superoxide production. Importantly, S-HAD-treated tumor cells but not the non-transformed cells were significantly more sensitive to cisplatin-induced apoptosis as indicated by Annexin V staining. Moreover, we found that adding S-HAD alone is sufficient to activate Bax in a cyclin C- and redox-dependent manner. This role appears direct as cyclin C co-immunoprecipitates with active Bax following S-HAD treatment. Finally, crosslinking revealed that Bax and cyclin C heterodimerize during cisplatin-induced apoptosis. The mechanism by which cyclin C promotes apoptosis may therefore proceed through direct and redox-sensitive activation of Bax.

P2132

Board Number: B275

Suppression of RAC1-driven malignant melanoma by Group A PAK inhibitors.

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Activating mutations in the RAC1 gene have recently been discovered as driver events in malignant melanoma. Expression of this gene is associated with melanocyte proliferation, and melanoma cells bearing this mutation are insensitive to BRAF inhibitors such as vemurafenib and dabrafenib, and also may evade immune surveillance due to enhanced expression of PD-L1. Activating mutations in RAC1 are of special interest, as small molecule inhibitors for the RAC effector p21-activated kinase (PAK) are in late-stage clinical development and might impede oncogenic signaling from mutant RAC1. In this work, we explore the effects of PAK inhibition on RAC1P29S signaling in zebrafish embryonic development, in the proliferation, survival, and motility of RAC1P29S-mutant human melanoma cells, and on tumor formation and progression from such cells in mice. We report that RAC1P29S evokes a Rasopathy-like phenotype on zebrafish development that can be blocked by inhibitors of PAK or MEK. We also found that RAC1 mutant human melanoma cells are resistant to clinical inhibitors of BRAF but are uniquely sensitive to PAK inhibitors. These data suggest that suppressing the PAK pathway might be of therapeutic benefit in this type of melanoma.

P2133

Board Number: B276

miR-195 regulates the response of non-small cell lung cancer to microtubule targeting agents by targeting CHEK1.

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Introduction: microRNAs (miRNAs) are a family of small non-coding RNAs (18-24 nt) that post-transcriptionally repress gene expression by direct binding to the 3’ untranslated regions (UTRs) of their targets. By targeting cancer-related genes, miRNAs have been shown not only to regulate cancer growth/progression, but also to modulate the response of cancer cells to chemotherapy. Such miRNAs are potential candidates for therapeutic intervention. Aiming to identify functional miRNAs in non-small cell lung cancer (NSCLC), we performed a high-throughput screen and found that miR-195 inhibits the growth of NSCLC cells and sensitizes them to microtubule-targeting agents (MTAs), a family of
chemotherapeutic drugs widely used for NSCLC treatment. The function and mechanism of miR-195 in NSCLC were demonstrated both in vitro and in vivo.

**Results**: We demonstrated that miR-195 synergizes with both an old-school MTA (paclitaxel) and a new-fangled one (eribulin) to repress the growth of NSCLC cells. Over-expression of miR-195 sensitizes NSCLC cells to paclitaxel and eribulin, while knockout of miR-195 confers resistance to paclitaxel and eribulin. Importantly, lung tumors with miR-195 over-expression are more sensitive to eribulin treatment than control tumors. Induced expression of miR-195 in lung tumors potentiates the efficacy of eribulin to repress tumor growth. Additionally, we showed that miR-195 directly targets CHEK1 to regulate the response of NSCLC cells to paclitaxel and eribulin. The direct and specific binding of miR-195 to the 3'UTR of CHEK1 was confirmed by luciferase reporter assay. Repression of CHEK1 using siRNAs and chemical inhibitor synergizes with paclitaxel and eribulin to repress the growth of NSCLC cells. Over-expression of CHEK1 contributes to the resistance to paclitaxel and eribulin in NSCLC cells. Analysis of TCGA data show that CHEK1 is significantly up-regulated in lung tumors compared to adjacent normal tissues and that its up-regulation is associated with worse recurrence-free and overall survival.

**Conclusions**: We report the identification of miR-195 as a sensitizer to microtubule-targeting agents in NSCLC, mediated by its repression of CHEK1. Mouse xenografts with induced or constitutive over-expression of miR-195 show that tumors with high miR-195 expression are more sensitive to drug treatment and that induction of miR-195 potentiates the efficacy of eribulin in repressing tumor growth. These results highlight the possible application of miR-195 expression as a biomarker to predict patient response to MTAs and the potential for delivery of miR-195 mimic as an adjuvant to chemotherapy.

P2134

**Board Number: B277**

TP53 gene status is a critical determinant of phenotypes induced by ALKBH3 knockdown in non-small cell lung cancers.

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Introduction: Although epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have revolutionized the treatment of EGFR-mutated non-small cell lung cancer (NSCLC), most patients develop acquired resistance either through secondary EGFR mutations or the activation of EGFR-independent pathways. Therefore, there is an urgent need for the identification and development of a novel therapeutic target molecule for NSCLC treatment. AlkB homolog 3 (ALKBH3) is a member of the AlkB family comprising nine paralogs (ALKBH1-8) and fat mass and obesity associated gene (FTO) and belongs to the 2-oxoglutarate and Fe2+-dependent dioxygenase superfamily. ALKBH3 demethylates 1-methyladenine (m1A) and 3-methylcytocine (m3C) in DNA/RNA and protects from DNA damage which caused by methylation. We have shown that ALKBH3 is overexpressed in prostate cancer, NSCLC, and pancreas cancer. ALKBH3 overexpression was detected in over 50% of NSCLC patients with poorer relapse-free survival. In vitro studies revealed m1A and m3C in DNA as the substrates for ALKBH3 and aberrant accumulation of methylation such as m1A and m3C in the genome may induce DNA damage-responsive signaling. Moreover, ALKBH3 knockdown induced powerful anti-tumor effect in tumor models of some cancer cells, including NSCLC cells. However, the underlying signal transduction mechanism of its anti-apoptotic effect on cancer cell proliferation is questionable. Methods: We evaluated the effect of TP53 on the phenotypes induced by ALKBH3 knockdown in NSCLC cells by establishing a TP53-knockout cell clone using A549 cells carrying wild-type TP53. Results: In this study, we found that ALKBH3 knockout induces cell cycle arrest or apoptosis depending on the TP53 gene.
status in NSCLC cells. TP53 gene encoding p53 is known to be frequently mutated in human cancers. Abnormality in TP53 gene is one of the most significant events in NSCLC and plays an important role in the tumorigenesis of lung epithelial cells. TP53 knockout not only promotes the ALKBH3 knockdown-induced DNA damage-responsive signaling but also shifts the phenotypes of ALKBH3-knockdown NSCLC cells from cell cycle arrest to apoptosis. These results suggest that the TP53 gene status is a critical factor for the phenotypic outcome of ALKBH3 knockdown in NSCLC cells, suggesting that ALKBH3 targeting may act as a novel approach for the treatment of NSCLC.

P2135
Board Number: B278
Akt 1 and PA28 do not have a synergistic effect on the Wnt pathway in cancer.
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Uncontrolled cellular proliferation is one of the defining hallmarks of cancer. One pathway that may contribute to enhanced proliferative signaling in cancer cells is the Wnt signaling pathway. In the absence of Wnt signals, GSK3β triggers β-catenin degradation. Previous research has shown that expression of β-catenin is increased in skin tumors, which may be related to the inhibition of GSK3β. Two additional proteins that inhibit GSK3β and also display increased expression in cancer are Akt 1 and PA28. The purpose of this research was to examine a possible synergistic relationship between Akt 1 and PA28 on GSK3β. Regulation of GSK3β directly affects β-catenin levels and its subsequent transcriptional targets that are important for cellular proliferation. In order to examine the Wnt pathway, total and phosphorylated version of GSK3β and Akt 1, and total β-catenin expression was measured in cell lines containing various expression levels of PA28, Additionally, the transcriptional targets of β-catenin, cyclin D1 and fgf18, were measured by qPCR. Interestingly, the lowest levels of β-catenin were identified in the two cancer lines, with the highest expression in the cells lacking the PA28 gene. Conversely, the transcriptional targets of β-catenin, cyclin D1 and fgf18, showed a different pattern of gene expression than the levels of β-catenin, suggesting additional mechanisms for transcriptional regulation. Levels of phosphorylated Akt 1 did not vary in these cell lines. Total expression and phosphorylation status of GSK3β did increase with transformation in cell lines, but it did not correlate with PA28 expression. In conclusion, there was insufficient evidence to suggest a synergistic relationship between Akt 1 and PA28 on the Wnt signaling pathway, and further research needs to be conducted in order to understand if PA28 is involved in the activation of cellular proliferation by the Wnt pathway in cancerous cells.

P2136
Board Number: B279
The Cdc42/Rac1 regulator CdgAP, a novel E-cadherin transcriptional repressor with Zeb2 in breast cancer, is regulated by RSK phosphorylation and binding to 14-3-3 adaptor proteins.
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Metastasis is the leading cause of death in breast cancer patients. The epithelial-to-mesenchymal transition (EMT) has a crucial role in metastasis and is highly critical for tumor cell dissemination. A set of transcriptional repressors of E-cadherin (CDH1) gene expression, including Snail1, Snail2 and Zeb2
mediate E-cadherin downregulation in breast cancer. Here, by using global gene expression approaches, we uncover a novel function for Cdc42 GTPase-activating protein (CdGAP) in the regulation of expression of genes involved in EMT. We found that CdGAP used its proline-rich domain to form a functional complex with Zeb2 to mediate the repression of E-cadherin expression in ErbB2-transformed breast cancer cells. In vivo, loss of CdGAP in ErbB2-transformed breast cancer cells impaired tumor growth and suppressed metastasis to lungs. The strong expression of CdGAP was correlated with poor prognosis in breast cancer patients. Furthermore, we have identified that CdGAP interacted with 14-3-3 adaptor proteins through its Ser1093 and Ser1163 residues in the C-terminal region, which sequestered CdGAP into the cytoplasm and inhibited its nucleocytoplasmic shuttling. Altogether, these data support a previously unknown nuclear function for CdGAP where it cooperates in a GAP-independent manner with transcriptional repressors to function as a critical modulator of breast cancer through repression of E-cadherin transcription. 14-3-3 binding to CdGAP may behave as an important negative regulator of CdGAP transcriptional activity by cytosolic sequestration, leading to the inhibition of EMT, cell motility and invasion of breast cancer cells. Targeting Zeb2– and 14-3-3-CdGAP interactions may represent novel therapeutic opportunities for breast cancer treatment.

P2137
Board Number: B280
Steroid receptors can facilitate the binding of each other and the pioneer factor FoxA1 to active enhancers in breast cancer cell lines through a dynamic assisted loading mechanism.
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Steroid receptors (SRs), such as the estrogen receptor (ER), play an important role in breast cancer development. The transcription factor (TF) FoxA1, has been implicated in ER binding patterns serving as a pioneer factor. However, the molecular interactions between ER and other TFs such as SRs are not well understood. Classically it has been proposed that pioneer factors have a slow residence time on DNA allowing chromatin to remain open. This permits access of SRs to binding sites in an ATP-independent manner, implying chromatin remodeling factors are not required to facilitate in the opening of chromatin at these sites. Here we established, by single-molecule tracking in living cells, that the DNA residence time of FoxA1 at specific sites is at a rate of ~8-10 seconds. This rate is similar to that of ER and the glucocorticoid receptor (GR). This finding is supported by the lack of a detectable FoxA1 DNase footprint together suggesting fast chromatin interactions. Complementing these results, we determined that SRs can alter the binding landscape of TFs, facilitating selective access to the chromatin by a mechanism termed dynamic assisted loading. To further investigate the functionality of this mechanism we characterized the chromatin landscape of ER, GR, and FoxA1 assisted loading sites in the MCF-7 breast cancer cell line. Here we utilizing ChIP-seq of H3K27ac and P300, both marks of active enhancers, and BrG1, ATPase subunit of SWI/SNF chromatin remodeling complex. Genome-wide analysis showed that the newly acquired ER, GR, or FoxA1 binding sites are highly associated with active enhancers due to an overall increase in H3K27ac and P300 at these sites. Most importantly, BrG1 binding patterns are correlated with ER, GR, and FoxA1 assisted loading sites. This indicates that the dynamic assisted loading mechanism requires ATP-dependent complexes. Together these results do not support a simplified model wherein a specific set of pioneer factors bind to closed chromatin and establish the binding landscape for other TFs. Rather, that many TFs have the potential to affect the binding landscape of other TFs. These newly acquired sites are associated with active enhancer marks, open chromatin, and chromatin remodeling factors. Suggesting these TFs can regulate the binding patterns of...
each through a bimodal switch, in a highly dynamic manner, with fast DNA residence times. In addition, this process involves Brg1, an ATP-dependent chromatin remodeling factor. This study has shifted our classical understanding of the functionality of pioneer factors in breast cancer, demonstrating multiple TFs have the capability to recruit and alter the response of other TFs via ATP-dependent processes.

P2138
Board Number: B281
Investigating the Epigenetic Regulation of the Breast Cancer Susceptibility Gene, BRCA1.
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Disrupted DNMT1 and DNMT3B, proteins that maintain methylation, function and amounts have been associated with aberrant DNA methylation levels in cancer. However, less is known about the downstream effect of the loss of DNMTs in specific genes related to breast carcinogenesis. In this investigation, DNMT1 and DNMT3B were knocked down utilizing siRNA and inhibited utilizing 5-aza-2’-deoxycytidine. Levels of both transcripts were determined by real-time qPCR. Levels of DNMT1 were found to be 27.32 +/- 19.56% in cells treated with DNMT1-6 siRNA. Transfection with DNMT3B-10 siRNA decreased DNMT3B mRNA to 46.64 +/- 22.34%. We found that levels of BRCA1 are aberrant when DNMT1 is knocked down but not when it is inhibited. Global DNA methylation was assayed by LUMA showing a decrease of about 20% when treated with 10um 5-aza which correlates with a percent decrease of 40% in enzyme activity. In addition, miRNA expression levels in knocked down and inhibited cells are also being investigated. Our study can further inform on the possible use of DNMT1 targeting drugs in breast cancer as well as suggest a possible crosstalk between the two proteins.

P2139
Board Number: B282
Understanding the Role and Regulation of the SENP1 SUMO Isopeptidase in Pancreatic Cancer.
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The small ubiquitin-related modifier, SUMO, is an essential posttranslational protein modification that controls nearly every aspect of cell function. Consequently, disruptions in the balance of SUMO pathway components are linked to abnormal cell function and disease, including the development and progression of human cancers. For example, it was recently proposed that misregulation of the SUMO isopeptidase, SENP1, contributes to the development and progression of pancreatic ductal adenocarcinoma (PDAC) (Ma et al. (2014) Tumor Biol. 35:12729-35). To further explore this finding, we first set out to validate that SENP1 expression levels were higher in PDAC as compared to normal tissues by characterizing SENP1 protein and mRNA levels in multiple PDAC cell lines. We were surprised to find that the SENP1 levels in these cells were not consistent with the published results. However, immunoblot analysis comparing SENP1 levels in the PANC-1 PDAC cell line to an immortalized “normal” pancreas cell line did show 60% more SENP1 in the PANC-1 cells. This finding is consistent with the hypothesis that elevated SENP1 plays a role in pancreatic cancer. To further test and validate this hypothesis, we are investigating SENP1 protein levels in PDAC patient tissues. To achieve this goal, we have generated formalin-fixed paraffin embedded faux-tissue samples using the PANC-1 PDAC cell line with or without SENP1 siRNA depletion. We are using these faux-PDAC tissue samples to validate the specificity of our SENP1 primary antibody and to develop a reproducible, robust and quantitative
immunohistochemical assay. This method will then be used to stain replicates of PDAC patient samples to evaluate SENP1 expression in the cancerous and adjacent normal tissues. In future studies, we aim to identify SENP1 substrates and understand how changes in the sumoylation of these proteins contributes to the development and progression of pancreatic cancer.

**Tumor Invasion and Metastasis 2**

**P2140**  
**Board Number: B283**  
**Matrix stiffness influences oral squamous cell carcinoma behaviour through EMT changes.**  
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A significant challenge in treating squamous cell carcinoma (SCC) is to completely eradicate neoplastic cells that have migrated and invaded the surrounding tissue. Squamous cells exist on a continuum from less aggressive, E-cadherin-rich epithelial, e.g. Cal27 and FaDu cell lines, to more invasive N-cadherin-rich mesenchymal phenotypes, e.g. SCC-9 and SCC-25; while in other tumor types, e.g. mammary, increased tissue stiffness contributes to epithelial-mesenchymal transition (EMT) and tumor progression, it is not clear whether this sensitivity applies to SCC given their phenotypic range. Thus we evaluated the structures that form and invasivity of SCC cells to determine their stiffness sensitivity across their initial phenotypic range using an organotypic model of oral cancer. To mimic the niche, we made a 3D gelatin-methacrylate (GelMA) extracellular matrix, which has tuneable matrix stiffness and mirrors the collagen-rich nice of SCC, and compared it across a range of stiffness to conventional collagen type I matrices. In the collagen gel after 21 days, the less invasive, more epithelial cell line Cal27 proliferated into cell layers without invading the collagen gel. However, an invasive, mesenchymal-like cell line SCC25 invaded the collagen gel and formed tumour islands and keratin pearls resembling what is seen in human specimens. When the GelMA system was stiffened from normal 2 to tumorigenic 20 kiloPascals (kPa, a unit of stiffness), differential cell behaviors were observed depending on the initial SCC phenotype, which together suggests for the first time that stiffness may affect SCC EMT.

**P2141**  
**Board Number: B284**  
**Differential Regulation of Mammary Cancer Invasivity due to Matrix Stiffness and Oncogenic Mutation.**  
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Mammary epithelial cells (MECs) form polarized 3D structures in vivo, i.e. acini, which can be mimicked in 3D extracellular matrix (ECM) cultures in vitro. Results from the past decade indicate that normal MECs are highly responsive to matrix stiffness, undergoing epithelial-to-mesenchymal transition (EMT) with increasing stiffness. However mammary tumors are a complex mixture of MECs with varying phenotypes and mutations and the adjacent stroma ranges from normal mammary stiffness, ~150 Pascal (Pa), to high malignant and stiff, ~5700 Pa. Thus we sought to understand how specific isogenic variants change MEC sensitivity to matrix stiffness. Pre-malignant MCF10A, Ras-transformed MCF10AT, MCF10DCIS obtained from ductal carcinomas, and MCF10CA1 cells derived from in vivo MCF10AT cells...
were utilized to span this spectrum of accumulating mutations. Consistent with previous in vitro 3D culture models of variable stiffness, cells were cultured on collagen-coated polyacrylamide gels with stiffness ranging from 150 to 5700 Pa. Though all cell lines were more proliferative and spread at 5700 Pa, only MCF10AT cells exhibited spread behavior at 150 Pa whereas other lines require 2- to 5-fold higher stiffness, e.g. MCF10CA1 and MCF10A, respectively. Nuclear localization of the EMT marker TWIST could be seen in all cell lines at 5700 Pa whereas in the softest niche, i.e. 150 Pa, only MCF10AT exhibited nuclear TWIST localization. Interestingly, MCF10AT cells displayed a uniquely pro-invasive phenotype regardless of stiffness despite lacking the additional mutations accumulated in MCF10DCIS and MCF10CA1 cell lines. Taken together, these data suggest a differential threshold to matrix stiffness on cellular phenotype that is largely dependent on the underlying oncogenic mutations present, a property that may be leveraged to better combat invasive breast cancer in humans.

P2142
Board Number: B285
The Morphological Characteristics of Carcinoma Migration Phenotypes are Differentially Regulated by the ROCK Isoforms.
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Rho-associated kinase (ROCK) drives migration by cancer cells through the extracellular matrix (ECM) via actomyosin contractility. To breach dense tissues, migrating cancer cells utilize invasive structures called invadopodia to proteolytically degrade the ECM. We previously found that the ROCK isoforms, ROCK1 and ROCK2, regulate invadopodia activity but through contractile and non-contractile mechanisms, respectively. Furthermore, we also found that both isoforms regulate invasion while ROCK2 was not necessary for migration. However, it was unclear whether these functional differences coincided with morphological properties that distinctly define the mesenchymal and amoeboid migration phenotypes. Therefore, we evaluated the physical characteristics of these cancer cells by quantitating cell size and shape as well as focal adhesion formation. Interestingly, we found that the ROCK isoforms differentially regulate cancer cell morphology resulting in intermediate phenotypes that share characteristics of both mesenchymal and amoeboid phenotypes. In particular, cancer cells were able to maintain a mesenchymal-like phenotype with ROCK2 inhibition despite a decrease in ECM degradation suggesting a hybrid phenotype still capable of migration. Overall, our data indicate that the ROCK isoforms may play unique roles in the phenotypic plasticity of mesenchymal carcinoma cells.

P2143
Board Number: B286
A novel Twist1-PKD1 axis promotes epithelial dissemination.
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Metastasis is initiated by tumor cells adopting an invasive, migratory program that allows them to disseminate and colonize distant organs. The minimal molecular requirement for metastasis is incompletely understood. We focused on elucidating the molecular mechanisms of cell detachment and migration downstream of Twist1, a transcription factor that is overexpressed in metastatic breast cancers and correlates with poor prognosis.
Using 3D organotypic culture of the mammary epithelium, we show that gain of Twist1 expression drives single cell dissemination without loss of epithelial identity. RNAseq of Twist1+ versus WT organoids revealed that Twist1 upregulates the expression of 107 genes, 9 of which are targetable with commercially available small molecules. We tested these inhibitors in our Twist1-induced dissemination assay. In particular, inhibition of protein kinase D1 (PKD1) blocked dissemination completely without affecting growth of WT epithelium. Knockdown of PKD1 in Twist1+ organoids confirmed its specific requirement for dissemination. We then investigated the cellular processes controlled by PKD1. Timelapse microscopy revealed that early pharmacologic inhibition of PKD1 prevented Twist1+ cells from protruding into the extracellular matrix (ECM) and detaching from the epithelium, while late PKD1 inhibition in disseminated cells abrogated their motility. The requirement of PKD1 for invasion and dissemination was also validated in organoids generated from a breast cancer mouse model (C3(1)-Tag) and in organoids isolated from primary human tumors. To study signaling downstream of the Twist1-PKD1 axis, we utilized phosphoprotein arrays which showed that PKD1 promotes the phosphorylation of the oncogene Myc, the tumor suppressor Brca1, the adherens junction protein β–catenin, the microtubule-associated protein Tau, and integrin partner FAK. Immunofluorescence staining confirmed that β–catenin phosphorylation (but not expression) increased in cells as they detached from the epithelium. Inhibition of FAK revealed that its activity was required for cells to disseminate. Moreover, PKD1 activity converged with microtubule depolymerization, likely through Tau phosphorylation. Microtubule depolymerization itself had a positive feedback on PKD1 as treatment with nocodazole, but not paclitaxel, weakened the anti-disseminative effect of the PKD1 inhibitor. Our data proposes a new working model whereby Twist1 drives epithelial dissemination by triggering PKD1 expression. In turn, PKD1 hijacks the cytoskeleton, thus forcing the cell to invade the ECM, then detach from the epithelium, and finally migrate with high persistence into the surrounding stroma. These results also highlight PKD1 as a new potential target for anti-metastatic therapy.

**P2144**

**Board Number: B287**

G1P3-induced mtROS augment caveolae mediated endocytosis of E-cadherin to promote breast cancer metastasis.

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Although a myriad of mechanisms are responsible for cancer metastasis, the mitochondria’s role in this process remains unclear. Our studies identified a role for G1P3, a mitochondrial localized survival protein, in cancer cell redox regulation. Moreover, G1P3-induced mitochondrial reactive oxygen species (mtROS) promoted the migration and invasion of breast cancer cells. In network based differential gene expression analysis, both epithelial to mesenchymal transition (EMT) and vesicle mediated endocytic pathways were enriched in G1P3 expressing cells (MCF-7G1P3). Since cell-cell adhesion protein E-cadherin suppresses EMT, we hypothesized that G1P3-induced mtROS augments endocytosis of E-cadherin to promote breast cancer metastasis. This hypothesis was tested by comparing the expression and localization of E-cadherin in vector control (MCF-7Vector) and MCF-7G1P3 cells. Although E-cadherin expression was similar in MCF-7Vector and MCF-7G1P3 cells, confocal imaging studies identified that in vector control cells, E-cadherin was predominantly localized on the plasma membrane, whereas in MCF-7G1P3 cells, the majority of E-cadherin was in endocytic vesicles. Compared to MCF-7Vector cells, E-cadherin was 1.5-fold lower in MCF-7G1P3 cell membrane fractions with a concurrent increase (6.45-fold) in cytosolic fractions (P≤0.05). Additionally, qRT-PCR and confocal immunofluorescence analysis identified
4.3-fold upregulation of Caveolin-1, a major mediator of endocytosis, in MCF-7G1P3 cells. Colocalization of E-cadherin with Caveolin-1 and early endosome antigen 1 (EEA1) was markedly increased in MCF-7G1P3 cells, suggesting caveolae mediated upregulation of E-cadherin endocytosis. Moreover, Mito-TEMPO, a mitochondrial specific ROS scavenger, reduced mtROS in MCF-7G1P3 cells, reversed G1P3’s effects on E-cadherin endocytosis, and restored the membrane localization of E-cadherin. Similarly, Methyl-beta-cyclodextrin, a caveolin inhibitor, suppressed the endocytosis of E-cadherin to reestablish its membrane localization in MCF-7G1P3 cells. Consequently, both mtROS and endocytosis suppression resulted in a significant reduction in the rate of migration (40% vs 12% wound closure in untreated vs treated cells) and invasion through matrigel (9.7-fold reduction, p<0.01). In summary, our results demonstrate a novel mechanism of G1P3-induced mtROS in breast cancer metastasis promotion through E-cadherin endocytosis. Considering the association between high expression of G1P3 and poor distant metastasis free survival (DMSF), interrupting G1P3 mediated E-cadherin endocytosis may prevent metastasis and improve clinical outcomes in breast cancer patients.

P2145
Board Number: B288
Prostate Specific Membrane Antigen promotes prostate tumor progression and survival by conferring resistance to hypoxic stress.
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Prostate cancer (PC) is the most commonly diagnosed cancer and is the second leading cause of cancer deaths among men in the United States. The ability of tumor cells to survive in the low oxygen (hypoxic) environment created by the expansion of primary tumors is fundamental to their growth in situ as well as metastatic progression that underlies the majority of disease-related mortality. Prostate Specific Membrane Antigen (PSMA), a type II transmembrane peptidase, is progressively upregulated in ~80% of tumors during PC progression where it correlates negatively with prognosis. We have previously reported that increased PSMA expression on tumor cells alters signal transduction mechanisms downstream of growth factor receptors to promote tumor progression. In addition, we found that expression of PSMA promotes increased survival in hypoxic environments and the current study was designed to determine these PSMA-dependent survival mechanisms. Using the CRISPR/Cas9 system, we engineered a panel of human and murine PC cell lines (LnCaP, 22rv1, TRAMP-C1) to lack PSMA expression. Interestingly, loss of PSMA (PSMAKO) in these highly metastatic PC cell lines resulted in a significantly lower proliferation rate than PSMA expressing cells (PSMAWT), formation of smaller anchorage-independent colonies in soft agar, a shift in the balance of RIPK3/MLKL family of necrosis proteins toward a decreased survival, and a decrease in the migration and intravasation/extravasation potential. Furthermore, in a NOD/SCID xenograft mouse model, LnCaP-PSMAWT cells formed large, well-vascularized tumors whereas LnCaP-PSMAKO showed no evidence of tumor growth, indicating that PSMA is a potent tumor promoter both in vitro and in vivo. Mimicking hypoxic conditions in vitro by treating both PSMAWT and PSMAKO cells with a HiF1 inhibitor, (CoCl2), produced drastic differences in the hypoxia-induced regulation of the calcineurin A and calpain-2 calcium-associated signal-transduction pathways as well as actin regulated cytoskeletal rearrangement highlighting PSMAs role in resistance to hypoxia as well as its potential contribution to a pro-tumor phenotype and increased metastatic potential. In support of this notion, in silico meta-analysis of publically available human gene expression data sets indicated that changes in PSMA and calcium associated genes correlates significantly with PC metastasis to bone and lymph. Dramatic changes in calcium levels are a primary response of many cells engaged in the formation of calcification process.
to hypoxia and typically stimulate cell death. These results suggest that PSMA expression on tumor cells confers resistance to hypoxia via changes in the calcium signal-transduction cascade, thus making the complete disruption of PSMA a promising therapeutic option in management and treatment of PC.

P2146

Board Number: B289

The role of antioxidant enzymes in the proliferation and survival of extracellular matrix-detached SKOV3 cells.

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Epithelial ovarian cancer (EOC) is one of the leading causes of cancer death in women. This could be attributed to the fact that approximately 70% of all cases are diagnosed after the disease has already metastasized, making EOC more difficult to treat. During metastasis, EOC cells shed from the primary tumor and accumulate in ascites before attaching and forming secondary tumors throughout the peritoneal cavity. Throughout this process, EOC cells survive and proliferate without anchorage to the extracellular matrix (ECM) in order to successfully metastasize; however, the specific molecular mechanisms behind the survival and proliferation of EOC cells in anchorage independence has yet to be unveiled. Here, we present data that suggests that the antioxidant enzymes Catalase, Peroxiredoxin 1, and Peroxiredoxin 2 are important and play different roles in the metastasis of SKOV3 cells. SKOV3 cells were genetically engineered to be deficient in Catalase, Peroxiredoxin 1, or Peroxiredoxin 2 through short hairpin RNA (shRNA) techniques, and we report that elimination of any of these antioxidant enzymes in SKOV3 cells results in decreased colony formation in soft agar. Furthermore, Catalase elimination in ECM-detached SKOV3 cells results in increased cell death at 48 hours; whereas elimination of Peroxiredoxin 1 or Peroxiredoxin 2 in ECM-detached SKOV3 cells results in decreased proliferation at 72 hours. Thus, our data reveal that while Catalase, Peroxiredoxin 1, and Peroxiredoxin 2 may be attractive targets for the design of chemotherapeutics aimed at eliminating metastatic ovarian cancer, each enzyme may play a different role in the success of EOC cells during the ECM-bereft metastatic cascade.

P2147

Board Number: B290

Role of NADPH Oxidase (NOX-1) in TNF-α mediated cellular response in regulation of cell death and survival.

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Tumor Necrosis Factor alpha (TNF-α) is known to regulate numerous cell signaling involves proliferation, differentiation, migration, inflammation, cell death and survival. TNF-α modulates NF-κB signaling pathway that plays a pivotal role in defining fate of cells for survival and death. Importantly, cellular redoxstasis also regulates various physiological processes and its imbalance leads to the pathophysiological consequences. NADPH oxidase (NOX) system is a potent pro-oxidant family of enzyme that generates ROS as a primary product. Additionally, NRF-2 is transcription factor that balances the NOX mediated intracellular ROS generation to maintain the cellular redoxstasis. Previous reports suggest that TNF-α modulates receptor interacting serine/threonine protein kinase 1 (RIP-1) and NOX activity and induces necrosis in fibroblast cells but the role of NOX mediated redox signaling in regulation of NF-κB activity remains unclear. In present study, we aimed to elucidate the role of NOX
machinery in TNF-α mediated NF-κB activation and downstream signaling to define fate of cells. In our study, we examined the activity and expression of NF-κB upon TNF-stimulation in HCT-116 cells. We found TNF-α activates NF-κB and translocates into the nucleus. We noticed that TNF-α efficiently induces NOX activity and ROS generation. Further, we inhibited NOX activity by Diphenyleneiodonium (DPI) and observed significant reduction of NF-κB expression and activity. Interestingly, we found that inhibition of NOX activity impedes downstream programmed necrotic cell death signaling by attenuating RIP-1 and anti-apoptotic protein cellular FLICE-like inhibitory protein (cFLIPL). Moreover, inhibition of NOX activity induces apoptotic cell death. Additionally, TNF-α sustains the expression of NRF-2 which may be regulated directly with the NOX activity. Collectively, our results suggest that NOX proteins play an essential role in modulation of NF-κB signaling to define fate of cell death and survival in cancer.

P2148
Board Number: B291
Effects of ASCT2 (alanine/serine/cysteine transporter 2 / SLC1A5) downregulation in human colon cancer HCT-116 cells.
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ASCT2 (alanine/serine/cysteine transporter 2 / SLC1A5) is expressed in colorectal adenocarcinomas and patient survival decreased with increased percentage of ASCT2-positive cancer cells (Anticancer Res 22:2555-2557,2002; Mol Imaging Biol 19:421-428,2017). The present study examined the effect of ASCT2/SLC1A5 gene down regulation by RNA interference on the expression and function of the transporter and cell proliferation of human colon cancer HCT-116 cells. The commercially available siRNA sequence Si00079730 targeting the human ASCT2 mRNA sequence and negative control siRNA commercial sequences NC-Si03650318 and NC-Si03650325 (Qiagen) were tested. The human colon carcinoma HCT-116 cells were transfected with siRNA complexed with Lipofectamine 2000® or Injectin®, and ASCT2 mRNA expression, protein abundance, inward transport of [14C]-L-alanine and the effect of siRNA sequences upon cell proliferation were evaluated after 72 h. The expression of ASCT2 in HCT-116 cancer cells was studied by means of immunoblotting and RT-PCR. Antibodies raised against ASCT2 and GAPDH were used, and images were obtained by scanning at both 700 nm and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences). Total RNA was converted to cDNA and qPCR analysis was made on the StepOnePlus instrument (Applied Biosystems). Primers for ASCT2 and for the endogenous control gene GAPDH were used. The inward transport of [14C]-L-alanine was examined by incubating cells with non-saturating concentrations (0.25 µM) of [14C]-L-alanine for 1 min. Cell proliferation was evaluated after 30 min incubation with the calcein-AM cell-permeant dye and fluorescence recorded using a 485/530 nm excitation/emission filter in the spectrofluorometer Spectramax Gemini EM (Molecular Devices). The siRNA sequence Si00079730 tested reduced ASCT2 mRNA expression to 30% when compared to the corresponding vehicle. ASCT2 protein expression was also reduced by approximately 50%, and this effect was associated with a 40% reduction upon the [14C]-L-alanine accumulation in HCT-116 cells. Moreover, ASCT2 downregulation markedly decreased (~70%) cell proliferation. The negative control siRNAs were devoid of effects on parameters measured. In conclusion, anti-ASCT2 siRNA decreased [14C]-L-alanine uptake and cellular proliferation in association with downregulation of ASCT2 expression and function. The decrease in cell viability and proliferation of cancer cells induced by anti-ASCT2 siRNAs is expected provide a decrease in tumor growth and metastasis potential in colon cancer.
P2149

Board Number: B292

Effects of LAT1 (L-type amino acid transporter 1 / SLC7A5) downregulation in human colon cancer HCT-116 cells.

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L-Type aminoacid transporter 1 (LAT1/SLC7A5) is highly expressed in cancer cells to support their continuous growth and proliferation and has been suggested as a marker of rectal cancer prognosis (Anticancer Res 30: 4223-4227,2010). The present study examined the effect of LAT1/SLC7A5 gene downregulation by RNA interference on the expression and function of the transporter and cell proliferation of human colon cancer HTC-116 cells. Two commercial siRNAs (SI31011000 (Qiagen) and HSS112005 (Invitrogen)) targeting human LAT1 mRNA sequences and negative control siRNA commercial sequences NC-SI03650318 and NC-SI03650325 (Qiagen) were tested. Cells were transfected with siRNAs complexed with Lipofectamine 2000* or Injctin*. LAT1 mRNA expression, protein abundance, inward transport of [14C]-L-leucine and the effect of siRNA sequences in cell proliferation were evaluated after 72 h. Total RNA was converted to cDNA and qPCR analysis was made on the StepOnePlus instrument (Applied Biosystems). Primers for LAT1 and for the endogenous control gene GAPDH were used. Antibodies raised against LAT1 and GAPDH were used, and images were obtained by scanning at both 700 nm and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences). The sodium-independent inward transport of [14C]-L-leucine was examined by incubating cells with non-saturating concentrations (0.25 μM) of [14C]-L-leucine for 1 min. Cell proliferation was evaluated after 30 min incubation with the calcein-AM cell-permeant dye and fluorescence recorded using a 485/530 nm excitation/emission filter in the spectrofluorometer Spectramax Gemini EM (Molecular Devices). [14C]-L-leucine accumulation in HCT-116 cells was reduced in the presence of the LAT1 inhibitor BCH in a concentration-dependent manner. Both siRNA sequences tested reduced LAT1 mRNA expression around 50% or more when compared to the corresponding vehicle. LAT1 protein expression was also reduced for at least 50%, and this effect was accompanied with a reduction upon the [14C]-L-leucine accumulation in HCT-116 cells. Moreover, LAT1 downregulation decreased cell proliferation, particularly when siRNA sequence HSS112005 was used. The negative control siRNAs were devoid of effects on parameters measured. In conclusion, the [14C]-L-leucine uptake was largely inhibited by the system L selective inhibitor BCH, which suggests that most of L-leucine uptake in HCT-116 cells is mediated by system L. Anti-LAT1 siRNA decreased [14C]-L-leucine uptake and cellular proliferation in association with downregulation of LAT1 expression and function. The decrease in cell viability and proliferation of cancer cells induced by anti-LAT1 siRNAs is expected provide a decrease in tumor growth and metastasis potential in colon cancer.
P2150

Board Number: B293

Insulin inhibits VEGF-induced endothelial permeability and metastasis by inhibiting TGase2 in the lung of diabetic mice.
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Insulin, which is derived from proinsulin, is an anti-hyperglycemic hormone, but its preventive roles in diabetic vascular permeability and metastasis are unknown. Thus, we investigated the preventive effect of insulin against endothelial cell growth factor (VEGF)-induced endothelial permeability and metastasis in human pulmonary microvascular endothelial cells (HLMVECs) and the lung of diabetic mice. VEGF elevated transglutaminase 2 (TGase2) activity through sequential elevation of intracellular Ca2+ and reactive oxygen species (ROS) levels in HLMVECs. Insulin, TGase inhibitor cystamine and TGase2 small interfering RNA (siRNA) prevented VEGF-induced vascular endothelial (VE)-cadherin disruption, which plays a critical role in modulating endothelial permeability. Furthermore, we showed that VEGF-induced pulmonary microvascular endothelial cell permeability is significantly prevented by insulin and cystamine. Consistently, in the lung of streptozotocin diabetic mice, VEGF-stimulated transaminating activity was inhibited by insulin replacement therapy (58.4 pmol/min/kg) using osmotic pumps. In addition, insulin prevented melanoma metastasis in the lung of streptozotocin diabetic mice. The role of TGase2 in VEGF-induced pulmonary vascular leakage and metastasis were further supported using diabetic TGase2-/- mice. Thus, our findings suggest that insulin inhibits VEGF-induced endothelial permeability and metastasis by inhibiting TGase2 in the lung of diabetic mice. Furthermore, TG2 may be a novel target for prevention of metastasis in diabetes.

P2151

Board Number: B294

Centrosome amplification triggers a non-canonical Senescence-Associated Secretory Phenotype and HIF1-α activation.
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Centrosomes promote the assembly and organization of the microtubule, which in turn supports many cellular processes including cell division and migration. In cancer, an abnormal increase in the number of centrosomes is commonly observed. However, tumours are extremely heterogeneous, which usually only have a subset of cells with supernumerary centrosomes. Therefore, whether centrosome amplification in a subpopulation of cells influences an entire population of cells remains an open question. Notably, centrosome amplification in a subset of cells within tissues is sufficient to promote spontaneous tumourigenesis, potentially through cell-autonomous induction of aneuploidy and invasion. But supernumerary centrosomes also trigger p53-dependent proliferation arrest in cells, thus potentially compromising their viability. Hence, how centrosome amplification promotes cancer progression despite triggering a proliferation arrest also remains incompletely understood. Here, we provide novel insights into these outstanding questions. We observe that most cells with centrosome amplification ultimately enter senescence. Senescence has long been recognized as a proliferation...
barrier that is activated in response to tumour initiation and a hallmark of premalignant tumours. Of note, recent evidence indicates that senescent cells can promote tumour invasion and metastasis in a cell non-autonomous manner, by up regulating the expression of cytokines and angiogenic factors - a phenomenon termed as the Senescence-Associated Secretory Phenotype (SASP). Indeed, we find that centrosome amplification increased the expression of extracellular proteins. While persistent DNA damage is the primary activator of SASP and that NF-κB is the major determinant of SASP, we show that centrosome amplification instead triggers an SASP independent of persistent DNA-damage and lacks a prominent NF-κB response. Therefore, indicating that centrosome amplification activates a non-canonical SASP program. Previously, we reported that centrosome amplification, through an increased nucleation of centrosomal microtubules, activates Rac-1 signaling and invasion in a cell-autonomous manner. Here, we find that centrosome amplification induced Rac-1 activation contributes to an increase in cellular ROS, which promotes the stabilization of HIF1α. Indeed, up regulation of cellular ROS is a common feature of senescent cells and may activate HIF1α. As most tumors only have a sub-population of cells possessing supernumerary centrosomes, we provide evidence that centrosome amplification exerts a non-cell autonomous impact by activating a non-canonical SASP and HIF1α.

P2152

Board Number: B295

Metastatic melanoma cells commandeer p53 activity to promote the survival of a therapy resistant subpopulation.

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Resistance to targeted therapy and chemotherapy increases with age. Previously, we showed that Wnt5A promotes resistance to therapy via the initiation of a pseudosenescent phenotype. Here, we show that Wnt5A correlates with wild type p53 expression in metastatic melanoma. Knocking down Wnt5A decreases the half-life of p53, and blocks a G2/M arrest following DNA damage. These data suggest that Wnt5A may promote the expression of p53 to drive the survival of a subpopulation of cells, which are highly invasive and therapy resistant. To determine if Wnt5A and p53 may be promoting the survival of metastatic melanoma in an aged microenvironment, we examined tumors from young and aged C57/BL6 mice generated using Yumm1.7 cells. We found that tumors in aged mice have a higher percentage of cells expressing p53 compared to tumors in young mice. When we expose human melanoma cells to conditioned media from aged fibroblasts, we see an increase in the percentage of slow cycling cells compared to melanoma cells exposed to conditioned media from young fibroblasts. Knocking down Wnt5A or p53 in melanoma cells decreased the percentage of slow cycling cells. When we isolate slow cycling cells, we see an increase in the expression of Wnt5A, p53, and p21. It may be these slow cycling cells, which are enriched in an aging microenvironment, that are driving resistance to targeted therapy in aged patients.
P2153
Board Number: B296
PLCy1 mediated migration and invasion of HEK293 cells stably expressing non small cell lung carcinoma associated EGFR mutants.
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Over a decade activating mutations in epidermal growth factor receptor (EGFR) playing a major role in predicting the treatment response of non small cell lung cancer patients to FDA approved tyrosine kinase inhibitors. Patients with mutations in exons19 and 21 of receptor kinase domain by and large have improved outcome in response to these drugs. Nonetheless, a majority of patients eventually develop secondary mutation; T790M in ATP binding site of exon20 region which confers drug resistance. Till date a large number of studies reported the prognostic implications of receptor sensitive and resistant mutations in non small cell lung cancer patients, but the role of these molecular alterations in lung tumor progression and tumor cell invasion remains still unclear. More studies on the regulatory role of mutants in tumor progression may enable to develop a novel class of drugs to target both sensitive and resistant mutant receptors. The current study was designed to investigate the signaling pathways recruited in response to both sensitive and resistant mutants independent of each other. HEK293 cells stably expressing receptor sensitizing mutants; L858R, L861Q of exon21 and resistant mutant T790M of exon20 and double mutant (L858R and T790M) were used in the study. Our experiments demonstrated increased phosphorylation of PLCγ1, c-Cbl, Stat, Mapk, Akt, Shc proteins in cells expressing both sensitizing and resistant mutant receptors as compared to wild type receptor. As PLCγ1 is known to be overexpressed in many tumor types and involved in metastatic development of tumor cells, we further investigated the mutant mediated PLCγ1 activation. Corresponding to EGFR and PLCγ1 phosphorylation, increased invasion and migration of cells expressing mutants was recorded by wound healing, transwell cells migration and invasion assays. Treatment of cells with Gefitinib, Afatinib, Osimertinib; first, second and third generation tyrosine kinase inhibitors reduced the phosphorylation of PLCγ1 in cells expressing receptor mutants. Significant reduction of in vitro migration and invasion of cells following treatment with tyrosine kinase and PLC inhibitors indicates the mutant receptor mediated PLCγ1 deregulation in HEK293 cells.

P2154
Board Number: B297
Myoferlin depletion reduces autocrine TGF-β1 production to regulate epithelial-mesenchymal transition in breast cancer cells.
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Tumor cells within an adenocarcinoma can undergo an epithelial-mesenchymal transition (EMT), enabling the cells to break free from the primary tumor and invade surrounding tissue and distant
organs (i.e., metastasis). Recently, myoferlin (MYOF), a protein involved in cell membrane function and repair, was found to be overexpressed in several invasive breast cancer cell lines as well as other types of cancer (e.g., pancreatic, squamous cell and melanoma). Using lentivirus-mediated RNA interference, depletion of myoferlin in the human breast cancer cell line MDA-MB-231 (MDA-231MYOFKD) reduced migration and invasion, and caused the cells to revert to an epithelial phenotype, in contrast to the vector control (MDA-231LVC) which maintained a mesenchymal phenotype. To test if the MET associated with loss of MYOF was permanent, MDA-231MYOFKD cells were treated with transforming growth factor β1 (TGF-β1, 2 ng/mL), a potent stimulus of EMT. After 48 hr of TGF-β1 treatment, MDA-231MYOFKD cells underwent an EMT as indicated by a more elongated, mesenchymal morphology, decreased expression of the epithelial marker E-cadherin, and increased expression of the mesenchymal marker vimentin. Additionally, studies on the effects of TGF-β1 treatment on cell motility in MDA-231MYOFKD cells revealed a decrease in directionality toward more random migration, similar to the highly invasive control MDA-231LVC and wild-type MDA-MB-231 cells. While these results indicated that an MET driven by MYOF depletion was reversible, the mechanism remains unknown. MDA-MB-231s growth and survival has been previously shown to be regulated in part by autocrine TGF-β1 signaling. Using this information, along with myoferlin being known to play a role in the secretion of another signaling factor, VEGF, we hypothesized that MYOF depletion may result in the misregulation of autocrine TGF-β1, prohibiting EMT. A TGF-β1 specific ELISA was used to measure the amount of TGF-β1 secreted into the culture medium. A significant decrease (20%) in TGF-β1 secretion was observed in medium from MDA-231MYOFKD when compared to MDA-231LVC. Similarly, RT-qPCR indicated a significant decrease (30%) in TGF-β1 mRNA expression levels in MDA-231MYOFKD cells. To test if TGF-β1 was necessary to maintain the mesenchymal phenotype, inhibition of the TGF-β1 signaling in MDA-231LVC cells with a pharmacological inhibitor of TGF-β receptor I/II kinases (LY2109761, 2 μM) elicited a decrease in the mRNA expression of the mesenchymal transcription factor Snail and increased E-cadherin mRNA expression. These results identify a novel pathway in the regulation of autocrine TGF-β signaling and establish a possible mechanism by which MYOF regulates the cellular phenotype and invasive capacity of human breast cancer cells.

**P2155**

**Board Number: B298**

Hepatitis B virus-human chimeric transcript HBx-LINE1 serves as a molecular sponge specific for hepatocellular miR-122 and promotes hepatocellular carcinoma progression via depleting miR-122.

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Chronic HBV carriers are at high risk for developing hepatocellular carcinoma (HCC) but the underlying mechanism remains unclear. Recent finding of viral-human hybrid RNA transcripts promoting hepatic cancer cell migration/invasion provides potential link between HBV infection and HCC progression. Here we report that a viral-human hybrid RNA transcript HBx-LINE1 serves as a molecular sponge specific for miR-122 and promotes HCC progression via depleting cellular miR-122. HBx-LINE1 was inversely correlated with miR-122 in HBV-positive HCC tissue and HBx-LINE1 over-expression in Huh7 cells decreased miR-122 level. HBx-LINE1 specifically bound to miR-122 through six miR-122-binding sites, and miR-122 over-expression completely reversed the effect of HBx-LINE1 on activating β-catenin signal pathway and decreasing E-cadherin. Furthermore, expression of HBx-LINE1 in mouse livers decreased miR-122 level and promoted hepatic injury, which was largely abolished by over-expression
of miR-122. In summary, our study provides the first evidence that viral-human chimeric transcripts can serve as a molecular sponge for a particular miRNA and modulate host cell function through depleting the cellular level of that miRNA. In specific, we report that HBV-human chimeric transcript HBx-LINE1 is a molecular sponge for miR-122, and play an important role in promoting HCC progression and activating β-catenin signaling pathway and EMT process in liver tumor cells through depleting cellular miR-122. This finding illustrates a new mechanism by which HBV modulates host cell function and also provides a potential novel therapeutic target for preventing or suppressing the progression of HCC associated with chronic HBV infection.

P2156
Board Number: B299
Hepatitis B virus X protein induces the development of hepatocellular carcinoma by stabilizing HIF-1α.
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Hepatitis B virus (HBV) infection is the primary cause of the development of various liver diseases including hepatocellular carcinoma (HCC). Among HBV proteins, it has been reported that hepatitis B virus X protein (HBx) is closely involved in the liver carcinogenesis, but its molecular mechanism remains to be elucidated.
In this study, we show that HBx enhances the stability of hypoxia inducible factor-1α (HIF-1α) by regulating VHL binding protein 1 (VBP1). HBx inhibits the interaction between VBP1 and Von Hippel-Lindau protein (pVHL), subsequently reduces the stability of pVHL which is responsible for the degradation of HIF-1α during normoxia.
HIF-1α is a key regulatory factor inducing the tumor formation and enhancing the epithelial-mesenchymal transition (EMT). Here, we also demonstrated that HBx promotes cancer development and EMT by stabilizing HIF-1α. Taken together, our data suggest that HBx stabilizes HIF-1α through interacting with VBP1, and induces the development of HCC.

P2157
Board Number: B300
Selected mitochondrial DNA landscapes activate the UPRmt to promote metastasis.
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Mitochondrial DNA (mtDNA) has been reported to alter the metastatic behavior of cancer cells. This phenotype was originally associated with specific reactive oxygen species (ROS)-generating mutations. Subsequently mild mtDNA mutations and even mtDNA polymorphism were shown to affect metastasis. Our data suggests that no common mtDNA mutation identifies metastatic cells; rather the metastatic potential of several ROS-generating mutations is largely determined by their mtDNA genomic landscapes, which can act either as an enhancer or repressor of metastasis. However, mtDNA landscapes of all metastatic cells are characterized by activation of the SIRT/FOXO/SOD2-axis of the mitochondrial unfolded protein response (UPRmt). ROS produced by mtDNA mutations can facilitate further mutation of mtDNA and leads to the oxidation of proteins thereby disrupting mitochondrial proteostasis. Metastatic cells with activating mtDNA landscapes may utilize the SIRT/FOXO/SOD2-axis of the UPRmt to handle this stress. Activation of the UPRmt in response to ROS and oxidative stress promotes a complex transcription program that results in increasing mitochondrial integrity and fitness.
Using SOD2 as a surrogate marker of the UPRmt, we found that compared to patient matched primary breast cancers, SOD2 is significantly increased in metastatic lesions. We propose that the ability of selected mtDNA species to activate the UPRmt is a process that is exploited by cancer cells to maintain mitochondrial fitness in the face of oxidative stress and facilitate metastasis.

P2158

Board Number: B301

Glioma mouse models reveal subtype specific cell dynamics.

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Glioblastoma (GBM; WHO Grade IV glioma) is the most common form of malignant glioma with median survival of 15 months and 5-year survival rate of less than 5%. GBM is currently incurable due to the high proliferation and migration rates associated with tumor cells, which allow them to invade healthy tissue and evade current therapies. Utilizing genomic analysis, many genetic drivers have been identified and high grade human glioma has been classified into four subtypes: classical, proneural, mesenchymal, and neural. However, how the different genetic drivers are influencing cell dynamics and consequentially impacting disease progression remains unknown.

PDGFb, platelet-derived growth factor B, has been shown to be expressed in GBM and was used to induce glioma in both mouse and rat models. Here we investigate the consequences of PDGFb overexpression on cell migration and proliferation relative to nRasG12V, a constitutively active nRas, overexpression. We hypothesize PDGFb overexpression will elicit distinct cellular dynamics from nRasG12V overexpression.

The Sleeping Beauty transposon system is used to induce tumor bearing mice using nRasG12V or PDGFb in combination with SV40-LgTA. Histological sections show that both nRasG12V and PDGFb generate high grade glioma. In addition, nRasG12V driven tumor cells are marked by significantly higher migration rate (~27µm²/hr) than PDGFb (~2µm²/hr), as measured ex vivo in mouse brain slices. Using Bioluminescence Imaging, the PDGFb cohort is estimated to have higher proliferation rate than nRasG12V. Higher proliferation rate also correlates with lower survival rate (median survival 31days PDGFb vs. 70 days nRasG12V). Lastly, RNA sequencing of healthy brain tissues and tumors from both cohorts reveals distinct expression profile between the two cohort: nRasG12V cohort resembles the mesenchymal human subtype and PDGFb cohort resembles the proneural human subtype.

Both nRasG12V and PDGFb overexpression leads to the development of high grade glioma resembling two of the human subtypes with distinct cellular dynamics, expression profile and survival rate. To explain the mechanism by which these two drivers are affecting cellular dynamics and tumor progression, further transcriptomic analysis is underway to highlight the pathways driving the observed cellular changes.
P2159

Board Number: B302

Characterization of anoikis-resistant endothelial cells after PIK3CA (phosphatidylinositol 4,5-bisphosphate 3-kinase, catalytic subunit alpha) gene silencing.
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The resistance of tumor cells to cell death by adhesion loss (anoikis) is a major cellular feature that contributes to tumor progression. Anoikis resistance is an important event in the metastatic cascade, a prerequisite for colonization and spread to distant sites. However, the mechanisms involved in anoikis regulation resistance are not fully elucidated. Various molecules are involved in the survival processes, cell adhesion and proliferation, including the phosphatidylinositol 3-kinase (PI3K). PI3Ks are lipid kinases that regulate signaling pathways important for neoplasia. PIK3CA, the gene encoding the p110α catalytic subunit of PI3K, was recently identified as novel mechanisms of inducing oncogenic PI3K signaling. This gene is frequently mutated in cancers. PI3K signaling pathway regulates several cellular processes and it’s one of the most frequently deregulated pathway in human tumors. Previous data from our laboratory showed that anoikis-resistant endothelial cells exhibit up-regulation of PI3k/Akt pathway and this is accompanied by high proliferation and low apoptosis rate. Aiming to clarify the role of the catalytic subunit of PI3K (p110α) in acquisition of anoikis resistance, we performed the silencing of the PIK3CA gene in anoikis-resistant endothelial cells (Adh1-EC). Silenced cells (miR-PIK3CA-Adh1-EC) were studied comparatively with parental endothelial cells (EC), EC resistant to anoikis (Adh1-EC) and EC transfected with the EJ-ras oncogene (EJ-ras EC) in relation to: gene and protein expression of PI3K, apoptosis, cell proliferation and ability to form colonies. After PIK3CA gene silencing, endothelial cells showed a decrease in the gene and protein expression of PI3K. This was accompanied by an increase in the apoptosis rate and a decrease in the cell proliferation. In addition, miR-PIK3CA-Adh1-EC cells displayed low capacity of colony formation in relation to anoikis-resistant EC. These results may help to understand the involvement of this gene in resistance to anoikis and tumor metastasis, contributing to the development of new compounds for treatment of cancer patients, using this macromolecule as a therapeutic target. Supported by FAPESP, CAPES and CNPq. Keywords: Endothelial cells; Anoikis resistance; PIK3CA gene.

P2160

Board Number: B303

Effect of MTA3 overexpression on B16 melanoma cells.
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Metastasis associated (MTA) genes function as corepressors or coactivators of a wide variety of cancer related factors. MTA3 expression influences metastatic progression of various cancer types. The effects of MTA3 expression on various metastatic properties are variable among different cancer types. The function of MTA3 in melanoma is unknown. MTA3 gene transfection via pcDNA3 expression plasmid was performed to study migration, proliferation, and invasion functions in the mouse melanoma cell lines B16F0 (low metastasis) and B16F10 (high metastasis). MTA3 overexpression lacks significant influence on melanoma cell migration and proliferation. MTA3 overexpression, however, decreased invasion through Matrigel in vitro for both B16F0 and B16F10 melanoma. MTA3 may have an important role in melanoma’s invasion processes and metastasis.
P2161
Board Number: B304

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Breast cancer is the leading cause of death among women around the world. Several epidemiological studies have established an association between ingestion of ω-6 fatty acids and breast cancer risk. Among these fatty acids, linoleic acid (LA) has been shown to induce a similar epithelial-mesenchymal transition (EMT) process in mammary non-tumorigenic epithelial cells MCF10A and migration and invasion in mammary tumorigenic cells MDA-MB-231. On the other hand, lactoferrins (Lf) has been highlighted as an alternative for the treatment of this disease. Lf is a multifunctional protein present in secretions, mainly in milk, and anticancer properties are attributed to it. However, there are reports indicating that the exposure to human Lf promotes a more aggressive phenotype in breast cancer cells.

The aim of our study is to establish the effect of human and bovine Lf on breast cancer cells MDA-MB-231 and MCF-7. We evaluated cell migration through wound closure assays, expression of proteins related to mesenchymal epithelial transition by Western blot and formation of focal adhesions by confocal microscopy. Our results demonstrate that bovine Lf decreases cellular migration induced by fetal bovine serum (FBS) and LA, and vimentin expression in MDA-MB-231 cells, as well as the stabilization of focal adhesions induced by FBS. In addition, human Lf enhances LA-induced cell migration, induces an increase in vimentin expression, and formation of focal adhesions in MDA-MB-231 cells. We also found that, human and bovine Lf behave in the same way in MCF-7 cells decreasing the expression of vimentin. Our results suggest that human and bovine lactoferrins modulate differentially cell migration and epithelial-mesenchymal transition process in breast cancer cells MDA-MB-231 and MCF-7. This research was partly funded by CONACYT (255429). N R-O and J R-R are recipients of Predoctoral training grants of CONACYT.

P2162
Board Number: B305
Extracellular vesicles from MDA-MB-231 cell stimulated with linoleic acid promote migration and invasion through Src/FAK and PI3K/Akt signaling pathways.

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Extracellular vesicles (EVs) are small membrane-enclosed sacs of endosomal and plasma membrane origin, which are secreted by normal and malignant cells. They are an important mode of intercellular communication by serving as vehicles for transfer of membrane and cytosolic proteins, lipids and RNA between the cells. Particularly, EVs from tumor cells mediate many stages of tumor progression including angiogenesis, escape from immune surveillance, invasion and metastasis. The secretion of EVs is mediated for a variety of stimulus, such as epinephrine, adenosine diphosphate, collagen, calcium ionophore and cytokines. The aim of our work was to determine whether EVs from MDA-MB-231 cells stimulated with linoleic acid were able to induce migration and invasion. Furthermore, we analyzed the role of Src/FAK and PI3K/Akt on these cell processes. EVs were obtained by differential centrifugation.
Activation of Src, FAK and Akt was analyzed by Western blot using phosphospecific antibodies (Abs). The migration was studied by scratch-wound assay and invasion was evaluated by the modified Boyden chamber method. Our results demonstrated that stimulation of MDA-MB-231 breast cancer cells with EVs promoted an increase in the phosphorylation of Src, FAK and Akt2. Treatment with the selective Src family kinase inhibitor pyrazolopyrimidine 2 (PP-2) markedly reduced the phosphorylation of both tyrosine (Tyr)-397 and Tyr-577 induced by EVs. In contrast, PP-2 did not prevent Akt Ser (serine)-474 phosphorylation stimulated by EVs. It demonstrated that activation of Akt is through a Src-independent pathway. In addition, EVs also induced the assembly of focal contacts and MMP-9 secretion, migration and invasion of MDA-MB-231 cells. In conclusion, our findings demonstrate that EVs from MDA-MB-231 cells stimulated with LA induce migration and invasion of MDA-MB-231 cells through Src/FAK and PI3K/Akt signaling pathways. This study was funded by CONACYT (Basic Science 255429 and FOSISS 261637).

P2163
Board Number: B306
Role of PI3K/Akt2 on migration induced by extracellular vesicles from MDA-MB-231 breast cancer cells stimulated with linoleic acid in MCF10A cells.
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Breast cancer is the most common cancer and the main cause of cancer death in women worldwide. In Mexico, it is the first cause of death by malignant neoplasms since 2006. Several studies suggest an association between a diet rich in fatty acids and risk of breast cancer. Linoleic acid (LA) is an omega-6 polyunsaturated fatty acid and the major PUFA in most diets. Recent studies demonstrate that LA induces an epithelial-mesenchymal transition-like (EMT) process in MCF10A mammary non-tumorigenic epithelial cells, as well as migration and invasion in MDA-MB-231 breast cancer cells. Extracellular vesicles (EVs) are membrane-limited vesicles secreted by normal and malignant cell. Particularly, EVs from MDA-MB-231 cells stimulated with LA promote migration of MCF10A cells. However the signal transduction pathways mediated by these EVs in MCF10A cells remain to be studied. Here, we demonstrate that EVs from MDA-MB-231 cells stimulated with LA promote migration and activation of Akt2 in MCF10A cells. Moreover, our findings show that these EVs promote migration through a PI3K/Akt-dependent pathway, and they also promote activation of FAK and Erk1/2. In summary, our findings demonstrate, for the first time, that EVs from MDA-MB-231 cells stimulated with LA induce migration through PI3K/Akt-dependent pathway in MCF10A cells. This study was funded by CONACYT (Basic Science 255429 and FOSISS 261637).

Cancer Therapy: Novel Techniques and Therapeutics

P2164
Board Number: B307
Fast constitutive turnover of the potential ADC target Prolactin Receptor (PRLR) is mediated by a 21-amino acid region in its cytoplasmic domain.
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Antibody-Drug Conjugates (ADCs), generated by linking a cytotoxic agent (such as a tubulin inhibitor, DM1) to a monoclonal antibody (mAb), represent a promising new class of cancer therapeutics. ADCs
exploit cancer-specific expression of cell surface proteins to enable selective delivery of cytotoxins to tumor cells. However, other properties that characterize good ADC targets have not been investigated in detail. The rate of target internalization and the route of intracellular trafficking are likely to be of particular importance. Previously, we compared two targets that are selectively over-expressed in breast cancer: the EGFR family member HER2 and the class I cytokine receptor family member prolactin receptor (PRLR). We have previously shown that PRLR ADC can effectively kill tumor cells that express fairly low levels of PRLR, whereas HER2 ADC conjugated with the same toxin is only effective in cells expressing very high levels of HER2. We discovered that rapid constitutive turnover of PRLR was the mechanism underlying the efficient target cell killing by a PRLR ADC. Hence, PRLR, but not HER2, contains sequence motifs that constantly direct it to lysosomes for degradation (Andreev, J et al. PMID 28108597). We have found that chimeric receptor containing HER2 extracellular and PRLR cytoplasmic and transmembrane domains (termed PRLRcytoTM) contains sequence(s) sufficient and necessary for rapid internalization of the receptor and for efficient cell cycle arrest by ADC. PRLR truncation mutants, unveiled a minimal 21-amino acid region (termed Cyto21-42) in PRLRcytoTM required for constitutive internalization and degradation of the receptor. Although cyto21-42 contains two candidate internalization signals (DAH^{283}LL and ^{292}LL), mutation of most residues within Cyto21-42 resulted in decreased constitutive turnover of the receptor, suggesting that the entire intact Cyto21-42 sequence is needed for the process. Interestingly, PRLR Cyto21-42 sequence is largely conserved in a closely related class I cytokine receptor – growth hormone receptor (GHR), which also undergoes constitutive lysosome-dependent internalization and degradation. CRISPR-based gene deletion experiments suggested that clathrin might not be required for PRLR internalization, and WB analysis showed lack of caveolin expression in T47D cells, suggesting the involvement of an unidentified clathrin- and caveolin-independent endocytic route. Taken together our data show that a 21-amino acid region in the PRLR cytoplasmic domain contains essential information for constitutive ligand-independent turnover of the receptor. This work is the first step towards defining the molecular architecture coupling potential ADC targets to the machinery of constitutive endocytosis and lysosomal degradation.

P2165
Board Number: B308
Characterization of a novel anti-cancer compound that targets ch-TOG/CKAP5.
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Our goal is to develop an anti-cancer compound that selectively kills cancer cells by targeting a selectable biomarker. Many of the cancer drugs that are currently in use are not selective for cancer cells over healthy cells, causing severe side-effects. Furthermore, patients often develop resistance to these drugs, leaving them with fewer alternatives. Thus, it is imperative to increase the repertoire of anti-cancer drugs targeting differentially expressed proteins, which confer a growth advantage for cancer cells. Through collaboration with a medicinal chemist, our lab is characterizing a family of small molecules that share a common scaffold and have several functional groups amenable to modification. The derivatives tested so far show a range in activity, from inert to highly active (IC_{50} < 100 nM), suggesting that the functional groups confer this change vs. the scaffold itself. One of the active compounds, C75, causes mitotic arrest in cancer cells (e.g. HCT116, A549, BTS49, HeLa) at ~2-3-fold lower concentrations in comparison to non-cancer cells (MCF10A, HFF1). Cancer cells treated with 100-300 nM of C75 arrest in pro/metaphase with disorganized or fragmented spindles. HCT116 cells, from colon colorectal adenocarcinoma, were particularly sensitive to C75. ch-TOG/CKAP5, a microtubule polymerase, is highly over-expressed in these cells. ch-TOG counteracts the function of MCAK, a microtubule depolymerase, to regulate spindle length during mitosis. Indeed, C75 phenocopies ch-TOG
knockdown, and similar to ch-TOG knockdown, the spindle phenotypes caused by C75 can be suppressed by MCAK RNAi. We also observed changes in endogenous ch-TOG localization in the presence of C75 using ch-TOG antibodies. Despite causing spindle phenotypes, ch-TOG becomes more highly enriched on the centrosomes and microtubules after C75 treatment. This suggests that C75 could bind to at least one of the TOG domains required for binding to free tubulin dimers, causing an increase in ch-TOG affinity to the polymerized microtubules. We are currently verifying that ch-TOG is the molecular target of C75, and determining if other cancer cells that are more sensitive to C75 also have high levels of ch-TOG, making it a suitable biomarker for a subset of highly progressive cancers.

P2166
Board Number: B309
Hyperthermia upregulates SLC22A16 expression and downregulates ABCG2 expression via ROS production and enhances the cytotoxicity of doxorubicin.
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Hyperthermia (HT) is a non-invasive cancer therapy. Treatment temperature between 41 to 44°C has no cytotoxic damage in normal cells, while the temperature does in cancer cells because of the underdeveloped vascular system. HT often used with other cancer therapy such as radiation-therapy and chemotherapy. However mechanisms of synergistic effects among these therapies remains unclear. The 42°C environment is a cellular mild heat stress generating O2- from mitochondrial electron transport chain. We have previously reported that the expression of ATP-binding cassette sub-family G member 2 (ABCG2), which is known as breast cancer resistant protein (BCRP), was suppressed by increasing mitochondrial ROS to induce cancer specific porphyrin accumulations. Since ABCG2 is a transporter of doxorubicin (DOX), we hypothesized that synergistic effect of HT and chemotherapy may be induced by down-regulation of ABCG2 expression via intracellular ROS increase. In this study, we elucidated whether HT with intracellular ROS increase by HT can enhance the cytotoxic effect of DOX for breast cancer cells. The murine breast cancer cell line, 4T1E was incubated at 37 or 42°C for 1h. Intracellular ROS generation was detected by electron spin resonance (ESR). Cytotoxicity of DOX was measured using the Cell Counting Kit 8. ABCG2 expression was analyzed by Western blotting. ESR signal peak with HT treatment became high as compared to without HT treatment, indicating intracellular ROS level was increased by HT treatment. Cell viability and ABCG2 expression were decreased by DOX exposure and by HT treatment. The enhancement of HT treatment effect by DOX is considered to be result of down-regulation of ABCG2 expression by ROS. When cells were exposed to DOX with 5-aminolevulinic acid (ALA), more severe cellular injuries were involved. Since it is known that porphyrin is introduced by ALA and is transported by ABCG2, we speculate that ALA worked as a competitive inhibitor of DOX excretion transporter to enhance cell death. Significant increase in cellular damage by HT treatment was shown by adding ALA, but not without ALA. These results suggest that cellular damage of HT treatment is due to ROS production induced by ALA. HT treatment involved intracellular ROS production and down-regulated the expression of ABCG2 protein. HT treatment also enhanced the cytotoxicity by DOX. Cell death by DOX was enhanced by combination with HT and ALA treatment, possibly via intracellular ROS generation, and was suppressed by additional antioxidants.
**P2167**

**Board Number: B310**

A novel anti-cancer drug disrupts or regresses a variety of multi-cellular tumor spheroids.

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We have been developing an anti-cancer compound that selectively kills cancer cells. Many anti-cancer drugs that are currently in use are not selective, causing harmful side-effects to the patient. Moreover, patients can develop resistance to these drugs. Thus, there is a need to expand the repertoire of anti-cancer drugs, especially those that are selective. Through collaboration with a medicinal chemist, we found several analogues of a family of small molecules that share a common scaffold, which kill cancer cells in the nanomolar range. The active derivatives cause mitotic arrest of cancer cells at concentrations ~2-3 fold lower in comparison to non-cancer cells, by causing cells to arrest in pro/metaphase with disorganized or fragmented mitotic spindles. To explore the effects of these compounds on cancer cells within a tumor microenvironment, we grew multi-cellular tumor spheroids (MCTS) from various cancer cell lines, and treated them with varying concentrations of C75, one of the more active derivatives. We grew MCTS’s from HeLa (cervix adenocarcinoma), A549 (lung carcinoma) and HCT116 (colon colorectal adenocarcinoma) cells. HeLa MCTS’s were completely disrupted within two days of a single treatment with 500 nM C75 in comparison to 1 uM C87, an inert derivative. A549 and HCT116 MCTS’s showed regression in growth in response to a single treatment with 500 nM or 1 uM of C75 in comparison to 1 uM C87. Stains for live and dead cells using fluorescein diacetate (FDA) and propidium iodide (PI), respectively, showed that as the tumors decreased in size, dead cells were shed from their periphery. Further, encapsulating C75 in polymeric nanoparticles caused MCTS disruption or regression in growth at lower concentrations in comparison free drug. Therefore, C75 appears to be effective at disrupting MCTS’s derived from multiple cancer cell types, and we are in the process of moving forward with in vivo studies.

**P2168**

**Board Number: B311**

Peptide antagonists of AGR2 inhibit cancer cell migration.

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The Anterior Gradient protein 2 (AGR2) encodes an endoplasmic reticulum disulfide isomerase that functions as a molecular chaperone. AGR2 is mainly expressed in epithelial cells and has been described as a cancer-associated protein, predominantly in adenocarcinomas. Increased levels of secreted AGR2 (sAGR2) has been correlated with poor prognosis in cancer patients, making it a potential biomarker. Additionally, sAGR2 may be a therapeutic target because neutralizing AGR2 antibodies have shown preclinical effectiveness in murine cancer models. As a result, sAGR2 is becoming recognized as a new theranostic target in cancer biology. We identified a novel peptide that binds sAGR2 by mRNA display. This method enables selection of peptides from a complex library (>109 unique members) and incorporates a protease incubation step to select for serum stable peptides. We performed six
successive rounds of enrichment using a 10-amino acid mRNA display library and identified several AGR2 binding peptides. One of these peptides (H10), demonstrated high affinity binding to AGR2 with an affinity binding constant (KD) of 6.4 nM measure on surface plasmon resonance analysis (SPR). We also investigated the therapeutic utility of H10 peptide and discovered that it inhibits the cancer cell viability at IC50 (16-20 µg/ml) in two cancer cell lines. Furthermore, we determined that 10 µg/ml of H10 peptide is sufficient to inhibit cancer cell migration in breast and prostate cancer cell lines. A control peptide does not show any appreciable activity in these cells.

**P2169**

**Board Number: B312**

Stressed out: DNA damage delivered at ultrahigh dose rates reduces cellular stress and apoptosis.

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The cellular response to DNA damage is critical for the maintenance of genome integrity. In response to DNA damage, eukaryotic cells rapidly activate a complex network of signaling pathways that localize the DNA lesion, sense the specific type of damage, arrest the cell cycle, and recruit DNA repair machinery. This DNA damage response (DDR) functions as a major barrier to tumorogenesis. Mutational or epigenetic inactivation of the DDR is a critical event that must occur during the early stages of tumor development in order for emerging cancers to bypass oncogene-induced senescence. Elegant work by the Bartek lab and others has demonstrated recruitment of the DNA damage machinery within seconds of microirradiation. Interestingly, most experiments using ionizing radiation occurs on the timescale of minutes. We are interested in determining if irradiating cells on a timescale faster than the activation of the DNA damage response could impact cell decision processes as compared to traditional timescales, in which cells accumulate damage in the setting DNA damage pathway activation. To that end, we modified a clinical linear accelerator to deliver up to 300Gy/s. Using U2OS cells, a p53 and RB WT cell line with intact G2 checkpoint, we did not observe any differences in recruitment of gamma-h2AX, activation and maintenance of the G2 and intra-S phase checkpoints in cells treated with ultrahigh dose rate and conventional radiation. We did, however, note a reduction in p53 stabilization and apoptosis in ultra-high dose treated cells, indicating that the treatment of cells with ultrahigh dose rate radiation improves cell survival, likely due to an overall reduction in cellular stress leading to a more cell survival.

**P2170**

**Board Number: B313**

9-Aminoaacididine inhibits ribosome biogenesis and synergizes with cytotoxic drugs to induce selective killing of p53-deficient cells.

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Common cancer treatments target rapidly dividing cells and do not discriminate between cancer and normal host cells. One approach to mitigating negative side-effects of cancer treatment is to temporarily arrest cell cycle progression and thus protect normal cells during cytotoxic treatments, a concept called cyclotherapy. We recently proposed that transient inhibition of post-transcriptional steps of ribosome biogenesis (RBG) can be used to selectively arrest p53-positive host cells and not p53-null cancer cells. In this study, we investigated whether cytoprotective RBG inhibition can be achieved through small
molecule treatment. We report that treatment of cells with 9-aminoacridine (9-AA) inhibits pre-rRNA processing as well as RNA Polymerase I (Pol I) transcription, in a dose-dependent manner. We propose a mechanism for 9-AA inhibition of RBG by disruption of binding between pre-ribosomes and small nucleolar RNAs (snoRNAs) required for rRNA maturation. Our data also indicate that the mechanism of Pol I inhibition by 9-AA is distinct from that of actinomycin D (Act D), an established Pol I inhibitor. We demonstrate in a model 3T3 cell system that low doses of 9-AA that reversibly inhibit RBG can be protective for p53-positive cells and used in synergy with anti-cancer agents camptothecin and methotrexate to selectively kill p53-negative cells. The ability of 9-AA to cause RBG inhibition, as well as the decoupling of the inhibition of Pol I transcription and pre-rRNA processing, positions acridines as a new tool for RBG and cyclotherapy research.

**P2171**

**Board Number: B314**


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Functional telomeres play an important role in genome stability. Maintenance of telomeres in cancer cells may differ from that in normal cells because of the specific expression of telomerase in cancer cells. Here we examine the role of heterogeneous nuclear ribonucleoproteins (hnRNP) A1 and A2 in telomere maintenance. Simultaneous suppression of both hnRNP A1 and A2, but not single suppression of hnRNP A1 or A2, produced dysfunctional telomeres and induced DNA damage responses in cancer cells, but not in normal immortal fibroblasts. Ectopic expression of human telomerase reverse transcriptase (hTERT) in normal fibroblasts (hTERT-immortalized normal fibroblasts) rendered the cells sensitive to the production of dysfunctional telomeres and inhibition of cell proliferation through depletion of hnRNP A1/A2. Our work demonstrates that telomerase-positive cancer cells employ factors for maintaining telomeres that differ from those used in telomerase-negative normal cells. We suggest that a strategy designed to inhibit telomerase-dependent maintenance of telomeres could be an attractive novel approach for telomerase-based cancer therapy.

**P2172**

**Board Number: B315**

HER2-targeting ADC containing cleavable linker for targeted therapy of HER2-positive gastric cancer.

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Human epidermal growth factor receptor 2 (HER2) is amplified and overexpressed up to 20% in gastric cancers, which leads to poor clinical outcome. Antibody-drug conjugate (ADC) is composed of the tumor-targeting capacity of monoclonal antibodies with the antitumor activity of cytotoxic agents. T-DM1 (trastuzumab-emtansine, drug antibody ratio (DAR) 3.5, non-cleavable) has been approved by the U.S. FDA as an ADC for HER2-positive metastatic breast cancer treatment and clinical trials in gastric cancer is currently underway. In spite of targeted therapeutic efficacy of T-DM1, patients that have
minimal or non-response to T-DM1 still remain and the main cause of resistance is not clear yet. In this study, we manufactured a new HER2-targeting ADC that can be adjust to accurate DAR (DAR2 or 4) by using cleavable linker. Cleavable linkers effectively release the drug by catalyzing in cancer cell, which induce bystander killing effect. To evaluate antitumor efficacy and mechanisms of action (MOA) in vitro, we performed cytotoxicity assay and cell cycle analysis in N87, HER2-positive gastric cancer cell line. As the results, the HER2-targeting ADC showed equivalent cytotoxic ability to T-DM1 and the ratio of G2/M arrest were 17.3, 30.6, 52.9 and 59.3% in treatment with 0, 0.01, 0.05 and 0.1 ug/ml of HER2-targeting ADC, respectively. Alpha-tubulin structure was investigated by immunofluorescence staining to confirm MOA of the drug and we observed irregular tubulin structure in vitro and in vivo. In vivo anti-cancer efficacy test, HER2-targeting ADC showed noticeable tumor growth inhibition, compared to trastuzumab and T-DM1. At day 56 of N87 transplantation, the treated/control tumor size (T/C) percentage values were 59.1 and 6.8 % in T-DM1 and HER2-targeting ADC (MMAF-DAR4) treated mice, respectively. In addition, HER2-targeting ADC inhibited in vivo tumor growth on patient-derived xenograft model of HER2-positive gastric cancer. Collectively, our results demonstrate that HER2-targeting ADC using cleavable linker exhibits a good MOA and effective anticancer efficacies in both of in vitro and in vivo. This study will contribute to develop a promising ADC for overcoming resistance to HER2 targeted therapy in HER2-positive gastric cancer.

P2173
Board Number: B316
The Anti-melanoma Effects of Heat Shock Protein Inhibitors.
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Effective BRAF inhibitor treatment for malignant melanoma loses its strength because of drug resistance. The increasing expression level of heat shock proteins (HSPs) induced by physiological stresses have been found to be associated with tumor initiation, growth and drug resistance. Hsp90 inhibitors CAY10607, Hsp70 inhibitor VER155008 and FDA-approved Vemurafenib were applied separately or in combination with each other to determine the cytotoxic effect on both Vemurafenib sensitive A375 and resistant A375VR melanoma cell lines. In cell viability assay, Hsp inhibitors displayed potent cytotoxicity against the human malignant melanoma cells and achieved a better therapeutic effect when combined with PLX4032. In wound healing assay, Hsp90 inhibitor CAY10607 significantly inhibited cell migration in the resistant cell line A375VR, and Hsp70 inhibitor VER155008 showed a synergistic effect with PLX4032 in inhibiting cell migration in the sensitive cell line A375. In apoptotic assay, Hsp70 inhibitor VER155008 promoted cell apoptosis significantly in both Vemurafenib sensitive and resistant melanoma cells, and the combination of Hsp70 inhibitor and Hsp90 inhibitor synergistically induced apoptosis in the melanoma cells. Furthermore, Hsp90 inhibitor CAY10607 suppressed the activation of Akt and Erk in both Vemurafenib sensitive melanoma cell line A375 and resistant cell line A375VR, while Hsp70 inhibitor VER155008 only inhibited the activation of Akt and Erk in the sensitive cell line A375. Therefore, Hsp inhibitors can serve as novel anti-cancer drugs by inhibiting cell migration, inducing cell apoptosis, and inactivating the PI3K/Akt and Ras-Raf-Mek-Erk pathways.
P2174
Board Number: B317
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Antibody–drug conjugates (ADCs) are attractive in cancer therapy because they can directly bind to cancer cells and provide anticancer activity. To kill cancer cells, the target antigens are required to be highly and/or selectively expressed on cancer cells and to be internalized by the cells. HER2, a member of the epidermal growth factor receptor (HER/EGFR/ERBB) family, is well known as an amplified antigen in invasive breast cancer. Trastuzumab emtansine (T-DM1) targeting Her2 is a successful ADC. However, because trastuzumab is used as the targeting antibody, this application is restricted to HER2-positive breast cancer patients. Therefore, novel antigens and targeting antibodies are required for the development of new ADCs. In this study we focused on CD239 that is a specific receptor for laminin α5, a subunit of laminin-511 that is a major component of basement membranes in various tissues. CD239, also known as the Lutheran blood group glycoprotein (Lu) or basal cell adhesion molecule (B-CAM), is an Ig superfamily transmembrane protein. The expression of CD239 is up-regulated in several carcinoma, suggesting CD239 is associated with tumor progression. Immunohistochemical study showed that CD239 was strongly expressed in a subset of human breast cancer tissues and cells. Recently we produced a human single chain variable fragment (scFv) specific to CD239 fused with human IgG1 Fc, called C7-Fc. Although C7-Fc antibody alone does not influence cellular functions, the antibody conjugated with a fragment of diphtheria toxin lacking the receptor binding domain (DT) can selectively kill breast cancer cells. Interestingly, DT-bound C7-Fc shows anticancer activity in CD239-highly positive SKBR3 cells but not in weakly positive cells. Furthermore DT-bound C7-Fc promoted regression of CD239-highly positive cell xenografts in nude mice. Our results show that CD239 is a promising antigen for ADC-based breast cancer therapy.

P2175
Board Number: B318
Dysregulation of human mitochondrial ClpP protease activity by acyldepsipeptides leads to apoptotic cell death.
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Acyldepsipeptides (ADEPs) are potential novel antibiotics. They have been shown to dysregulate the activity of the highly conserved tetradecameric bacterial ClpP protease leading to cell death. Here, we identified several ADEP analogs that are potent dysregulators of the human mitochondrial ClpP (HsClpP). These ADEPs interact tightly with HsClpP, causing the protease to unspecifically degrade model substrates. The ADEP-HsClpP co-crystal structure was solved for one of the compound analogs revealing a highly complementary binding interface at the pockets formed by two HsClpP neighbouring subunits with the HsClpP in the compact conformation. Dysregulation of HsClpP activity by these ADEPs was

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found to have cytotoxic effects in multiple human cell lines as a result of the activation of the intrinsic, caspase-dependent apoptosis. Given that HsClpP is highly expressed in multiple types of cancers and has important roles in cell metastasis, our findings suggest a novel therapeutic potential of ADEPs in cancer treatment.

P2176

**Board Number: B319**

**Biomarker studies for identification of USP7i sensitive cancer cell lines.**

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Ubiquitination is a regulatory post-translational protein modification that plays important roles in most, if not all, cellular pathways. The conjugation of ubiquitin is a reversible process and can be antagonized by deubiquitinating enzymes (DUBs), also known as isopeptidases. Given its critical role, malfunctions of the ubiquitin pathway are normally associated with human diseases, such as cancers and neurodegenerative diseases. Having a role in multiple cancer pathways, ubiquitin specific peptidase 7 (USP7) is the most well characterized DUB and an attractive therapeutic target for oncology treatment. Progenra has developed a series of selective, covalent, irreversible inhibitors of USP7 that have been widely used to study the biological roles of USP7. These USP7 compounds have been shown to induce degradation of many USP7 substrates in cells as well as inhibit tumor growth in vivo in various models including multiple myeloma, neuroblastoma, lung cancer and colon cancer. However, it remains unclear which tumor types are most sensitive to and what patient population are most likely to benefit from USP7i-treatment. In order to identify potential biomarkers for tumor and patient selection, we have used a variety of cellular assays to investigate the efficacy of our USP7 compounds in tumors with various USP7 expression levels as well as either wild type or mutant versions of p53, and the results will be discussed.

P2177

**Board Number: B320**

**Toxicological Effects of NCKU-21, a Phenanthrene Derivative, on Cell Growth and Migration of A549 and CL1-5 Human Lung Adenocarcinoma Cells.**

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Background: Chemotherapy insensitivity continues to pose significant challenges for the treatment of non-small cell lung cancer (NSCLC). The purposes of this study were to investigate whether NCKU-21 (3, 6-Dimethoxy-1, 4, 5, 8-phenanthrenetetraone) has potential activity to induce effectively toxicological effect in different ethnic NSCLC cell lines, A549 and CL1-5 cells, as well as to examine its anticancer mechanisms. Methods: Cell viability and cell-cycle distribution were analyzed using MTT assay and flow cytometry in NCKU-21-treated cells. NCKU-21-induced cell apoptosis were verified by Annexin V-FITC/propidium iodide (PI) double staining and measurement of caspase 3 activity. Western blot and wound healing assay were applied to evaluate the regulations of NCKU-21 on signaling pathways and cell migration, respectively. The molecular interaction between target protein and NCKU-21 was predicted and performed by molecular docking. Results: The results indicated that NCKU-21 markedly induced cytotoxic and cytostatic effects to reduce cell viability via cell apoptosis and cell-cycle arrest in tested NSCLC cells. Activation of AMP-activated protein kinase (AMPK) and protein expression of p53.
were also increased in both NSCLC cells stimulated with NCKU-21. However, PI3K-AKT activation repressed by NCKU-21 was found in CL1-5 cells but not in A549 cells. In addition, increases of phosphatidylserine externalization and caspase 3 activity also confirmed apoptotic effect of NCKU-21 in both NSCLC cells. Moreover, cell migration and translational levels of gelatinases, matrix metallopeptidase-2 (MMP-2) and MMP-9, were obviously reduced in both NSCLC cells after NCKU-21 incubation. Experimental data obtained from molecular docking suggested that NCKU-21 can bind to the catalytic pocket of MMP-9 and then has potential to suppress MMP-9 activity. Conclusion: Our results suggest that NCKU-21 can effectively reduce cell growth and migration as well as induce apoptosis in A549 and CL1-5 cells, whose toxicological effects may be partly modulated through PI3K-AKT inhibition, AMPK activation, p53 up-regulation, and gelatinase inhibition.

P2178

Board Number: B321

_In Vitro_ Cytotoxicity Study of Mitochondria Targeted IR780-based NanoGUMBOS in Breast Cancer Cells.

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Heptamethine dye (HP) IR780, has been widely investigated as a mitochondrial toxin for cancer research due to its chemotherapeutic properties, as well as its near-infrared fluorescence allowing for deep tissue penetration. However, the non-selective uptake of this compound leads to toxicity in normal cells as well, which limits its further biological applications. The study presented here focuses on the use of GUMBOS, a group of uniform materials based on organic salts, for selective toxicity as observed previously with Rhodamine 6G-based nanomaterials (nanoGUMBOS) (Magut et al.). NanoGUMBOS display enhanced and uniform properties at the nanoscale level similar to typical nanodrugs; however, they can serve as the drug themselves rather than the drug carrier giving them several distinct advantages. Herein, a simple counter-ion variation strategy has been proposed to design different IR780-based nanoGUMBOS towards enhanced selective antineoplastic activity. These nanomaterials were synthesized by directly nanoengineering hydrophobic IR780-based nanoGUMBOS using a reprecipitation method, without addition of other materials, which allowed the use as a carrier-free nanodrug. Our in-vitro studies indicate an enhanced selective toxicity of all these nanoGUMBOS towards breast cancer cells as a result of improved cellular uptake as compared to the parent IR780 dye. The mechanism of toxicity for the nanoGUMBOS was also examined by study of their sub-cellular localization as well as using a mitochondrial toxicity assay. Analysis of data from these studies revealed that all nanoGUMBOS primarily accumulate in the mitochondria of cancer cell and dysfunction mitochondria to induce cell apoptosis. In order to elucidate other factors that contribute to the observed enhanced toxicity, we further studied the internalization pathways of all nanoGUMBOS. The anions were observed to play a role in regulating the internalization process of nanoGUMBOS by enhancing the interactions between nanoparticles and their transportation receptors on cancer cells, which can eventually affect the final therapeutic outcome of these nanoGUMBOS. From these studies, we demonstrate tunable chemotherapeutic properties of IR780-based nanoGUMBOS through simple variation of counter ions of GUMBOS, which may provide a promising strategy for future design of better nanomedicine for cancer treatment.
P2179

Board Number: B322

Selective Killing of Cancer Cells by Mixed-Charge Gold Nanoparticles Targeting Endo-Lysosomal System.

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Cancer remains a leading cause of death worldwide because most of the cancers acquire genetic mutations that protect them from therapies designed to trigger classical caspase-dependent apoptosis. Meanwhile, there has been increasing interest in designing novel therapies that would target alternate cell death pathways. Here, we show that gold nanoparticles (NPs) functionalized with a mixture of positively [+] and pH-sensitive, negatively [-] charged ligands, so-called mixed charge [±/±] NPs, selectively kill cancer cells while being non-toxic to non-cancerous cells. Cellular uptake of [±/±] NPs is dependent on net surface charge with NPs of corresponding [±/±] charge ratios uptaken equally well in all cell types. Combination of confocal reflection and fluorescence microscopy imaging reveals that the main mechanism of uptake is through endocytosis -- via Rab5-labeled compartments into Lamp1-labeled lysosomes — and is similar in cancer and non-cancerous cells. In contrast, [±/±] NPs, but not purely cationic NPs, selectively compromise the integrity of the membranes of cancer lysosomes. The optimal destabilization of cancer lysosomes is determined by the fraction of [+] and [-] charged ligands attached to the NPs. The selective destruction of cancer lysosomes is attributed to pH-dependent aggregation -- MC NPs are stable at neutral pH, but aggregate rapidly at pH ~ 5.5 -- inside endolysosomal compartments leading to increased osmotic pressure, swelling of the lysosomes and leakage of their contents into cytosol triggering apoptosis-like cancer cell death. Taken together, these results show that tuning of the surface charge balance by functionalization of NPs with a combination of, [±], and pH-sensitive [−], charged ligands could be a useful design strategy for “smart” pH-sensing anticancer therapeutics that selectively destabilize cancer lysosomes.

P2180

Board Number: B323

Nanoparticle-Neural Stem Cells for Targeted Ovarian Cancer Treatment.

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One of the drugs used to treat ovarian cancer is cisplatin. However, cisplatin kills healthy tissue in addition to cancer cells. It has been shown that neural stem cells (NSCs) accumulate near ovarian tumors and are used for efficient tumor targeting. If free cisplatin is loaded into NSCs, the NSCs will die. To prevent cytotoxicity, silica nanoparticles (SiNPs 100nm) are used as a protective carrier. The SiNPs encapsulate cisplatin and are then loaded into NSCs. The big picture is to maximize efficiency of tumor targeting using NSCs and minimize toxicity to these NSCs using SiNPs. When tested in vivo, the SiNPs leaked cisplatin before reaching the tumor. The objective of this project is to optimize the stability of SiNPs without cisplatin in efforts of achieving efficient drug loading and delivery. To do this, the concentration of tetraethyl orthosilicate (TEOS), one of the main components of SiNPs, was varied. We hypothesized that the more TEOS added, the more stable the particles will be. The reasoning is that
more TEOS means more carbon in the chemical structure of SiNPs, and thus an intact SiNP results in a stable particle. Using the microemulsion method, six batches of SiNPs were synthesized: 200, 400, 800, 1000, 1400, and 1800 µL TEOS. Then, the SiNPs were placed in cell media and phosphate buffered saline (PBS) for an evaluation of their stability. This step is required because the NSCs grow in cell media and the SiNPs will be in PBS when injected into mice. In order for the particles to effectively carry the drug without leakage, they must be stable in both cell media and PBS. Lastly, the SiNPs were analyzed for their porosity using the transmission electron microscope (TEM). Our results align with our hypothesis: more TEOS equates to more stable SiNPs. In the TEM images, white spots were observed in the SiNPs for the 200-800 µL TEOS batches. The white spots are pores, which indicate instability. However, the 1000-1800 µL TEOS batches had no white spots. Based on the qualitative TEM results, SiNPs with a higher TEOS concentration are stable in cell media and PBS. Our next step was to determine if organic substances, other than TEOS, resulted in stable SiNPs. The organic substance phenyl trimethoxysilane (PTMS) was added to the 400 µL TEOS unstable SiNPs. The stability of these SiNPs was then checked in cell media and PBS. TEM imaging proved that the SiNPs do not form pores in either solution with the added PTMS. Thus, more organic substance made the unstable SiNPs stable. We concluded that the ultimate factor that determines the stability of our microemulsion-synthesized SiNPs in PBS and cell media is the concentration of organic substance. Based on these data, we will now load the stable SiNPs with cisplatin for studies in vivo.

P2181
Board Number: B324
Internalization and Uptake of Targeted Molecular Imaging Agents (TMISs) Through Clathrin-Mediated Endocytosis.
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Targeted molecular imaging agents (TMIs) that bind to biomarkers on cancer cells and illuminate the presence of cancer can be a powerful tool in the battle against cancer. Purpose: The goal of this research is to develop specific targeted molecular probes for detecting prostate cancer and understand their interaction with cells. Methods: The TMIs used are near-infrared fluorescent binding agents that will illuminate cancer cells for cancer detection. The Cy5.5-DCL-DSS-K-NH2 (B1) TMIA that has been developed has a fluorophore conjugated to a urea inhibitor moieties bridged by a linker group. The urea inhibitor will act as the ligand for Prostate Specific Membrane Antigen (PSMA) and bind to it. The cell line used in this research to evaluate the uptake of TMAs is C4-2, a PSMA-positive prostate cancer cell line. Results: It is believed molecules binding to PSMA on cell membranes are taken up by receptor-mediated endocytosis via clathrin-coated pits. Therefore, the uptake of the B1 TMIA is investigated in order to better understand the process of molecule internalization by PSMA. The internalization and uptake of B1 TMIA was evaluated by observing the co-localization of B1 and transferrin-receptor complex using confocal laser scanning microscopy (CLSM). Confocal images of the stained C4-2 cells (PSMA+) at various time-points showed the process of internalization and co-localization of B1 TMIA and fluorescein-tagged transferrin receptor, which indicate that B1 TMIA is being taken up by the cells through clathrin-mediated endocytosis. Antibody tagged with fluorescein to transferrin receptor was used as the control due to the fact that its endocytosis is well-understood and has become the model for studying clathrin-mediated endocytosis. Confocal images of the stained C4-2 cells at various time-points showed the process of internalization and co-localization of B1 TMIA and transferrin, which indicate that B1 TMIA is being taken up by the cells through clathrin-mediated endocytosis the same as

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Antibody-drug-conjugates (ADCs) induce cell killing by specifically delivering a toxin to the cytosol or nucleus of cancer cells. ADC efficacy requires both the ADC target to be expressed on the cell surface and to traffic efficiently to lysosomes. We have previously reported that HER2, the target of the clinically approved ADC ado-trastuzumab emtansine (T-DM1), does not traffic efficiently to lysosomes. In contrast, the prolactin receptor (PRLR), another potential ADC target in breast cancer, is rapidly and constitutively internalized into lysosomes, where it is degraded (Andreev et al., 2017). Accordingly, cell killing by HER2-ADC requires ~10^6 HER2 surface receptors/cell, while PRLR ADC cell killing requires only ~30,000 PRLR surface receptors/cell. Although the faster PRLR internalization dramatically compensates for lower surface expression, cells with less than 10,000 PRLR surface receptors are not effectively killed by PRLR-ADC. On the post-translational level, the number of surface PRLR is determined by the relative rates of receptor delivery to the cell surface and internalization. Identification of proteins that control delivery of PRLR to the cell surface or that regulate PRLR internalization might provide biomarkers that help predict the efficacy of PRLR-ADC in breast cancers. CRISPR-based genome editing can be used to specifically inactivate genes with fewer off-target effects than siRNA or shRNA approaches. Arrayed CRISPR screens allow studying the effect of gene inactivation on cell surface expression or internalization of a cargo protein, such as PRLR. Here we studied the role of 151 genes in the trafficking of fluorescent anti-PRLR antibody in the breast cancer cell line T47D. To this end we combined an arrayed CRISPR screen, high content microscopy and quantitative imaging of the amount of fluorescent antibody internalized into endocytic vesicles. The largest inhibition of anti-PRLR internalization observed in this screen was caused by knockout of the coatamer subunit alpha (COPA), a protein involved in trafficking between the endoplasmic reticulum and the Golgi complex. Validation experiments confirmed an inverse correlation between the degrees of COPA KO and anti-PRLR internalization. Surface biotinylation assays in COPA KO cells showed decreased PRLR surface expression without reduction in total PRLR expression, suggesting impaired biosynthetic delivery, rather than reduced internalization. This study suggests that COPA is necessary for efficient intracellular accumulation of anti-PRLR antibodies and establishes a platform to use arrayed CRISPR screens to study the trafficking machinery responsible for the internalization of other therapeutic antibodies.
P2183
Board Number: B326
Potential lung cancer therapy by the development of stigmasterol-solid lipid nanoparticles as drug delivery system.
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Lung cancer is the second most common cancer in the US and is by far the leading cause of cancer death. Development of new therapies for this type of cancer using nano-formulations has become one of the most important strategies to minimize the lack of drug inactivation after administration into patients. An alternative to increasing the effectiveness of the drugs is by the development of drug delivery systems using solid-lipid nanoparticles (SLN). Stigmasterol (Stg), an unsaturated phytosterol found in plant fats or oils, has shown potent anti-carcinogenic properties. Herein, we developed a Stigmasterol-SLN (≈200 nm) nano-formulation by the use of a microemulsion method. This size is compatible with tumor accumulation through the irregular vasculature observed in cancer tissue. Our system demonstrated strong cytotoxicity on lung cancer A549 cells with an IC50 of 11 µM after 24 h. The mechanism of action of the Stg-SNL has been confirmed to involve activation of caspase-3/7 and is necrosis independent. Stability of the Stg-SLN formulation in the liquid phase was investigated by incubation at 37ºC by for 3 days. More in vitro studies will be completed to confirm the selectivity of Stg-SLN. In conclusion, we have designed a system for phytosterols delivery from nanoparticles with low poly-dispersity which could accumulate in cancerous tissues.

P2184
Board Number: B327
A novel bicyclic binder of a prostate cancer marker Glutamate Carboxypeptidase II.
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Targeting to cancer cells can be achieved by small-molecular ligands of known cancer markers. The discovery of novel binders is possible through phage display of variable peptide libraries. We describe the discovery and assessment of a bicycle peptide binder of glutamate carboxypeptidase II, a membrane metalloprotease expressed in prostate cancer cells.

For this study, we used phage library displaying bicyclic peptides with differently sized loops developed by Christian Heinis. We performed phage display selection and analyzed the final phage pool using next generation sequencing. The resulting phage clone was isolated and prepared using the same cyclization reagent. The binding was assessed in biochemical assays, mostly using ELISA. Alternative cyclization reagents have been used to test the importance of the specific reagent for the binding of the target protein.

Since the next generation sequencing revealed that 74% of the resulting pool was composed by single candidate binder, it was relatively easy to isolate the phage clone. The preparations of the phage candidate with different linkers show on ELISA that binding is strictly dependent on the same cyclization that was used in the display itself. The inhibitory properties of the resulting peptide are being analyzed.
The study shows that in principle, bicycle phage display can be used to discover novel binders to cancer markers. The prepared bicyclic peptide could then be used for the functionalization of nanoparticles for therapeutic purposes.

P2185
Board Number: B328
A human full length preparation of ERCC1-XPF suitable to test compounds targeted to DNA repair mechanisms.
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Different human recombinant and native preparations of ERCC1-XPF have been obtained in order to assess kinetic parameters of the enzyme and the effect of compounds directed to target DNA repair mechanisms. Truncated recombinant forms of ERCC1-XPF can be obtained in high yield, however, some of these shorter sources of ERCC1-XPF have been found to contain low molecular weight contaminants which are needed for the nuclease activity. A similar scenario has been found for reconstituted native ERCC1-XPF systems where additional enzymes were required for DNA nicking. Here, we decided to express, purify and validate a full length, wild-type recombinant ERCC1-XPF protein from a bicistronic expression plasmid introduced in E. coli (Bowles B. et al Nuc. Acids Res. (2012) 40 (13): e101). The purpose was to obtain a source that could be used to test compounds directed against ERCC1-XPF. Methods: ERCC1-XPF purification involved three main steps: nickel agarose, heparin Sepharose and filtration on a Superdex column. ERCC1-XPF incision activity was measured with a 5’-6-FAM, 3’-dabcyl stem-loop oligonucleotide substrate. Results: a close-to-homogeneity preparation of ERCC1-XPF could be obtained following these procedures with a yield of 20-50 ug protein/L culture. The enzyme showed an specific activity of 2.76 nmol product/min x mg protein, which, although it is somehow lower than a previously reported activity of a similar preparation of the enzyme (8.2 nmol product/min x mg protein), it is still several orders of magnitude higher than the activity found in truncated forms of ERCC1-XPF. Enzyme activity could be inhibited with low concentrations of specific nuclease inhibitors. Conclusion: An active, full length ERCC1-XPF was obtained. Yield and ease of obtaining have to be considered before generalizing its use for high throughput screening.

P2186
Board Number: B329
Human radon exposure and a medium throughput system to study alpha particle irradiation in cellular assays.
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Radon represents the greatest single lifetime source of ionizing radiation (IR) exposure in humans. Radon emits alpha particle radiation a form of high linear energy transfer (LET) IR believed to cause significantly greater genomic instability than low LET IR such as x-rays and gamma-rays. The impact of alpha particle radiation on genomic instability is an understudied area, and by understanding the molecular and cellular consequences of this is likely to have a significant impact on human health. Here, I present data on human household radon exposure in the Canadian province of Alberta. Approximately 3,500 residential homes were radon tested for 90+ days, as per national guidelines. Subsequently remediated homes were retested to determine efficacy of radon reduction techniques in

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this region. Homes had an average indoor air reading of 126 Bq/m$^3$ radon, equating to an effective absorbed radiation dose of 3.2 mSv/yr. 48% of households were ≥100 Bq/m$^3$ and 12.4% were ≥200 Bq/m$^3$, with homes measuring as low as <15Bq/m$^3$ and as high as 3,441 Bq/m$^3$. Newer homes had on average >30% higher radon compared to homes built prior to 1992. To study the impact of alpha radiation in a higher throughput manner, we have developed a unique Am241 irradiation system to expose cells to alpha particles in a 96 well plate-based assay. Using this setup, we present a spatiotemporal study of alpha particle induced DNA damage responses and repair. We measure DNA double strand breaks caused by alpha particles using a 3D analysis of the nuclear DNA damage γH2AX signaling response. We’ve demonstrated a persistence in the DNA damage response significantly compared to gamma rays, and are exploring a panel of previously reported radioprotective compounds to investigate possible interventions for alpha particle exposure in humans. This work demonstrates that radon and alpha particle irradiation is a genuine public health concern, and legitimizes efforts to understand the consequences of radon exposure to human health.

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**Board Number: B330**

Validation of label-free impedance-based technology for potency assessment of immune cell-mediated cytolysis and immune checkpoint modulation.

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In vitro characterization of reagents efficacy for cancer immunotherapy reagents is a necessary step before moving to expensive animal models and clinical studies. Currently in use potency assays like Chromium-51 release, ATP-based luminescence cell proliferation monitoring or flow cytometry are difficult to implement in a high throughput environment and are based on end point methodologies that are unable to capture the full dynamic of the immune response. Here we present the validation of an impedance-based platform for monitoring cytotoxic activity of immune cells in the context of cancer immunotherapy assays. The technology detects cell death and proliferation of adherent cells by measuring changes in conductance of microelectrodes embedded in 96 and 384-wells cell culture plates, without the use of labeling or cell modification. Immune cells are unable to bind the microelectrodes; hence the technology directly monitors adherent target cells proliferation/survival without signal interference by effector cells. Furthermore, we adapted the approach to B cell leukemia suspension cells through adhesion mediated by coating antibodies. We provide a large set of validation including most of the tolls that are currently utilized in the immunotherapy space: CD19 Car T models, EpCAM/CD3 BiTE antibodies and immune checkpoint inhibitor combination therapies. Based on our observation, PD-1 specific antibody can dose-dependently modulate the potency of human-derived PBMC against PC3 prostate cancer cell lines. Using PD-1 checkpoint antibody in combination with antibodies directed against other checkpoint proteins including Tim-3, Lag-3 and in combination with PDL-1 antibody, we can demonstrate either additive effects or no net increase in potency. Data comparison with Annexin V staining/Flow Cytometry shows perfect correlation between the drop in impedance signal and % of apoptotic cells, as well as correlation between immune checkpoint protein expression in PBMC populations and immune blockade. In summary, we have developed a quantitative and reproducible functional assay that can be efficiently used to characterize immune cytotoxic response across the temporal scale, an aspect that is otherwise very difficult to assess with more canonical end point assays. Thanks to the availability of 384-wells format and minimal sample handling, the technology is ideally suited for applications in large drug screening campaigns or therapeutic protocol validation directly on patient samples.
P2188
Board Number: B331
Pro-inflammatory macrophages-derived extracellular vesicles facilitate anticancer drug delivery to metastatic ovarian cancer.
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Over 80% of ovarian cancers are diagnosed at an advanced-stage (stages III and IV), when peritoneal metastasis has commonly occurred. Primary tumors can often be treated with radiation or surgery, however, cancer cells spreading within the body are resistant to conventional therapy. Therefore, how to completely eliminate ovarian cancer cells remains a great clinic challenge to address. In this study, we developed a new therapeutic approach to treat metastatic ovarian cancer on the basis of pro-inflammatory macrophages-derived extracellular vesicles (M1-type EVs) which were encapsulated with anticancer drug doxorubicin. M1-type EVs were produced by differentiated macrophages RAW 264.7 cells stimulated with lipopolysaccharide (LPS) and interferon-\(\gamma\) (IFN-\(\gamma\)). In contrast to synthetic liposomes, cellular uptake efficacy of M1-type EVs by human ovarian carcinoma SKOV3 cells was significantly higher in vitro. Moreover, M1-type EVs were more likely to adhere to both primary and metastatic ovarian cancer cells in peritoneum and extra-ovarian pelvic organs (i.e., colon, liver and bladder) than in non-target normal tissues. Thus, accumulation levels of doxorubicin delivered by M1-type EVs to primary and metastatic tumors were highly enhanced. It prevented non-specific distribution of toxic drug in the liver where liposomal nanoparticles showed remarkable accumulation leading to severe adverse effect. This approach facilitated doxorubicin to suppress ovarian cancer growth and metastasis, and thereby improved the overall survival rate. Taken together, all the results demonstrated that M1-type EVs may represent nature shuttles for anticancer drug delivery to advanced ovarian cancer.

P2189
Board Number: B332
Acoustic Force Cytometry: high throughput cell-cell avidity screening and sorting.
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Communication between cells consist of a multitude of receptors interacting simultaneously. The intensity of interactions plays an important role in various instances, the immune system being one of the most prominent examples. Techniques to study the cell-cell interactions are therefore highly valuable both in fundamental research as well as in the development of cellular therapies. Immune cell therapies hold great promise as an effective novel treatment for a wide range of diseases such as cancer and autoimmune diseases. However, the response rate to this kind of therapies can still be improved due to the lack of tools to quantitatively select and sort the most effective immune cells at high throughput. Here, we employed acoustic force manipulation, a technology with a proven track record in the study of molecular interactions, to query the interactions of live cells. This platform, termed acoustic force cytometry (AFC), provides a novel, high-throughput technology capable of measuring thousands of cell-cell interactions in parallel.

The AFC platform is being developed to provide an accurate method to screen and sort cells based on their direct cell-cell avidity to specific targets such as proteins, peptides, viruses or other cells such as...
tumor cells. As a proof of concept, we aimed to assess the interactions between T cells and tumor cells. We validated the AFC technology by analyzing the cell-cell avidity of T cells transduced with different tumor recognizing T cell receptors with respect to patient-derived tumour cells. AFC is a lab-on-a-chip technology that employs ultrasounds to generate controllable forces on individual cells in a label-free, non-contact and high-throughput way. These data demonstrate the potential of the AFC in profiling T cell-tumor cell interaction and pave the way to quantitative cell-cell avidity studies.

P2190

Board Number: B333


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In the last two decades, nanoparticles have experienced an increased interest both in terms of diagnostics and in relation to therapy as drug delivery for various diseases including cancer therapy. In our research, we have worked with Falcarindiol incorporated nanoparticles and solid lipid nanoparticles (SLNs) to function as drug delivery systems in cancer therapy. In the first study, we examined the cellular uptake and effect in hMSC cells of (i) free Falcarindiol by white light microscopy and fluorescence microscopy, and (ii) Falcarindiol incorporated nanoparticles using fluorescence and confocal microscopy. Falcarindiol has been found to prevent and reduce the number of neoplastic lesions in the colon and to increase the number of cholesteryl-ester lipid droplets in treated cells, an effect seen when using other anticancer drugs. By examining the uptake of free Falcarindiol we were able to confirm previous results found using Raman spectroscopy showing that Falcarindiol leads to an increased number of lipid droplets in the treated cells. The nanoparticles designed in this study consisted of PEGylated monolayer of the phospholipids DSPC and cholesterol. From the uptake study of these nanoparticles, we found that they can function as stable drug carriers for Falcarindiol.

The second study concerned the synthesis of antibody conjugated SLNs in the sub-500 nm range to function as an active and targeted drug delivery system in cancer therapy. The SLNs were formulated using a one-step solvent injection method where the organic phase, containing the lipid, and a reagent, was injected into an aqueous solution containing albumin and antibodies (anti-EGFR) resulting in the formation of SLNs where both albumin and antibodies were covalently bound to the surface. In this study, we used this method to make SLNs with diameter down to 89 nm and a zeta potential of -29 mV. We further did in vitro cellular uptake of the SLNs and examined the uptake in different cell lines using confocal microscopy and flow cytometry. The results indicated that (i) SLNs were internalized by the cells, (ii) by incorporating hydrophobic molecules into the SLNs the unspecific uptake of SLNs could be reduced and, (iii) conjugation of the antibody (anti-EGFR) initiated an elevated cellular uptake. Based on the findings, we, therefore, conclude that the SLNs produced in this study may function as an active targeted drug carrier for hydrophobic therapeutics in cancer therapy. Additionally, due to the properties of the SLNs, developed in this study, we plan to study their potential as cancer drug carriers.
P2191  
**Board Number: B334**  
Combined nanotherapy based on MAPK kinase inhibitor and clinically approved cytotoxic agent for colorectal cancer.  
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Colorectal cancer (CRC) is highly prevalent worldwide and despite notable progress in its treatment still, leads to significant morbidity and mortality. Notwithstanding the high efficiency of chemotherapeutic agents used to treat CRC, their low specificity often produces a range of dose-limiting side effects. Cancer cells have developed different strategies to escape cell death induction by chemotherapeutics. Consequently, therapies intending to interfere signal transduction pathways using a single drug may be insufficient to accomplish the tumor recession. Several studies have described that the upregulation of p38 mitogen-activated protein kinase (MAPK) in CRC represents a critical factor during the cell migration, and invasion, as well as, in the maintenance of cell metabolism promoting cell survival. Then, the combined therapy against different pathways encourages excellent potential for CRC. Recently, the use of hybrid nanoparticles (lipid-polymer) as drug delivery system for cancer therapy has become one of the most promising strategies against cancer. Moreover, the combined drug therapy loaded-nanoparticles could boost the therapeutic effect. Here, we combine the cytotoxic drug 5-FU and the p38 inhibitor skepinone-L into PLA–DHPE-PEG nanoparticles (NPs) prepared by the single emulsion method, and we evaluated their cytotoxic effect and cellular internalization in HTC-116 and SW480 cells (colorectal cancer cell lines). NPs analyzed by transmission electron microscopy exhibited spherical shape nanoparticles with a homogeneous size. The quantification of size by dynamic light scattering (DLS) confirmed the diameter ~100 nm in both, empty and loaded nanoparticles. The superficial charge of particles evaluated using DLS displayed negative charge in both, distilled water and PBS. Also, the stability performed in PBS for 96 hours showed a uniform diameter and absence of agglomerates. Cell viability was measured by the cell counting kit-8 (CCK-8) in cell lines treated with non-loaded drug combined therapy, and loaded-nanoparticles for 48 hours. The IC50 value was calculated for each treatment using the GraphPad Prism 6.0 software from triplicated experiments. The obtained results exhibited that combined therapy based on 5-FU + skepinone-L loaded polymeric nanoparticles exhibited significant cytotoxic effect than non-loaded drugs on HTC-116 and SW480 cells. The uptake of NPs studied on HTC-116 and SW480 cells at 0, 2, 4, 8, 16 and 24 hours of incubation by confocal microscopy displayed a time dependence internalization rate in the cell line evaluated. The results suggest that combinatorial therapy using nanoparticles improves effectiveness and may decrease side effects of CRC therapy.
**P2192**  
**Board Number: B335**  
**Novel Conjugation Chemistry for Antibody-Drug Conjugates.**  
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Antibody-drug conjugates (ADCs) have been an attractive target of research in the development of cancer therapeutics. Conventional linkers for drug conjugation to the antibody utilize N-hydroxysuccinimide ester chemistry with amines (N-terminal amine or lysine) or maleimide reaction with thiols (cysteine). Given the relative abundance of amines in proteins and the level of circulating thiols in the body, these chemistries carry the drawbacks of producing heterogeneous products with different pharmacokinetic properties and/or premature release of the drug due to retro reaction. There is thus a growing interest within the field of ADCs toward conjugation chemistries that are site-specific, efficient, and can produce stable conjugates. Here, we demonstrate the potential of 2-formylphenylboronic acid (2fPBA) chemistry as a novel conjugation strategy for meeting these needs. Recent reports have shown that 2fPBA exhibits fast reaction kinetics and selectivity toward \(\alpha\)-aminothiol and \(\alpha\)-aminoimidazolidine to form stable boron-nitrogen heterocycles (BNHs). The bioorthogonality of these reactions is herein demonstrated further by selective protein labeling within a complex mixture (i.e. cell lysate). In addition, we demonstrate efficient protein-small molecule as well as protein-protein coupling via this chemistry. Finally, the BNH bond is found to be stable over time as well as in the presence of biologically relevant molecules. Altogether, the results show promise of utilizing 2fPBA chemistry with \(\alpha\)-aminothiol and \(\alpha\)-aminoimidazolidine in the linker design for ADCs.

**Tumor Microenvironment 1**

**P2193**  
**Board Number: B336**  
**Investigating the role of microenvironmental stress in transcriptional control and cancer progression.**  
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Oxidative stress (OS), resulting from an imbalance in reactive oxygen species generated and cleared by antioxidants in the body, is a feature of the tumor microenvironment involved in cancer progression. How OS leads to chromatin alterations and epigenetic modifications that drive cancer progression by influencing gene transcription is not well understood. We have been studying the structural protein, nuclear mitotic apparatus (NuMA) for its function at the boundary of nuclear architecture and chromatin organization with an impact on major aspects of cellular homeostasis, such as phenotypically normal differentiation; nucleolus-based stress response and DNA repair. Through mass spectrometry analysis we have identified the transcriptional coactivator lens epithelium derived growth factor (LEDGF), which is also protective against OS, as an interacting partner for NuMA. Our preliminary investigation indicated that LEDGF expression was decreased in invasive triple negative breast cancer (TNBC) compared to the preinvasive stage. It is known that increased H3K9me2 expression represses LEDGF and correlates with increased expression of chromatin protein, remodeling and spacing factor (RSF-1) that also binds to NuMA from our findings. RSF-1 is a transcriptional repressor when overexpressed under OS and is a marker of aggressive breast cancers. Our central hypothesis is that NuMA-LEDGF-RSF1 interaction
controls cancer progression under microenvironmental stress by influencing transcriptional regulation. To test our hypothesis we use the HMT-3522 model of TNBC progression that in 3D cell culture mimics the phenotypically normal luminal epithelium (non-neoplastic S1 cells), ductal carcinoma in-situ (DCIS; S1-derived S2 cells) and invasive ductal carcinoma (IDC; S2-derived T4-2 cells). Induction of OS with 250 μM H2O2 in S1 cells decreases NuMA expression, yet NuMA interaction with LEDGF is strengthened compared to control, but in S2 cells NuMA-LEDGF interaction is weakened under OS when compared to control. NuMA and RSF-1 follow a similar expression pattern as LEDGF with lower expression in T4-2 cells compared to S2 cells, and increased expression under OS in S2 cells. However, prolonged exposure to ROS (25 μM H2O2 for four weeks) results in a loss of NuMA and LEDGF and increase in expression of RSF1 in preinvasive TNBC, hence mimicking the changes observed in vivo. These results highlight the need to investigate whether RSF-1 over-expression under chronic OS in the S2 cells triggers H3K9me2 increase, and consequently loss of LEDGF, hence pushing cells toward an invasive phenotype. Also, important is to understand whether loss of RSF-1 or LEDGF controls the interaction of NuMA with either of these co-transcription factors and cancer progression.

P2194
Board Number: B337
Oscillatory HIF-1α induction promotes proliferation of hypoxic cells through a lactate dependent quorum autophagy response.
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Response to hypoxia is a highly regulated process controlling multiple cell and tissue level functions. However, we still know little about dynamic single cell responses to hypoxic conditions. Here, using fluorescent reporters of hypoxia response factor-1 alpha (HIF-1a) activity, we show that hypoxic responses in individual cells can be highly dynamic and variable across the population. These responses fall into three classes, including oscillatory activity. We identify a molecular mechanism that can account for all three response classes, demonstrating that the oscillations of HIF-1a activity and abundance are controlled by the reactive oxygen species-dependent chaperone-mediated autophagy in a subset of respiring cells. Furthermore, we find that the oscillatory response is modulated by the abundance of extracellular lactate in a quorum sensing-like mechanism. We show that oscillatory HIF-1a activity can help overcome hypoxia-mediated inhibition of cell division, suggesting a mechanism for aggressive behavior in a subset of hypoxic tumor cells.

P2195
Board Number: B338
Environmental availability of cystine drives usage of glutamine as a TCA cycle substrate and causes glutamine addiction.
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The tri-carboxylic acid (TCA) cycle is primarily known for its role in generating NADH from the oxidation of pyruvate, which is used to power ATP production by oxidative phosphorylation. Beyond ATP production, the TCA cycle also supplies biomass precursors needed for the growth and proliferation of
cells. Thus, supplying the TCA cycle with carbon is an essential metabolic feature of proliferating cells. Many mammalian cancer cell lines depend on extracellular glutamine as a major TCA cycle substrate to support their proliferation in vitro. However, recent studies have suggested that some cells that depend on glutamine to fill the TCA cycle and proliferate in culture rely much less on glutamine catabolism for these functions when growing as tumors in vivo. This has led to the conclusion that environmental differences between tumors and cell culture influence the extent of glutamine catabolism. We sought to identify such environmental differences that cause differential dependence on glutamine for TCA cycle maintenance. We hypothesized that differences in available nutrients between standard culture conditions and tumors could account for differences in glutamine utilization. Therefore, we cultured cancer cell lines in 100% adult bovine serum, a condition that more closely reflects the nutrients available to cells in vivo. Cells cultured in adult bovine serum have decreased glutamine catabolism and rely less on glutamine metabolism for proliferation compared to growth under standard tissue culture conditions. The small molecule nutrient component of adult bovine serum was responsible for the differential utilization of glutamine. By analyzing the nutrient differences between bovine serum and media, we find that levels of a single nutrient, cystine, can account for the differential dependence on glutamine in these different environmental contexts. We show that increasing cystine availability to cancer cells growing as tumors in vivo increases glutamine catabolism in these tumors. Lastly, we find that cystine levels dictate glutamine dependence via the cystine/glutamate antiporter xCT/SLC7A11, and that environmental cystine levels in conjunction with xCT/SLC7A11 expression are necessary and sufficient to drive increased glutamine anaplerosis. Collectively, these results define important determinants of glutamine metabolism and glutamine dependence in cancer cells, and highlight that cellular metabolism is determined not only by cellular genetics, but by the interplay of the cell with the nutrient environment.

P2196
Board Number: B339

One carbon metabolism-mediated protein methylation triggered by histidine regulates the filaments formation and preservation of CTP synthase.

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CTP synthase form compartmentalized filament in respond to environmental nutrients status. However, the physiological role of filaments and mechanisms for filaments assembly are not understood. Here, we demonstrate that CTP synthase form filaments under glutamine deprivation in response to the influx of histidine. The protein level of CTP synthase could be preserved in the presence of histidine, which associated with rapid growth following nutrients replenishment. Tetramer conformation based filament formation reflected the downregulation of CTP synthase enzyme activity. Induction of activating transcription factor 4 (ATF4) by nutrients restriction, which was essential for filament formation. Comprehensive analysis identified that methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) involved one carbon metabolism regulated by ATF4, which affected filaments formation. One carbon metabolism mediated protein methylation triggered by histidine was critical for filament formation. Collectively, we demonstrate that CTP synthase filament formation through one carbon flux mediated protein methylation could be one of strategies for cancer cells to recovery quickly after stress relief and maintain homeostasis under adverse environments.
Galectins are multifunctional soluble beta-galactoside-binding proteins capable of modulating cell differentiation, proliferation, survival, and adhesion. These cellular events are critical in fundamental biological processes such as embryogenesis, angiogenesis, neurogenesis, immunity, and tissue repair. The expression pattern of twelve members of the human galectin family changes substantially in differentiated cells, tumor cells, and cells exposed to environmental stress stimuli such as oxidative stress [1,2]. The level of galectins in circulation also changes under conditions of physiological (e.g. physical exercises) and pathological (e.g. cancer) stresses. These findings suggest that galectins are potential biomarkers and regulators of cellular and organismal stress responses. However, molecular mechanisms that are responsible for regulating the complex galectin network in cells are not well known. The goal of our study was to examine a role of global stress-associated glycosylation of intracellular proteins with O-linked beta-N-acetylglucosamine (O-GlcNAc) as a new signaling paradigm for controlling galectin gene expression in human cells. Changes in the galectin expression profiles were compared between several human cell lines (MCF7, MDA-MB-231, HT-29, and HL-60) treated with biochemical inhibitors of two enzymes which add (O-GlcNAc transferase, OGT) or remove (O-GlcNAcase, OGA) O-GlcNAc residues. The expected modulation of global O-GlcNAc levels by drugs and stress stimuli was confirmed and quantified using immunodot blot analysis with an anti-O-GlcNAc antibody, RL2. Remarkably, a significant decrease of O-GlcNAc was noticed in differentiated HL-60 cells (1.3% DMSO treatment) and HT-29 cells (crowding stress). Quantitative RT-PCR assay revealed both cell-specific galectin expression profiles and significant differences between cells treated with OGT versus OGA inhibitors. Pearson correlation analysis showed a positive correlation between the expression levels of specific galectins and O-GlcNAc, although no or negative correlations were observed in other cases. We conclude that O-GlcNAc-mediated regulation of galectins may interfere with other signaling systems as, for instance, COX-2-positive MDA-MB-231 breast cancer cells were less sensitive to OGT/OGA inhibitors in comparison with COX-2-negative MCF7 cells. In sum, our findings provide novel evidence that O-GlcNAc-mediated signaling pathways are involved in remodeling galectin networks in human cells in association with cellular stress responses and cellular differentiation.


P2198

Board Number: B341

LIPIIDIMAGINGIN PROSTATE CANCER.

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Prostate cancer remains one of the most commonly diagnosed cancers in males. Distribution and metabolism of lipids is known to be altered in cancer cells in particular prostate cancer. Though a
number of approaches have described these changes in lipids, visualisation of cellular lipid distribution and metabolism is still restricted by the availability of specific imaging tools. Fluorescent complex ReZolve-L1TM has been showed to have an affinity towards cholesterol, phosphatidylethanolamine, sphingosine and sphingomyelin and localise that is consistent with these lipids in cells and tissues. By utilising this imaging agent to investigate lipid distribution at the cellular level, we revealed a different pattern of staining in malignant prostate cancer compared to non-malignant cells. Following lipid loading there was also significant differences in lipid staining by ReZolve-L1 revealing an altered cell response to lipid availability between malignant 22RV1 and non-malignant PNT1a cells. This suggested that malignant prostate cancer cells had enhanced uptake of exogenous lipids compared to non-malignant cells. Biochemical analysis confirmed that there were significant differences in lipid profiles for malignant prostate cancer cells when compared to non-malignant prostate cells, and this was also confirmed by IR spectroscopy. These findings demonstrate that ReZolve-L1TM lipid staining could be utilised to aid in understanding lipid distribution and metabolism at a cellular level. Further ReZolve-L1 may provide a tool for prostate cancer diagnosis/prognosis. This is important as early accurate detection and appropriate intervention will have a significant impact on patient morbidity and mortality.

P2199
Board Number: B342
Optimization of a Chromatin Immunoprecipitation Assay to Assess Target Genes of the FOXC2 Transcription Factor in Melanoma.
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characterized by rapid growth and metastasis. Increased expression of the transcription factor FOXC2 has been associated with more aggressive melanoma outgrowth and metastasis. We sought to optimize a chromatin immunoprecipitation assay to identify novel gene targets bound by FOXC2 in B16-F1 melanoma, specifically investigating genes regulating cell adhesion proteins and genes regulating tumor invasion and metastasis that we have been found to exhibit altered expression in wild-type B16-F1 versus a novel CRISPR-Cas9-engineered B16-F1ΔFocc2cell line with a homozygous disruption of the Foxc2 gene. Wild type B16-F1 chromatin was ChIPed using an anti-FOXC2 Ab, an anti-Histone H3 positive control Ab, and a normal goat IgG negative control Ab. Enrichment of known FOXC2 gene targets in the promotor regions of Itgb3 and PAI-1 was measured by qPCR for each condition. We demonstrated consistent enrichment of gene targets by anti-Histone H3 ChIP and anti-FOXC2 ChIP. We also reduced background from 14% of Input to 5.5% of Input by altering our protocol to accommodate a preclearing step and magnetic beads of a different protein-binding subtype. While background remains higher than our goal of 0.01% of Input, current work is focused on generating a novel CRISPR-Cas9-engineered variant of the wild-type B16-F1 melanoma with a homozygous disruption of the DNA-binding domain sequence of the Foxc2 gene for use as a strong negative control in further comparative ChIP-qPCR studies.
P2200
Board Number: B343
Optogenetic manipulation and monitoring of YAP signalling in tumours.
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It is known that the complexity of the cancer is created by a fine interplay between the cancer cells and their microenvironment. Cancer cells interact with various stroma components during tumour growth and development of metastasis. Many signalling pathways influence cross talk between cancer cells and their environment. For example, YAP/TAZ has already been implicated to be an important determinant of cancer progression. However, so far no method provided means of activating signalling specifically in the subset of tumour cells.
We use optogenetic approach to specifically modulate YAP signalling both in vitro and in vivo. By using blue light illumination we can control signalling pathways in space and time. We are developing two different methods to modulate signalling: by translocation of a downstream transcription factor to the nucleus (opto-release) or by controlling expression of transcription factor from light inducible promoter. The specific activation of YAP signalling through the light inducible promoter is followed by in-depth analysis of the possible phenotypic changes in cancer cells and cancer associated fibroblasts (CAFs). Additionally, the opto-release tool is used alongside mathematical modelling to study YAP protein dynamics and nucleocytoplasmic shuttling on single cell level in cancer cells. Finally, we investigate YAP nucleocytoplasmic shuttling machinery. The optogenetic tools allow detailed and previously unachievable analysis of YAP signalling in cancer cells and CAFs.

P2201
Board Number: B344
A low affinity leukotriene B4 receptor-2 regulates proliferation of malignant Colon Cancer Cells.
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Inflammation and inflammatory mediators are intimately linked with colorectal cancer progression through complex pathways. However, the mechanism by which leukotriene B4 (LTB4) and 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE) contribute to colorectal cancer progression remains elusive. In this study, we determined that the low-affinity leukotriene B4 receptor-2 (BLT2) and its ligands leukotriene B4, 12-HETE were highly up-regulated in KRAS mutated SW480 and LOVO colorectal cancer cells and affect the proliferation through the activation of Phosphoinositide 3-kinase (PI3K)-Protein kinase B (Akt) and the subsequent up-regulation of Cyclin D1. BLT2 depletion with siRNA and LY255283 clearly down-regulated the proliferation of KRAS mutated colorectal cancer cells and further increased G1 phase cell cycle arrest by down-regulating the PI3K-Akt cascade. Enlarged tumour formation due to the KRAS mutation of LOVO and SW480 cells in xenografted mice was also obviously reduced by treatment with the BLT2 inhibitor in vivo. Our study elucidates that BLT2 is a novel contributor to inhibit growth effect in KRAS mutated colorectal cancer cells and thus may be a potential therapeutic target for the treatment of KRAS mutated colorectal cancer.
P2202
Board Number: B345
Transmembrane 4 L six family member 5 (TM4SF5) forms complex with SLC members for cell survival and homeostasis.
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TM4SF5 is one of the transmembrane 4 L 6 family which has similar domain structure and function with tetraspanin. Tetraspanin associates and interacts with several partner proteins in plasma membrane to form TERM (Tetraspanin-enriched microdomains). Protein complex formed by TM4SF5 contribute to cell adhesion, invasion and migration. To investigate other diverse roles of TM4SF5 and TERM, mass spectrometry analysis was performed. Mass spectrometry data show TM4SF5 interacts with many SLC (Solute Carrier) family. However, the roles and significance of TM4SF5 to regulate SLC family remain unclear. We focused on the complex formation of TM4SF5 with an antiporter which imports cysteine into cells and exports glutamate. TM4SF5 upregulates a CD44 variant, which increases protein stability of the antiporter. In addition, TM4SF5 interact directly with the antiporter together with the CD44 variant. TM4SF5 increases intracellular GSH dependent on the antiporter and the CD44 variant. In addition, TM4SF5 attenuates cellular ROS level in response to extracellular and intracellular ROS stimuli. Moreover, RNA-seq data show TM4SF5 upregulates antioxidant genes in cell line and knockout mouse model. In conclusion, our study reveals the novel mechanism of SLC regulation and its function contributing to cell survival and cellular homeostasis.

P2203
Board Number: B346
Large-scale expansion and banking of novel 2D and 3D cancer models from the Human Cancer Models Initiative: Results of a pilot study.
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The Human Cancer Models Initiative (HCMI) is an international collaboration between the National Cancer Institute, Cancer Research UK, Wellcome Trust Sanger Institute, and the foundation Hubrecht Organoid Technology, devoted to the development of approximately 1,000 novel human primary tissue derived tumor models. These models will utilize advanced in vitro cell culture techniques that better mimic human in vivo tumor physiology than traditional cancer cell lines, and will be supported by clinical and genomic data. Some of these next-generation cancer models will be derived from tumors that are rare, lack effective treatments, originate from patients from underrepresented populations, or are otherwise not well represented amongst currently available cancer cell lines. While the laboratories supporting the HCMI project are focused on early-stage model development, little attention has been given to issues of scale up for expansion, the need for quality control assays, and the standardization of protocols. Additionally, the advanced culture techniques being utilized to generate these models pose challenges for typical large-scale expansion and banking, including the requirements for specialized feeder cells, growth in undefined extracellular matrices, complex media formulations containing multiple growth factors and small molecules, and use of undefined conditioned media from multiple other cell lines. Herein we describe the results of a pilot project to determine the feasibility of performing large-scale expansion and banking of novel cancer models derived from esophageal, colon, pancreatic, and mammary tissues. We found significant laboratory to laboratory differences in protocols, reagents and culture conditions even for models derived from the same culture technology.
and primary tissue. Tissue to tissue and donor to donor variation was also evident in model characteristics, including morphology, growth rates and genetic instability. All the models tested were amenable to scale up from multiwell plates to flasks, though more specialized consumables were required than with typical continuous cell lines. Conversely, population doubling times and yields per cm² for many models were similar to traditional 2D cell lines, though viability as assessed by trypan blue exclusion was often low (<80%). Models exhibited tissue-like architecture and cell-type relevant markers as assessed by immunocytochemistry and immunohistochemistry. Multiple commercially available cryopreservation media were able to support cryopreservation and recovery. Our results suggest that despite challenges, next-generation in vitro models are suitable for large-scale manufacture and distribution to support researchers in a variety of applications in the field of cancer biology.

P2204
Board Number: B347
TISSUE PROTEOME ANALYSIS OF HORMONE RECEPTOR-POSITIVE BREAST CANCER.
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Breast cancer (BC) is the most threatening cancer type among the women in many countries. Despite of the major developments in medical sciences and technologies, the incidence and mortality rates of BC is still increasing, making it the number one cause of death among women. Detection of BC at an early stage increases the chance of survival and reduces the burden of the disease. Although there are certain BC biomarkers routinely used for prognostic and predictive purposes, none of these biomarkers are useful for the early detection of the BC. Therefore, the early diagnosis of BC still remains an important challenge and there is a great need for the early-diagnostic biomarker(s). The aim of this study is to discover a (or more than one) biomarker molecule(s) that will allow early diagnosis of the BC. For this purpose, tissue samples from BC patients were collected for analysis. Patients with invasive or infiltrative ductal carcinoma have been included to this study. Based on their clinicopathological data, patients were classified into 3 molecular subtypes: Luminal A (LA), Luminal B (LB), Luminal B-like Her2+ (LB-Her2+) and each group contained 34, 33, and 14 patients, respectively. The control group was created using part of the healthy breast tissues obtained from the same BC patients. Protein extracts were prepared and protein pools for each group were created and subjected to 2-DE coupled with MALDI-TOF/TOF. Comparative 2-DE analysis revealed the presence of a total of 39 differentially regulated protein spots among the groups. However, of these, 37 proteins displayed changes in their abundance for at least 2-fold when the BC groups were compared with the control group. The highest level of differential regulation was observed in proteins monoglyceride lipase, carboxypeptidase B, Pyruvate kinase isozymes M1/M2, Stress-70 protein, L-lactate dehydrogenase B chain, Carbonic anhydrase 1 and Aldo-keto reductase. The proteome profiles of LA, LB, and LB-like Her2+ subtypes were more conserved indicating that the subtyping of hormone receptor positive tumor tissues was not profoundly reflected onto the regulations occurring at the proteome level.
P2205

Board Number: B348

The importance of cellular oxygenation measurements in the analysis of hypoxia-induced signalling and related metabolic adaptation.

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There is a growing appreciation that typical cell culture conditions reflect a hyperoxic condition for the majority of cell types. In an effort to more accurately reflect the in vivo condition, a growing number of researchers are conducting in vitro measurements at what are deemed to be more physiologically relevant oxygen concentrations (1-8% O₂). The importance of oxygen concentration in in vitro model design is underscored by observations that O₂ levels influence a wide range of cellular phenomena, including metabolic poise and senescence while the development of localised hypoxic environments are implicated in a variety of disease including stroke, and cancer. Impaired O₂ supply can reduce O₂ concentrations to below normal physiological levels, initiating a graded series of complex interconnected adaptive responses mediated by regulators including the hypoxia-inducible factor (HIF). These processes are particularly relevant within solid tumours, where oncogene-driven proliferation causes nutrient and oxygen deprivation, aberrant angiogenesis, and the activation of O₂-sensitive survival pathways. Despite the importance of cellular oxygenation, research using in vitro models makes the assumption that oxygenation can be defined by simply altering the oxygen condition applied i.e. cells cultured in a 5% O₂ environment are assumed to have an intracellular O₂ level of 5%. To test this key assumption, cellular oxygenation measurements are performed using a nanoparticulate intracellular oxygen probe (MitoXpress-Intra), while tumour hypoxia/oxygenation is modelled on a fluorescence plate reader equipped with an atmospheric control unit (CLARIOstar). Data is presented illustrating that the depth of hypoxia experienced by the cell model is impacted significantly by respiratory activity, and that this additional oxygen deprivation is a dynamic process, effected by respiratory substrate availability. Data is also presented demonstrating that, in in vitro models, additional respiration-induced hypoxia directly impacts glycolytic flux and metabolic pathways. Together these data invalidate the assumption that cellular oxygenation can be inferred from ambient oxygen measurements. If ignored, the significant and dynamic deviations between ambient O₂ and oxygenation at the cellular level leads to erroneous conclusions regarding the relationship between oxygen concentration, HIF stabilisation and related metabolic adaptations. This in turn can impair the physiological relevance of experimental observations.

P2206

Board Number: B349

Tunneling Nanotubes (TNTs) mediate bidirectional transfer of specific vesicular cargo and proteins between leukemia and stroma cells.

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Tunneling nanotubes (TNTs) have been recently recognized as a new form of intercellular connection and communication. They allow for the transfer of various cellular components, including proteins, RNAs, vesicles and organelles, such as mitochondria, between two distant cells. They have been proposed as an important way by which signals propagate in cancer, neurodegeneration and many other situations. Here we have studied the presence and role of TNTs formed between chronic myeloid leukemia (CML) and stroma cells. Using different microscopy methods, including live imaging and 3D reconstruction, we have found that leukemia cells possess very limited capacity to form TNTs between each other (1 TNT/100 cells). However, upon co-culture, the number of TNTs formed between leukemia and stroma cells significantly increased (35 TNTs/100 cells) and they constituted almost 27% of all formed TNTs. This correlated with leukemia cells receiving vesicular cargo, transferred specifically when cells could be in direct contact. Vesicular exchange, quantified by tracking vesicles labeled with DiD by flow cytometry, was more efficient when stroma cells served as donor and leukemia as acceptor cells (51.4 +/- 3% of leukemia cells received cargo after 24 hours), compared to the opposite direction (20 +/- 2.1% of stroma cells). Functionally, separate analysis of leukemia acceptor cells (DiD+) which received fluorescently-labeled vesicles from stroma and those which did not exchanged cargo (DiD-) indicated that transferred vesicles may provide protection from apoptosis caused by imatinib, a first line drug in CML treatment, resulting in decrease of apoptotic population by 40% (n=4, p<0.005). Importantly, trans-SILAC MS identification and quantification of proteins transferred from donor to acceptor cells showed that different groups of proteins are exchanged bidirectionally between leukemia and stroma cells. In conclusion, TNTs formed between leukemia and stroma cells facilitate the intercellular transfer of vesicular cargo and specific proteins, which may be important in facilitating drug resistance in cancer cells.

This work has been financed by the National Science Centre grants: UMO-2013/10/E/NZ3/00673 (to K.P.) and UMO-2015/17/B/NZ3/00557 (to J.W.)

P2207

Board Number: B350

3-D Carcinoma Cell Culture Model for in vitro Evaluation of Anticancer Drugs.

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3-dimensional (3D) culture of human lung carcinoma cells can favorably replace the conventional 2-dimensional (2D) culture for a closer simulation of in vivo conditions. Lung adenocarcinoma alveolar basal epithelial cells (A549) are known to grow 2-dimensionally in tissue culture dishes. Cells can be cultures in vitro in an adaptable 3D environment using alginate beads or specialized cell culture dishes to form spheroids. In this project, we grow lung cells 3-dimensionally using alginate beads. The alginate beads incorporating lung cells were formed and maintained in mammalian cell culture media. The cell viability of A549 cells was studied using trypan blue assay as needed. We are utilizing our 2-D versus 3-D cell culture models to do a variety of assays testing the efficacy of chemotherapeutic agents. We are also exploring them as an excellent alternative for long-term, low-cost cell propagation.
Chromatin and Chromosome Organization

P2208
Board Number: B352
CRISPR-Based DNA Imaging in Living Cells Reveals Cell Cycle-Dependent Chromosome Dynamics.
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In contrast to their well-studied condensation and folding during mitosis, the dynamics of interphase chromosomes are less understood. We developed a multicolor and sensitive system, CRISPR-Sirius, allowing us to track the dynamics of pairs of loci on a single human chromosome in real time throughout interphase. We measured the inter-locus distances from kilobase to megagase scales as compared to the DNA physical map and found cell cycle stage-dependent changes in the inter-locus distance. In addition, we resolved two distinct modes of dynamics: local saltatory movements as well as longer-range domain translational movements. The magnitude of both of these dynamic substantially increased from early to late G1, whereas the domain translational movement was greatly reduced in early S. The local saltatory fluctuations decreased slightly in early S and declined further in late S. These unanticipated movements are indicative of both a compression-relaxation dynamic of the chromosome fiber operating concurrently with changes in the extent of long-range movements of an observed domain. It should now be possible to address the relationship these dynamic phenomena to DNA replication and repair, epigenetic modification, transcription regulation, and stem cell differentiation.

P2209
Board Number: B353
Measuring local chromatin compaction using fluorescence lifetime imaging.
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The linear length of genomic DNA is many orders of magnitude greater than the size of the nucleus. To fit a eukaryotic genome into the nucleus, DNA undergoes several different levels of compaction. DNA compaction also affects protein accessibility of DNA elements and thus can contribute to transcriptional regulation. Traditional size estimation methods to study compaction, such as DNA volume measurements or proximity of distant chromosomal loci are limited in their resolution by the wavelength of light. This presents a large-scale picture that aggregates many different compaction processes. Thus data regarding local changes in compaction has been difficult to obtain. To circumvent this problem, we apply a new microscopy technique that uses changes in fluorescence lifetime of DNA binding dyes to measure local changes in DNA compaction. We have shown that the lifetime faithfully responds to a number of established protocols that induce DNA compaction, such as nucleosome formation, and addition of magnesium or spermine. We also used the sensor to study the impact of linker histone H1, related remodeling complex and condensin on local chromatin structure. The implication in the differential regulation of the local and global chromatin compaction will be discussed.
P2210
Board Number: B354
Structure and dynamics of the Polycomb body.
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The subcompartmentalization of the cell nucleus into nuclear domains seems to be the fundamental principle that regulates the functioning of the nucleus. Our research has been concentrated on one of the nuclear domains known as a Polycomb (PcG) bodies. The PcG body is observed by fluorescence microscopy as a nuclear subcompartment in the form of a fluorescent PcG focus enriched in Polycomb repressive complex 1 (PRC1) proteins. In our work, we were first interested in the structural basis of the PcG body in mammalian (primarily human) cells. Using correlative light-electron microscopy, we visualized the fine structure of the PcG body. We observed that the so-called PcG body (PcG focus) corresponds to a local accumulation of heterochromatin structures. To strengthen chromatin nature of PcG foci, crowding experiments were carried out. We observed that the behaviour of PcG foci in cells grown under changed molecular crowding conditions vastly differs from the behaviour of proteinaeous nucleoplasmic bodies, but is in harmony with the behavior of chromatin domains. Further, we were interested in the dynamics of PRC1 proteins within PcG foci and the structural changes of the PcG chromosomal domain during DNA replication. Dynamics of PRC1 proteins within PcG foci was high and, interestingly, we observed the presence of PRC1 proteins on replicating PcG domains and the asynchrony in replication timing of PcG foci. We also detected a decompaction of the PcG chromosomal domains during their replication. Together, our findings show that the PcG body is a chromosomal domain, the dynamics of PRC1 proteins in PcG foci is high, and the replication of the PcG chromosomal domain is accompanied with changes in chromatin structure within the PcG focus. This work was supported by the grants GACR P302/12/G157, UNCE 204022 and OPPK CZ.2.16/3.1.00/24010 and Progres Q28.

P2211
Board Number: B355
Condensin II drives large-scale chromatin folding and genome compartmentalization in Drosophila.
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Proper nuclear organization is essential for development and organism viability. Both chromosome confirmation capture (3C)-based methods and fluorescence in situ hybridization (FISH) have shown that chromosomes occupy distinct nuclear domains called chromosome territories (CTs). The fact that CTs have been observed in a broad range of species suggests that their formation may play a role in vital cellular processes, such as transcription and DNA repair. In support of this, CT disruption has been seen in several human diseases, including numerous forms of cancer, correlating CT loss with gene misexpression and genome instability. However, the genes involved in the formation and maintenance of CTs remain unknown. Using a combination of forward and reverse genetics, we address these fundamental questions in Drosophila, targeting their relatively small number of chromosomes with whole-genome, multi-color, and chromosome arm-specific Oligopaint FISH probes. Our data reveal that Drosophila cells form largely non-overlapping CTs, indicating that heterologous chromosomes minimize their intermingling during interphase. Additionally, the use of whole-genome Oligopaints on metaphase
chromosome spreads illustrate that large chromosomal rearrangements can be correlated with the spatial orientation of DNA during interphase. Finally, we have identified the condensin II complex as an essential factor that inhibits interchromosomal interactions and facilitates chromosome folding during CT formation. Collectively, these analyses show that Drosophila cells and Oligopaint FISH provides a powerful platform for assessing CT function and characterizing the regulatory pathways that control chromosome packaging and positioning in a metazoan.

P2212

Board Number: B356

BAC transgene arrays as a system to dissect regulatory elements of large-scale chromatin organization.

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Dissecting mechanisms regulating large-scale chromatin organization in mammalian cells is difficult largely due to the complexity of the mammalian genome and the lack of efficient ways both to manipulate the sequence and then to visualize the consequences of these manipulations on chromatin folding and nuclear localization. Adding to these difficulties is the significant functional redundancy of cis elements controlling large-scale chromatin organization. Here we show how bacterial-artificial chromosomes (BACs) may be a powerful tool to dissect determinants of large-scale chromatin organization. BACs can integrate into the mammalian genome as tandem arrays of up to mega-base pairs in size. These arrays have much lower DNA complexity than endogenous genomic regions of comparable size, yet they retain near normal levels of expression for the genes contained within the BAC genomic inserts. Moreover, BAC DNA sequences can be efficiently manipulated by BAC recombineering within E.coli. We reconstituted differentially compacted chromatin regions in mouse NIH 3T3 cells by altering the genomic inserts within the BAC scaffold. BAC arrays formed from a BAC harboring an actively transcribed Dhfr-Msh3 gene locus (DHFR BAC) showed extended, fiber-like structures. In contrast, BACs harboring a transcriptionally silenced HBB locus (HBB BAC) or a gene desert region formed BAC arrays with noticeably more condensed structures. Manipulation of these BAC sequences revealed interesting clues to determinants of large-scale chromatin organization. For example, deleting large regions encompassing the Dhfr-Msh3 divergent promoter increased the compaction of these BAC arrays. Based on ongoing experiments, this increased compaction does not appear to be simply the consequence of decreased transcription. For example, overall transcription of the BAC array, assayed by RT-qPCR, remained high and transcriptional inhibition did not change the structure of arrays made from intact DHFR BACs. Our current working hypothesis is that these promoter regions have cis elements and bound trans factors that produce large-scale chromatin decondensation at a distance.

As another example, our work demonstrated that reporter mini-genes containing a strong housekeeping gene promoter from a gene expressed at similar levels in many cell types appeared imperious to differences in large-scale chromatin structure of these BAC arrays. Interestingly, this reporter mini-gene changed the nuclear localization of the HBB BAC transgene array from the nuclear periphery to interior, and possibly certain epigenetic marks. These results suggest an experimental path forward to identify separable pathways that control large-scale chromatin compaction versus nuclear localization.
P2213
Board Number: B357
DNA Helicase and temperature regulation of mitotic chromosome condensation.
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Chl1 DNA helicase plays multi-faceted roles in sister chromatid cohesion. For instance, Chl1 associates with both the cohesion establishment acetyltransferase Eco1/Ctf7 and the DNA polymerase processivity factor PCNA that supports Eco1/Ctf7 function. Mutation in Chl1 results in precocious sister chromatid separation and cell aneuploidy, defects that arise through reduced levels of chromatin-bound cohesins which normally tether together sister chromatids. Key insight into the mechanism through which Chl1 helicase promotes the tethering of DNA segments in trans (between sister chromatids) was revealed through its role in recruiting Scc2 (cohesin deposition component) to DNA. A single report suggested that Scc2 might also direct the deposition onto DNA of the cis tethering condensin complex. Here, we developed a streamlined rDNA condensation assay and then tested the ability of Chl1 to promote cis tethering. The results reveal that chl1 mutant cells exhibit moderate condensation defects both within the rDNA locus and genome-wide. We then further assessed the roles for both Chl1 and Scc2 to promote condensin recruitment to DNA. Importantly, chl1 and scc2-4 mutant cell condensation defects appear primarily to occur through reduced chromatin binding of cohesin. Consistent with a role for Scc2 specifically in cohesin deposition, scc2-4 mutant cell condensation defects are irreversible – in contrast to prior findings that condensin-dependent condensation defects are reversible. We thus term Chl1 a novel regulator of both chromatin condensation and sister chromatid cohesion through cohesin-based mechanisms. Combined with findings that Chl1 functions during S phase and resolves DNA secondary structures, these results reveal an exciting interface between DNA structure and cohesin complex functions. Surprisingly, our streamlined condensation assay uncovered a heretofore dramatic change in rDNA conformation in response to temperature. This apparent hypercondensation occurs in wildtype cells, independent of mutation maintained at 37°C, compared with cells maintained at 23°C. Neither accelerated condensation, elevated recombination nor reduced transcription appear to promote this reduction in rDNA loop structure. This observation impacts current views of chromatin structure based on conditional mutant gene analyses and significantly extends our understanding of physiologic changes in chromatin architecture in response to hyperthermia.

P2214
Board Number: B358
Genome organization and metastatic potential in breast cancer.
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The human genome exhibits a higher-order organization and architecture within the highly compartmentalized nucleus. For instance, association of chromatin with the nuclear periphery as the so-called Lamina Associated Domains (LADs) has been implicated in gene regulation, particularly correlating with gene repression. Such organization and compartmentalization is intimately associated with epigenetic modifications to both DNA and histone proteins modifications that distinguish heterochromatin from euchromatin. However, the mechanisms involved in dysregulation of chromatin and overall nuclear architecture in breast cancer remain largely unknown. Like most carcinomas, breast
cancers develop through multiple genetic and epigenetic changes that culminate in oncogenic transformation, deregulated cell growth and, for a subset of these, metastatic potential. Large changes in nuclear morphology or ‘nuclear grade’ are key criterion for pathologic diagnosis of many cancers, including breast cancer, and gross changes in nuclear morphology are generally associated with tumor progression. By using high throughput genomic data, such as DNA Adenine Methylation Identification (DamID) to identify LADs, combined with RNA-seq, we intend to decipher the inter-relationship of genome organization and dysregulation of transcription in breast cancer by comparing normal epithelial breast (MCF10A, HMECs) with -breast cancer cell lines (MCF7, MDA-MB231). We also decrypt the relationship between genome organization and metastatic potential by analyzing single colony clones (SCCs) from MDA-MB231 with different tumorigenic and metastatic potentials. Our preliminary data show that 20%-25% of up-regulated mRNAs were found in regions where LAD organization was disrupted. In particular, ESM-1 and SPANX loci were upregulated and no longer associated with the nuclear lamina in the highly metastatic clones. Intriguingly, SPANX, ESM1, and HMGA1 genes have all been linked with a highly aggressive and invasive phenotype in breast cancer. Genes not in LADs were also differentially regulated including HMGA1 (High Mobility Group protein 1) which is involved in chromatin decomposition and the formation of distorted DNA structures. These early results suggest that LADs and chromosome organization become disrupted during tumor progression, providing molecular insights into pathological findings correlating highly disorganized nuclei with poor prognosis.

P2215
Board Number: B359
Lamina associated domain dynamics during cell cycle investigated by super-resolution microscopy.
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3D nuclear organization and compartmentalization are important for regulation of gene expression. Lamina Associated Domains (LADS) are regions of chromatin associated with or in close proximity to the nuclear lamina. These regions are enriched in heterochromatin and genes found within them are largely inactive. Lamin A and C are hypothesized to be necessary for maintenance of this organization, at least in more differentiated cell types. Using murine fibroblast cells expressing the m6A tracer system and a GFP-coupled single chain antibody to detect LMNA/C we are able to study the dynamics of LADs and lamin protein using live cell imaging. Intriguingly, during the cell cycle we observed changes in LAD localization and organization consistent with a loss of organization during mitosis and robust reorganization in early G1. During G1 phase the LADs are organized at the periphery of the nucleus and associate with the nuclear lamina. In prophase, LADs collapse together at the periphery of the nucleus before moving to the center as full chromatin condensation occurs. LADs do not immediately return to the periphery after division but return several hours into G1 by an undetermined mechanism. Importantly, LADs maintain self-association throughout cell cycle. Imaging this system using super resolution microscopy, including the Nikon N-SIM and 3i lattice light sheet microscopes, revealed novel structural features of the LADs at a much higher resolution. Rather than appearing as a continuous layer within the nuclear lamina, the LADs form globules that associate with the lamina and each other in a dynamic fashion. These results emphasize the role of self-association in maintaining LAD organization and contribute to our overall understanding of LAD and nuclear organization.
P2216
Board Number: B360
Extracellular divalent ions rescue aberrant nuclear morphology through a novel heterochromatin formation pathway.
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The nucleus is a dynamic organelle responsible for maintaining the structural and functional organization of the genome. Proper nuclear morphology is critical for gene localization, gene expression, differentiation, and cellular function. In several human diseases, including progeria, cancers, and dystrophies, nuclear morphology becomes severely altered and exhibits protrusions termed “blebs”. Nuclear blebs are associated with altered lamina content and chromatin compaction. It is widely believed that lamina perturbations are responsible for bleb formation, but the role of chromatin compaction state in this process is poorly understood. We find a novel signaling pathway through which extracellular divalent ions (Mg+2 and Ca+2) alter histone modification state, which then dictates nuclear blebbing independent of lamins. Treating mammalian cells with histone deacetylase inhibitors increases euchromatin levels and leads to nuclear blebbing without altering lamin content. Conversely, treating histone deacetylase inhibitor induced blebs with divalent ions rescues nuclear blebs in a dose dependent fashion. Immunofluorescence confocal imaging reveals that treatment with divalent ions significantly increases the heterochromatic marks H3K27me3 and H3K9me2,3. Micromanipulation force measurements of isolated nuclei also reveal that nuclear morphology is tightly coupled with chromatin-based nuclear rigidity, which is determined by histone modification state. Furthermore, divalent ions also rescue aberrant nuclear morphology associated with lamina perturbations. Nuclear blebbing associated with lamin B1 depletion as well as nuclear morphological abnormalities in Hutchinson-Gilford Progeria Syndrome caused by mutant lamin A can be rescued via treatment with extracellular divalent ions. Immunofluorescence imaging reveals that treatment with extracellular divalent ions does not alter lamin levels, but instead restores heterochromatin markers that are depleted in these cells. Interestingly, even abnormal morphology of nuclei in the breast cancer model MDA-MB-231 can be partially rescued through increased extracellular divalent ions. Therefore, chromatin compaction through histone modification state is a major determinant of nuclear morphology and presents a potential therapeutic target.

P2217
Board Number: B361
Characterization of nucleolar-localized H4 histone variant, H4G.
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Histone variants are the non-allelic variants of core canonical histones. Numerous studies have showed their diverse roles in chromatin regulation. The most studied histone variants are those of H3 and H2A. Compared to H1, H2A, H2B and H3, histone H4 is considered to be the only one without histone
variants. We identified a primate-specific histone H4 variant HIST1H4G (H4G). The H4G protein has 85% protein sequence identity with canonical H4. We detected H4G RNA expression in malignant breast cancer cell lines MCF7, MCF7/LCC1 and MCF7/LCC2 but not in benign breast epithelial cell line MCF10a. Immunostaining of tagged-H4G shows its subcellular localization in nucleolus and cytoplasm whereas canonical H4 localizes in the nucleus. We further identified that amino acids in the third alpha helix of globular domain are responsible for H4G nucleolar distribution. In addition, we found that a nucleolar protein nucleophosmin1 (NPM1) interaction with H4G through the third alpha helix leads to H4G nucleolar localization. In conclusion, our research has characterized a new nucleolus localized histone variant H4G and identified NPM1 as a chaperone targeting H4G to nucleolus.

**Epigenetics and Chromatin Remodeling**

**P2218**

**Board Number: B362**

Hippocampal Neuron Stimulation Promotes Intracellular Tip60 HAT Dynamics with Concomitant Genome Reorganization and Synaptic Gene Activation.

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Coordinated transcription of genes within mammalian nuclei in response to external stimuli is a highly orchestrated process involving the interplay between epigenetics-mediated chromatin remodeling and RNA polymerase-mediated transcription. Recently, the emergence of the concept of transcription factories (TFs), characterized as specialized nuclear subcompartments enriched in hyperphosphorylated RNAPII and transcriptional regulatory proteins, suggests the potential for an additional mechanism directing coordinated and efficient gene transcription. While these nuclear transcriptional ‘hot spots’ have been implicated in the transcriptional co-regulation of partner genes in other cell types, its presence in hippocampal neurons and its role in activity-dependent transcriptional control within the brain remains relatively unexplored. Furthermore, while previous findings indicate a functional relevance of histone acetylation in activity-dependent gene expression, the full array of histone acetyltransferases (HATs) involved in this process remain to be determined. Our findings reveal that the HAT Tip60 shuttles into the nucleus following extracellular stimulation of rat hippocampal neurons and by utilizing chromatin immunoprecipitation (ChIP), further show that Tip60 binding patterns on activity-dependent synaptic plasticity genes are altered in response to extracellular stimulation. Combining immunocytochemistry with DNA fluorescent in situ hybridization, we show that neuronal stimulation alters the localization of these genes within the nucleus, corresponding to the mobilization of these co-regulated genes to RNAPII-rich TFs. Lastly, we show that Tip60 is found within the same TFs as the co-regulated synaptic plasticity genes following neuronal stimulation. Taken together, these data suggest that specific, directed compartmentalization of target genes, HATs and transcription machinery within hippocampal nuclei occurs following the introduction of external stimuli thereby enabling efficient, coordinated gene transcription. Our findings provide insights into a fundamental physiological gene expression paradigm governing transcriptional activation of co-regulated genes in response to external stimuli in hippocampal neurons.
P2219
Board Number: B363
β-actin dependent global chromatin organization and gene expression programs control cellular identity.
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During differentiation and development, cell fate and identity are established by waves of genetic reprogramming. Although the mechanisms are largely unknown, during these events dynamic chromatin reorganization likely functions to ensure that multiple genes involved in the same cellular processes are co-regulated depending on the nuclear environment. Emerging evidence suggests that changes in chromatin dynamics and genome organization may be dependent on nuclear actin and its function as part of chromatin remodeling complexes. In this study we determined the functional impact of β-actin on genome organization and gene activity. Using high content profiling of embryonic fibroblasts from a β-actin knockout mouse, we report major chromatin rearrangements and changes in histone modifications such as methylated H3K9. Genome-wide H3K9Me3 landscape changes correlate with gene up-regulation and down-regulation in β-actin knockouts. As part of the BAF complex, β-actin regulates the ATPase subunit Brg1. Compatible with this findings, we found a general loss of chromatin association at multiple genomic sites by the BAF chromatin remodeling complex subunit Brg1 in the absence of β-actin. Transcriptional profiling by RNA-seq revealed that actin-dependent chromatin reorganization was concomitant with the up-regulation of sets of genes involved in angiogenesis, cytoskeletal organization and myofibroblast features in β-actin knockouts. Some of these genes and phenotypes were gained in a β-actin dosage-dependent manner. Moreover, reintroducing an NLS-containing β-actin in the knockout cells affected nuclear features and gene expression. Collectively our results suggest that, by affecting the genome wide organization of heterochromatin through the chromatin-binding activity of the BAF complex, β-actin plays an essential role in the determination of gene expression programs and cellular identity.

P2220
Board Number: B364
PRC2-Ezh1 mediates transcription of hippocampal genes.
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Methylation in lysine 27 of histone H3 (H3K27me3) is mainly known as a critical component of gene silencing mechanisms during cell differentiation. This methylation is mediated by the widely-distributed Polycomb Repressive Complex 2 (PRC2). PRC2 methylase activity lays in both Ezh1 and Ezh2 catalytic subunits. Ezh1 is the main form in postmitotic cells, exhibits lower enzymatic efficiency, and has been also found associated with transcriptionally active promoters. Here, we have analyzed global profiles of H3K27me3 and H3K27Ac enrichments using publicly-available ChIP-seq data (http://www.ncbi.nlm.nih.gov/gds) from mouse hippocampal tissue. Ezh1 genomic distribution in hippocampi as well as in FACS-purified hippocampal neural nuclei was also assessed by Chip-seq. The data were clustered according to the expression profiles of putative targets. Our results indicate that
Ezh1 isoforms are differentially partitioned within nuclear compartments, with a distribution profile that changes during neuronal maturation. Ezh1 is found enriched at transcriptionally active gene promoters and enhancers and poorly associated with H3K27me3-marked genomic regions. Co-immunoprecipitation analyses show an association between soluble Ezh1 and RNA-Pol II. Together, our results provide a new layer of complexity among the epigenetic mechanisms that control genes associated with hippocampal neuronal function. We propose that PRC2-Ezh1 contributes to transcriptional activity of a selected group of neural genes by interacting, together with RNA Polymerase II, with their promoters and enhancers. FONDECYT-3150694/FONDECYT-1170878/FONDAP-15090007

P2221
Board Number: B365
Chromatin dependent glucocorticoid receptor plasticity within the cancer genome.
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Upon hormone stimulus, type I nuclear receptors (NRs) bind to their hormone ligand, enter the nucleus, and interact with regulatory elements in the DNA to elicit changes in transcriptional activity. NRs are required throughout development and in adult life, and NR activities are frequently targeted in the treatment of human diseases. Activation of Glucocorticoid Receptor (GR) with the synthetic corticosteroid dexamethasone is commonly used to promote fetal lung development and to combat auto-immune and inflammatory disorders. Elucidating the genetic and transcriptional mechanisms by which GR and other NRs perform their myriad functions is critical for human health and disease treatment.

Brg1, the catalytic ATPase of the human SWI/SNF complex, is critical for hormone-induced transcriptional regulation by GR. Previous studies from our lab demonstrated that upon DNA-binding, GR recruits Brg1 to remodel the local chromatin architecture to alter transcriptional output. Our work significantly expands this model and suggests that Brg1 may act on hormone-regulated genes prior to hormone stimulus and GR binding. Using ChIP-seq, we have generated a genome-wide map of Brg1 and GR chromatin interactions in untreated and dexamethasone-treated cells. We show that Brg1 interacts with many GR binding sites in untreated cells and is present at these sites upon dexamethasone treatment and subsequent GR binding. Using these and other data sets, we have identified three classes of GR binding sites that are differentially associated with hormone-dependent transcriptional events. These classes of binding site showed distinct patterns of chromatin accessibility, histone modification, and transcription factor binding. Examination of GR binding in Brg1-deficient cells revealed that disruption and restoration of Brg1 expression selectively alters the chromatin environment at these classes of GR binding sites. Taken together, these data suggest that GR elicits the transcriptional response to hormone via multiple distinct mechanisms that are dependent on specialized chromatin environments. Our classification of these genomic environments provides a more profound understanding of nuclear receptor function that will allow new approaches in drug development and disease treatment.
Tissue specific gene expression plays a critical role in regulating energy metabolism, and understanding the transcriptional mechanisms by which metabolically active tissues such as liver and adipose respond to caloric imbalance holds the key to better treatments for diseases such as obesity and diabetes. In order to better elucidate the transcriptional circuitry underlying these diseases, we set out to map the enhancer proteome in liver tissue, naive mesenchymal stem cells, and differentiating adipocytes using ChIP with selective isolation of chromatin-associated proteins (ChIP-SICAP). ChIP-SICAP targeting of H3K27ac, a well-characterized histone mark of active enhancers, uncovered roughly 500 proteins including sequence-specific transcription factors (TFs) and numerous members of co-activator and co-repressor complexes that define the landscape of active chromatin in hepatocytes and differentiating adipocytes. This analysis revealed a pivotal role for CEBP TFs in establishing enhancers in both settings. Strikingly in liver, both CEBPalpha and CEBPbeta rank within the top-five proteins enriched over IgG control, and they are the top-ranked TFs. To gain further insight into the roles of CEBPs, we performed ChIP-SICAP for CEBPbeta and identified a subset of the proteins comprising the H3K27ac proteome. Interestingly, although CEBP-bound chromatin is enriched for both the nuclear receptor GR and the bZip protein NFIL3 in both liver and differentiating adipocytes, other co-distributing TFs, such as TEADs and HNF1a, show strict tissue-specificity. Moreover, we observe that chromatin remodelers and transcriptional co-activators such as BRD2 and MED8 are dynamically recruited to CEBP-bound chromatin during the course of adipogenic differentiation, suggesting these factors may play important roles in the establishment of active chromatin at sites of CEBP binding. In contrast, repressor proteins such as transducin-like beta 1 X proteins and HDACs appear to be more constitutively associated with CEBP-bound chromatin in both liver and differentiating hMSCs. Together the enhancer proteomes revealed by ChIP-SICAP analyses suggesting novel insights into the establishment of tissue-specific gene expression programs.

Mutations in CDCA7, the SWI2/SNF2 family protein HILLS (LSH), or the DNA methyltransferase DNMT3b, cause Immunodeficiency–Centromeric instability–Facial anomalies (ICF) syndrome. While DNA methylation defects presumably cause this disease, little is known about the molecular function of CDCA7 and its functional relationship to HILLS and DNMT3b. Systematic analysis of how the cell cycle, H3K9 methylation, and the mitotic kinase Aurora B affect chromatin in Xenopus egg extracts revealed that HILLS and CDCA7 comprise a complex whose chromatin binding is inhibited by Aurora B. Although
HELLS alone fails to remodel nucleosomes, we demonstrate that the HHELLS-CDC7 complex possesses nucleosome remodeling activity. Furthermore, CDC7 is essential for loading HHELLS on chromatin, and CDC7 harboring patient ICF mutations fails to recruit the complex to chromatin. Together, our study identifies a unique bipartite nucleosome remodeling complex where the functional remodeling activity is split between two proteins, delineating the pathway defective in ICF syndrome.

**P2224**

**Board Number: B368**

**Measuring DNA Methyltransferase Activity in Breast Cancer Cells.**

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Epigenetics is the study of “heritable changes in gene expression not attributed to DNA sequence,” or as Adrian Bird described: “inheritance, but not as we know it” (Bird, 2007). The epigenetic mechanism of DNA methylation, or the addition of a methyl groups to the promoter regions of genes, is mediated in humans by the the DNMT1 enzyme. Abnormal methylation patterns influenced by DNMT1, such as the hypermethylation of tumor suppressing genes (TSGs) and/or hypomethylation of cancer-causing genes (oncogenes), can lead to carcinogenesis and cancer progression. Abnormal methylation is also a hallmark of breast cancer, a leading cause of death among women worldwide. The exact activities and functions of DNMT1 in breast cancer are not clearly understood. In this work, the activity and function of DNMT1 in the MCF-7 breast cancer cell line will be studied. We hypothesize that DNMT1 activity will be increased in MCF-7s in comparison to normal breast cancer cells. To date, standard curves using Bovine Serum Albumin (BSA) protein have been generated to define a linear range of 0-50 ug for quantifying protein in MCF-7 nuclear extracts. Furthermore, we have observed lower DNMT1 activity in MCF-7 nuclear extracts compared to commercially-provided pure DNMT1 with the use of an Enzyme-Linked Immunosorbent assay kit (ELISA). Future work will be done to observe DNMT1 activity in MCF-7s treated with varying concentrations of 5-azacytidine, a known DNA methylation inhibitor, with respect to normal breast cancer cells. This work holds importance in establishing DNMT1 as a potential therapeutic target for breast cancer treatment.

**P2225**

**Board Number: B369**

**Dissecting the synthetic lethality between htz1Δ and RPB2-2<sup>5L</sup>: the interplay between RNA Pol II and the nucleosome dynamics.**

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Histone H2A.Z, coded for by the HTZ1 gene in *Saccharomyces cerevisiae*, is a highly conserved variant of histone H2A with many reported important roles in chromosome segregation, transcription regulation, maintenance of heterochromatin-euchromatin boundaries, DNA repair, cell cycle control and resistance to genotoxic stress, among others. Unlike its homologues in other species, the protein is not essential in yeast, as deletion of this gene is not lethal. We previously reported that Htz1 has a role in transcription elongation, but the mechanism of this is not yet understood. We uncovered a synthetic lethality between a HTZ1 null (htz1Δ) and a mutation in the second largest subunit of the RNA pol II (rp2-2). That is, either mutant is viable individually but the combination is lethal suggesting that Htz1 and Rpb2 work together to facilitate an essential function. Moreover, the synthetic lethal phenotype is dominant, i.e. an extra wild type copy of the *RPB2* gene in the *rp2-2* mutant does not alleviate the dependence on

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Htz1, suggesting that rpb2-2 could stall on the elongation template in the absence of Htz1. In order to study the mechanism of the Htz1 role in transcription elongation, we have focused our efforts on the \(htz1Δrpb2-2^{SL}\) synthetic lethality. A second site suppressor analysis of the \(htz1Δrpb2-2^{SL}\) synthetic lethal uncovered links to \(set2\), which encodes a protein that methylates H3K36 in RNA polymerase II transcribed regions of the genome. It has been shown that in cells lacking Set2, initiation of RNA pol II transcription occurs inappropriately within the protein-coding regions of genes, rather than in the proximal promoter regions; a phenotype that has been referred to as “cryptic” initiation. It is thought that chromatin integrity in the wake of the polymerase passage is compromised in the absence of me-H3K36 and downstream modifications. We tested our strains for cryptic initiation phenotypes and have found that \(htz1Δ\) cells exhibit a mild cryptic initiation phenotype and \(rpb2-2\) has a strong phenotype. A plausible explanation for these results is that the Rpb2-2 mutant polymerase is particularly prone to aberrant transcription initiation and when nucleosome dynamics is altered in the absence of Htz1, the effect is exacerbated.

P2226
Board Number: B370
Investigating the role of NuA4 and Swr1 in regulating RNA splicing in \(Saccharomyces cerevisiae\).
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Eukaryotes undergo transcription and splicing simultaneously, allowing for coordination and regulation of these two processes. Currently, there are a few known connections between chromatin modification, which regulates transcription, and splicing found in yeast and metazoan, yet there is still much to study. The NuA4 histone acetyltransferase works with Swr1 chromatin remodeling enzyme to promote transcription. First, NuA4 acetylates histone H4, which is recognized by Swr1. Next, Swr1 inserts the histone variant H2A.Z, which is subsequently acetylated by NuA4. Interestingly, in \(Saccharomyces cerevisiae\), both NuA4 and Swr1 play a key role in the transcription of the ribosomal protein genes, which make up a large fraction of the intron containing genes. Thus, NuA4, Swr1, and H2A.Z are excellent candidates for coordinating transcription and splicing. Indeed, a role for H2A.Z in splicing was recently identified. Previous experiments from our lab revealed genetic interactions between genes encoding splicing factors and \(NUA4\) and \(SWR1\). Additionally, we found that deletion of the \(NUA4\) and \(SWR1\) proteins impact the splicing of mRNA. We are now testing whether NuA4 acetyltransferase activity is important for RNA splicing. We generated double mutants that contained a deletion of a splicing factor gene, as well as a point mutation in the catalytic domain of gene encoding the Esa1 subunit of NuA4, and showed that \(ESA1\) shows multiple genetic interactions with various splicing factor genes. We are now utilizing these strains to further elucidate the impact of NuA4 on splicing. Moreover, using a directed genetic screen, we show that mutations that alter the catalytic activity of NuA4 display genetic interactions with \(SWR1\), supporting the possibility of independent functions of each complex in RNA splicing. Together, our data implicate both NuA4 and Swr1 in regulating RNA splicing, and our future work will focus on investigating the specific mechanism(s) by which NuA4 and Swr1 impact splicing.
P2227

Board Number: B371

Elucidating the function of histone H4 acetylation in RNA splicing in *Saccharomyces cerevisiae*. A.G. Mendizabal¹, J.S. Kopew¹, N. Paripati¹, T.L. Kress¹; ¹Biology, The College of New Jersey, Ewing, NJ

The ability of RNA splicing and transcription to take place simultaneously allows for efficient coordination and regulation within cells. Few known connections exist between chromatin modifications, which regulate transcription, and RNA splicing in the yeast *Saccharomyces cerevisiae*, as well as in metazoa, leaving much to explore. Our previous research has shown that genetic interactions exist between splicing protein genes and the NuA4 complex genes in *S. cerevisiae*. In addition, we found that deletion or mutation of these NuA4 complex genes have impact RNA splicing. NuA4 plays a key role in regulating transcription by acetylating histone H4; following this, the Swr1 chromatin remodeling complex is recruited to the acetylated histone and ultimately deposits H2A.Z in place of H2A, allowing for transcriptional activity. Given these findings, our current research is focused on elucidating whether specific acetylation of histone H4 is required for coordinating splicing with transcription. We constructed an array of double mutant yeast strains comprising a deletion of an essential splicing factor gene and a point mutation in either H4K5, H4K8, H4K12, or H4K16 preventing chemical modification and conducted genetic interaction studies to identify synthetic sick/lethal, or suppressive genetic interactions between the two genes. The majority of the double mutant growth phenotypes reveal synthetic sick/lethal interactions when compared to their single mutant parent strains, suggesting a functional link between H4 acetylation and RNA splicing. RT-qPCR was used to determine whether the mutation of H4 resulted in different levels of pre-mRNA splicing. Our data show that there is generally higher accumulation of pre-mRNA in the histone point mutant strains when compared to the wild type, and that this trend is most pronounced in the H4K12 and H4K16 point mutants. In addition, mutation of H4 worsens splicing defects observed in splicing factor deletion strains, consistent with the genetic interactions observed. Our future research will focus on identifying the specific mechanism by which the histone H4 acetylation is functioning to regulate RNA splicing.

P2228

Board Number: B372

Age-related changes in diurnal non-coding RNA correlates with changes in genome-wide facultative heterochromatin. J. Park¹, W. Belden¹; ¹Animal Sciences Department, Rutgers University, The State University of New Jersey, New Brunswick, NJ

The circadian rhythm controls timed choreography of gene expression to maintain normal cell physiology and metabolism. The predominant regulatory mechanism of the circadian rhythm is a transcriptional negative feedback loop that facilitates, and is facilitate by circadian-regulated facultative heterochromatin. The long-term consequence of disrupted diurnal rhythm, or mutations in core clock genes, cause accelerated aging and an increased incidence in age-related diseases. To understand the mechanisms underlying age-related changes to circadian transcription, we performed a combined RNA-seq and ChIP-seq at two diurnal time-points for three different age groups. Our analysis revealed that the core clock genes remain rhythmic expression regardless of age, but circadian output, including numerous ncRNAs, changes dramatically with age. In addition, there is both diurnal and age-related change in Histone H3 lysine 9 trimethylation (H3K9me3) that correlates with the changes in gene...
expression. Collectively, the data suggest a model where age-related changes in diurnal gene expression occur, in part, due to age-related redistribution of rhythmic facultative heterochromatin. The age-related redistribution of rhythmic facultative heterochromatin is potentially mediated by changes in diurnal lncRNA expression creating an interlocked circadian-chromatin regulatory network that undergoes age-dependent metamorphosis.

P2229
Board Number: B373
Neurodegenerative Disease Proteinopathies Are Connected To Distinct Histone Post-Translational Modifications.
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Amyotrophic Lateral Sclerosis (ALS) and Parkinson’s disease (PD) are devastating neurodegenerative diseases involving the progressive degeneration of neurons. No cure is available for patients diagnosed with these diseases. A prominent feature for both ALS and PD is the accumulation of protein inclusions in the cytoplasm of degenerating neurons; however, the particular protein comprising these inclusions varies. The RNA-binding proteins TDP-43 and FUS are most notable in ALS, while α-synuclein aggregates into Lewy bodies in PD. In both diseases, genetic causes fail to explain the occurrence of a large proportion of cases and, thus, both are considered mostly sporadic. We aim to understand the role of epigenetics in ALS and PD. In particular, we are interested in delineating histone post-translational modification profiles in both yeast and human ALS and PD models. Histones from cell models recapitulating FUS, TDP-43, and α-synuclein proteinopathies are probed for different histone modifications. Remarkably, we find distinctive changes in histone modification profiles for each proteinopathy model. We detect the most striking changes in the context of FUS aggregation: changes in several histone marks support a global decrease in gene transcription. We also detect more modest changes in cells overexpressing TDP-43 and α-synuclein. Our results highlight a great need for the inclusion of epigenetic mechanisms in the study of neurodegenerative disease. We hope our work will pave the way for discovery of more effective therapies to treat patients suffering from ALS, PD, and other neurodegenerative diseases.

Nucleocytoplasmic Transport

P2230
Board Number: B375
Nuclear Envelope As A Physical Barrier In Electrotransfection.
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Electrotransfection is one of the widely used gene delivery approaches because it is simple to use, safe, and can result in successful delivery to difficult-to-transfect cells. The main limitation of this technology is low efficiency for some types of cells, compared to viral gene delivery approaches. This is caused partly by a lack of understanding of how plasmid DNA (pDNA) enters cells and travels to the nucleus for successful transgene expression. For exogenous molecules, such as pDNA, to enter the nucleus of a mammalian cell, they must overcome three physical barriers: (1) plasma membrane, (2) cytoplasm, and (3) nuclear envelope. Among them, the last one is probably the strongest barrier to gene transfer. To understand mechanisms of nuclear entry of electrotransfected pDNA, the current study investigated
effects of nuclear envelope break down (NEBD) on electrotransfection efficiency (eTE). The NEBD occurs when cells enter the M phase of the cell cycle. COS7 and HCT116 cells were synchronized in G2-M phase through treatment with nocodazole or released from double thymidine block for eight hours prior to electrotransfection. As a result, a large fraction of cells entered the M phase when electrotransfection was performed. The dissolution of the nuclear membrane was visualized after 30 minute treatment with FM64 dye, which binds to lipids that localize to the nuclear membrane via diffusion and membrane trafficking during this timeframe. Additionally, overexpression of Lamin A fusion protein, which is in direct contact with the inner nuclear membrane, was used to visualize the NEBD. Results from the study showed that both nocodazole treatment and eight-hour release from double thymidine block synchronized cells to G2-M phase. NEBD was observed prior to electrotransfection. The eTE was greatly enhanced by the treatments. These results indicated that eTE depended on the percentage of cells in the M phase within a period after electric pulse application, or the percent of cells that can be successfully transfected may be critically limited by the number of cells in a mitotic state where the nuclear membrane is dissolved. This study confirmed that the nuclear membrane was a critical barrier in delivery of pDNA via electrotransfection, and that dissolution of this barrier could significantly increase transfection efficiency. The significance of the study is two-fold. One is that the study provides new insight on mechanisms of nuclear entry of genes delivered by electrotransfection. The second is that results from the study can be used to develop novel strategies for significantly improving efficiency of electrotransfection, which could make electrotransfection a more competitive method for gene delivery in the clinic.

P2231
Board Number: B376
Characterizing mRNA export at high resolution in individual nuclear pores in single cells.
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The export of mRNA from the cell nucleus is one of the pillars of the gene expression pathway in eukaryotes. Conventional light microscopy does not allow high resolution analysis of specific and functional interactions that exported mRNA molecules undergo as they pass from the nuclear side of the nuclear pore complex (NPC), through the inner channel of the pore, and then out to the cytoplasmic side. Such limitations hinder our understanding of the biology of mRNA export within the context of gene expression and its regulation, and require the innovation of new approaches. We set out to characterize interactions involved in the various stages of mRNA transition through the nuclear pore at the level of individual NPCs. Using different types of mRNA export blocks together with single-molecule mRNA tracking in living human cells we could detect the regions of the NPC at which the mRNAs were stuck. Treatments that led to mRNA export blockage were the addition of wheat germ agglutinin (WGA), knockdown of the mRNA export factor NXF1/Tap (Mex67 in yeast), knockdown of Nup153, and the use of a dominant negative Dbp5 helicase. Our results suggest that the initial mRNA binding to the NPC does not require NXF1/Tap, whereas mRNA passage through NPC and release into the cytoplasm does. In accordance, super-resolution microscopy showed that NXF1/Tap is consistently occupying positions within the cytoplasmic side of the NPC. Measurements performed within individual nuclear pores using super-resolution STED microscopy, showed the positioning of NXF1/Tap in individual pores during regular and export blockage conditions. We then could further pinpoint these interactions with specific nucleoporins using a FLIM-FRET approach for measuring protein-protein interactions inside single NPCs. Altogether, these approaches have allowed the detection and measurements in intact cells, of specific interactions taking place between NXF1/Tap and mRNAs, and between NXF1/Tap and proteins within...
the NPCs. We have found that specific interactions at the cytoplasmic side of the NPC are crucial for the release of the mRNA to the cytoplasm, and have identified positions within the NPC that are mechanistically fundamental for the directional flow of mRNA and NXF1/Tap from the nucleus into the cytoplasm.

**P2232**

**Board Number: B377**

**Identification of a small, PH domain-embedded region responsible for the nuclear-cytoplasmic distribution of RGNEF.**

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Rho Guanine Nucleotide Exchange factor (RGNEF) is a 190 kDa protein implicated in both cancer and amyotrophic lateral sclerosis (ALS) pathologies. One area of interest in cancer research – and increasingly in ALS research – is the role nuclear-cytoplasmic shuttling plays in the disease mechanism. RGNEF has previously been reported to localize at low levels to the nucleus despite its large molecular weight, suggesting active transport likely plays a role. Here we have identified a 23 amino acid region composed of both a nuclear localization signal (NLS) and nuclear export signal (NES) that is capable of regulating RGNEF nuclear-cytoplasmic distribution.

We created a series of mutant constructs and observed their localization using confocal microscopy. First, deletion of the Pleckstrin Homology (PH) domain of RGNEF was sufficient to eliminate nuclear localization of the endogenous protein. In silico analysis was used to identify two clusters of basic residues that were located on the exterior of the PH domain and were accessible for karyopherin interaction. We predicted this region would constitute a bipartite NLS. Site-directed mutagenesis in three different constructs – endogenous RGNEF, a vector designed to exclude the fusion protein from the nucleus (pHM830), and a vector designed to direct the fusion protein to the nucleus (pHM840) – all consistently showed that these residues were both necessary and sufficient for nuclear localization of a protein of 160 kDa or greater molecular weight.

We were able to extrapolate from this set of data the presence of an NES. Expression of the PH domain in the pHM840 vector was sufficient to significantly reduce nuclear localization of the fusion protein compared to empty vector. We found that the linker region of the bipartite NLS contained a large cluster of hydrophobic residues, which were also accessible for karyopherin interaction. When considered as a linear amino acid sequence the NES is flanked by the NLS, though molecular modeling shows us that the two signals are independent of one another. We confirmed that the region promotes nuclear export in an exportin-1 dependent manner by treating cells with leptomycin B (LMB) and observing accumulation of the fusion protein in the nucleus.

To date, this is the first time a NES has been described embedded within a NLS. Given the role it plays in determining the nuclear localization of the protein we have termed this 23 amino acid region the multisignal transport (MT) region. As an RNA binding partner, the nuclear-cytoplasmic distribution of RGNEF may have profound effect on ALS and particularly cancer pathology. Future directions should focus on how the dual, opposing roles of this region are regulated to maintain healthy nuclear-cytoplasmic balance.
P2233
Board Number: B378
The role of specific sequence patterns of FG Nups on transport through the NPC.
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Nuclear Pore Complex (NPC) is the largest protein complex inside the cell that is responsible for bidirectional transport of cargo through the nuclear envelope. It is made up of ~30 different types of proteins that are called nucleoporins (Nups). Nups can be categorized into two major groups, FG Nups and non-FG Nups. FG Nups are disordered proteins that are rich in Phenylalanine-Glycine repeats and are major role players in nucleocytoplasmic transport. While it is commonly believed that the FG Nups enable transport process through transient and weak interactions with cargo complex, the mechanism by which the FG Nups enable fast yet selective transport has remained unknown. This question has motivated recent bioinformatics and computational biology studies on sequence features of FG Nups that may help understand the underlying reason for FG Nups’ specific function. We previously showed that there are evolutionarily conserved features in sequences of FG Nups that affects their conformation and dynamics in the NPC. These sequence patterns are long regions in the disordered region of FG Nups that only contain positively charged residues. These regions are low in charge density, and are located toward the N terminus of FG Nups. We called these patterns Like Charge Regions (LCRs). In our recent study, using bioinformatics techniques and in-house codes, we compared FG Nups with other disordered protein datasets, namely Disprot, IDEAL and MobiDB-Lite disorder predictions of Uniprot database, and showed that LCRs are specific to FG Nups, and are not found in other disordered proteins. These new results highlight the possible role of LCRs in transport process. In our last study, by using a previously developed coarse-grained molecular dynamics model we studied the role of LCRs in the conformation of FG Nups. In our current study, we added a representation of cargo complex to the system, and repeated the study. In this coarse-grained model, FG Nups are modeled as chains of beads with one-bead-per-amino acid and the cargo complex has been roughly modeled as a sphere with hydrophobic patches on it. Our results show that presence of LCRs is effective in the location of cargo inside different cross sections of NPC. Mutating the positive charges in LCRs to Alanine causes the cargo to move differently in the NPC and cover a different amount of volume inside the NPC. The difference in the mutated and wildtype results is dependent on the type of Nup that is being simulated. In conclusion, our results suggest that LCRs are evolutionarily conserved patterns that can be found only in sequences of FG Nups, and are important both in the conformation of FG Nups in the NPC and in the dynamics of cargo complex in the NPC.

P2234
Board Number: B379
Quantitative analysis of nuclear translocation of ERK by using a novel single molecule technique.
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Nuclear translocation of ERK is a key process in the signal transduction of MAP-kinase pathway. It has been shown to be the rate-limiting step and its non-linear kinetics has suggested to be essential to trigger digital response of cell fate decision from the analog input of growth factor signal (Y. Shindo, K. Iwamoto, K. Mouri, et. al., Nat. Commun., 2016). The observed non-linear kinetics suggests underlying positive-feedback mechanisms, but the molecular mechanisms still remain unclear. To elucidate the
mechanism, we wanted to build quantitative models of this process, which requires to measure parameters such as concentration of ERK in cytoplasm and nucleoplasm, diffusion coefficient, binding frequency of ERK to a nuclear pore, and the import and export rates through the nuclear pore. Fluorescent correlation spectroscopy or FCS has been used for the measurement of the concentration and the diffusion coefficient of protein in cytoplasm, but the signal from only a single point in the sample is measured at once. However, it would be beneficial to make simultaneously in the cytoplasm and in the nucleoplasm of the same cell for the analysis of the nuclear transport. Here we report the development of a new multi-point FCS method using laser scanning confocal microscope. Line scan image sequence is taken at high frequency by using the resonant scanner. Thus, the autocorrelation is calculated for each pixel, yielding the concentration and the diffusion coefficients for the diffraction limited spots along the line. In this presentation, we will first describe the principle of this method, and its validity compared with conventional single point FCS and raster scan image correlation spectroscopy (RICS). Finally, we will discuss on the latest results on the quantitative parameters of ERK translocation measured by this method, and the extension to the FCCS (fluorescent cross correlation spectroscopy) for the quantitative measurement of protein-protein interaction.

P2235
Board Number: B380
Super-resolution microscopy reveals the transport route of transmembrane proteins into the nucleus.
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The transport routes and mechanisms used by transmembrane proteins to reach the nucleus across the nuclear envelope remains in dispute. Using high-speed super-resolution microscopy, we have solved the dispute by identifying two distinct transport routes for different membrane proteins as they transport through the peripheral or central channels of the nuclear pore complexes.

P2236
Board Number: B381
High nuclear export efficiency and conformational changes of pre-ribosomal subunits revealed by high-speed super-resolution microscopy.
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The nuclear envelope (NE) in eukaryotic cells provides a barrier separating nascent pre-ribosomal subunits assembled in the nucleus from matured ribosomes translating proteins in cytoplasm. Nuclear pore complexes (NPCs) embedded in the NE function as the sole gatekeepers for pre-ribosomal subunits exiting the nucleus. However, the basic nuclear export mechanism for pre-ribosomal subunits through the NPCs remains obscure. Here we combined a high-speed super-resolution microscopy with Förster resonance energy transfer (FRET) to obtain real-time single-molecule trajectories for pre-60S and pre-40S through the NPCs in live HeLa cells. Remarkably, we found that approximately two thirds of all NPC-interacting pre-ribosomal subunits successfully reach the cytoplasm during their export time of $\sim$14 ms for pre-60S and $\sim$8 ms for pre-40S. Moreover, the unexpectedly high nuclear export efficiency, about two-fold over that of message RNAs, may be enhanced by multiple species of transport receptor recruited for pre-ribosomal subunits. Finally, dual-channel single-molecule FRET tracking provides
evidence that both pre-ribosomal particles undergo conformational changes during their export through the NPCs.

P2237
Board Number: B382
Karyopherins regulate nuclear pore complex barrier and transport function.
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Nucleocytoplasmic transport of nuclear localization signal (NLS)-specific cargos involves karyopherin receptors (e.g., Kapα and Kapβ1), a RanGTP gradient, and nuclear pore complexes (NPCs) that perforate the nuclear envelope (NE). Kapβ1 binds specifically to intrinsically disordered proteins called Phe-Gly nucleoporins (FG Nups) within each NPC to facilitate this selective transport. Otherwise, the FG Nups are widely accepted to form a permeability barrier that hinders the passage of non-specific macromolecules. However, because Kapβ1 also binds Kapα in the cytosol to ferry NLS-cargoes and RanGTP in the nucleus to deliver the cargo, the binding of one partner may functionally impact on another to orchestrate the entire transport continuum. To this end, NPC barrier selectivity, Kap traffic and NLS-cargo release are inexplicably linked yet how they are simultaneously regulated under equilibrium conditions remains incoherent and poorly understood. Here, we show using quantitative modelling and functional analysis of permeabilized cell assays that Kapα is essential for facilitating Kapβ1 turnover from NPCs in a RanGTP-dependent manner that is directly coupled to NLS-cargo release and NPC barrier function. This is underpinned by the binding affinity of Kapβ1 to the FG Nups, which is comparable to RanGTP-Kapβ1, but quantitatively stronger for Kapα-Kapβ1. On this basis, RanGTP is ineffective at releasing standalone Kapβ1 from NPCs. Depleting Kapα-Kapβ1 by RanGTP further abrogates NPC barrier function whereas adding back Kapβ1 rescues it. Therefore, the FG Nups are necessary but insufficient for NPC barrier function. Based on these findings, we reveal that Kapα is more than an adaptor for NLS-cargo but in fact underpins functional NPC control by mediating Kapβ1 occupancy and turnover. Thus, a deregulation of Kapα leads to defects in NCT that might result in cellular dysfunction. To conclude, Kaps constitute integral constituents of the NPC whose barrier, transport and cargo-release functionalities converge under Kap-centric control.

P2238
Board Number: B383
A novel role for the Calcineurin phosphatase at the nuclear pore.
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Calcineurin (CN) is the Ca2+/Calmodulin-activated serine/threonine protein phosphatase with a well-defined role in immune cell activation as well as critical roles in the brain, heart, and other tissues. CN-substrate engagement is mediated via conserved docking surfaces on CN that bind to Short Linear Motifs (SLiMs), termed PxxiIT and LxVP, in substrate proteins. Recent work in our lab has employed SLiM-directed approaches to identify the spectrum of CN substrates and interactors, thereby mapping the human CN signaling network and expanding our understanding of CN-dependent processes in...
human cells. Using Proteomic Peptide Phage Display analysis (ProP-PD), which directly selects for CN-binding SLIms from the human proteome, we identified many novel PxlIT- and LxVP-containing peptides from a diverse group of proteins that were not previously known to be regulated by CN. One such sequence is a PxlIT-containing peptide from NUP153, a peripheral nucleoporin that comprises part of the nuclear basket of the nuclear pore complex (NPC). Using in vitro and in vivo binding assays, we have shown that this conserved, novel PxlIT sequence is required for the interaction between CN and NUP153. The NUP153 PxlIT sequence lies within a heavily phosphorylated region of NUP153 that mediates many protein-protein interactions which are important for proper nuclear transport. Building upon previous studies demonstrating ERK phosphorylation of this region of NUP153, we have shown that CN dephosphorylates these ERK phosphosites on NUP153 in a PxlIT-dependent manner in vitro and that a NUP153 PxlIT mutant shows altered dephosphorylation in vivo. Together, these data suggest that NUP153 is a bona fide substrate of CN and establishes a role for CN dephosphorylation at the nuclear pore. In addition to our ProP-PD analyses, we have also employed computational and BioID/MS approaches to identify hundreds of novel CN substrates and interactors. These analyses have uncovered many additional CN-binding nucleoporins and nuclear transport factors, such as NUP50, another nuclear basket-associated nucleoporin that binds to CN via a conserved PxlIT sequence. Consistent with these observations as well as previous studies that demonstrate a role for Ca\(^{2+}\) in nuclear transport, we observe co-localization of CN to the nuclear rim as well as CN-dependent changes in nuclear transport of a GFP reporter. Altogether, these data demonstrate a novel point of regulation for CN in human cells and suggest a broader and previously uncharacterized role for CN in regulating nuclear transport.

P2239

**Board Number: B384**

**Soluble host factors CPSF6 and CypA determine the HIV-1 nuclear import pathway.**

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Transport of the human immunodeficiency virus type 1 (HIV-1) pre-integration complex (PIC) through the nuclear pore complex (NPC) is prerequisite to virus replication. The viral capsid protein (CA), a PIC component, directly interacts with specific nucleoporins (Nups), including the phenylalanine-glycine (FG) rich Nup153. N74D mutation of CA reduces Nup153 binding and HIV-1 dependence while increasing reliance on Nup155, indicating an ability to exploit secondary nuclear transport routes. Here we find that CA extends this discrimination to Nup35 and POM121, with the loss of either impairing infection under conditions where Nup153 is also utilized. The coordinate requirement of Nup35, Nup153, and POM121 enabled the investigation of controlling mechanisms. We show HIV-1 use of Nup35, Nup153, and POM121 is regulated by CPSF6 and cyclophilin A (CypA) interaction with CA. Both factors show a differential concentration gradient in cells with CypA enriched at the cytoplasmic face of the nuclear membrane, and CPSF6 predominantly localized to the nucleus. CypA binding in particular directs HIV-1 toward the Nup153-dependent nuclear entry pathway, whereas HIV-1 switches to a Nup155-dependent pathway in the absence of this interaction. This dual tropism in the utilization of nuclear entry pathways likely reflects the challenges of transporting a megadalton protein and nucleic acid complex that is susceptible to binding of competing factors in the cytoplasm prior to docking at the NPC. We propose that the HIV-1 core, comprised of multimeric CA in association with the viral nucleic acid and enzymatic proteins, directly functions as a nuclear transport receptor and exploits successive FG interactions to achieve transfer through the NPC. Notably, soluble non-karyopherin factors are key regulators of this process.

Monday-235
The Ran (Ras-related nuclear protein) GTPase plays critical roles in multiple cellular processes, including nucleocytoplasmic transport, nuclear envelope (NE) assembly and mitotic spindle assembly. During interphase, high levels of GTP-bound Ran (Ran-GTP) within nuclei and of GDP-bound Ran (Ran-GDP) in cytosol determine the direction of transport to and from the nucleus. After mitotic NE breakdown, Ran-GTP is concentrated near mitotic chromatin while Ran-GDP is more abundant distal to chromosomes. This pattern is essential because it spatially controls spindle assembly. RanBP1 and RanBP3 are conserved Ran-GTP binding proteins that can form complexes containing RCC1 (Regulator of chromatin condensation 1). RCC1 is a chromatin bound protein that is the sole exchange guanine nucleotide factor for Ran, converting Ran-GDP to Ran-GTP. RanBP1 and RanBP3 have also been implicated in transport cargo loading and unloading from nuclear transport receptors of the karyopherin family. The in vivo functions of these proteins remain somewhat enigmatic, particularly for RanBP1 because knockout mice lacking this protein are viable, albeit with male infertility. To test the role of RanBP1 and RanBP3 in shaping Ran-GTP gradients in somatic cells, we have employed CRISPR/Cas9 to insert sequences encoding an auxin-induced degron (AID) into the RanBP1 and RanBP3 genes of DLD-1 human cells that stably express the Transport Inhibitor Response 1 (TIR1) protein. Auxin family hormones bind TIR and promote its interaction with AID domain. TIR acts as a substrate recognition subunit for SCF-mediated ubiquitination, resulting in rapid auxin-dependent proteasomal degradation of AID-tagged substrates in a manner that is reversible and tunable. AID-tagged RanBP1 or RanBP3 can be rapidly degraded in a regulated fashion. This system allows us to test the influence of RanBP1 and RanBP3 on Ran gradients using previously developed Förster (fluorescence) resonance energy transfer (FRET)-based assays, to assess their influence RCC1 dynamics quantitatively, and to determine the extent of disruption in interphase nuclear trafficking and mitotic progression in their acute absence.

Nuclear transport factors NTF2 and importin α regulate nuclear size in Xenopus. In general, importin α levels positively regulate nuclear size, while increased NTF2 concentrations decrease nuclear size. Comparing melanoma cell lines that represent different stages of disease, we found that NTF2 expression levels inversely correlate with nuclear size. Consistent with this trend, normal melanocytes exhibited the highest levels of NTF2 and smallest nuclei. Based on transient transfections of different melanoma cell lines, as well as HeLa and MRC5 cells, we find that increased NTF2 expression leads to reduced nuclear size. We have generated a stable WM983B melanoma cell line that allows for titratable doxycycline-induced expression of NTF2. The largest reduction in nuclear cross sectional area (~10%) was observed with 20 ng/ml doxycycline which causes a 5-fold increase in NTF2 levels. These cells
showed downregulation of TSG101, which was performed using bafilomycin, leading to the degradation of vesicles. To begin to understand the mechanism of action of NTF2 in these assays, we performed RNAseq to determine how nuclear size impacts gene expression. These results will be presented and used for future FISH studies to examine how nuclear size affects gene position of differently-expressed genes.

We have also performed in vivo experiments in which NTF2-inducible WM983B melanoma cells were injected subcutaneously or intravenously into RAG2 and NSG mice. Tail vein injected RAG2 mice exhibited fewer lung metastases when treated with doxycycline, indicating that in vivo metastatic potential is reduced upon NTF2 overexpression. We are now planning to test a combination therapy in which we reduce nuclear size and treat with the well-established melanoma drug vemurafenib. Most melanoma patients with BRAF mutations show a good response to this drug, however relapse often occurs with drug-resistant melanoma cells expressing high levels of the cancer stem cell markers JARID1B, CD271, and fibronectin. According to our RNAseq data, cells expressing higher levels of NTF2 show reduced gene expression of JARID1B and fibronectin. Thus increasing NTF2 expression in drug-resistant tumors may improve prognosis and survival time.

P2242
Board Number: B387
Mechanistic Study of the Attenuation of Androgen Receptor Expression Level by TSG101-ART27 Interaction.
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The androgen receptor (AR) signaling pathway plays a vital role in the normal development and function of male reproductive organs. Dysregulation of the AR signaling pathway have been linked to diseases such as prostate cancer. Here, we demonstrate that tumor susceptibility gene 101 (TSG101) regulates AR transactivation via interacting with ART-27 protein, an AR interaction partner. In LNCaP cells, TSG101 overexpression recruits ART-27 to TSG101-decorated cytoplasmic vesicles and leads endogenous AR to TSG101-containing vesicles, resulting in reduced AR protein level and AR transactivation activity downregulation. Immunofluorescence microscopy demonstrated that TSG101-decorated cytoplasmic vesicles were associated with late endosomes or lysosomes and AR could be found within the Lamp2 positive TSG101 vesicles upon lysosomal protease inhibitor treatment. Furthermore, chloroquine or bafilomycin A1 treatment was able to restore TSG101-mediated AR expression reduction and depletion of ART-27 expression compromising AR co-localization in TSG101 vesicles. Based on this data, we conclude that the localization of AR to TSG101-containing cytoplasmic vesicles is mediated by ART-27, and TSG101-induced downregulation of AR expression associated with the late endosome/lysosome degradation pathway.
P2243

Board Number: B388

Structural determinants of Dnase1L3 that alter its localization during inflammation.
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One debilitating autoimmune disease that affects about 1.5 million Americans is Systemic Lupus Erythematosus (SLE). Although SLE is a multifactorial disease, one cause of SLE in humans and mice is disruption of Dnase1L3. Dnase1L3 is a secreted endonuclease that degrades chromatin in apoptotic microparticles. However, Dnase1L3 can also act intracellularly to promote inflammation. Dnase1L3 switches from extracellular to intracellular localization, and what domains are necessary for intracellular activity are unknown. To understand how this secreted protein functions intracellularly, we expressed Dnase1L3 both as a tdTomato fusion in mammalian cells to study trafficking and in bacteria to solve the structure. We found that lipopolysaccharide increased the nuclear localization of tdTomato-Dnase1L3, suggesting that inflammation can alter the nuclease trafficking. To purify recombinant Dnase1L3, we generated a His6-Maltose Binding Protein-Dnase1L3 fusion protein. We compared expression of this fusion between E. coli BL-21, and E. coli Rosetta-gami cells, and purification between either Nickel-NTA beads or amylose affinity resin. We found optimal expression using E. coli Rosetta-gami cells and purified the fusion protein to 97.89% purity using amylose resin, compared to 1.87% purity using Nickel-NTA beads. We removed the His6 tag and Maltose Binding Protein using Tobacco Etch Virus (TEV) protease and further purified Dnase1L3 using S-resin and size exclusion chromatography. Purified Dnase1L3 was active, based on nuclease assays. We confirmed the identity and size of Dnase1L3 by Western blot. Overall, we developed a purification method to generate high-purity Dnase1L3. We also found that inflammation perturbs the trafficking of tdTomato-Dnase1L3. Our combined structure-function approach will enable us to relate disease causing mutations in Dnase1L3 to the intracellular trafficking and structure of Dnase1L3. The structural information may provide key binding site information that will facilitate a greater understanding into the mechanisms behind SLE and characterize Dnase1L3 prior to use in SLE treatment.

P2244

Board Number: B389

Tau protein disrupts nucleocytoplasmic transport in Alzheimer’s disease.
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Tau protein, which normally functions to stabilize microtubules, is the major constituent of neurofibrillary tangles in Alzheimer’s disease (AD). The accumulation of tau in neuronal soma correlates with neuronal loss. However, the mechanism underlying tau associated neurodegeneration remains unclear. We now show that hyperphosphorylated tau can interact with nucleoporins (nups), protein constituents of the nuclear pore complex (NPC) and affect its functional integrity. Pathological tau leads to disruption of nuclear pore complex proteins, reduction of NPC complexes, cytoplasmic mislocalization of nups, and impairs nuclear export and import, in vitro, in tau overexpressing transgenic mouse models.
and in human Alzheimer tissue. Correspondingly, nuclear pore component, Nup98, surprisingly colocalizes with neurofibrillary tangles in neuronal soma, and both in vivo and in vitro directly interacts with tau and facilitates its aggregation. These data support the hypothesis that phospho-tau directly interacts with nuclear pore complex constituents, leading to their mislocalization and to disruption of nuclear pore function, raising the possibility that nuclear pore dysfunction contributes to tau induced neurotoxicity in Alzheimer’s disease.

P2245

Board Number: B390

ROCK-dependent phosphorylation of NUP62 regulates p63 nuclear transport in squamous cell carcinoma.
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p63, more specifically its Np63 isoform, plays essential roles in squamous cell carcinomas (SCCs); yet the mechanisms controlling its nuclear transport remain unknown. Nucleoporins (NUPs) are a family of proteins building nuclear pore complex (NPC) and mediating nuclear transport across the nuclear envelope. Recent evidence suggests a cell-type-specific function for certain NUPs; however, the significance of NUPs in SCC biology remains unknown. In this study, we report that nucleoparin 62 (NUP62) is highly expressed in stratified squamous epithelia, which is further elevated in SCCs. Depletion of NUP62 inhibited the proliferation potential and augmented differentiation of SCC cells. This loss of dedifferentiation was associated with defects in Np63 nuclear transport. We further found that differentiation inducible Rho kinase reduced an interaction between NUP62 and Np63 by phosphorylation of phenylalanine-glycine regions of NUP62, resulting in attenuated Np63 nuclear import. Our results characterize NUP62 as a key gatekeeper for Np63 and uncover its lineage-specific function to control cell fate through regulation of Np63 nuclear transport in SCC.

The Nuclear Envelope and Nuclear Pore Complexes 1

P2246

Board Number: B391

A nuclear localization signal is sufficient to target membrane proteins to the nuclear envelope in plants.
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Among protein targeting pathways, the journey of a transmembrane protein to the inner nuclear membrane (INM) is likely the least understood. However, understanding trafficking to the INM has increased in importance, as many INM proteins have been identified with roles in chromatin organization, nuclear morphology, meiosis, and nuclear movement, and have further been implicated in human disease. Two primary theories of how proteins reach the INM have been described, both with experimentally demonstrated backing: a. passive lateral diffusion coupled with local nucleoplasmic retention, and b. signal-driven nuclear import. In some cases of signal-driven import, nuclear localization signals (NLSs) have been implicated. All currently available data on INM targeting stem from the

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eukaryotic supergroup Opisthokonta (including animals and fungi). Here, we ask if an NLS-driven pathway for INM protein targeting exists in another supergroup, the Archaeplastida (plants). The SV40 Large T Antigen NLS was fused to the Arabidopsis thaliana ER tail-anchored protein PICL tagged with GFP (“NLS-PICL”). Subcellular targeting of fusion proteins was assessed after transient transformation of Nicotiana benthamiana leaf epidermal cells and the enrichment of the protein at the NE compared to the ER was quantified. Addition of this NLS was sufficient to enrich PICL at the NE. To differentiate between association with the outer versus inner nuclear membrane, we applied high-resolution Airyscan confocal microscopy. NLS-PICL displayed a localization indistinguishable from an INM or INM-associated protein, indicating that addition of an NLS is sufficient to target a tail-anchored ER membrane protein to the INM.

Alternative NLSs- five monopartite and four bipartite- were then tested for their ability to enrich chimeric membrane proteins at the NE. NLS fusions resulted in various degrees of NE enrichment. Based on these data, the NE enrichment of one monopartite and one bipartite NLS were analyzed by Airyscan confocal microscopy. Chimeric membrane proteins fused with either NLS localized to the INM.

Dependence of INM targeting on the spacing between NLS and TMD is currently being tested. Rules for plant INM targeting thus defined will be used to (a) artificially target novel functions to the plant INM, and (b) generate bioinformatic search criteria to identify unknown plant INM proteins.

**P2247**

**Board Number: B392**

**Biochemical fractionation of Xenopus extract to identify components limiting for nuclear growth,**

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Nuclear size dramatically reduces during early *Xenopus laevis* embryogenesis. From fertilization to gastrulation (stages 10-12), there is a ~16-fold reduction in the volume of individual nuclei. The regulatory mechanisms responsible for these nuclear size reductions have not been fully elucidated. We are interested in the contribution of cytoplasmic volume to the regulation of nuclear size during early embryogenesis. We use microfluidic devices to encapsulate *X. laevis* stage 10 embryo extracts, generating cytoplasmic droplets of defined size and shape. Nuclei in droplets expand to a new steady-state size after ~3 hours. We find that nuclei exhibit similar growth trends in spherical and flattened droplets of comparable volume, indicating droplet shape minimally affects nuclear growth. Additionally, nuclear volume increases by 1.4- to 3-fold in droplets ranging in volume from 0.02 nL - 0.5 nL, with larger increases occurring in larger droplets. These data indicate that the volume of embryonic cytoplasm is limiting for nuclear growth. However, in larger than ~0.5 nL droplets, the increase in nuclear volume reaches a threshold of ~3-fold. Compared to stage 8 blastomeres (~0.8 nL) in vivo, final nuclear volume in ~0.8 nL droplets is two times less than for nuclei in stage 8 embryos. Furthermore, when gastrula-stage nuclei are treated with stage 5 embryo extract and encapsulated in gastrula-cell-sized droplets (~0.08 nL), the average nuclear volume increases ~3-fold more than in gastrula-stage embryo extract droplets. These results suggest that both cytoplasmic composition and volume contribute to nuclear size scaling. We are now testing the hypothesis that limiting components in later stage embryo extract contribute to nuclear size scaling. We developed an in vitro assay in which clarified cytosol from egg extract causes stage 10 nuclei to grow. The cytosolic nuclear growth activity is heat-sensitive and dependent on importin alpha-mediated nuclear import, and we have ruled out several potential candidates, including nuclear lamins. Fractionation of cytosol through gel filtration and anion
exchange chromatography results in two active fractions, and proteomic analysis of these fractions has generated a list of candidate proteins that are limiting for nuclear growth in our assay. We are currently testing these putative nuclear size regulators both in vitro and in vivo.

P2248
Board Number: B393
SPOP regulates the levels of the nuclear pore protein NupJ.
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Cell processes like growth and division are tightly regulated. One such mechanism of regulation is ubiquitination. Ubiquitination can change a protein’s localization or activity, or it can mark the protein for degradation by the ubiquitin proteasome system. The final step of ubiquitination, transferring ubiquitin to the target protein, is mediated by E3 ligases and their substrate adaptors, proteins that allow E3 ligases to be selective in choosing their targets. Understanding the targets of E3 ligases and substrate adaptors, then, is crucial to understanding cell regulation and disease mechanisms linked to misregulation of protein levels and activity. SPOP is a Cul3 E3 ligase substrate adaptor whose targets, such as c-Myc, DAXX, and ERG, are crucial for cell cycle progression and proliferation. Through a mass spectrometry screen, we identified SPOP as a potential regulator of NupJ, a nuclear pore protein. Knockdown of SPOP via siRNA in HeLa cells leads to increased protein levels of NupJ via immunoblotting, and SPOP and NupJ both co-localize at the nuclear membrane via immunofluorescence microscopy. Moreover, co-immunoprecipitation assays demonstrate that SPOP and NupJ bind to each other in vitro. Similar to overexpression of NupJ, siRNA against SPOP leads to an increase in the number of nuclear envelope defects. Our results suggest that SPOP targets NupJ for ubiquitin-mediated proteasomal degradation.

P2249
Board Number: B394
Actin facilitates nuclear envelope breakdown by separating nuclear membranes from the lamina in starfish oocytes.
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In Metazoa, the nuclear envelope (NE) disassembles and reassembles in every division. This requires a concerted disruption of links between the NE components: the nuclear membranes, their resident proteins, nuclear pores (NPCs) and the underlying lamina, an intermediate filament scaffold stabilizing the NE. A phase of sudden and complete rupture of the NE appears to be a conserved feature of NEBD across animal species. This sudden rupture, which is crucial to the timely capture of chromosomes by spindle microtubules, appears to involve mechanical forces that in cultured mammalian cells are generated by microtubules tearing the NE – although the exact way the force is transduced and the type of membrane rearrangements brought about, remain completely unknown.

We have recently uncovered that in the starfish oocyte it is rather a transient Arp2/3 nucleated F-actin shell assembling on the inner side of the NE that drives rapid rupture of the large oocyte nucleus during meiosis (Mori et al. 2014). Through immunostaining and live cell imaging, we have found that this transient cortex-like structure assembles within the lamina and sprouts dynamic spike-like protrusions
towards the nuclear membranes. As consequence, the nuclear membranes appear to separate from the underlying lamina, which at this time are still intact and maintain a tight-knit network, as confirmed by super resolution microscopy (SMLM). We used a correlative light-electron-microscopy (CLEM) approach to gain more insight into the morphological changes within the nuclear membranes that might transpire from the observed separation of NE layers. To this end, we used fluorescence imaging to capture oocytes at the moment of shell formation, just prior to NE rupture, a time-window of ~1-2 minutes. These correlative EM micrographs reveal the morphology of NE membranes at the exact sites of destabilization. We observe fibrous spikes (presumably F-actin) underlying protrusions in the NE membrane. Moreover, we find that these protrusions are in areas devoid of NPCs, in contrast to adjoining stretches of the NE, which are packed with pores. Based on these data, we propose that F-actin shell with its spikes puts force on the weakened NE areas – presumably stripped of nuclear pores prior to this stage of NEBD – and as a consequence separates the membranes from lamina. This in turn leads to destabilization of the NE and results in breaks. Thereby, our data for the first time provide detailed characterization of the dramatic reshaping of the NE during its breakdown.

P2250
Board Number: B395
Mixing of parental genomes after fertilization in C. elegans involves fusion and fenestration of pronuclear membranes.
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In a fertilized embryo, the nuclear envelopes (NEs) of the maternal and paternal pronuclei must disassemble to allow mixing of two haploid genomes, leading to the formation of a single diploid nucleus in the zygote. The regulation and mechanics of this process are poorly understood but they are clearly important as premature or delayed disassembly could result in failure to incorporate chromosomes in a zygotic nucleus. It is assumed that NEBD occurs once the two pronuclei are in close apposition, but the precise order of events, and in particular the architecture of the pronuclear membranes, is unclear. To address this, we used the C. elegans embryo and followed NE dynamics and architecture using two orthogonal techniques, live cell fluorescence microscopy and 3D electron microscopy, specifically Focused Ion Beam - Scanning Electron Microscopy (FIB-SEM). After fertilization, the maternal and paternal pronuclei migrate towards each other and form a stable interface where NEs are closely juxtaposed. The NE between maternal and paternal pronuclei is breached via a membrane fenestration in the vicinity of the chromosomes only after the maternal and paternal chromosomes have aligned on their respective metaphase plates. This membrane gap then expanded as chromosomes moved in opposite directions in anaphase. Using FIB-SEM we visualized the 3D architecture of the NE fenestration through the four juxtaposed pronuclear membranes. We found that breaching the nuclear membranes between the two pronuclei involves the formation of membrane gaps that span all four membranes, the most prominent of which is in the vicinity of the aligned chromosomes. Moreover, we detected multiple sites where the membranes of the two pronuclei physically interact, forming membrane junctions. Our preliminary data suggest that NE fenestration involves membrane fusion between the two pronuclei at multiple sites, leading to the formation of gaps across all four membranes. We further speculate that the presence of the chromosomes, and perhaps signals emanating therefrom, contribute to the expansion of NE gaps in the vicinity of the chromosomes.
We previously reported that inactivation of the conserved Polo-like kinase 1 (PLK1) results in a failure in pronuclear membrane fenestration and inhibition of parental chromosome mixing in the *C. elegans* zygote (Rahman et al., MBoC 2015). Recently we identified five additional genes that upon down-regulation by RNAi fail to fenestrate the pronuclear membranes. We are currently investigating how these genes contribute to the timely NEBD after fertilization in the *C. elegans* embryo.

**P2251**
**Board Number: B396**
Basket nucleoporins “fingerprints”.
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Exchange of molecules between the cytoplasm and the nucleus occurs through conduits called nuclear pore complexes (NPCs). NPCs consist of roughly 30 distinct proteins (nucleoporins), forming a central channel with filaments extending into the nucleus and cytoplasm. Besides macromolecular trafficking, nucleoporins participate in the control of gene expression, chromatin maintenance and mitotic progression. In particular, Nup153, Tpr, and Nup50 localize to nucleoplasmic filaments, and they are collectively called the basket nucleoporins. The nucleoplasmic filaments have been proposed to serve as a platform for RNA modification and export, as well as for chromatin remodeling. Their action in these processes offers a rich variety of possible mechanisms for biological regulation via nucleoporins and for coordination amongst cellular functions. However, understanding the roles of individual basket nucleoporins in vertebrate cells is difficult because their depletion by RNAi requires an extended incubation, during which the all processes may be increasingly disrupted, potentially resulting in a highly pleiotropic phenotypes, many of which are secondary consequences of nucleoporin loss. To circumvent this problem, we created CRISPR/Cas9-targeted cell lines, where the basket nucleoporins Nup50, Nup153, and Tpr are endogenously targeted with Auxin Inducible Degron (AID) tags. The AID-tagged nucleoporins localize correctly, are functional within NPCs and are rapidly degraded upon Auxin addition (<2 hours). To assess the role of each nucleoporin, we followed cell growth in the absence and presence of Auxin, as well as nuclear trafficking and the immediate response in gene expression profile (RNA-sequencing). Moreover, we assessed the interdependence of the basket components, and associated with the basket proteins (SENP1, SENP2, MAD1) on each other, on the stability of the assembled nuclear pore, and ability to reform the nuclear pore post mitosis. Overall, our data indicate that basket nucleoporins play distinct, individual roles in nuclear function and gene expression. This system will allow us to dissect their roles at a molecular level.

**P2252**
**Board Number: B397**
Analysis of individual subunits within the Nup107-160 complex of the Nuclear Pore.
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The nuclear pore complex (NPC) acts as a conduit for transport between the interphase cytoplasm and nucleus. NPCs consist of roughly 30 distinct proteins (nucleoporins), forming a channel through the nuclear envelope. The central domain of NPCs consists of three co-axial rings that each display a lattice-
like arrangement, and that are called the cytoplasmic ring, inner ring, and nucleoplasmic ring, respectively. The Nup107-160 complex contains nine core nucleoporins (Nup37, Nup85, Seh1, Sec13, Nup96, Nup107, Nup133 and Nup160), with a tenth subunit called ELYS required for chromatin recruitment. The Nup107-160 complex forms the scaffold underlying the cytoplasmic and nuclear rings. Due to this major structural role, Nup107-160 complex organization has been intensely studied. Interestingly, the Nup107-160 complex also associates with kinesinocores in metazoan mitosis, where it plays a transport-independent role in spindle assembly and chromosome segregation.

In vivo analysis of individual vertebrate Nup107-160 complex members during interphase and mitosis has been problematic because their abundance and stability makes them difficult to manipulate with standard methods (e.g., RNAi). The extended time required for depletion causes progressive defects in both interphase and mitotic functions that can produce adverse secondary consequences. Moreover, the levels of non-targeted subunits decrease during extended RNAi depletion, possibly suggesting that they become unstable when the larger complex is absent. To address this problem, we have used CRISPR/Cas9 to add Auxin-Induced Degron (AID) tags along the genomic locus of the individual subunits in human DLD-1 cells. AID-tagged nucleoporins assemble into functional NPCs, and they are degraded rapidly (<4 hours) after auxin addition, with minimal impact on the stability of other Nup107-160 complex members. We are currently using these cells to analyze the in vivo functions of Nup107-160 complex components. We have assessed the roles of Nup107-160 complex subunits in nuclear trafficking through comparison of nuclear import and export in the absence and presence of auxin. We will also discuss how individual complex members contribute to the structural stability of NPCs, and the inter-dependence between subunits for Nup107-160 complex persistence at existing NPCs, as well as for spindle function and post-mitotic NPC assembly.

P2253

Board Number: B398

Nucleoporin-dependence of Karyopherin Dynamics in Mammalian Cells.
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Nuclear pore complexes (NPCs) are essential in eukaryotes, and they facilitate gated transport of macromolecules between the interphase cytoplasm and nucleus. NPCs consist of roughly 30 distinct proteins called nucleoporins (Nups), forming a channel through the nuclear envelope. The barrier function of NPCs is established by a subset of nucleoporins (FG Nups) that possess tandem repeats of motifs based on a Phe-Gly core. These FG repeats also enable selective nuclear-cytoplasmic trafficking through binding to karyopherins, a family of Ran-GTP binding receptors that mediate nuclear import and export of cargo proteins and RNAs. Karyopherins form multiple low-affinity hydrophobic interactions with FG Nups, allowing them to selectively traverse NPCs. An intriguing possibility is that specific karyopherin-Nup interactions give rise to distinct import pathways. However, it has been technically difficult to examine the requirements of individual vertebrate Nups for particular karyopherin transport events in vivo. The abundance and stability of Nups makes them problematic to manipulate with standard methods (e.g., RNAi): The extended time required for depletion causes progressive defects in both interphase and mitotic functions that can produce adverse secondary consequences, as well as disrupt the localization and stability of other Nups over time. To address this problem, we have used CRISPR/Cas9 to add Auxin-Inducible Degron (AID) tags at the genomic loci encoding individual Nups in human DLD-1 cells. We find that AID-tagged Nups assemble into functional NPCs, and they are degraded rapidly after Auxin addition. Here, we examine the localization and dynamics of karyopherins within cells after Auxin-induced depletion of individual Nups, in order to establish the distinct impacts of Nup loss.

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on different transport pathways. In particular, we focus on the evolutionarily conserved, essential karyopherins, Importin-β, Transportin, RanBP5, Crm1, and CAS which are mediators of major cellular import and export pathways. We have quantified the relative nuclear-cytoplasmic distributions of these receptors in fixed control and Nup-depleted cells by immunofluorescence, as well as mCherry-tagged karyopherin relocalization by live cell imaging before and after Nup degradation. Additionally, we are exploring changes in steady state dynamics of karyopherins following Nup-degradation using Fluorescence Recovery After Photobleaching. Depletion of a subset of Nups results in altered karyopherin transport dynamics, suggesting a specific requirement of karyopherins for these Nups during import and export. Together, these data provide useful insights into the specific relationship between individual Nups and specific import and export pathways.

P2254

Board Number: B399

Functional analysis of nucleoporins on the cytoplasmic face of the nuclear pore complex.

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Nuclear Pore Complexes (NPCs) mediate bidirectional transport of molecules between the cytoplasm and nucleus. NPCs consist of around 30 distinct proteins (nucleoporins) that form a channel through the nuclear envelope, with filaments extending into the nucleus and cytoplasm. Nucleoporins associated with the cytoplasmic filaments (CFs) include RanBP2 (also known as Nup358), Nup214, Nup88 and Aladin. RanBP2 binds the SUMO1-modified form of the Ran GTPase activating protein (RanGAP1•SUMO1), and the SUMO conjugating enzyme Ubc9 in a stable complex (RRSU complex). CFs interact with transport complexes as they enter and exit the nucleus, and CFs interact with components of the microtubule cytoskeleton. During mitosis, some CF components localize on mitotic spindles and play important roles in spindle assembly or function. In particular, the RRSU complex associates to mitotic kinetochores in a Crm1- and Ran-dependent manner, and this recruitment is important for the formation of spindle-kinetochore attachments. Nup214 and Nup88 have likewise been reported to have important mitotic roles. It is difficult to assess the functions of individual vertebrate nucleoporins using conventional gene knockdown or knockout systems because they are abundant and highly stable proteins, because they have important roles in both interphase and mitosis, and because they are often encoded by essential genes. To circumvent these problems, we are using CRISPR/Cas9 to biallelically insert an auxin-induced degron (AID) tag into CF nucleoporins genes in DLD-1 cells. AID-tagged CF nucleoporins correctly local in functional NPCs. Upon auxin treatment, they are rapidly and specifically degraded, allowing us to assay changes in cellular functions. We are particularly investigating nuclear transport, gene regulation, mitotic progression, and post-mitotic nuclear envelope and NPC re-assembly. Our results will be discussed in comparison to earlier reports, and we will discuss how these proteins individually contribute to NPC function during the cell cycle in higher eukaryotes.
P2255
Board Number: B400
Torsin A expression in budding yeast reveals a connection to conserved lumenal domains of the nuclear pore complex.
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Early onset hereditary dystonia is a neurodegenerative disease caused by a 3 bp (ΔE) deletion in the DYT1 gene, which encodes the AAA+ ATPase TorsinA (TorA). TorA is an atypical ATPase that localizes to the endoplasmic reticulum/nuclear envelope lumen: its ATPase activity is stimulated by binding to the lumenal domains of either the integral inner nuclear membrane protein LAP1 or the resident ER protein, LULL1; however, its substrates and function remain ill defined. Accumulating evidence supports that TorA functions at the nuclear envelope, perhaps during nuclear pore complex (NPC) biogenesis: expression of TorA-ΔE in developing neurons and the knockout of all Torsin alleles in cell culture results in the formation of nuclear envelope herniations over NPC-like structures. As the NPC biogenesis pathway is thought to be well conserved, we hypothesized that the machinery that Torsin acts on is likely also present in budding yeast: morphologically similar nuclear envelope herniations have long been observed upon perturbing NPC biogenesis in this organism. We therefore expressed TorA-GFP and TorA-ΔE-GFP in S. cerevisiae and observed a discrete accumulation of these fusion proteins at the nuclear envelope, likely at spindle pole bodies. Consistent with the idea that TorA impacts nuclear envelope function, genetic backgrounds with deletions of conserved integral nuclear envelope proteins were sensitive to TorA or TorA-ΔE overexpression. Most remarkably, an unbiased affinity purification strategy revealed that TorA-GFP interacts with Pom152, a transmembrane domain-containing nucleoporin that possesses a well conserved cadherin-like lumenal domain. As TorA-ΔE-GFP failed to interact with Pom152, our data are consistent with a model in which TorA-ΔE-linked nuclear envelope herniations might be a result of an inability of TorA to interact productively with the NPC biogenesis machinery. Our data therefore provide new avenues to interrogate the dystonia-disease mechanism while further suggesting the existence of structural motifs that have been well maintained through evolution.

P2256
Board Number: B401
Chm7/CHMP7 is recruited to nuclear envelope herniations through the winged helix domain of Heh1/LEM2.
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The endosomal sorting complexes for transport (ESCRT) are recruited to the nuclear envelope upon the imposition of genetic nuclear pore complex (NPC) assembly blocks (Webster et al. Cell. 2014; Webster, Thaller et al. EMBO. 2016). In particular, yeast strains with nuclear envelope herniations (Wente and Blobel, JCB. 1993; Scarcelli et al. JCB. 2007) exhibit a striking accumulation of the ESCRT Chm7/CHMP7 suggesting that ESCRTs might seal off defective NPCs in a quality control mechanism that remains to be fully elucidated. It is unclear, for example, whether Chm7 is even recruited to NE herniations let alone...
whether it plays a direct role in their biogenesis or resolution. Here, we take advantage of correlative light electron microscopy to show that a gain of function mutant of Chm7 (chm7\textsuperscript{OPEN}) accumulates at nuclear envelope herniations. Affinity purification of chm7\textsuperscript{OPEN} further confirms the importance of the Lap2-emerin-MAN1 (LEM) domain integral inner nuclear membrane protein, Heh1, and specific nucleoporins, as physical interactors. We also further refine the molecular determinants of Chm7 recruitment to conserved amino acid residues in the C-terminal winged helix domain of Heh1, which are also required to support viability of CHM7-dependent genetic backgrounds. Ongoing efforts are examining the morphology of the nuclear envelope in strains lacking CHM7, which show discontinuities consistent with ruptures. These data suggest an emerging relationship between herniations and loss of nuclear envelope integrity.

P2257

**Board Number: B402**

Towards defining the interactome of LEM-domain proteins and ESCRTs during nuclear pore complex biogenesis.

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The biochemical interactions that contribute to de novo nuclear pore complex (NPC) assembly and endosomal sorting complexes required for transport (ESCRT)-mediated NPC assembly-surveillance remain ill defined. For example, in previous work, we determined that the budding yeast Lap2-emerin-MAN1 (LEM)-domain containing integral inner nuclear membrane proteins, Heh1 and Heh2, recruit the ESCRTs to the nuclear envelope, likely to sites of NPC assembly (or misassembly) (1, 2). However, the mechanism by which the LEM domain proteins and/or the ESCRTs identify de novo NPC assembly sites remains unknown. To shed light on this question, we have used an affinity-pulldown strategy to isolate native complexes of Heh1, Heh2 and the nuclear envelope-specific ESCRT, Chm7/CHMP7 from both wildtype cells and those where NPC assembly is genetically inhibited. These experiments show that Heh2 stably interacts with either a subset of NPCs and/or intermediates in the NPC assembly process whereas the interaction between Chm7, Heh1 and nuclear pore complex proteins (nucleoporins/nups) can be stimulated by imposing NPC assembly blocks. These data provide a foundation to identify the molecular mechanism that trigger ESCRT-recruitment to the nuclear envelope.

References:

2. Webster BM, Thaller DJ, Jäger J, Ochmann SE, Borah S, Lusk CP (2016) Chm7 and Heh1 collaborate to link nuclear pore complex quality control with nuclear envelope sealing EMBO J. 35:2447-2467

P2258

**Board Number: B403**

DNA-origami based platforms for investigating the properties of FG-nups within nuclear pore complex-like architectures.

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The Nuclear Pore Complex (NPC) is the major conduit of macromolecular transport between the nucleus and cytoplasm. At the core of this massive protein complex is a central channel filled with intrinsically disordered proteins rich in Phe-Gly (FG) amino acid residues in repetitive motifs. A clear understanding of the mechanisms underlying the NPC’s selective permeability properties remain to be fully defined and are likely dependent on the collective properties of these “FG-nups”. Here, we use a technique called DNA-origami to generate a ring-like scaffold with dimensions that mimic the native NPC central channel. By engineering “anchor points” with 8-fold radial symmetries for recombinantly produced FG-nups, these Nucleoporins Organized by DNA” or “NuPODs allow the systematic examination of the collective properties of FG-nups within relevant 3D architectures, with precise stoichiometries and spatial positioning. So far, we have successfully assembled up to 48 copies of either the FxFG nup, Nsp1, or the GLFG nup, Nup100. Using transmission electron and super-resolution microscopy, these FG-nups exhibit unique morphologies; high speed atomic force microscopy shows that both FG-nups exhibit remarkable mobility within the DNA rings. We will ultimately utilize NuPODs as a platform for investigating increasingly complex networks of FG-nups and their ability to function as a permeability barrier.

P2259
Board Number: B404
Nuclear Pore Selective Barrier Dynamics as Revealed by High-Speed Atomic Force Microscopy in Colorectal Cancer Cells.
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Nuclear pore complexes (NPCs) are the sole gates embedded in the nuclear envelope (NE), acting as a key regulator of transport between the cytosol and the nucleus. NPCs consist of ∼30 proteins, termed nucleoporins. About one-third of nucleoporins harbor unstructured, intrinsically disordered phenylalanine-glycine repeats (FG-Nups), which involved in transport selectivity. Because the barriers insert deeply in the NPC, they are nearly inaccessible. Several in vitro barrier models have been proposed; however, the dynamic FG-Nups protein molecules themselves are imperceptible in vivo. We show here that high-speed atomic force microscopy (HS-AFM) is a potential tool to visualize nanotopographical changes of the nuclear pore central channel in colorectal cancer cells. Furthermore, using MLN8237/alisertib, an apoptotic and autophagic inducer currently being tested in relapsed cancer clinical trials, we showed the functional loss of nucleoporins, particularly the deformation of the FG-Nups barrier, in dying cancer cells. We propose that the loss of this nanoscopic resilience is an irreversible dying code. These findings not only illuminate the potential application of HS-AFM as an intracellular nanoendoscopy but also might aid in the design of future nuclear envelope-targeted nanodrug delivery tailored to the individual patient.

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P2260  
Board Number: B405  
Complete 3D mapping of FG domains for all eleven FG-Nups in living cell NPCs using super-resolution microscopy.
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Nuclear Pore Complexes (NPCs) are large macromolecular gateways that serve to regulate the passage of particles between the nucleus and cytoplasm of eukaryotic cells. The two primary components of the NPC are the structural scaffold nucleoporins and the phenylalanine-glycine (FG) motif containing nucleoporins (FG-Nups). While the former has been well studied with electron microscopy to reveal their spatial positions and structure, FG nups, however, due to their dynamic nature and lack of secondary structure, have thus far not been individually mapped in their native environment: a living cells NPC. These FG-Nups compose the NPCs permeability barrier. Here we present a complete three-dimensional mapping of the FG domains for all known FG-Nups in living cells obtained through super-resolution microscopy for the first time. Dynamical ranges of motion for FG-Nups within native NPCs are also revealed.

P2261  
Board Number: B406  
Correlative Light and Electron Microscopy at the Nuclear Envelope Herniations.
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Nuclear envelope herniae (herniations) are protrusions of the outer and inner nuclear membrane that enclose nuclear pore complexes (NPCs). They have been observed as a result of genetic manipulation of nuclear envelope associated proteins and nucleoporins in both yeast (Wente and Blobel, 1993; Scarcelli et al, 1996; Murphy et al, 1996) and human cells (Laudermilch et al, 2016). More recently, ESCRT components have been found to play a major role in the surveillance of nuclear envelope integrity, including the formation of herniae (Webster et al, 2014; Webster, Thaller et al, 2016). Nevertheless the exact origin and potential physiological role of nuclear envelope herniations remains unknown.
We have used correlative light and electron microscopy CLEM (Kukulski et al, 2011) to understand the fate of the herniae in various yeast strains and under conditions of impaired NPC assembly. We show that anchor away (Haruki et al, 2008; Colombi et al, 2013) of the newly synthesized nuclear pore proteins Nup192 (inner ring complex) and Nup84 (Y-complex) induce herniae formation after 3-5 hours. We show that FG-nucleoporins of the inner ring are present at the herniae sites. Prospectively, we will structurally analyze herniations by cryo-electron tomography and subtomogram averaging to address if the underlying NPC is fully or partially assembled.

References
Webster BM, Thaller DJ, Jager J, Ochmann SE, Borah S, Lusk CP (2016) Chm7 and Heh1 collaborate to link nuclear pore complex quality control with nuclear envelope sealing. EMBO J 35: 2447-2467.

P2262
Board Number: B407
Opening windows into the Cell: taking the next step in Structural Biology.
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Studying the molecular machinery of cells with molecular detail in the cellular context is a great challenge for cell and structural biology. It requires the integration of dynamic and structural information coming from different ranges of spatial resolution and imaging modalities. In order to do so, a comprehensive workflow is needed covering live-cell imaging, vitreous freezing, cryo-fluorescence microscopy, sample thinning and high-resolution electron cryo-tomography.

As most cell types (and particular the nuclear region) are too thick to be studied in the cryo-TEM the sample needs to be thinned in order to render it accessible to cryo-TEM imaging. Thinning by mechanical means has been notoriously difficult and artifact prone in the past why we follow the approach to apply cryo focused-ion-beam (cryo-FIB) milling to thin vitreously frozen specimens. Using a FEI DualBeam™ microscope, regions of interest are thinned down to the appropriate thickness of 200-300 nm while maintaining cryogenic conditions. The thinned cellular sample is then transferred to an automated, high-throughput cryo-TEM for high-resolution imaging by electron cryo-tomography

P2263
Board Number: B408
On the micro rheology of the Nuclear Pore Complex: Nature evolved a novel super nanopore.
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The nuclear pore complex is the unique gateway to the cell nucleus and is allegedly filled with intrinsically disordered proteins (IDPs) rich in Phe-Gly repeat domains. While the microdynamics of these IDPs is under debate and there are several groups actively involved in the field, there is no information and/or consensus about their micro rheology. Here, unprecedentedly, we deep dive into the micro rheology of IDPs confined inside the NPC. Our results indicate that the IDPs rich in FG repeat domains show an intriguing rheological behavior, which uniquely stems from the interplay of biophysical factors including hydrophobicity, charge, the ratios of positive and negative charge content to hydrophobicity content, geometrical confinement, chains' lengths, channel wall permeability, and the
chains’ end-tethering. It appears that among these factors, the latter plays the dominant role in shaping the mechanical spectrum of FG-meshwork, particularly in low-frequency domains. The frequency-dependent viscosity of the FG-meshwork is reminiscent of pseudo-plasticity, and thus, the FG-repeats form a shear-thinning polymeric meshwork. The meshwork poses a super-viscous environment to the inert (non-specific) cargos while behaving like less viscous liquid for nuclear-transport-factor-bound cargos.

**P2264**

**Board Number: B409**

Genome-wide screen indicates that the spatial organization of genomes is dynamically regulated by transcription factors.

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Hundreds of genes physically interact with the nuclear pore complex (NPC) and many inducible genes reposition to the nuclear periphery when they are activated. In budding yeast, interaction with the NPC is controlled by cis-acting targeting elements (or “DNA zip codes”) and requires both NPC proteins and transcription factors (TFs). Here, we describe the mechanistic dissection of the function of TFs and transcriptional regulators/mRNA export factors in mediating targeting to the nuclear periphery. Further, we have performed a global screen of all ~200 yeast transcription factors to test the generality of this phenomenon. Each TF was tagged with the DNA binding domain from LexA and crossed against a strain having the LexA binding site integrated at a locus that normally localizes to the nucleoplasm. The position of this locus with respect to the nuclear envelope was scored using confocal microscopy. Approximately 50% of the tested transcription factors were sufficient to cause localization to the nuclear periphery. The major pathway by which this targeting was mediated required the NPC; CRISPR-mediated mutations in the NPC protein Nup2 blocked peripheral targeting by >95% of these TFs. This suggests that controlling gene positioning within the nucleus is an important and unappreciated function of TFs and that interaction with the NPC is the major pathway by which genes localize at the nuclear periphery. The TFs identified in the screen regulate over 1000 genes, including housekeeping genes as well environmentally or developmentally regulated genes. The TFs are found in all transcription factor families in budding yeast and a large subset (50%) of the transcription factors identified have human homologs. A human TF was able to mediate targeting to the nuclear periphery in yeast, suggesting that the molecular mechanism of gene recruitment to the NPC has been deeply conserved and likely plays a fundamental role in genome organization.

**P2265**

**Board Number: B410**

Sphingolipid homeostasis is critical for nuclear envelope integrity.

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The structure and integrity of the nuclear envelope depends on complex interactions between a diverse group of nuclear envelope proteins, the nucleoplasm and cytoplasm. However, the roles that specific lipid molecules play in the formation and integrity of the nuclear envelope remain poorly understood. Here we show that inhibition of de novo synthesis of sphingolipids leads to abnormal nuclear envelope morphology in *Saccharomyces cerevisiae*. Moreover, we find that in human cells, inhibition of sphingolipid synthesis not only affects nuclear envelope structure but also promotes the formation of
micronuclei and increases genomic instability. Our studies reveal an important role for sphingolipids in regulating nuclear envelope integrity, and also suggest a possible mechanism for how downregulation of sphingolipids may promote tumorigenesis as a result of increased genomic instability.

P2266
Board Number: B411
Nuclear envelope defects on S. japonicus lagging chromosomes.
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The nuclear envelope (NE) is a highly organized membrane structure separating chromosomes from the cytoplasm in eukaryotes. In metazoans, the NE disassembles in mitotic prophase, and reassembles in late anaphase/telophase. If a lagging chromosome persists until telophase, NE assembly on the lagging chromosome can lead to the formation of an aberrant nuclear structure called a micronucleus. Micronuclei are prone to disrupt the NE, which can result in massive localized DNA rearrangements termed chromothripsis. This highlights the importance of understanding NE assembly on lagging chromosomes, which would be greatly facilitated by studies in a facile model system. However, NE disassembly and reassembly do not occur in organisms that undergo closed-mitosis, such as S. cerevisiae and S. pombe, making it difficult to utilize them in aforementioned studies. Here we use S. japonicus, the dimorphic yeast that undergoes semi-open mitosis, to investigate the NE on lagging chromosomes. We found that although lagging chromosomes were enclosed by NE components, they could not assemble a functional NE, and would spontaneously lose NE components over time. We are testing several possible mechanisms underlying this instability. One possibility is that NE sealing could be defective on lagging chromosomes. Recent studies suggested that Chmp7, a protein likely to be a fusion of ESCRT-II and ESCRT-III proteins, directs the assembly of ESCRT-III filaments on chromatin to seal the newly assembled NE after DNA segregation. We found that deletion of S. japonicus chmp7 impaired the import of NLS-GFP, suggesting that the function of Chmp7 in NE integrity may be conserved. Interestingly, chmp7\textsuperscript{-}\textDelta cells were viable. In addition to investigating how Chmp7 might affect NE sealing around lagging chromosomes, we have also isolated suppressors of chmp7\textsuperscript{-}\textDelta to define parallel NE sealing mechanisms.

P2267
Board Number: B412
Analysis of Gp210 function in Drosophila melanogaster.
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Although Gp210 was the first nuclear pore complex (NPC) protein identified 35 years ago, a role in supporting nucleocyttoplasmic transfer has remained elusive. Despite being one of the few transmembrane NPC proteins, a number of studies suggest that Gp210 is not required for assembly or localization of the NPC, consistent with observations that not all cells express Gp210. Recent inquiries into Gp210 function have uncovered roles in a variety of cellular processes including regulation of gene specific transcription in muscle cells, maintenance of ER homeostasis and in cellular differentiation. A particularly interesting and surprising finding is that some functions of Gp210 do not require the protein to be localized to the NPC. We present an in-depth study of Gp210 function in Drosophila melanogaster, reporting on gp210 mutant phenotypes in multiple tissues and cell types during various stages of the Drosophila life cycle.
Our initial investigation takes advantage of two existing P-element insertions into the gp210 locus which significantly decrease transcript and protein levels. Despite strong knock-down from these hypomorphic mutations, both alleles are surprisingly viable and fertile as homozygotes, and do not have a decreased lifespan. Moreover, preliminary studies indicate that these two gp210 alleles display age dependent loss of motor function similar to wild-type animals. We are currently generating a null allele and tissue specific knock downs of Gp210 function using transgenic CRISPR/Cas9 approaches. Our preliminary results have also led to the interesting discovery that Gp210 mutants have an increase in Nuclear Envelope Budding (NEB) events, however it is unclear if this phenotype is due to increasing or blocking of this process. During NEB, large granules containing RNA presumably bud through the nuclear envelope in a process akin to the nuclear egress of Herpesviridae nucelocapsids, however, the cellular function of this pathway is still enigmatic. We are testing a number of possibilities to determine the mechanisms underlying the apparent increase in NEB events in gp210 mutants. Additionally, we find that mutant animals display altered movement behavior, and in accordance with other studies, loss of Gp210 function does not grossly alter the localization of the NPC but does lead to elevated levels of ER stress in some cell types. Ultimately, these in-vivo studies will add to our understanding of this enigmatic NPC protein.

P2268
Board Number: B413
Aurora B-mediated exclusion of HP1α from late-segregating chromatin prevents formation of micronuclei.
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In Drosophila neuroblasts, late-segregating acentric chromosome fragments that would otherwise form micronuclei are instead incorporated into telophase daughter nuclei by passing through Aurora B kinase-dependent channels in the nuclear envelope. However, the mechanism by which Aurora B kinase activity mediates channel formation remains unknown. Here, we show that localized concentrations of Aurora B preferentially phosphorylate H3(S10) on acentrics and their associated DNA tethers to exclude association with the heterochromatin component HP1α. Additionally, we show that Aurora B-mediated exclusion of HP1α from endonuclease- and irradiation-induced acentrics results in localized inhibition of nuclear envelope reassembly both on acentrics and on the main daughter nuclei at the sites of acentric entry. These data suggest a model in which Aurora B-mediated phosphorylation of H3(S10) on acentrics and their associated tethers prevents HP1α recruitment and subsequent nuclear envelope reassembly on the lagging acentrics and their tethers. This ultimately results in nuclear envelope channel formation and reintegration of the late-segregating acentrics into the main nuclei, avoiding micronucleation. In support of this model, we find that HP1α also plays a role in global nuclear envelope dynamics by specifying the site of initiation of nuclear envelope reassembly. Taken together, these results demonstrate that mitotic HP1α-chromatin interactions influence nuclear envelope reassembly and maintain genome integrity in response to endonuclease- or irradiation-induced chromosome fragments that would otherwise form micronuclei.
Membrane Fission and Coat Proteins

P2269
Board Number: B415
The Structural Basis of an ESCRT-III Membrane Assembly.
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The Endosomal Sorting Complexes Required for Transport (ESCRT) mediate critical membrane remodeling events throughout the mammalian cell cycle, including HIV budding, cytokinetic abscission, and sealing of the nuclear envelope, among other roles. ESCRT-III proteins assemble into membrane-binding filaments to catalyze these reactions, but the structures and functions of these assemblies remain poorly understood. Our collaborative team recently determined the first atomic-resolution structure of an ESCRT-III filament – a hetero-polymer consisting of CHMP1B and IST1/CHMP8. Our previous structure demonstrated how one of these ESCRT-III subunits, CHMP1B, transitions from a “closed” to an “open” state to form an interlocked and domain-swapped filament. Moreover, we and others have shown that the CHMP1B-CHMP8 copolymer participates in non-canonical, positive-curvature membrane fission pathways. Very recent work on other ESCRT-III proteins indicated that the mechanisms of opening and assembly we reported are conserved, but also raised questions regarding membrane binding and remodeling activities, as well as and the generality of hetero-polymerization. To address these gaps in our understanding, we have determined the high-resolution structure of a membrane-bound CHMP1B-CHMP8 assembly by cryo electron microscopy (cryoEM). We find that CHMP1B induces a high degree of curvature alone, and that deposition of the CHMP8 strand further constricts the membrane tubule by more than 2-fold – almost to the fission point. Notably, the distance between outer leaflet lipid headgroups is ~10 nm and the distance between inner leaflet lipid headgroups is reduced to only ~4 nm. Conserved residues along helix α1 of CHMP1B serve as the major membrane binding surface and exploit both electrostatic as well as hydrophobic interactions with the convex leaflet of the membrane tubule. Our atomic-resolution cryoEM study reveals the structural mechanisms governing ESCRT-III assembly, membrane-binding, and positive-curvature membrane deforming activities. By analogy with the structural features of other ESCRT-III proteins, we propose general rules for membrane-shaping by the ESCRT-III family.

P2270
Board Number: B416
A new model for COPII-mediated cargo export from the endoplasmic reticulum.
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Both COPII and COPI are considered as analogous sets of coat protein complexes mediating the formation of membrane vesicles translocating in opposite directions to different destinations within the secretory pathway. Here we provide evidence for a fundamentally different model for the function of
the COPII coat during protein export from the endoplasmic reticulum (ER). We used live cell microscopy combined with a range of well-defined pharmaceutical and genetic perturbations of ER-Golgi transport. Primarily, ER to Golgi transport of a cargo membrane protein was visualized in living intact cells coexpressing fluorescently tagged Sec24 isoforms. Further COPII localization and function were analyzed and characterized in living intact cells by uncoupling vesicle fission from the preceding cargo sorting and accumulation processes in ERESs (using BFA and Nocodazole treatment). Finally, perturbation at the interface of COPII-cargo using mutagenesis of Sec24 was applied resulting in cargo accumulation in the ER, functionally establishing the localization of COPII at the ER-ERES boundary. Together, these data support the hypothesis that rather than generating coated vesicles, COPII mediates the sorting of transport-competent proteins by dynamic binding to stable elongated membranes that comprise the ER-ERES boundary. The finding that COPII is absent from nascent vesicles and thus does not limit carrier size or shape, resolves the dispute of how large cargo molecules or complexes can be accommodated in ER-derived vesicle. We propose that cargo sorting and concentration is driven by ER to ERES-directed treadmilling of the membrane bound COPII coat-cargo complex. This treadmilling movement is generated by restricting COPII recruitment to the ER side of the boundary by the activated GTP-bound ER-localized Sar1 GTPase. Cargo-protein interaction with its neighboring membrane environment contributes to direct this process. These findings transform our understanding of the role of coat proteins in ER to Golgi transport.

P2271
Board Number: B417
“Clusterase” model of dynamin-mediated membrane fission.
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Dynamin is a mechanochemical GTPase essential for membrane fission during clathrin mediated endocytosis. Dynamin forms washer ring-shaped/helical complexes at the neck of endocytic pits and their structural changes coupled with GTP hydrolysis drive membrane fission. Dynamin and its binding protein amphiphysin cooperatively regulates membrane remodeling during fission (Yoshida et al., EMBOJ 2004), but its precise mechanism remains elusive. In this study, we analyzed structural changes of dynamin-amphiphysin complexes during membrane fission using electron microscopy and high-speed atomic force microscopy (HS-AFM). In vitro incubation of liposomes with recombinant human dynamin 1 and amphiphysin resulted in the formation of membrane tubules decorated with dynamin 1-amphiphysin ring complexes. The addition of GTP to the membrane tubules lead to the formation of multiple constriction sites within 30 sec., then eventually the membrane fission occurred and numerous vesicles were formed within 1min. Interestingly, dynamin 1-amphiphysin ring complexes were transiently formed clusters prior to membrane fission. HS-AFM analyses clarified that regularly spaced dynamin-amphiphysin rings were dynamically rearranged to form clusters upon GTP hydrolysis and membrane constriction occurred at protein-uncoated regions between the clusters. Furthermore, we found a novel function of amphiphysin in size control of the dynamin 1-amphiphysin clusters to enhance biogenesis of endocytic vesicles. Our new approaches using combination of EM and HS-AFM clearly demonstrates dynamics of dynamin-amphiphysin complexes during membrane fission suggesting a novel “clusterase” model of dynamin-mediated membrane fission.
P2272
Board Number: B418
Structural Basis of Mitochondrial Receptor Binding and GTP-Driven Conformational Constriction by Dynamin-Related Protein 1.
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Mitochondrial inheritance, genome maintenance, and metabolic adaptation all depend on organelle fission by Dynamin-Related Protein 1 (DRP1) and its mitochondrial receptors. DRP1 receptors include the paralogs Mitochondrial Dynamics 49 and 51 (MID49/MID51) and Mitochondrial Fission Factor (MFF), but the mechanisms by which these proteins recruit DRP1 and regulate its activities are unknown. Here we present a cryoEM structure of human, full-length DRP1 bound to MID49 and an analysis of structure-and disease-based mutations. We report that GTP binding allosterically induces a remarkable elongation and rotation of the G-domain, Bundle-Signaling Element (BSE) and connecting hinge loops of DRP1. In this nucleotide-bound conformation, a distributed network of multivalent interactions promotes DRP1 copolymerization into a linear filament with MID49, MID51 or both. Subsequent GTP hydrolysis and exchange within the filament leads to receptor dissociation, shortening through disassembly, and concomitant curling of DRP1 oligomers into closed rings. The dimensions of the closed DRP1 rings are consistent with DRP1-constricted mitochondrial tubules observed in human cells. These structures are the first views of full-length, receptor- and nucleotide-bound dynamin-family GTPases and—in comparison with nucleotide-free crystal structures—teach us how these molecular machines perform mechanical work through nucleotide-driven allostery.

P2273
Board Number: B419
Escaping the bar: modulating dynamin function and T-tubule maintenance through SH3-PRD domain interaction.
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Eukaryotic cells harness multiple endocytosis pathways to regulate cell signaling and nutrient uptake to cope with different physiological demands. Among those different endocytic machineries, a membrane fission GTPase dynamin has been demonstrated to be a key regulator for several endocytic routes. However, fission activity of dynamin-2, a ubiquitously expressed isoform of dynamin, is curvature sensitive. In muscle cells, invagination of cell membrane forms highly curved T-tubules that are enriched in phosphatidylinositol-4,5-bisphosphate (PI4,5P2), a phospholipid that is strongly bound by PH domain of dynamin-2. The muscle-specific form of BIN1/Amphiphysin 2, a Bin/Amphiphysin/Rvs (BAR) domain containing protein, is highly expressed in T-tubule and is important for its biogenesis. BIN1 could bind to dynamin-2 PRD domain through its SH3 domain. These criteria make T-tubule a perfect platform for dynamin-2 scission. We previously discovered that Centronuclear myopathy-associated dynamin-2 mutants could indeed induce T-tubule fragmentation. Yet T-tubule integrity is preserved in physiologically normal cells, suggesting that there must be a mechanism to regulate dynamin function. Despite its membrane-curvature-generation ability, we find that BIN1 would actually inhibit dynamin function in mediating membrane fission in vitro. This inhibition is due to SH3-PRD domain interaction, as truncated BIN1 lacking the SH3 domain would cause opposite result; it promotes GTP-dependent
membrane scission of dynamin. In turns, membrane tubulation by BIN1 is inhibited in the presence of dynamin. This reciprocal inhibition happens through regulation of protein assembly on the membrane. We propose that phosphorylation in PRD domain of dynamin-2 would serve as a mechanism to modulate SH3-PRD domain inhibition. We generate dynamin-2 phosphomimetic mutant with lower binding affinity to SH3 domain of BIN1 and show that this mutant could partially relieve inhibition of both dynamin-mediated membrane fission and BIN1 membrane tubulation caused by SH3-PRD domain interaction. Together, our results provide a new insight into the regulation mechanism between dynamin and its binding partners via binding affinity alteration. Our next step is to observe whether dynamin function in other endocytic pathways is controlled through similar mechanism, particularly clathrin-mediated endocytosis (CME) which happens generally in all cells and GLUT4 endocytosis that is specifically expressed only in muscle and fat cells.

P2274
Board Number: B420
Identification of cargoes trafficked by the retromer and CCC complexes using quantitative proteomics.
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The retromer (VPS35-VPS29-VPS26) and CCC (CCDC22-CCDC93-COMMD) complexes function at endosomes to sort and traffic cargoes along specific intracellular routes. Cargoes regulated by these complexes include sorting receptors, ion transporters, and signaling receptors, that are directed either to the plasma membrane via the recycling pathway or the Golgi via the retrograde pathway. The precise delivery of cargoes to their appropriate destinations is critical for normal cellular function, and aberrant retromer- or CCC-mediated sorting results in cargoes mistrafficked to the lysosome and abnormal cellular function. In fact, VPS35 and CCDC22 have been identified as genetic risk factors of Parkinson’s disease and X-linked intellectual disability, respectively, where the incorrect trafficking of yet unknown cargoes due to compromised function of the retromer and CCC complexes contributes to the cellular physiology of these disease states.

In this study, we used an organelle enrichment strategy in combination with multiplexed tandem mass tag (TMT)-based quantitative proteomics to systematically profile retromer and CCC cargoes. Plasma membrane proteins were isolated using selective cell surface biotinylation followed by streptavidin enrichment, while Golgi and lysosome fractions were isolated by sequential density ultracentrifugation. The isolated organelle protein fractions were digested using Lys-C and trypsin, and the resulting peptide fragments were labeled with isobaric TMT and subjected to multi-notch mass spectrometry. Through the comparison of the proteomes of the plasma membrane, Golgi, and lysosome in control versus VPS35 or CCDC22 null cells, we have identified a comprehensive set of cargoes that are trafficked by the retromer and CCC complexes in the recycling and retrograde pathways. Our proteomic dataset includes several previously unidentified cargoes of the retromer and CCC complexes such as members of the GOLGIN family of tethering factors of the Golgi and the plasma membrane transporter SLC7A1, respectively. We are in the process of expanding our proteomic analyses to include mutated variants of VPS35 and CCDC22 to identify which cargoes, if any, are mistrafficked to the plasma membrane or Golgi in disease states. Our study has provided a high confidence set of retromer and CCC cargoes that facilitates our understanding of the biology of these sorting complexes, how their perturbation disrupts cellular function, and provides novel targets for therapeutic intervention in disease states.
Rab GTPases

P2275
Board Number: B421
A Rab32 trafficking pathway that prevents bacterial infections.
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Host defense mechanisms protect complex organisms against the attack of microbes. Intracellular bacterial pathogens such as Salmonella, evolved sophisticated, often redundant, strategies to overcome host defense and cause infection. Despite sharing many strategies to overcome host defense, different Salmonella serovars show different pathogenic behaviors, suggesting the existence of unknown bacterial killing mechanisms. We previously identified a host trafficking pathway to the bacterial intracellular vacuole that prevents the human-restricted bacterial pathogen Salmonella Typhi from surviving in mouse macrophages and therefore infecting mice. This pathway depends on the host GTPase Rab32 and its guanine nucleotide exchange factor BLOC-3. We showed that in contrast to Salmonella Typhi, the broad-host pathogen Salmonella Typhimurium infect mice by counteracting the Rab32 trafficking pathway through the delivery of two type-III-secretion effectors: GtgE, which is a specific protease cleaving Rab32; and SopD2, which is a Rab GTPase activating protein (GAP). A Salmonella Typhimurium strain deficient for both of these effectors is unable to infect mice, yet it is fully virulent in BLOC-3 deficient mice. Therefore, the Salmonella Typhimurium effectors GtgE and SopD2 act redundantly to neutralize a powerful host defense pathway that can prevent bacterial infections. However, the Rab32-dependent mechanisms directly involved in bacterial killing are still unknown. Our new results indicate that Salmonella is not the only pathogen susceptible to this pathway and suggest that other intracellular pathogens have to counteract this host-defense pathway to be able to survive inside the host cell and to cause infection.

P2276
Board Number: B422
Regulation of connecdenn/DENND1 guanine nucleotide exchange factor activity by Arf5.
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The DENN (differentially expressed in normal and neoplastic cells) domain is an evolutionary conserved protein module found in all eukaryotes and serves as a GEF (guanine nucleotide exchange factor) for Rab GTPases to regulate diverse cellular functions. Connecdenn 1/2 are DENN domain-containing proteins known to act as GEFs for Rab35. Disruption of connecdenn/Rab35 activity causes alterations in endosomal trafficking including defects in the recycling of multiple cargo proteins from early endosomes to the plasma membrane and an enlargement of early endosomes. These alterations lead to important changes in cellular function, and yet our understanding of the regulation of connecdenn GEF activity is incomplete. Here, we have discovered a novel interaction between the DENN domain of connecdenn 1/2 and Arf5, a class II Arf GTPases. Remarkably, while the DENN domain has no GEF activity towards Arf5, the binding of Arf5 to the DENN domain greatly enhances the GEF activity towards Rab35. Moreover Arf5 knockdown decreases the activity of Rab35 in cells with subsequent enlargement of early endosomes. This study reveals an unanticipated regulatory mechanism of connecdenn GEF activity towards Rab35 and provides an unprecedented link between Rabs and Arfs.
**P2277**

**Board Number: B423**

Arf6 is a negative regulator of axonal elongation in cultured rat hippocampal neurons.

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The axonal elongation is a critical process for neuronal development that involves many cellular events, such as endosome recycling, membrane trafficking and cytoskeletal remodeling. Members of several families of GTPases act as regulators of these processes. Previous work from our group has identified the small GTPase Rab35 as a positive regulator of axonal elongation, through a mechanism dependent on Map1b. Additionally, it has been reported that there exists an interplay between the small GTPases Rab35 and Arf6 acting in cellular events such as cytokinesis, phagocytosis and the coordination of adhesion and migration. Therefore, in this work, we studied the effect of dominant negative (DN) and constitutively active (CA) forms of Arf6 upon axonal outgrowth in cultured rat hippocampal neurons. We also used SecinH3, a pharmacological inhibitor that targets the cytohesin family of Arf-GEFs, to evaluate its effect on axonal elongation. Overexpression of Arf6-DN and treatments with SecinH3 induced a phenotype on cultured neurons that resembled the effect of Rab35 upon axonal growth. Treatment with SecinH3 is also capable of rescuing the phenotype observed after Map1b knockdown. Additionally, either the overexpression of Arf6-CA or Epi64B, a Rab35 GAP that interacts with Arf6-GTP induced significantly shorter axons. Finally, co-transfection of wild type Rab35 and Arf6 produced a phenotype indistinguishable from control neurons. These results suggest that active Arf6 acts as a negative signal for axonal elongation, possibly through interactions with Rab35 regulators and point to a balance between the functions of Arf6 and Rab35 during axonal outgrowth.  

Supported by FONDECYT 1140325 and FONDAP 15150012 To CG-B

**P2278**

**Board Number: B424**

Cdk5-dependent phosphorylation of GRAB, a guanine nucleotide exchange factor for Rab8, regulates neuronal migration in the developing cerebral cortex.

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The migration of newborn neurons from their birthplace to their final destination is critical for the construction of brain structure. Recently, it is demonstrated that the migration of developing cortical neurons is precisely regulated under the membrane traffic. Rab GTPases are major regulators of the membrane trafficking. There are more than 60 members in Rab family, each of which is localized to distinct membrane components in promoting respective membrane traffic. However, it is not fully understood how these Rabs act cooperatively in migrating neurons during corticogenesis. Cyclin-dependent kinase 5 (Cdk5) is a neuron specific Ser/Thr protein kinase that is activated by binding either p35 or p39 regulatory subunit. It plays an important role in a variety of neuronal functions including neuronal migration during brain development. Cdk5-p35 is also involved in membrane traffic, but it is unknown whether Cdk5-p35 regulates membrane traffic in migrating neurons. Recently, we have shown that Cdk5-p35 phosphorylates GRAB, a guanine nucleotide exchange factor for Rab8, at Ser169 and...
Ser180, and this phosphorylation regulates the Rab11-Rab8 cascade. Because Rab11 is involved in cortical neuronal migration, we hypothesized that Cdk5-p35 regulates neuronal migration via GRAB activity by phosphorylation. Herein, we examined and found that GRAB is a novel regulator of neuronal migration in developing cerebral cortex. In utero electroporation studies revealed that GRAB knockdown arrested most neurons in intermediate zone of cerebral cortex. The knockdown of Rab8A/B or Rab11A/B showed similar phenotypes to the GRAB knockdown, suggested that GRAB regulates neuronal migration through the cascade of Rab8A/B and Rab11A/B. We examined the role of Cdk5-dependent phosphorylation of GRAB by introduction of wild-type GRAB (WT GRAB), non-phosphorylatable GRAB mutant S169/180A (2A GRAB), or phospho-mimic GRAB mutant S169/180D (2D GRAB) in migrating neurons together with GRAB-knockdown vector. The expression of WT GRAB fully restored neuronal migration in GRAB knockdown cortices, whereas 2A or 2D GRAB could not. These results suggested that GRAB regulates neuronal migration in developing cerebral cortex via Rab8 and Rab11 in a Cdk5-dependent manner.

P2279
Board Number: B425
Disruption of Rab8a and Rab11a causes formation of basolateral microvilli in neonatal enteropathy.
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Misplaced formation of microvilli to basolateral domains and intracellular inclusions in enterocytes are pathognomonic features in congenital enteropathy associated with STX3 mutation. While the demonstrated binding of Myo5b to the Rab8a and Rab11a small GTPases in vitro implicates cytoskeleton-dependent membrane sorting, the mechanisms underlying the microvillar location defect remain unclear. By selective or combinatorial disruption of Rab8a and Rab11a membrane traffic in vivo, we demonstrate that transport of distinct cargo to the apical brush-border rely on either individual or both Rab regulators, whereas certain basolateral cargos are redundantly transported by both factors. Enterocyte-specific Rab8a and Rab11a double knockout mouse neonates showed immediate postnatal lethality and more severe enteropathy than single knockouts, with extensive formation of microvilli along basolateral surfaces. Notably, following an inducible Rab11a deletion from neonatal enterocytes, basolateral microvilli were induced within 3 days. These data identify a potentially important and distinct mechanism for a characteristic microvillus defect exhibited by enterocytes of patients with neonatal enteropathy.

P2280
Board Number: B426
Intracellular logistics of LIS1, cytoplasmic dynein, and unconventional microtubules.
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Lissencephaly is a devastating serious neurological disorder caused by defective neuronal migration. The LIS1 (platelet-activating factor acetylhydrolase, isoform 1b, subunit 1; official symbol PAFAH1B1, for
platelet-activating factor acetylhydrolase, isoform 1b, subunit 1) gene was identified as the gene mutated in individuals with lissencephaly. LIS1 was also found to regulate cytoplasmic dynein function and localization. Cytoplasmic dynein drives the movement of a wide range of various cargoes towards the minus ends of microtubules. LIS1 is known to be required for the dynein activity, but the underlying mechanism is poorly understood. We have previously already reported that the following; 1) that LIS1 mediates anterograde transport of cytoplasmic dynein to the plus end of cytoskeletal microtubules (MTs) as a LIS1-cytoplasmic dynein complex on unconventional microtubules (we call them transportable microtubules, t-MTs) in a kinesin-1-dependent manner. LIS1 forms an idling complex with cytoplasmic dynein, which is transported to the plus ends of cytoskeletal MTs by kinesin motors. We have also shown that Mammalian mammalian NUDC (mNUDC) interacts with kinesin-1 and is required for the anterograde transport of the LIS1/cytoplasmic dynein/tMTs complex by kinesin-1. mNUDC is also required for anterograde transport of a dynactin (P150Glued)-containing complex. However, the cytoplasmic dynein complex and the dynactin complex are separately transported to the plus-end of cytoskeletal MTs. 3) Small GTPase Rab6a mediates LIS1 release from the LIS1—cytoplasmic dynein complex followed by dynein activation at the plus-end of cytoskeletal MTs. On the other hand, another small GTPase, ADP-ribosylation factor-like 3 (Arf3), and cytoplasmic dynein light chain DYNNL1/LC8 regulate the unloading of dynactin (P150Glued)-bound cargo from the dynein motor. On the other hand, we previously found that inhibition of calpain protects LIS1 from proteolysis, resulting in the augmentation of LIS1 amounts levels and the a functional improvement in LIS1-deficient mice, implying. This means that calpain inhibition is a potential therapeutic intervention for lissencephaly. Neuronal protein alpha-synuclein A neuronal protein alpha-synuclein has been shown to beis linked to Parkinson’s disease (PD), but their causative role in PD how synucleins play a causative role is unclear not clear. We have also have found that alpha-synuclein is required for the creation of unconventional microtubules named transportable microtubules (t-MTs), which function as carriers enabling anterograde cytoplasmic dynein transport. Live-cell imaging demonstrated the co-trans

P2281
Board Number: B427
The small GTPase Rab10 regulates the formation of tubular endosomes.
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Eukaryotic cells contain the endomembrane system, which is crucial for cellular homeostasis and various cellular events. A striking tubular-shaped organelle, so-called tubular endosome, is one of the components of the endomembrane system that is often considered to be involved in recycling of internalized proteins back to the plasma membrane. However, how the formation of tubular endosome is regulated and why its tubulation is necessary remain largely unknown. To address these issues, we focused on the small GTPase Rab, a conserved switch molecule for membrane trafficking by cycling between a GDP-bound inactive form and a GTP-bound active form. The active Rab drives a variety of membrane trafficking steps, such as vesicle budding, transport, and fusion, by recruiting its specific effector protein. Although Rab family consists of ~60 members in mammalian cells, which Rab regulates tubular endosomes is poorly understood. In this study, we performed a systematic Rab localization screening by expressing EGFP-tagged Rabs in HeLaM cells and identified Rab10 as a prime candidate for the crucial regulator of tubular endosomes. We found that knockout (KO) of Rab10 disrupts the formation of tubular endosomes and re-expression of Rab10 in Rab10-KO cells perfectly rescues the phenotype. We then performed live-cell time-lapse imaging of EGFP-tagged Rab10 and showed that EGFP-Rab10-marked tubular endosomes are highly dynamic structures, whose dynamics requires microtubules. To further determine the mechanism by which Rab10 regulates the formation of tubular
endosomes, we searched for microtubule-associated Rab10-binding proteins by using PSI-BLAST and successfully identified KIF13A/B, kinesin-3 type motors, as candidates for novel Rab10 effectors. We are now investigating whether KIF13A/B actually regulates the formation of tubular endosomes. We will discuss the possible molecular mechanisms for the formation of tubular endosomes and their physiological significance based on our findings.

P2282
Board Number: B428
Rab4 and Rab14 effector Rabip4’ interacts with lysosomal small GTPase Arl8b and promotes cargo trafficking to lysosomes.
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Lysosomes receive and degrade extracellular and intracellular cargo delivered by endocytic, phagocytic and autophagic pathways. Recent studies have shed light on novel lysosome functions in regulating processes such as cell migration, nutrient sensing, bone remodeling, antigen presentation etc. that are regulated by lysosome positioning within the cell. The small GTPase Arl8b regulates both cargo delivery to lysosomes and lysosome distribution by recruiting its downstream effectors SKIP, PLEKHM1 and HOPS complex that mediate microtubule-dependent motility of lysosomes and fusion with late endosomes, respectively. Since the discovery of Arl8b, an ever-increasing number of its interaction partners have come into light, inclusive of RUN-domain containing proteins SKIP and PLEKHM1. Here, we identify a Rab4 and Rab14-interacting and RUN domain containing protein Rabip4’, as an interaction partner for Arl8b. Using GST-pulldown assay and purified protein interaction assay we show that RUN domain of Rabip4’ is sufficient for its interaction with GTP-bound Arl8b. Immunofluorescence studies show that Arl8b and Rabip4’ localize on LAMP1-positive endosomes as well as on mature phagosomes. Notably, Arl8b depletion led to displacement of Rabip4’ from the endosomal and phagosomal membranes, rendering it more cytosolic. The membrane localization of Rabip4’ in Arl8b depleted cells was rescued upon transfection of siRNA resistant Arl8b construct. Similar localization was also observed of the Rabip4’ mutant lacking Arl8b-binding, reinforcing that Arl8b regulates Rabip4’ membrane localization. Rabip4’ has separate binding sites for Rab4, Rab14 and Arl8b; we propose that it may act as a linker between these small GTPases as the co-expression of these proteins resulted in triple co-localization of Rab4/Rab14, Rabip4’ and Arl8b. Finally, depletion of Rabip4’ in macrophage cell line RAW264.7 impaired lysosomal membrane acquisition on latex bead containing phagosomes. LAMP1 acquisition around latex bead/bacteria (E.coli) containing phagosomes was delayed in cells depleted of Rabip4’ as compared to Control siRNA treated cells, indicating that Rabip4’ regulates phago-lysosome fusion. Together our results suggest that Rab4 and Rab14 effector, Rabip4’ interacts with Arl8b and this interaction possibly regulates cargo delivery to lysosomes.
P2283
Board Number: B429
Role of Small GTPases on differentiation of pre-adipocyte.
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Awareness in adipogenesis has increased noticeably over the past few years with the emphasis on the connection between extracellular signals and the transcriptional cascade that regulate adipocyte differentiation. Differentiation of murine pre-adipocytes is a highly regulated process that requires specific and selective regulation of numerous of protein activities. As many Small GTPases, Rab5 plays a critical role in numerous signaling transduction pathways. However, the role of Rab5 in adipogenesis still remains not fully understood. In this study, we prepared 3T3-L1 mouse pre-adipocytes with Rab5-wild type (WT) and several Rab5 mutants. We found that overexpression of Rab5-WT and Rab5-Q79L, the GTPase defective mutant, inhibited adipogenesis; on the contrary, Rab5-S34N, the GTP binding defective mutant, did not inhibit adipogenesis. In concordance with these results, adipogenesis markers, such as ACC, FABP4, PPARγ and C/EBPα showed consequent results. In cells expressing Rab5-Q79L mutant, but not in cells expressing Rab5-S34N mutant, the expression of PPARγ and C/EBPα were down regulated as compared with GFP expressing cells. In addition, we observed reduced Akt and MAPK signaling in Rab5-Q79L mutant expressing cells, but increased signaling in Rab5-S34N mutant expressing cells. In conclusion, our results showed an unexpected role of Rab5-WT in pre-adipocyte differentiation, which is important in coordinating a complex network of transcription factors, cofactors and signaling intermediates from numerous pathways.

P2284
Board Number: B430
Early-sorting endosomes in cancer cells show drastically altered morphology, EGFR signaling and degradation but maintain significant organization of regulatory Rab-GTPases and recycling function.
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Many promising anti-cancer therapies target membrane receptors that are endocytosed into dynamic early endosomes. These sorting organelles house signaling receptors, such as EGFR, and function as essential junctions for the endocytic recycling and lysosomal pathways. However, it remains unclear how the trafficking through these structures is modified in different forms of cancer. Using a combination of fixed and live-cell fluorescent confocal and super-resolution microscopy (3D STORM, STED, and Fast Airyscan), we studied the early-sorting endosome dynamics in the non-cancerous MCF10A cells and breast cancer cell lines (triple-negative MDAMD231 and ER positive T47D cells). We quantified transferrin (TF) recycling and the trafficking of lysosomally-destined ligands EGF and LDL by pulse-chase experiments with fluorescently labelled ligands. Quantitative 3D rendering of early-endosome antigen-1 (EEA1) endosomes indicates that MDAMD231 and T47D cells have fewer endosomes compared to MFC10A cells. However, the vesicles were significantly larger in the cancer cells, which resulted in minimal differences in the per cell endocytic volume between MCF10A and MDAMB231 cells. In all the cell lines, colocalization of early-endosome antigen 1 (EEA1) positive vesicles with the Rab5 and Rab4 GTPases was high; there was no significant colocalization of EEA1 with the late
endosome and autophagy markers Rab7 and LC3. 3D STORM imaging revealed that TF was present in recycling tubules of MCF10A cells, and similar structures were not observed in MDAMB231 or T47D cells. Live-cell super-resolution imaging showed us that TF was sorted into budding vesicles in MDAMB231 cells. The recycling rate of TF was similar between MCF10A and MDAMB231, while TF recycling was significantly delayed in T47D cells. We found EGF and LDL remained in the large EEA1 vesicles of cancer cells for prolonged periods, with EGFR signaling and MMP14 present in the MDAMB231 cells. Together, our data indicates that despite the gross morphological changes of the endocytic structures in cancer cells, many aspects of the pathway are conserved including endocytic volume per cell, endocytic recycling of TF, and the gradient of early and recycling Rab-GTPases. However, there may be differences in the mechanisms of recycling with regards to endocytic tubular formation vs. budding. Our data suggests that the adaptations of the early-endosomes in cancer may be causing delayed maturation of early-endosomes and therefore prolonged receptor signaling and delayed lysosomal degradation.

P2285
Board Number: B431
TRAPP complex substrate specificity is mediated by the Rab GTPase hypervariable domain.
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In eukaryotic cells virtually every step of membrane transport is coordinated by Rab GTPases, which recruit effectors that facilitate vesicle formation, transport, tethering, and fusion. Rab GTPase activity is controlled by guanine nucleotide exchange factors (GEFs), which convert inactive GDP-bound Rabs to their active GTP-bound state. In yeast the Rab GTPases Ypt1 and Ypt31/32 control entry to and exit from the Golgi complex, and thus are of critical importance to the secretory pathway. The GEFs that activate Ypt1 and Ypt31/32 are the multi-subunit complexes TRAPPIII and TRAPPII, respectively. The TRAPP complexes activate these two different Rabs through a shared active site. This indicates that these complexes use a novel mechanism for substrate specificity involving complex-specific regulatory subunits.
The C-terminal hypervariable domain (HVD) has the highest degree of sequence divergence between distinct Rab GTPases, and has been proposed to function as subcellular targeting signal through an unknown mechanism. An apparently competing hypothesis is that GEFs are responsible for subcellular targeting of Rabs via their localized activation. Using a series of Ypt1 and Ypt31/32 HVD chimeras, we have found that the Ypt1 HVD plays a significant yet non-essential role in enabling TRAPPIII catalyzed nucleotide exchange. In contrast, the more highly conserved Ypt31/32 HVD is essential for activation by TRAPPII. These findings support a unifying mechanism in which the Rab HVD functions as a targeting signal by mediating GEF substrate specificity through direct interaction between the GEF and the HVD.

P2286
Board Number: B432
The RAB2B-GARIL5 complex promotes cytosolic DNA-induced interferon responses.
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The innate immune system senses viral nucleic acids by pattern-recognition receptors and protects the host from viral infection. Pattern-recognition receptor, cyclic guanosine monophosphate–adenosine
monophosphate synthase (cGAS), detects viral genomic DNA in the cytosol and activates downstream signal transducer, stimulator of interferon genes (STING), to establish an antiviral state. STING is an ER-resident protein that translocates to the Golgi apparatus to induce expression of type I interferon (IFN) and IFN-inducible genes after cytosolic double-stranded DNA stimulation. Although the regulatory mechanism for the cGAS-STING signaling axis has been revealed, the positive regulator of this signaling axis that functions on the Golgi apparatus has not been identified. We show here that RAB2B, a Golgi-resident small GTPase, is involved in the cGAS-triggered innate immune response. RAB2B recruits its candidate effector molecule GARIL5 to the Golgi apparatus, where it colocalizes with STING after cytosolic dsDNA stimulation. The RAB2B-GARIL5 complex promotes expression of type I IFN and IFN-inducible genes, and limits replication of vaccinia virus, a DNA virus. The present study revealed a positive regulator of the cGAS-STING signaling axis and highlights the importance of the Golgi apparatus in the host defense response to DNA virus.

P2287
Board Number: B433
Defining the PI3Kβ-Rab5 Interface.
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The class IA PI 3-kinase PI3Kβ is a heterodimer consisting of a p85 regulatory subunit and a p110β catalytic subunit. PI3Kβ has been implicated in the regulation of endocytic trafficking and macroautophagy in response to serum starvation, and binds to the endosomal small GTPase Rab5. Previous reports have suggested that the BCR domain of p85α binds Rab5GTP. However, using bacterially expressed and purified p85α, as well as p85α expressed in HEK293T cells, we could not detect p85α binding to GST-Rab5GTP. In contrast, Rab5 binding to the p110β catalytic subunit was readily detected. We have previously defined two amino acids in the helical domain of p110β, whose mutation disrupts binding to Rab5GTP. Our current studies focus on the further characterization of the p110β-Rab5 binding interface, which will provide an opportunity to develop modulators of the p110β-Rab5 interaction. We have developed an in vitro binding assay, which uses bacterially expressed and purified recombinant GST-Rab5 GTP and HEK293T cell expression of wild type or mutated p110β. We evaluated 36 mutations in the p110β helical domain, and identified 9 residues that reduced Rab5 GTP binding by more than 50%. These residues localize primarily to two α-helices in the p110β helical domain, which sit below the GBγ binding loop. This data will help to define the Rab5 interface within p110β and allow us to develop a high throughput assay to selectively screen small molecule inhibitors of Rab5-PI3Kβ binding in the future.
Epidermal growth factor (EGF)-mediated activation of EGF receptor (EGFR) leads to internalization and endosomal trafficking of the receptor. Feedback-regulated cross-talk between EGFR signaling and transport maintains cellular homeostasis; and the loss of spatiotemporal regulation of EGFR signaling leads to diseases such as cancers. However, the precise mechanism whereby ligand-dependent EGFR signaling controls individual endosomal transport steps, remains largely unknown. In this work, we integrate experimental measurements with mathematical modeling to identify key proteins mediating the coupling between ligand-concentration-dependent receptor signaling, and activities of Rab5 and Rab7 GTPases, which regulate early and late endosomal trafficking, respectively. In order to build a predictive, rule-based computational model, we experimentally measured a number of critical input parameters such as activation rates and protein copy numbers. Consistent with previous literature, we observed faster and higher levels of EGFR phosphorylation, as well as greater EGFR degradation in HeLa cells upon activation with high EGF dose (100 ng/mL), compared to low EGF dose (10 ng/mL). We measured rapid Rab5 activation in response to high EGF, mimicking EGFR phosphorylation kinetics, which was followed by robust Rab7 activation. Interestingly, Rab7 activation upon stimulation with low EGF dose was below detection. These results indicate that Rab7 activation plays a key role in shunting EGFR for degradation in response to high EGF stimulus. To probe the roles of regulatory proteins involved in Rab5-to-Rab7 conversion, we accurately measured copy numbers of GTPase activating proteins (GAPs) and Guanine nucleotide exchange factors (GEFs) controlling Rab5 and Rab7 activities using PRISM-SRM (high-pressure, high-resolution separations coupled with intelligent selection and multiplexing for selected reaction monitoring) technique. Measured protein copy numbers of Mon1A and Mon1B are almost 10-fold higher than previously reported. These new copy numbers are in line with Ccz1 copy numbers, thereby supporting Mon1 and Ccz1 forming an obligate heterodimer to act as a Rab7 GEF. Further experimental verification of model predictions regarding effects of modulating Mon1 and Ccz1 levels on EGF-mediated Rab7 activation kinetics is in progress. Proteomic analyses also revealed that while total copy numbers of two Rab5 GEFs, RIN1 and Rabex5, together are constant, the two Rab5 GEFs were differentially expressed in cells overexpressing Rab5 versus Rab7. These data suggest that the two Rab5 GEFs may have different roles in determining kinetics and route of EGFR transport. Taken together, our results demonstrate that Rab7 activation in response to stimulation with high dose of EGF drives...
All eukaryotes contain organelles that perform specialized functions essential for life. The Golgi complex, an organelle that is important for transport of cellular material, serves as the “Grand Central Station” of the cell by receiving, then sorting proteins and membranes to various locations. Many steps of vesicular trafficking at the Golgi complex are regulated by several families of small GTPases, which modulate between an active and inactive state. One particularly interesting GTPase is Rab1, which also has an established role in autophagy. Rab1 is activated by a guanine nucleotide exchange factor (GEF), a process that is conserved from mammals to yeast. Discordantly, mammals contain two protein complexes named TRAPPII and TRAPPIII (transport protein particle) which activate Rab1; while in yeast, four (TRAPPI - TRAPPIV) have been implicated as GEFs in Ypt1 (Rab1 homolog) activation. Yeast TRAPP complexes share a core set of six subunits. Additional subunits are thought to regulate the complex by adding substrate and compartmental specificity. TRAPPII contains four more subunits, while TRAPPIII and TRAPPIV include one additional subunit. Importantly, the precise functions of the accessory subunits remain unclear. We demonstrate that TRAPPII and TRAPPIII are the only two complexes present in yeast cells, which is consistent with findings in mammalian cells, by quantifying isolated TRAPP complexes and simultaneously monitoring the localization of all TRAPP complexes in vivo. We also find that, using an in vitro fluorescent nucleotide exchange assay, TRAPPIII activates Ypt1 more effectively than the other complexes. Concurrently, we notice that Trs85 localizes to the Golgi complex and that Ypt1 is mislocalized in vivo in the absence of the TRAPPIII-specific subunit, Trs85. Additionally, using an in vitro membrane binding assay, we find that Trs85 has an affinity for membranes. These findings suggest that there are only two TRAPP complexes in yeast, and that TRAPPIII is involved in trafficking at the Golgi complex in addition to its established role in autophagy. Taken together, our model suggests that the main role of Trs85 in the TRAPPIII complex is to bring the catalytic core close to a membrane surface, localizing the active site to the right compartment in an orientation that facilitates localization and activation of Ypt1.

Endocytic Trafficking 1

The mechanistic target of Rapamycin complex 1(mTORC1) is an evolutionarily conserved serine/threonine kinase that controls organismal growth, and have been found deregulated in several multi-faceted diseases including cancer and neurodegeneration. Here we show that overexpression of TFEB induces cellular endocytosis and formation of endosomes carrying activated protein kinase B (pT308-Akt), the amino acid transporter SLC38A9 and the tethering molecule RagD, crucial components of the lysosomal nutrient sensing complex, which stimulates mTORC1 activity. We further observed that these TFEB-induced endosomes en route to lysosomes were required to dissociate TSC2 and tether
mTORC1 to lysosomal membranes, this way restoring its activity during prolonged starvation. Finally, we find that TFEB-triggered endocytosis is required for lysosomal biogenesis and autophagy flux. Our results identify TFEB-mediated endocytosis as a critical process to regulate mTORC1 activity, lysosomal biogenesis and sustain autophagy.

P2291
Board Number: B437
Functional analysis of PI3P effector candidate SNX in Entamoeba histolytica.
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Phagocytosis and phagosome maturation are indispensable for the parasitism and pathogenesis of Entamoeba histolytica. Phosphoinositides (PIs) are well known regulators of membrane traffic during phagocytosis and phagosome maturation in model organisms. Previously we reported that one of the PIs, phosphatidylinositol 3-phosphate (PI3P), is localized on phagosomes and involved in phagosome maturation, however, PI3P downstream effectors known from model organisms are not conserved in E. histolytica. To identify unique PI3P effectors in E. histolytica, we conducted proteomic analysis of the isolated phagosomes from the transgenic strain expressing GFP-Hrs-FYVE, which binds to and is thus expected to compete for PI3P. Compared to the phagosomes from the parental strain, recruitment of two components of the retromer complex, Vps26 and Vps35, was not detected in the GFP-Hrs-FYVE expressing strain, suggesting that retromer recruitment to phagosomes is regulated via PI3P. It is well established in other organisms that the retromer complex is composed of Vps26, Vps29, Vps35, and PI3P-binding sorting nexins (SNXs). SNXs interact with PI3P via Phox homology (PX) domain. In silico analysis of the E. histolytica genome identified two PX domain-containing proteins, which were designated as EhSNX1 and EhSNX2. Biochemical and localization studies showed that both of them specifically bound to PI3P and localized on phagosomes. However, data from immunoprecipitation analysis suggested temporal, weak, or no interaction between EhSNXs and retromer complex. To understand the role of EhSNX1 and EhSNX2, transcriptional silencing of corresponding genes is underway.

P2292
Board Number: B438
Syntaxin-6 defines a cellular compartment distinct from the trans-Golgi network that accumulates internalized somatostatin receptor 2.
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Syntaxin-6 defines a cellular compartment distinct from the trans-Golgi network that accumulates internalized somatostatin receptor 2
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Somatostatin receptor 2 (SSTR2) is the major receptor mediating the effects of somatostatin. Upon somatostatin binding, SSTR2 rapidly traverses the endosomal system arriving in a juxta-nuclear compartment thought to be the trans-Golgi network (TGN). Using AtT20 cells expressing endogenous
SSTR2, we now demonstrated that consistent with previous reports, activated receptor rapidly internalizes in transferrin-labeled vesicles before shifting away from transferrin-labeled endosomes and into a syntaxin-6-enriched compartment. However, both confocal and stimulated emission depletion (STED) microscopy reveal that neither internalized SSTR2 nor syntaxin-6 co-localize with the TGN markers PIST and TGN38. Furthermore, neither nocodazole treatment, which leads to dispersed Golgi ministacks, nor Brefeldin A treatment, which leads to dispersion of the TGN, have any influence on the juxtanuclear localization of SSTR2 and syntaxin-6, which remain co-localized. Both STED and electron microscopy reveal that syntaxin-6 and SSTR2 localize to tubulo-vesicular juxta-nuclear structures. Sucrose gradient fractionation confirmed that the bulk of syntaxin-6 and SSTR2 signals are overlapping and distinct from TGN38. This compartment may serve as a reservoir for SSTR2 recycling since the association of SSTR2 and syntaxin-6 is reduced when the receptor recycles back to the cell surface. Our results indicate that upon ligand binding, SSTR2 accumulates in an ill-defined syntaxin-6-enriched compartment and strongly suggest that syntaxin-6 should not be used as a marker of the TGN. The identity of this compartment will provide clues about the protein machinery involved in SSTR2 cytoplasmic retention and recycling.

P2293
Board Number: B439
Involvement of the HPV E6 protein in the trafficking of several cellular SNX27 cargoes through the PDZ binding motif.
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A subset of high-risk Human Papillomaviruses (HPVs) are the causative agents of a large number of human cancers, of which cervical is the most common. Two viral oncoproteins, E6 and E7, contribute directly towards the development and maintenance of malignancy. A characteristic feature of the E6 oncoproteins from cancer-causing HPV types is the presence of a PDZ binding motif (PBM) at its C-terminus, which confers interaction with cellular proteins harbouring PDZ domains. We have shown that this motif allows E6 interaction with Sorting Nexin 27 (SNX27), an essential component of endosomal recycling pathways. This interaction is highly conserved across E6 proteins from multiple high-risk HPV types and is mediated by a classical PBM-PDZ interaction but unlike many E6 targets, SNX27 is not targeted for degradation by E6. Rather, in HPV-18 positive cell lines the association of SNX27 with components of the retromer complex and the endocytic transport machinery is altered in an E6 PBM-dependent manner. Analysis of a SNX27 cargo, the glucose transporter GLUT1, reveals an E6-dependent maintenance of GLUT1 expression and alteration in its association with components of the endocytic transport machinery. Since E6 interaction with SNX27 can alter the recycling of cargo molecules, one consequence of which is modulation of nutrient availability in HPV transformed tumour cells, we decided to analyse in detail which other targets involved in the trafficking pathway could be affected by the SNX27-HPVE6 interaction. With this aim we generated a Hela cell line expressing GFP-SNX27 and analyse the SNX27 proteom interaction profile from these clones in the presence or absence of E6. Several cargoes were immunoprecipitated such as the PBM containing proteins Emilin2 and Tanc2, both involved in cell growth, migration and transformation pathways.
Microvillus Inclusion Disease (MVID) is a rare form of congenital diarrhea resulting from inactivating mutations in Myosin Vb (MYO5B). The underlying cause of MVID associated diarrhea is currently unknown. We hypothesize that loss of MYO5B results in aberrant expression of key apical enterocyte membrane transporters that promote the absorption of water. Our lab has generated a mouse model of MVID with a germline deletion of MYO5B (MYO5B KO). Duodenal tissue was collected from neonatal (3-5 day old) MYO5B KO mice and wildtype (WT) littermates. Immunofluorescence staining was performed to determine the localization of apical transporters and proteins in intestinal tissue of MYO5B KO and WT mice. Human biopsies from healthy individuals and from individuals harboring a Navajo specific mutation in MYO5B resulting in MVID were immunostained to examine the localization of brush border proteins. Transgenic mice expressing a LifeAct-EGFP peptide, labeling F-actin were crossed with MYO5B KO mice to derive MYO5B KO;LifeAct-EGFP mice. Enteroids were generated from duodenal tissue of neonatal MYO5B KO, WT, MYO5B KO;LifeAct-EGFP and LifeAct-EGFP to determine the functional capacity of CFTR in vitro. Immunostaining for phosphorylated ezrin (P-ezrin) showed numerous P-ezrin positive inclusions in the duodenum of MYO5B KO mice, no inclusions were observed in WT mice. To assess the localization of key transporters known to regulate water absorption, we performed immunofluorescence staining for the NHE3, SGLT1 and CFTR. WT mice showed apical localization of NHE3, SGLT1 and CFTR. In contrast, MYO5B KO mice showed decreased apical expression and increased subapical expression of NHE3 and SGLT1. NHE3 and SGLT1 were present in intracellular inclusions in MYO5B KO mice. MYO5B KO mice had CFTR positive inclusions, but CFTR was still present on the apical membrane of enterocytes. Measurement of luminal Cl- showed a significant increase in Cl- in intestinal flushes of MYO5B KO compared to WT mice. Consistent with these findings, MVID patient biopsies showed decreased apical expression of NHE3 but CFTR remained on the apical membrane. MYO5B KO derived enteroids had decreased apical expression of SGLT1 however CFTR was expressed on the apical membrane in MYO5B KO and WT enteroids. A forskolin swelling assay demonstrated that CFTR was functional in MYO5B KO;LifeAct-EGFP and LifeAct-EGFP derived enteroids. Collectively, these data suggest that decreased apical expression of NHE3 and SGLT1 may be responsible for the dysfunction in water absorption in individuals with MVID. Furthermore, CFTR may be trafficked and recycled predominantly through a MYO5B-independent pathway leading to exacerbated water loss by continued secretion of chloride into the intestinal lumen by apical CFTR.

Amyloid Precursor Protein (APP) undergoes complex processing involving two separate pathways. Misprocessing at the terminal step of one pathway by the gamma -secretase complex leads to the
production of different-sized versions of the peptide a-beta, which have been implicated in the onset of Alzheimer’s disease. Neither the cellular factors that may contribute to this misprocessing nor the exact cellular location where the misprocessing occurs are clearly understood, but evidence suggests that cholesterol may influence the misprocessing. Previously, we found that a phospholipase A2 plays a role in the intracellular trafficking of LDL-derived free cholesterol, and inhibition of this PLA2 with ONO RS-082 (ONO) results in the accumulation of cholesterol in the Endocytic Recycling Compartment (ERC) when cells are exposed to certain levels of LDL. Immunofluorescence microscopy with an antibody that recognizes different forms of APP revealed that treatment of cells with ONO resulted in the accumulation of APP in the ERC even when Rab11 was knocked down. Furthermore, immunoblotting showed the processing of APP was altered when cells were treated with ONO. To further investigate factors involved in the localization and processing of APP, cells were grown in fetal bovine serum containing different levels of LDL. Populations of these cells were stressed by nutrient starvation, and the localization of APP was compared to the localization of Rab11 using immunofluorescence microscopy. In stressed cells, treatment with ONO altered the distribution of APP even under conditions in which the distribution of cholesterol was not significantly altered. In stressed cells grown in LDL levels of 6 mg/dl, there was little or no colocalization between APP and Rab11, but following ONO treatment significant colocalization in a compact, juxtanuclear pattern, which was similar to the response of unstressed cells grown in 15-20 mg/dl LDL. In 15 mg/dl LDL, stressed cells exhibited partial colocalization between Rab11 and APP, but APP was also localized to the Golgi complex. When stressed cells were treated with ONO, the number of cells exhibiting a compact, juxtanuclear pattern increased. In stressed cells grown in 25mg/dl LDL, the compact pattern of colocalization was observed in control cells. After ONO treatment, this same pattern could be discerned, but the overall distribution of rab11 and APP was more diffuse, with punctate structures scattered in the cytoplasm. These results are consistent with evidence that the rab11 endosome serves as a hub for APP trafficking. They also suggest that cholesterol levels affect APP processing and that cell stress may also play a role.

P2296
Board Number: B442
Reduced expression of the Endosomal Sorting Complex Required for Transport (ESCRT)-associated factor HD-PTP/PTPN23 in mice causes reduced fat accumulation and increased mortality.
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The Endosomal Sorting Complexes Required for Transport (ESCRTs) facilitate a unique form of membrane deformation enabling the budding of membranes away from the cytoplasm (e.g. intraluminal vesicle formation during multivesicular body sorting) or the scission of a membrane tubule from within the structure (e.g. abscession during cytokinesis). This unique machinery has been implicated in a number of cellular processes including cytokinesis, receptor down-regulation via MVB sorting, exosome biogenesis, autophagy, and plasma membrane repair. Greater understanding of the contributions of the ESCRTs to animal physiology has been confounded by the observations that multiple ESCRT knockout mouse models have exhibited embryonic lethality. The mammalian Bro1 Family proteins ALIX/PDCD6IP and HD-PTP/PTPN23 are ESCRT-associated factors that appear to exhibit
unique contributions to different ESCRT-driven processes. While the ALIX knockout mouse model was viable with a normal lifespan, the HD-PTP knockout mouse exhibited embryonic lethality, suggesting HD-PTP may play a more central role in critical ESCRT-driven processes. To gain further insights into the unique contributions of HD-PTP to mammalian physiology, a murine HD-PTP hypomorphic animal model (HD-PTP\textsuperscript{hyp/o}) was generated. This allele reduced HD-PTP expression more than 80% in both mouse embryonic stem cells and mouse embryonic fibroblasts. HD-PTP\textsuperscript{hyp/o} animals were viable but exhibited a number of unexpected phenotypes. Reduced HD-PTP expression resulted in increased mortality with 6-month survival approximately 25%. The HD-PTP\textsuperscript{hyp/o} animals were reduced in mass compared to wild-type littermates. Fat mass was particularly affected evident at 6-weeks as assessed by whole body composition analysis. Mass analyses of individual fat tissues and histological examinations supported this observation of reduced fat accumulation. Reduced vacuolization within the HD-PTP\textsuperscript{hyp/o} liver, consistent with reduced lipid accumulation, was also observed, and Oil Red O staining of isolated HD-PTP\textsuperscript{hyp/o} and wild-type hepatocytes indicated reduced lipid droplet area with reduced HD-PTP expression. Oleate loading of HD-PTP\textsuperscript{hyp/o} mouse embryonic fibroblasts similarly exhibited reduced lipid droplet area compared to wild-type cells. Reduced lipid droplet area was also evident in HD-PTP\textsuperscript{hyp/o} brown adipose tissue and inguinal white adipose tissue in vivo. These results support the conclusion that reduced HD-PTP expression perturbs lipid accumulation in multiple tissues and suggest that the defect in lipid accumulation may occur in a cell autonomous manner.

**P2297**

**Board Number: B443**

**Genome-wide siRNA screen identifies GBF1-interacting protein UNC50 as a differential regulator of Shiga toxin trafficking.**

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Shiga toxins 1 and 2 (STx1 and STx2) are potent bacterial toxins that induce cell death by targeting ribosomal function and shutting down protein synthesis. This mechanism relies on retrograde transport to endoplasmic reticulum, from where toxin’s catalytic subunit is translocated into cytosol. During early endosome-to-Golgi transport the toxins evade the lysosomal degradation pathway. Targeting this trafficking step has therapeutic promise, but mechanisms are unclear for STx2, which is more potent and is typically associated with poor clinical prognosis. We performed a viability-based, genome-wide siRNA screen in HeLa cells to identify host factors required for early endosome-to-Golgi trafficking of STx1 or STx2. We identified S64, S35, and 196 hits that were required for toxicity induced by STx1-only, STx2-only, or both toxins, respectively. Depletion of Golgi localized UNC50 prevented early endosome-to-Golgi trafficking of STx2, in turn routing it to lysosomes for degradation. UNC50 was also required for optimal Golgi delivery of STx1. Mechanistic assays revealed that UNC50 acted by recruiting GBF1, an ADP-ribsylation factor guanine nucleotide exchange factor (ARF-GEF), to the Golgi. These findings provide new insight into STx2 intracellular transport mechanisms, and may advance efforts to generate therapeutically viable toxin trafficking inhibitors.
P2298
Board Number: B444
Interrogating the thiol-disulfide redox status of the mammalian cell surface by ratiometric fluorescence imaging.
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The exofacial/extracellular thiol/disulfide (SH/SS) redox status has been shown to play critical roles in a range of mammalian cell behaviors, including adhesion, migration and invasion. Two proteins, whose activities potentially oppose each other, the disulfide-generating Quiescin-sulfhydryl oxidase (QSOX), and the disulfide reductase activity of protein disulfide isomerase (PDI), are believed to contribute to extracellular SH/SS status. However the range of their substrates, and the possible synergy between these oxidoreductases remain cryptic. To investigate exofacial SH/SS status in a range of mammalian cells in culture, we have developed conventional confocal and super-resolution fluorescent imaging methods that can quantitatively assess the status of cell surface proteins using impermeant fluorescent maleimide reagents and selective reduction of exofacial disulfides. Using these approaches, effects of extracellular QSOX and PDI on surface SH/SS can be observed and quantitated. We will also present the impact of components of the extracellular matrix, and their in vitro mimics, on the exofacial SH/SS status of a range of mammalian cell types.

P2299
Board Number: B445
PI4K2A controls PI4P production and Rab7-cycling in late endosomal compartments.
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Vesicular trafficking between various organelles requires proper sorting of cargos in endocytic compartments. During this process, different Rab small G proteins mark the endosomes at different stages of their maturation. In addition, endosomes also change their phosphoinositide profiles during their conversion where both 3-phosphorylated and 4-phosphorylated inositol lipids play important roles. Type II phosphatidylinositol 4-kinases, PI4K2A and PI4K2B are localized in various endocytic compartments and have been implicated in the trafficking of various cargoes. In this study, we analyzed the effect of PI4K2A gene deletion on endosomal PI4P levels and cargo sorting. PI4K2A knockout HEK293 cells were created by CRISPR/Cas9 genome editing. PI4K2A-deficient cells showed no compensatory increase in PI4K2B and PI4KB, other PI4Ks acting on endosomes. A bioluminescence resonance energy transfer (BRET) method was developed to measure PI4P levels in different Rab-positive compartments. These measurements showed that PI4P levels were highest in Rab7-positive compartment, and PI4K2A had the biggest contribution to the PI4P in this pool. PI4K2A knockout didn’t affect the degradation of EGFR, or the endocytosis or trafficking of the transferrin receptors. PI4K2A deletion, however, caused tubulation of Rab7-positive compartments similar to those observed in cell expressing constitutively active Rab7. Current experiments are focused on understanding the mechanism by which PI4P affects the GTP-GDP status of Rab7 and how these changes affect the function(s) of late endosomes.
P2300
Board Number: B446
Regulation of membrane scission in yeast endocytosis.
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Endocytosis involves rearrangement of the plasma membrane in a controlled sequence: a flat membrane forms an invagination that undergoes scission to produce a vesicle. In yeast, endocytic sites are very stereotypic, recruiting over 50 proteins, most with mammalian homologues, to effect these membrane shape-changes and produce cargo-filled vesicles. These proteins can be assigned to separable modules based on their role in the endocytic timeline. Here we study the scission module of yeast endocytosis by combining correlative light and electron microscopy with live-cell imaging of fluorescently tagged proteins in wild type and mutant cells.

A mechanistic understanding of scission remains incomplete although several hypotheses have been proposed and proteins that influence the scission process have been identified: BAR proteins interact with the invagination neck concomitant with scission, and phospholipid phosphatases are known to affect scission dynamics. We find that localization of the yeast BAR protein complex Rvs161/167 (Rvs) is influenced by an interaction of the Rvs167 SH3 domain, aside from its expected BAR-membrane interaction. The SH3 domain also affects the number of Rvs molecules at endocytic sites and progression of the invagination, suggesting that the domain is more important for the function of Rvs than has been assumed. Rvs allows invaginations to grow to around 160nm before scission, while deleting it results in either scission failure or causes scission at half that length. We thus conclude that Rvs prevents premature vesicle scission, and both BAR and SH3 domains are important for its role as a regulator of the scission machinery.

We have tested current models of membrane scission and find that although Rvs plays an important role, scission timing is independent of the number of Rvs molecules at endocytic sites, suggesting that the recently proposed mechanism of BAR-induced protein friction on the membrane is not likely to cause scission. Deleting yeast synaptojanin Inp51 or dynamin Vps1 also does not influence scission dynamics: it is likely that interfacial forces at lipid boundaries are not sufficient, and forces exerted by dynamin are not required, to produce vesicles. We propose a mechanism of membrane scission in which Rvs is recruited to endocytic sites by both BAR and SH3 domains, and the SH3 domain of Rvs167 regulates actin forces on the invagination neck, modulating scission timing.

P2301
Board Number: B447
Elucidating the Endocytosis Mechanism of Cx36.
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Gap junctions are clusters of channels that directly connect the cytoplasms of apposing cells and permit the passage of hydrophilic molecules less than approximately 1.5kD in size. The channels are comprised of proteins called connexins (Cxs) which are four-pass transmembrane spanning proteins with their N- and C-termini residing in the cytoplasm. Twenty-one connexins exist in the human genome and are expressed tissue specifically.

Cx36 is of great interest to the gap junction and neuroscience fields because it is expressed in the brain where it forms electrical synapses. Furthermore, it is also expressed in the pancreas where it is needed
for the synchronous release of insulin into the blood stream from pancreatic beta-cells. A better understanding of Cx36 promises to aid in an improved understanding of endocytosis, neuron communication, and diabetes.

An important aspect of gap junction regulation involves its turnover and endocytosis. The regulation of connexin endocytosis has been shown to be critical for proper tissue function. Despite the fact that there is significant phylogenetic distance and amino acid diversity between connexins, most seem to undergo endocytosis in a similar manner. The majority of connexin endocytosis research has been conducted on Cx43, the most commonly expressed connexin, and has been shown to involve the clathrin mediated endocytosis (CME) pathway. This study addresses the turnover of Cx36, an important phylogenetically distant cousin of Cx43.

We have shown that gap junctions comprised of Cx36 form circularized Annular Gap Junctions during their endocytosis in a manner very similar to the more commonly studied Cx43. We demonstrated through cycloheximide half-life assays that Cx36-GFP exists in the membrane for approximately 2 hours. We have also shown through clathrin knockdown experiments evidence that suggests that clathrin is required for Cx36 endocytosis. Further research into the endocytosis mechanism is ongoing at this time.

P2302
Board Number: B448
NECAPs are negative regulators of the AP2 clathrin adaptor complex.
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Eukaryotic cells internalize transmembrane receptors via clathrin-mediated endocytosis, but it remains unclear how the machinery underpinning this process is regulated. We recently discovered that membrane-associated muniscin proteins such as FCHO and SGIP initiate endocytosis by converting the AP2 clathrin adaptor complex to an open, active conformation that is then phosphorylated. Here we report that loss of ncap-1, the sole C. elegans gene encoding an adaptiN Ear-binding Coat-Associated Protein (NECAP), bypasses the requirement for FCHO-1. Biochemical analyses reveal AP2 accumulates in an open, phosphorylated state in ncap-1 mutant worms, suggesting NECAPs promote the closed, inactive conformation of AP2. Consistent with this model, NECAPs preferentially bind open and phosphorylated forms of AP2 in vitro and localize with constitutively open AP2 mutants in vivo. NECAPs do not associate with phosphorylation-defective AP2 mutants, implying that phosphorylation precedes NECAP recruitment. We propose NECAPs function late in endocytosis to inactivate AP2.

P2303
Board Number: B449
QUANTITATIVE IMAGING AND STATISTICAL ANALYSIS OF THE DYNAMICS OF CLATHRIN-DEPENDENT AND -INDEPENDENT ENDOCYTOSIS REVEALS A DIFFERENTIAL ROLE OF ENDOPHILINA2 IN DYNAMIN2 RECRUITMENT.
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Eukaryotic cells internalize cargos specifically through clathrin-mediated endocytosis (CME) or clathrin-independent processes (CIE). EndophilinA2 was shown as preferentially implicated in CIE although
initially involved in CME. Here, we investigated the native interplay of endophilinA2 and dynamin2 during CME as compared to CIE. We developed an unbiased integrative approach based on genome-engineering, robust tracking methodology and advanced analytics. We statistically identified several CME and CIE subpopulations corresponding to abortive, active and static endocytic events. Depletion of dynamin2 strongly affected active CME and CIE events, whereas the absence of endophilinA2 impacted only CIE. Accordingly, we demonstrated that endophilinA2 is needed for dynamin2 recruitment during CIE, but not in CME. Despite these differences, endophilinA2 and dynamin2 acted at the latest stage of endocytosis within a similar stoichiometry in both mechanisms. Thus, we propose a conserved function of dynamin2 and endophilinA2 in vesicle scission, but a differential regulation of their recruitment during CME and CIE.

P2304
Board Number: B450
Kidney proximal epithelial cells apically express the neuronal-surface P-antigen (NSPA) cross-targeted by anti-ribosomal P antibodies from lupus patients.
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Anti-ribosomal-P proteins (anti-P) antibodies from patients with systemic lupus erythematosus are potentially pathogenic in the brain, skin, liver and kidney epithelial cells. We recently disclosed a pathogenic mechanism in the brain involving anti-P cross-reaction with a cell surface protein of unknown function, which we called neuronal-surface P-antigen (NSPA). Because anti-P antibodies have been associated with lupus nephritis here we explore whether NSPA is an anti-P target also in the kidney. Immunoblot and RT-PCR revealed the expression of NSPA in the kidney. Histochemistry of β-galactosidase reflecting the activity of NSPA promoter in knock-in mice revealed NSPA expression mainly in the proximal epithelial tubule cells (PTEC). Immunohistochemistry showed NSPA polarized to the apical domain of PTEC. Cell surface domain-specific biotinylation assays and transfection of mCherry-NSPA showed a similar predominant apical distribution in MDCK cells. Immunization of mice with recombinant P0 ribosomal protein bearing the P-antigen generated high titers of anti-P antibodies and showed renal juxtaglomerular interstitial infiltration. These results indicate that NSPA has apical sorting information and suggest that anti-P antibodies must be filtered through the glomerulus to reach its cell surface antigen causing damage in the kidney.

P2305
Board Number: B451
TrkA signaling endosomes: Association with Rab7 and Rab11 and dynamic fusion and fission events as possible mechanisms of signaling endosome diversification.
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Protein trafficking is involved in all aspects of neuronal function including development, axon/dendrite growth, and synaptic function. Neurons of the peripheral nervous system in particular are in distinct
need of specialized protein transport due to the great length of their axons and need to coordinate axon growth and presynaptic development with dendritic growth. One receptor that is critical for the development of the sympathetic nervous system, TrkA, has been shown to undergo complex trafficking events. In sympathetic neurons, TrkA is expressed during development and encounters its high-affinity ligand, Nerve Growth Factor (NGF), once the axon reaches its final target tissue. TrkA binds NGF and the complex is internalized into a signaling endosome. The NGF-TrkA signaling endosome is critical in the axon for outgrowth and branching, but it is also transported retrogradely to the soma and to dendrites where it is necessary for survival signaling and synapse formation, respectively. We hypothesize that the multitude of NGF-dependent outputs, such as survival and synapse development, relies on functional and molecular diversification of the signaling endosome. We thus determined the trafficking steps and association of signaling endosomes with distinct effector proteins, especially members of the small GTPase family of Rab proteins. Indeed, we find that signaling endosomes are molecularly diversified into Rab7- and Rab11-positive populations in vitro and in vivo. This diversification happens early since retrograde axonal transport carriers can be Rab7 or Rab11 positive. Additionally, we find that signaling endosomes continue to exist in multiple distinct pools as they transport into dendrites. Surprisingly, TrkA-NGF signaling endosomes undergo dynamic sorting events, including fusion and fission in dendrites. These dynamic events might drive diversification of signaling endosomes and suggest a role for sub-populations of these endosomes to function independently in distinct signaling pathways.

P2306
Board Number: B452
ApoER2 and Its Ligand Reelin Follow a Clathrin-Independent Endocytosis.
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Reelin is an extracellular glycoprotein with essential roles in the formation of laminated brain structures formation such as neocortex, hippocampus and cerebellum, during vertebrate embryonic development, as well with roles in learning and memory in the adult. Reelin receptors are members of the apolipoprotein E receptors and include ApoER2 and VLDLR. The disruption of ApoER2 results in cognitive and motor dysfunction, conditions that are more severe than the ones resulting from VLDLR disruption. Although, the reelin/ApoER2 signaling pathway has been widely described, little is known about its endosomal trafficking. Our laboratory has previously demonstrated that, in the absence of ligand, ApoER2 is internalized through a clathrin-dependent endocytosis (CDE) depending on the NPxY cytoplasmic motif that is recognized by the adaptor protein Dab2. Besides CDE there are clathrin-independent endocytosis (CIE) pathways, including one controlled by the small GTPase Arf6 which regulates membrane trafficking and actin cytoskeleton dynamics. Here we show for the first time that, in HeLa cells, ApoER2 and reelin are internalized using a clathrin-independent endocytosis (CIE) pathway, specifically by Arf6-pathway. In clathrin-silenced cells ApoER2 is still internalized in the presence of reelin, being the receptor able to arrive to degradative Rab7-compartment. Altogether, our results suggest that ApoER2 has at least two different routes of internalization, the already demonstrated CDE and the Arf6-pathway. We also found a similar spatial pattern of expression of endogenous ApoER2 and Arf6-overexpressed in hippocampal primary culture neurons where both proteins were found within axon dilation. Therefore, our working model is that reelin modifies ApoER2 endosomal dynamics by facilitating its traffic toward degradative pathways by Arf6-CIE. Funded by Fondecyt Regular 1150444, Chile and Vicerrectoria de Investigación PUC.
P2307

Board Number: B453

Alpha-arrestins Aly1 and Aly2 regulate trafficking of the glycerophosphoinositol transporter, Git1 and impact lipid homeostasis in cells.
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Cells must reorganize proteins at the plasma membrane (PM) in response to the extracellular environment in order to grow and survive. Protein trafficking adaptors that recognize select protein cargo and mark them for removal from or targeting to the PM are critical regulators of the PM proteome. A family of trafficking adaptors known as the alpha-arrestins has an emerging role in this regulation. Alpha-arrestins bind cargo proteins and recruit a ubiquitin ligase, which ubiquitinates the cargo protein to mark it for endocytosis. To date a limited number of alpha-arrestin–cargo pairs are known and the mechanisms by which alpha-arrestins control selective protein trafficking are relatively unclear. Our studies suggest a novel role for alpha-arrestins in maintaining phosphatidylinositol (PI) homeostasis. We find that alpha-arrestins Aly1 and Aly2 control endocytosis of the glycerophosphoinositol (GPI) transporter, Git1. GPI is a metabolite whose breakdown releases inositol which can be used for the synthesis of PI. Loss of Aly1 and Aly2 results in retention of Git1 at the PM, increased uptake of GPI, and sensitivity to exogenously added GPI. Aly1 and Aly2 mutants that are unable to bind the ubiquitin ligase Rsp5 fail to internalize Git1 from the PM, demonstrating that Aly1- and Aly2-mediated trafficking of Git1 requires Rsp5. Internalization of Git1 additionally requires well-established clathrin-mediated endocytic components, such as End3 and Vrp1. The AP-2 complex is also important for Git1 endocytosis; We have previously shown that Aly1 and Aly2 interact with AP-2 and we are currently assessing the role of this interaction in regulation of Git1. Interestingly, dephosphorylation of Aly1 by the protein phosphatase calcineurin, which controls other key regulators of sphingolipid homeostasis, is required for optimal Aly1-mediated trafficking of Git1. Consistent with a broader role for these alpha-arrestins in controlling sphingolipid balance in cells, loss of Aly1 and Aly2 results in sensitivity to myricin, a chemical that inhibits the first step in sphingolipid biosynthesis, and resistance to aureobasidin A, a chemical that blocks sphingolipid biosynthesis at a later step by preventing inositol-containing ceramide production. Preliminary data suggests that Aly1 and Aly2 may regulate Git1 through distinct mechanisms, with Aly1 being more important for ligand-induced endocytosis of Git1 while Aly2 is more important in the constitutive endocytosis of Git1. These findings implicate the alpha-arrestins as new players in regulating the recycling of the PI catabolite, GPI, into cellular metabolism, including PI biosynthesis, which likely impacts sphingolipid metabolism.

P2308

Board Number: B454

Screening of the ScUbl yeast deletion library for modifiers of Aly1- or Aly2-mediated resistance to rapamycin in an undergraduate lab course.
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Cells respond to cues in their extracellular environment by selectively redistributing proteins. This reorganization is imperative for cell survival and is regulated, in part, by alpha-arrestins. How then is alpha-arrestin-mediated trafficking controlled? We know that modification by ubiquitination plays a role in modifying alpha-arrestin function. To help us identify specific alpha-arrestin regulators, we generated and utilized a unique yeast gene deletion library called the Saccharomyces cerevisiae Ubiquitin

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Interactome (ScUbl) library. ScUbl contains all the non-essential genes annotated as important for ubiquitination and ubiquitin interaction. We used this library as part of the BIOL 371w Cell & Molecular Biology laboratory course to screen for gene deletions that altered the growth phenotypes associated with over-expression of alpha-arrestins. Specifically, the undergraduates transformed the ScUbl library with plasmids over-expressing alpha-arrestins Aly1 and Aly2 and then assessed these transformants for gene deletions that either increased or decreased cells sensitivity to rapamycin, an inhibitor of TORC1 function that mimics nitrogen starvation. Three replicate screens of the ScUbl library were evaluated for changes in rapamycin sensitivity when over-expressing Aly1 or Aly2 identified 45 and 39 hits respectively as having >1 or < -1 Z-scores. Of these, 11 candidates are found in both the Aly1 and Aly2 screens. From this, Atg7 was chosen as a strong candidate and further assessed. We show that in cells lacking Atg7, Aly1 and Aly2 electrophoretic-mobility is altered, and demonstrate that this is due to a change in phosphorylation status of the alpha-arrestins. These data suggest that alpha-arrestins are regulated by changes in the autophagy pathway and we are currently working to define that role.

P2309
Board Number: B455
Systematic analysis of the molecular architecture of endocytosis reveals a nanoscale actin nucleation template that drives efficient vesicle formation.
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Clathrin-mediated endocytosis is an essential cellular function in all eukaryotes. It is a highly efficient process that is driven by a self-assembled macromolecular machine of over 50 different proteins in tens to hundreds of copies. How so many proteins can be organized to produce endocytic vesicles with high precision and efficiency is not understood. Here, we developed high-throughput superresolution microscopy to reconstruct the nanoscale structural organization of 23 endocytic proteins from over 100,000 endocytic sites in yeast. We found that after stochastic initiation assembly proceeds by a radially ordered recruitment according to protein function. WASP family proteins formed a circular nano-scale template on the membrane to spatially control actin nucleation during vesicle formation. Mathematical modeling of actin polymerization showed that this WASP nano-template allows actin polymerization to create sufficient force for membrane invagination, and substantially increases the efficiency of the budding reaction. Such nanoscale pre-patterning of actin nucleation may represent a general design principle for force generation in other membrane remodeling processes such as cell migration and division.

P2310
Board Number: B456
NBEAL2 is required for retention of endocytosed and megakaryocyte synthesized α-granule cargo proteins.
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Platelets are blood cells that facilitate clotting to prevent bleeding from wounds. Platelet function is linked to secretion of a wide variety of proteins carried in α-granules. Platelets and their granules are
produced by megakaryocytes, bone marrow-resident cells that can each release several hundred platelets into circulation. Most α-granule cargo is synthesized by megakaryocytes (e.g. Von Willebrand factor; VWF), but some is endocytosed from plasma, including fibrinogen. In Gray platelet syndrome (GPS), individuals lacking expression of NBEAL2 (neurobeachin-like 2) have platelets that lack α-granules and their protein cargo. The function of NBEAL2 is poorly understood. In previous studies we established Nbeal2/- mice as an animal model of GPS, and observed abnormalities in megakaryocyte development that included externalization of VWF. In this study we investigated the role of NBEAL2 in α-granule cargo protein trafficking by comparing the uptake, release and intracellular localization of labeled fibrinogen in megakaryocytes cultured from wild type (WT) and Nbeal2/- mice. While initial uptake of fluorescently-labeled fibrinogen was similar in WT and Nbeal2/- megakaryocytes, the latter showed considerably less retention of fibrinogen as assessed via flow cytometry and confocal fluorescence microscopy. Using biotinylated fibrinogen, we observed that neither WT nor Nbeal2/- megakaryocytes degraded endocytosed protein, but Nbeal2/- cells released it back to the medium much more rapidly than WT cells. Using confocal fluorescence microscopy to track fluorescently-labeled fibrinogen within megakaryocytes, we observed sequential colocalization of fibrinogen with subcellular compartments positive for RAB5, RAB7 and P-selectin in both WT and Nbeal2/- cells. In WT megakaryocytes the association of fibrinogen with the P-selectin positive compartment persisted, but this association was brief in Nbeal2/- megakaryocytes, as fibrinogen passed on to a RAB11-positive compartment and was subsequently lost from the cells. We also observed colocalization of VWF with RAB11 in Nbeal2/- megakaryocytes, indicating a common route of exit for both endocytosed and endogenously-synthesized α-granule cargo via recycling endosomes. A strong colocalization of NBEAL2 with P-selectin was observed in human megakaryocytes, supporting the conclusion that NBEAL2 associates with α-granules and/or their precursors, and is required for the proper loading and/or retention of granule cargo.

P2311

Board Number: B457

Clathrin Light Chain A is Specifically Required for Efficient Cell Spreading and Migration.

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The highly conserved clathrin heavy chain is the structural component of the clathrin triskelion. Its canonical function in membrane trafficking is well established, while additional roles have been more recently appreciated. In vertebrates, the triskelion also contains two clathrin light chains (CLCs, denoted CLCa and CLCb), each also highly conserved but with only approximately 60% identity, and with distinctive tissue distributions suggestive of exclusive functions. Yet, the few putative activities of CLCs, including coordination of actin with endocytosis, regulation of coat assembly, and G-clathrin formation and mediation of integrin recycling (PMCID: PMC4050264), appear to be shared by both CLCs. Only recently has a CLCb-specific role in regulation of endocytosis rates and dynamics been identified (PMID:28171750), while a distinct function for CLCa has remained elusive. Here we show that in detached cells, CLCa is uniquely responsible for promoting efficient cell spreading after plating, and for full mobility in subsequent cell migration. Selective depletion of CLCa using multiple siRNAs reduced spreading of HeLa, H1299 and HEK293 cells by ≥50% compared to untreated or scrambled siRNA controls. Depletion of CLCb did not inhibit spreading, and exogenous expression of siRNA-resistant CLCa but not CLCb could rescue in CLCa-depleted cells. While the initial isotropic phase of spreading is reduced in the treated cells, cell attachment is not: indeed, surface levels of active and inactive β1-integrins that largely drive attachment are unaffected by CLCa depletion. However, CLCa was required for efficient

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integrin-mediated activation of Src and for targeting of phosphorylated FAK and paxillin to properly positioned focal adhesions. Depletion of CLCa, but not CLCb, also altered intracellular distributions of clathrin, actin, WAVE2, inactive β1-integrin, and EEA1-decorated sorting endosomes. We propose that CLCa plays key roles in targeting of endosomal components and functional actin recruitment during early phases of cell spreading, with important implications for understanding normal and neoplastic cell detachment, migration and reattachment.

Establishment and Maintenance of Polarity

P2312
Board Number: B459
Suppressors of pam-1: Uncovering the role of PAM-1 in regulation of anterior-posterior polarity in the one-cell C. elegans embryo.
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Anterior-posterior axis establishment occurs in the one-cell C. elegans embryo, triggered by the sperm-donated centrosome through two redundant mechanisms. First the centrosome cues rearrangement of the actin-myosin cortical cytoplasm, which reorganizes the cell contents through cortical flows. In the absence of flows, microtubules from the centrosome can cue loading of the cortical PAR-2 protein to the posterior cortex to polarize the cell. In our recent work, we have shown that the PAM-1 puromycin-sensitive aminopeptidase is required for centrosome positioning at the cortex and that sustained contact between the centrosome and the cortex enhances polarity establishment through both mechanisms. pam-1 mutants produce nonviable embryos with a sparse cortical microfilament network, compromising polarity establishment through the first pathway. Longer centrosome contact with the posterior cortex can rescue polarity by enhancing both the cortical flow and the PAR loading pathways. To learn more about PAM-1’s mechanism of action, we have identified suppressors of pam-1 that raise the hatch rate from 2% to 35-50%. By looking at the actin-myosin cytoskeleton, centrosome dynamics, and polarity landmarks in these suppressed strains, we hope to learn more about the cross-talk between these two polarity establishment pathways and the role of PAM-1 in regulation of centrosome positioning and polarity establishment.

P2313
Board Number: B460
Suppressor screening to identify new regulators of anterior-posterior axis establishment in Caenorhabditis elegans.
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Polarity is established in the one-cell C. elegans embryo shortly after fertilization and determines the anterior-posterior body axis. This polarization is cued by the sperm-donated centrosome, which triggers changes in the posterior cortex. The PAM-1 aminopeptidase plays a role in axis polarization through regulation of the cortical cytoskeleton and centrosome positioning. PAM-1 is a highly conserved aminopeptidase that targets unknown proteins for degradation. In order to identify novel regulators of centrosome positioning and targets of the PAM-1 aminopeptidase, we took advantage of the maternal-effect embryonic-lethal phenotype of pam-1 mutants to conduct a suppressor screen. We have identified six suppressors, which significantly increase the pam-1 hatch rates from 2% to between 25-
75%. Most of the suppressors are recessive and can suppress both missense and nonsense alleles of *pam-1*. Using DIC microscopy we have found that each suppressor significantly rescues the polarity defects, such as the lack of pseudocleavage and the symmetric cleavage, found in *pam-1* mutants. Four of our suppressors have been mapped, three to different positions on chromosome I and one to chromosome II. We are currently using whole genome sequencing and RNAi to identify the suppressor mutations. Future characterization of these suppressors is likely to uncover new insights into the role of this aminopeptidase in centrosome positioning and polarity establishment and may lead to identification of targets of the aminopeptidase.

P2314
Board Number: B461
Deciphering the role of centrosomes in symmetry breaking of the *C. elegans* zygote.
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The newly fertilized *C. elegans* zygote exhibits uniform contractions of the acto-myosin cortical network driven by RHO-1 (RhoA). Thereafter, symmetry of the system is broken, as evidenced by the clearance of the RHO-1 guanine-nucleotide-exchange factor (GEF) ECT-2 from the cortex next to centrosomes, which results in local RHO-1 inactivation and cortical relaxation¹,². This is followed by anteriorly-directed flows of the acto-myosin cortex and segregation of anterior and posterior PAR proteins into distinct cortical domains, with PAR-6 on the anterior and PAR-2 on the posterior³. It has been shown that laser-mediated removal of centrosomes prevents polarity establishment⁴, but the mechanisms through which centrosomes instruct symmetry breaking remain to be elucidated. Intriguingly, we observed that zygotes from a mutant that lacks centrosomes undergo spontaneous symmetry breaking, leading to the formation of a PAR-2 domain either on the anterior, on the posterior or on both sides (bipolar phenotype). Analogous phenotypic manifestations have been found in embryos depleted of the centrosomal proteins SPD-2 or SPD-5⁵,⁶. Likewise, depletion of the Aurora A kinase AIR-1, which is normally recruited by SPD-2/SPD-5 during centrosome maturation, results in spontaneous symmetry breaking. Moreover, we established that AIR-1 kinase activity is essential for proper symmetry breaking. Whether this reflects merely the known role of AIR-1 in centrosome maturation or whether the kinase is required for phosphorylating a particular substrate modulating symmetry breaking remains to be determined.

Overall, our findings demonstrate that *C. elegans* zygotes possess intrinsic polarization cues that do not necessarily need mechanochemical signaling from centrosomes to be activated, and that centrosomes are critical for ensuring proper spatio-temporal regulation of these cues in an AIR-1 dependent fashion.

References:
Hamill DR, Severson AF, Carter JC, Bowerman B; *Developmental Cell* (2002)
P2315
Board Number: B462
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The epithelial membrane trafficking system is both complex and robust owing to multiple sorting mechanisms and parallel transport routes to ensure the delivery of cargo to its correct destination. Among the membrane organelles, Rab-11-enriched apical recycling endosomes (Rab-11-AREs) mediate recycling and sorting of both apically and basolaterally endocytosed membrane components, and can function as secretory transport intermediates between the Golgi complex and plasma membrane[1]. Yet, it remains an open question to elucidate the molecular networks coupling Rab-11-ARE positioning to establishment and maintenance of epithelial polarity in an epithelium in vivo.

Here, taking advantage of the powerful genetic tools available for C. elegans, using high-resolution live imaging of the C. elegans intestine, we demonstrated that the microtubule cytoskeleton and the associated minus-end motor complex, including cytoplasmic dynein and dynactin complexes, were required for the apical positioning of RAB-11-AREs. Strong basal accumulation was observed for the GFP-RAB-11 upon mirotubule or dynein-dynactin machinery disruption, while depletion of the plus-end kinesin UNC-116 caused increased apical distribution of Rab-11-AREs. Depletion of the tethering complex Exocyst components increased the apical aggregation of Rab-11-AREs more or less, suggesting a tethering problem of the AREs to the apical membrane. SEC-15, one of the exocyst components, was always perfectly located with RAB-11, which strongly suggested that SEC-15 was recruited by Rab-11 as an effector to the AREs at the quite earlier stage of AREs transport toward the apical membrane, instead of at the later stage upon Rab-11-AREs already located underneath the apical membrane. Recently, clathrin adaptor complex AP-1, instead of the epithelial-specific AP-1B, was reported to be required for the apical sorting and apicobasal polarity maintenance in the C. elegans intestine[2]. We found that depletion of AP-1 subunits resulted in the cytosol distribution of Rab-11 and SEC-15. We further determined whether these Rab-11-AREs are involved in the post-Golgi sorting or the baso-to-apical transcytotic sorting of apical membrane proteins.

In conclusion, we identified an ensemble comprising the cytoskeleton, polarity machinery and membrane traffic system that coordinates to regulate the apical sorting and apicobasal polarity maintenance in vivo.


P2316
Board Number: B463
Assessing a role for membrane trafficking in polarization of the intestinal epithelium.
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During organogenesis, epithelial cells must become polarized to ensure proper organ function. Polarization begins with an initial symmetry breaking cue, followed by the establishment and maintenance of distinct apical and basolateral domains. While many proteins have been identified that
are required during polarity establishment and/or maintenance, far less is understood about how symmetry is initially broken in epithelial tissues in vivo, or how proteins become sorted into polarized domains. In vitro, membrane trafficking contributes to symmetry breaking, with endosomes directed to sites of cell-cell contact that define the future apical surface. Membrane trafficking has also been shown to regulate the localization of proteins to apical or basolateral domains during polarity establishment and maintenance. We are using the C. elegans embryonic intestine to investigate the role of membrane trafficking in epithelial symmetry breaking and polarity establishment in vivo. The polarizing embryonic intestine consists of 16 epithelial cells with their future apical surfaces oriented towards a central midline that will become the intestinal lumen. Prior to polarization, cells of the intestinal primordium divide parallel to the future apical surface. Proteins destined for the apical surface cluster at lateral membranes and then reposition along with centrosomes approximately ninety degrees to the presumptive apical surface. This somewhat atypical pattern of polarization makes the C. elegans intestine a good system for studying mechanistic aspects of polarization in vivo as there is a clear spatial and temporal separation between symmetry breaking and polarity establishment. Previous reports found that C. elegans intestinal cells become highly endocytic prior to the establishment of polarity and our preliminary evidence suggests that membrane trafficking is required for polarization. Therefore, we hypothesize that membrane trafficking is required in vivo for symmetry breaking, establishing a flow of proteins toward the future apical surface, which may be defined by sites of cell-cell contact. Consistent with this hypothesis, we observe that basolateral proteins first move to the apical surface of the intestine during normal polarity establishment and then become relocalized to basolateral surfaces. These data suggest that apical is the default localization pattern in polarizing intestinal cells and that the subsequent sorting of apical and basolateral proteins is a critical step in polarity establishment. As previous evidence suggests that apical endosomes are essential for maintaining polarity in the C. elegans intestine, we hypothesize that these membrane trafficking pathways are also essential for sorting of proteins during the establishment of polarity.

P2317
Board Number: B464
Induction of apically mistrafficked epieregulin is sufficient to disrupt apico-basolateral polarity in MDCK cells in 3D.
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Overexpression of the EGF receptor (EGFR) ligand, epieregulin (EREG), has been linked to metastasis. We previously reported that basolateral trafficking of EREG is regulated by a conserved tyrosine residue within a YXXΦ motif (Y156ERG) in its cytoplasmic domain. Constitutive overexpression of mutant (Y156A)EREG in MDCK cells resulted in its apical mistrafficking and transformation as determined by formation of invasive tumors upon subcutaneous injection into athymic nude mice (PNAS 110: 8960-5, 2013). We have also identified human cancer mutations within the trafficking motif of EREG (R147stop), which we now show leads to EREG apical mistrafficking. Using an inducible (Tet-ON) system, we have generated MDCK cells expressing wild-type and Y156A-mutant EREG. Both wild-type and mutant EREG induction increased size of MDCK cysts in 3D Matrigel cultures; however, only Y156A EREG induction in fully formed cysts led to formation of ectopic lumens and inward growth that correlates with the transformed phenotype observed in nude mouse xenografts. Furthermore, we show that EGFR activity and ligand cleavage is required for EREG mistrafficking-induced ectopic lumen formation as pre-incubation with the irreversible EGFR kinase inhibitor, EKI-785, or broad specificity metalloprotease...
inhibitors, BB94 and GM6001, abrogates formation of ectopic lumens. When observed with live cell confocal microscopy, the ectopic lumens appear to form de novo rather than budding from the central lumen. Two possible hypotheses for ectopic lumen formation are being tested and the results will be presented: 1) EREG mistrafficking compromises the fidelity of cell division and/or 2) EREG mistrafficking provides an anti-apoptotic luminal environment leading to decreased clearance of luminal cells.

P2318
Board Number: B465
Leptin signaling disrupts tight junctions in the mammary gland.
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Obesity is a highly prevalent and modifiable breast cancer risk factor. Chronic inflammation and elevated growth factors and cytokines are linked to tumor aggressiveness and account for poorer prognoses in breast cancer patients with high body mass index. In contrast, the mechanisms linking obesity to breast cancer initiation are poorly understood. Here, we show with a mouse model of obesity that a high fat diet leads to the mislocalization of Par3, a regulator of cellular junctional complexes defining epithelial polarity. Epithelial polarity loss is recapitulated in a 3D co-culture system combining breast acini with human mammary adipose tissues. We further establish that polarity loss results from a paracrine effect of leptin, an adipokine linked to obesity that we find elevated 2.3 fold in breast tissues from obese vs. normal weight women at high risk for breast cancer. PI3K/Akt signaling downstream the leptin receptor leads to afadin phosphorylation and relocalization away from apical polarity complexes, resulting in reduced cortical actin, reduced apical tight junctions, and increased epithelial permeability. Loss of apical polarity by leptin leads to mitotic spindle misalignment and sensitization to proliferative stimuli in epithelial cells, which are both primordial for cancer initiation. The results define a novel role for afadin in maintaining epithelial polarity and provide a molecular basis for alterations in epithelial architecture during obesity-mediated cancer initiation that may be leveraged for targeted cancer prevention approaches.

P2319
Board Number: B466
Regulation of epithelial junctions and polarity by the Scribble/SGEF/Dlg complex.
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Establishment of epithelial cell polarity is essential for growth, differentiation and morphogenesis. Conversely, loss of cell polarity is a hallmark of many disease states, including cancer. The concerted action of three conserved protein complexes – PAR, Crumbs and Scribble – controls the establishment of cell polarity. In addition, the Rho-family of small GTPases, which regulate actin dynamics, plays a key role in the development of polarity. How polarity complexes regulate Rho GTPase function remains unknown.

Monday-285
Our results show that SGEF, a RhoG-specific guanine-nucleotide exchange factor (GEF), interacts simultaneously with two members of the Scribble polarity complex – Scribble and Dlg – and functions a bridge that mediates the formation of a ternary complex. We have also identified the domains that mediate the interactions among these three proteins. SGEF binds to Scribble PDZ1 domain through a novel internal PDZ binding motif located at its N-terminus. We have solved the crystal structure of Scribble PDZ1 in complex with a peptide encoding the binding domain in SGEF. A different region in SGEF N-terminus binds to Dlg GUK domain. In addition, we found that SGEF is targeted to cell-cell junctions in both mammalian cells and Xenopus embryos, where it colocalizes with ZO-1. Silencing SGEF expression delays the formation of tight junctions and disrupt normal junction architecture. In 3D MDCK cysts, SGEF KD interferes with the establishment of polarity, with cysts showing altered lumen formation. On the other hand, SGEF overexpression alters junction architecture and dynamics, promotes apical constriction, and increases the tension at the junctions. Taken together, our results suggest that Scribble and Dlg bind to SGEF using unique interaction motifs, and that this complex by virtue of localizing RhoG activity to cell-cell junctions and modulating cell polarity is essential for the dynamic assembly and maintenance of cell-cell junctions in cells and in organisms.

P2320
Board Number: B467
Epithelial Membrane Protein 2 and Adenomatous Polyposis Coli interactions regulate apical-basal polarity.
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Adenomatous Polyposis Coli (APC) is a well-known negative regulator of the Wnt pathway; however, it also regulates polarity proteins, such as Dlg and Scribble. Loss of apical-basal polarity disrupts several cellular processes including epithelial structure and intracellular signaling, and is an early marker for tumor development. We previously demonstrated that APC knockdown (APC<sup>KD</sup>) in Madin-Darby Canine Kidney (MDCK) cells altered cyst size and inverted polarity in 3D culture. Through microarray analysis we made the novel observation that epithelial membrane protein 2 (EMP2) expression was increased upon APC loss. Interestingly, knockdown of EMP2 in APC<sup>KD</sup> cells decreased cyst size and restored apical polarity. These data suggest a previously unknown role for EMP2 in regulating polarity and cyst size; however, how EMP2 influences APC-mediated polarity remains unknown. In this study, we seek to investigate both the mechanism by which APC regulates EMP2 expression and the signaling modalities downstream of EMP2 that control APC-mediated polarity. First, we investigated transcriptional activation of EMP2 using bioinformatics, transcription factor DNA/protein arrays, and luciferase reporter assays. EMP2 promoter screens (ConTra v2 webserver) identified binding sites for signal transducer and activator of transcription 1 and 3 (STAT1 and 3), and E2F transcription factor 1 (E2F1). Interestingly, APC<sup>KD</sup> cells exhibited increased expression of STAT1 and E2F1 in DNA/protein arrays compared to controls. Additionally, STAT3 activation was increased in APC<sup>KD</sup> cells compared to controls in reporter assays. These studies identified STAT3, STAT1, and E2F1 as possible APC-mediated transcriptional regulators of EMP2. Future studies will continue to dissect the transcriptional mechanism of EMP2 regulation through ChIP assays and reporter assays using mutant EMP2 promoter constructs. Next, we assessed the mechanism(s) downstream of EMP2 using 2D gel analysis and mass spectrometry. Cell lysates were ran on 2D protein gels and spots that exhibited changes upon APC loss and were restored
by either EMP2 knockdown or APC reintroduction were chosen for mass spectrometry. Analysis identified the spots as filamin A, plectin isoform type 2, 3-hydroxyacyl-CoA dehydrogenase type-2, histone H3.3, and histone H2A type 1-E-like providing possible downstream pathways of APC and EMP2. Future studies will validate the 2D gel data with western blotting and investigate the signaling pathways involved in calcium/phospholipid binding and DNA binding and histone folding, processes identified with cluster analysis, as downstream mechanisms of EMP2. Understanding the interaction of APC and EMP2 and the influence on apical polarity will identify key players in APC disease progression.

P2321
Board Number: B468
Developing an assay to analyze the molecular events leading to establishment of polarity during polarized growth.
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Controlling the site of secretion is an essential aspect of cell biology. In walled eukaryotic cells, such as yeasts and plants, growth is dictated by secreting growth material to specific subcellular sites and thus these cell types are particularly amenable model systems to analyze the molecular basis of polarized secretion. While the mechanisms that direct the growth machinery to these specific sites have been studied in detail in fission and budding yeasts, they remain largely underexplored in many other cell types that do not have obvious homologs to the key fungal molecular players. To gain insights into this process in plants, we focused on the filamentous juvenile tissue of the moss *P. patens*, which grows via targeted secretion to the apex of the cell. While numerous cytoskeleton elements and their regulators have been identified as essential for this form of growth in moss, the exact mechanism by which the apex of the cell is selected as the site for growth has yet to be determined. The filamentous tissue of *P. patens* branches, thereby establishing new sites of polarized secretion. However, predicting when a new branch will emerge is challenging. It is known though, that *P. patens* branching is inhibited in the dark and in far red light. Upon illumination with white light, new branches emerge, thus providing a potential tool to accurately time polarity establishment. Here, we cultured and imaged *P. patens* juvenile tissue in microfluidic devices that allow long-term time-lapse recording. After a 4-day dark treatment, unbranched filaments initiate branching at 18±3.2 hrs after light exposure. Surprisingly, on a similar time scale, the majority of the previously established growing tips generate a new tip at a slightly altered angle after a short period of bulging. This phenomenon suggests that dark treatment depolarizes the original tip-growing machinery, which must then re-establish after exposure to light. Thus, dark treatment could be developed into an assay enabling visualizing of the onset of polarized growth and the recruitment of the tip-growth machinery. In future studies, known players including but not limited to actin, microtubules and cytoskeletal regulating proteins will be examined for their potential role in the initiation of tip growth.
**P2322**

**Board Number: B469**

Dissecting Roles for a Signalling Mucin and Effector Cdc42p-MAP Kinase Pathway in Polarity Establishment and Metabolic Reprogramming.

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In *Saccharomyces cerevisiae*, cell polarity and bud morphogenesis are multifaceted events governed by the concerted actions of key regulatory proteins. These proteins receive inputs from multiple signal transduction pathways to control morphogenesis. One such pathway that regulates the morphogenetic response to changing nutrient availability is the filamentous growth Mitogen Activated Protein Kinase (fMAPK) pathway. The fMAPK pathway is triggered by nutrient limitation and elicits a foraging-type response where ovoid yeast-form cells polarize into filaments of elongated cells. The plasma membrane regulators of fMAPK pathway include the mucin-like glycoprotein Msb2p and the tetraspan protein Sho1p. Msb2p and Sho1p’s interactions with the polarity GTPase, Cdc42p and its GEF, Cdc24p, respectively are required for the activation of fMAPK pathway. Exactly how the fMAPK pathway controls morphogenetic events is not clear. In this study, we identify new roles for the fMAPK pathway in bud emergence and metabolic reprogramming. We also show that Msb2p and Sho1p regulate aspects of filamentous growth through fMAPK-dependent and fMAPK-independent mechanisms. New Msb2p-interacting proteins were identified that might account for the how Msb2p directly impacts cellular processes during filamentous growth. Collectively, the study uncovers new functions for a signalling mucin and its cognate pathway in regulating morphogenesis and metabolism. Receptors that directly promote their cognate pathway’s goals may be a widespread biological principle.

**P2323**

**Board Number: B470**

Cellular Polarity is Directed by Electrophoresis and Electrically Driven Water Flow.

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Weak, DC electric fields (EFs) are critical components of development, regeneration and repair. EFs direct cellular polarity and migration (galvanotaxis), giving rise to the use of externally applied EFs to manipulate cellular polarity in the applications of tissue engineering and repair of the epidermis and other soft tissues. It is not clear how cells can sense and respond to weak EFs less than 150 mV/mm, which mediate polarization of cellular structure and promote directional migration, most commonly towards the cathode. A few biophysical mechanisms have been postulated to explain the directional response observed during galvanotaxis. The predominant hypothesis involves two opposing forces exerted on the surface macromolecules in the cellular boundary layer by the EFs, electrophoresis and electro-osmosis which is electrically driven water flow towards the cathode under physiological conditions. We show that in the absence of EFs, zebrafish keratocytes migrate randomly with an asymmetric index near zero, but migrate cathodally with an asymmetric index of -0.82 in an applied EF, compared to a completely cathodal response of -1. Under similar conditions, fluorescently labelled cell surface proteins, including Concanavalin A (Con A) receptors, randomly distribute in the absence of EFs but electromigrate towards the cathode in the presence of EFs. Con A labelled receptors have 60% greater accumulation on the cathode side of cells. We are attempting to impair electro-osmosis with neutral, viscous polymers and thereby reduce cathodal electromigration of surface receptors and impair
cathode-directed migration according to our hypothesis. We are also screening known plasma membrane surface proteins with known physical characteristics that may be good candidates for the electric field receptor. Our efforts aim to bring the field closer to identifying the putative electric field receptor; a fundamental, outside-in signaling receptor that controls cellular polarity of different cell types and directs cell migration in weak EFs.

P2324
Board Number: B471
Mechanisms connecting the conserved protein kinases Kin1, Pom1, and Ssp1 in fission yeast cell polarity and division.
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Connections between the protein kinases that function within complex cell polarity and division networks are poorly understood. Rod-shaped fission yeast cells grow in a highly polarized manner, and genetic screens have identified multiple protein kinases required for polarized growth and cell shape in this organism. Kin1, the sole MARK/PAR-1 family kinase in fission yeast, regulates cell polarity and cytokinesis through unknown mechanisms. Here, we show that the CaMKK-like Ssp1 acts as an upstream activator of Kin1 by directly phosphorylating the Kin1 activation loop to promote cell polarity. To define the downstream targets of Ssp1-Kin1 signaling, we performed large-scale phosphoproteomics screens that identified Kin1 substrates in cells. We found that Kin1 phosphorylates itself and the endocytic adaptor protein Pal1 to promote growth at cell tips, and these proteins were interdependent for localization to growing cell tips. Additional Kin1 substrates for cell polarity and cytokinesis (Tea4, Mod5, Cdc15 and Cyk3) were also directly phosphorylated by a second cell polarity kinase, the DYRK-family member Pom1. Interestingly, Kin1 and Pom1 were enriched at opposite ends of growing cells, and they phosphorylated largely non-overlapping sites on shared substrates. Combined inhibition of both Pom1 and Kin1 led to synthetic defects in Cdc15 and Cyk3, confirming a non-redundant functional connection through shared substrates. Thus, connections between protein kinases can be direct (Kin1-Ssp1) or indirect (Kin1-Pom1), and contribute to dynamic control of robust cellular processes such as polarized growth and division. These findings identify connections between Kin1 and other conserved protein kinases in the polarity and division network, providing insights into similar mechanisms in other higher organisms.

P2325
Board Number: B472
Drosophila neural stem cells are polarised by their daughter cells.
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Drosophila neural stem cells (neuroblasts) divide asymmetrically to both self-renew and generate Ganglion Mother Cells (GMCs) daughter cells. A noticeable feature of larval neuroblasts is the fact that their division orientation is maintained from one cell cycle to the next, even when isolated from their niche in neuroblast/GMCs clusters. This relies on an apical microtubule network acting as an intrinsic polarising cue which disruption, however, only partially affects division orientation maintenance. Here, we used live imaging of cultured Drosophila larval brains, genetics and laser ablation to demonstrate that the GMC acts as an additional - this time extrinsic - polarity cue also participating to neuroblasts.
division axis maintenance. We further investigated the molecular mechanism underlying this polarity cue by performing a small-scale RNAi screen and uncovered the involvement of midbody and midbody-associated structures components, which likely allow neuroblasts to distinguish their latest daughter cells from the previous ones.

P2326

Board Number: B473

Characterising the molecular mechanism of the fission yeast memory-based growth polarity landmarks Rax1 and Rax2.

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Fission yeast are rod-shaped cells that grow in a highly polarised manner, exclusively from their cell tips. Following cytokinesis, daughter cells first grow in a monopolar fashion from their ‘old’ ends (those present in the mother cell). At a specific cell size, cells also start growing from their ‘new’ ends (those formed during cytokinesis), in a bipolar fashion. Polarity landmarks localised at the cell tips co-ordinate this growth. When the polarity landmark Tea1 is deleted, both daughter cells grow exclusively in a monopolar fashion. However, the two daughter cells have different growth patterns: one daughter cell grows from its old end, while the other daughter cell grows from its new end. The daughter cell that grows from its old end is always the cell that inherited the cell tip that was previously growing in the monopolar mother cell. This suggests that there is a memory-based landmark for cell growth. We identified the proteins Rax1 and Rax2 as being part of this landmark. In the absence of Tea1 and either Rax1 or Rax2, cells “lose memory” of past growth and are no longer able to grow from a previously growing tip. Instead, they grow exclusively from the ends formed during cytokinesis. The molecular mechanism of how the Rax1/Rax2 landmark plays a role in the re-initiation of growth at a previously growing tip is currently unknown.

We have used live-cell imaging to show that Rax1 and Rax2 are localised at the cell tips, and septum during cytokinesis, and that their localisation is co-dependent. Rax1 and Rax2 are both transmembrane proteins. Rax2 has a large extracellular domain and a small cytoplasmic tail at its C-terminus. Truncating the whole cytoplasmic tail results in loss of Rax2 localisation and function. Smaller truncations have mild effects on Rax2 localisation and function.

We fused a His-TEV-Biotin (HTB) tag to the Rax2 C-terminus and optimised cross-linking and purification of Rax2 and its interacting partners. Mass spectrometry analysis identified ~800 proteins that were detected exclusively in the cross-linked Rax2-HTB pull down. Notable identified proteins included known polarity landmarks, secretory proteins and proteins involved in cytoskeleton maintenance. To identify interacting proteins that are functionally important to the role of Rax1 and Rax2 in polarised growth, we are currently carrying out mass spectrometry in different genetic backgrounds.
P2327
Board Number: B474
MARK2 regulates directed cell migration and cytoskeleton polarization through modulation of MYPT1-Myosin II activity.
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Directed cell migration requires establishment of cell polarity to spatially coordinate adhesion formation, to the acto-myosin cytoskeleton and microtubules to drive cell movement. MARK2 (Microtubule affinity-regulating kinase, also known as PAR1b) is well known to regulate front-back cell polarity and the microtubule cytoskeleton. However, the role of MARK2 in polarization of actin, myosin II and focal adhesions (FAs) remains less clear. A previous study from our lab using mass spectrometry identified MARK2 as a component of FAs. Thus, we hypothesized that in addition to regulating microtubules, MARK2 may promote polarity in cell migration by regulating FAs and their associated acto-myosin cytoskeleton. To test this, we analyzed GFP-MARK2 localization and the effects of its disruption or overexpression on cell polarity, FA and acto-myosin dynamics in cell migration. We found in U2OS cells that MARK2 localized at FAs and actin stress fibers, in addition to its reported association with microtubules and cell protrusions. Using siRNA and CRISPR technology, we found that MARK2 depletion increased FA size, in addition abolishing cell polarization, and slowed persistent migration. Conversely, overexpression of MARK2 resulted in smaller FAs at the cell edge, reduced cell migration and caused myosin IIA displacement from the cell edge to the center. We also found that overexpression of MARK2 induced inactivation of two key proteins in involved in myosin IIA regulation; 1) increased T696 phosphorylation of the myosin phosphatase MYPT1, and 2) increased S885 phosphorylation of GEF-H1. Using Co-immunoprecipitation experiments we found that MYPT1-MYC and MYPT1-GFP pull down endogenous MARK2 as well as MARK2-GFP. These findings indicate that MARK2 not only regulates microtubule stability through phosphorylation of MAPs as reported, but also influences the acto-myosin cytoskeleton and FAs. Our results further suggest that this effect on FAs and the cytoskeleton could possibly be achieved by inactivating GEF-H1 in the front of the cell and/or inactivating myosin phosphatase in the back of the cell, overall affecting cell polarization, FA turnover and directed cell migration.

P2328
Board Number: B475
Exploration and stabilization of Ras1 zone during fission yeast mating: a mechanism with positive and negative feedback regulation.
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Directional cell growth or migration requires detection and interpretation of shallow chemical gradients. Recent studies in budding and fission yeasts suggest that the decoding of gradient extracellular signal happens locally through the GTPase Cdc42 polarity complex on the cell membrane. Cdc42 is a main regulator of cell polarity which prior to mating forms a dynamic polarity patch that explores the cell periphery in discrete jumps and performs a stochastic local sampling of the extracellular signal. It has been shown that upon deletion of Gap1, the GTPase-activating protein for Ras1, the lifetime of the
These constants, of occurs more 3D could we mathematical modeling to study the underlying mechanism of Ras1 patch appearance and disappearance through Gap1 inhibition. We developed a set of reaction-diffusion equations for the substrate Ras1-GDP, activator Ras1-GTP, and inhibitor Gap1. We solved the equations numerically on a 3D geometry of the fission yeast cell. We illustrated that by tuning positive and negative feedback rate constants, exploratory patch behavior can be reproduced. By locally increasing the positive feedback at the site of the gradient signal, the patch stabilizes. Our model predicts how the Ras1 patch size is regulated through positive and negative feedback.

Neuronal Degeneration - ALS, HSP and SCA

P2329
Board Number: B477
Enhanced Outgrowth and Regeneration in Adult Motor Neurons from Amyotrophic Lateral Sclerosis Mouse Models.
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Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by motor neuron cell death. Though ALS specifically affects motor neurons, certain groups of them are more resistant to disease progression. The majority of ALS studies have focused on the cellular mechanisms that cause loss of viability, less is known about the neurons that do survive. In this study, we address the latter by culturing spinal motor neurons from adult ALS mouse models at various states of disease progression. Surprisingly, we found that in comparison to age-matched controls, motor neurons cultured from SOD1G93A ALS mouse models display increased outgrowth and branching. They also display an increase in the number and size of actin-based structures such as growth cones and filopodia. The most substantial increase in regeneration occurs in a SOD1G93A ALS mouse model with a lower copy number of the transgene that yields delayed disease onset. This phenotype occurs independently of SOD1 enzymatic activity and is cell autonomous. Further, the enhanced outgrowth occurs before the mice become symptomatic, though the effect increases with disease progression. These results indicate that the surviving motor neurons in ALS are primed for regeneration well before an individual gets sick, in response to cellular stress. Understanding this mechanism of cellular resistance could result in new therapeutic targets for the treatment of ALS.

P2330
Board Number: B478
Characterizing SMA patient-derived mutations to connect proteomic environment with disease-related phenotypes.
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Spinal muscular atrophy (SMA) is a recessive neurodegenerative disease and the leading genetic cause of death in young children. While it is known that SMA is caused by reduced amounts of functional Survival Motor Neuron (SMN) protein, the aberrant molecular mechanisms that lead to motor neuron degeneration and muscle loss are unclear. SMN’s canonical role is in snRNP biogenesis, however it has been implicated in many other functions such as mRNA transport, cytoskeleton organization, and endocytosis.

To further elucidate the pathophysiology of SMA, our laboratory has developed an allelic series of human patient-derived Smn mutations in Drosophila. These missense mutations cover all three functional domains of SMN protein and exhibit disease-related phenotypes. Our objective is to use this model to compare disease phenotypes to proteomic environments that arise from mutations in the various domains and residues. To assess disease-related phenotypes we performed assays for viability and muscle function through locomotion.

Viability assays show that these mutant lines cover a spectrum of severities. Crawling assays also show a spectrum of locomotor defects, but they do not always correlate with viability. Using the viability and locomotion phenotypes, we have analyzed a subset of fly lines by whole proteome analysis. These lines include mutations in all three functional domains of SMN and varying severities of viability and locomotion defects. The results of this analysis are expected to provide insight into how loss of Smn function affects other proteins and pathways, and which effects depend on specific functional domains. Overall, using characterization and proteomic analysis of these patient-derived mutation lines helps us better understand how the implicated pathways and functions of SMN fit into the disease etiology, and which domains/residues are important for these functions.

P2331
Board Number: B479
Rho guanine nucleotide exchange factor (RGNEF) as a pro-survival factor on in vitro and in vivo models.
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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive loss of motor function due to loss of motor neurons. RNA metabolism has been found to play a central role in the etiology of the pathology of this disease. Specifically, RNA-binding proteins have been found to form intracellular inclusions, a key hallmark of ALS pathology. We have described a RNA-binding protein called Rho Guanine Nucleotide Exchange factor (RGNEF) that has been observed to form aggregates in the spinal cord neurons of both sporadic and familial cases of ALS. RGNEF co-localizes with other ALS-related RNA-binding proteins, including TAR DNA-binding protein 43 (TDP-43), a toxic ALS-linked RNA-binding protein with broad functions in RNA metabolism. We have determined that RGNEF is a survival factor under stress conditions. Comparison of different fragments of RGNEF revealed that its amino-terminal Leucine-rich domain plays a critical role in RGNEF’s survival activity in HEK293T cells. In MTT assays where HEK293T cells were exposed to osmotic stress, RGNEF lacking the Leucine-rich domain conferred no protection in comparison to full-length RGNEF. Additionally, we have established an in vivo model of RGNEF transgenic fly using the UAS-Gal4 expression system. This transgenic fly line was characterized through negative geotaxis (climbing assay) and lifespan. Interestingly full-length RGNEF expressed in flies produces an important improvement in their lifespan. This confirms our in vitro results showing RGNEF as a pro-survival factor and opens the door to analyze the role of the Leucine-rich domain of RGNEF in vivo exploring RGNEF as a modifier of ALS pathology.
P2332
Board Number: B480
A new genetic mouse model for SPAST-based Hereditary Spastic Paraplegia reveals the importance of toxic gain-of-function mechanisms.
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Mutations of the SPAST gene, which encodes for the microtubule-severing protein spastin, are the most common cause of Hereditary Spastic Paraplegia (HSP), a debilitating neurological disease, typically adult-onset, in which corticospinal degeneration leads to spasticity and gait deficiencies. Haploinsufficiency is the most prevalent opinion as to the mechanism of the disease, but several lines of evidence suggest a different mechanism, namely gain-of-function toxicity of the mutant spastin proteins. SPAST has two start codons, producing a full-length isoform called M1 and a slightly shorter isoform called M87. The region specific to M1 is hydrophobic and may cause mutant or truncated forms of M1 to misfold and produce cytotoxic effects. Here we report a new genetic mouse that expresses human M1 and M87 spastins harboring a pathogenic HSP mutation (C448Y), while still also expressing endogenous rodent spastin. The particular mutation was chosen in part because previous studies indicated that the mutant spastin does not act in dominant-negative fashion to reduce endogenous spastin activity, and this conclusion was supported by observations on the mouse. For example, staining for acetylated tubulin suggest a decrease in microtubule stability, which is the opposite of the expectation of a loss-of-function mechanism. Expression of the mutant proteins was detected by Western blotting from fetus to adult, but spasticity-like tremor and gain defects were only identified in adults. No symptoms inconsistent with HSP were observed. Results of histological and tracer studies were consistent with dying back of corticospinal axons, which is characteristic of HSP. Cultured newborn cortical neurons from the mouse showed no cellular morphological defects compared to wild-type counterparts, but displayed defects in lysosome transport that were worse in homozygotes than heterozygotes and worse yet when endogenous mouse spastins was mostly depleted by siRNA. These results indicate that the toxic gain-of-function effects of the mutant spastins are present in cells long before behavioral symptoms are apparent in the animal. Interestingly, the defects in lysosome transport were not correctable when the human mutant spastins were mostly depleted by siRNA, which is consistent with previous indications that only vanishingly small amounts of the mutant protein, presumably M1, are needed to elicit the cellular pathology. We posit that the HSP phenotype in humans is produced by toxic gain-of-function mechanisms, but that haploinsufficiency can exacerbate the symptoms. The mouse will be useful for testing therapies. (LQ and EP are co-first authors. The work was supported by grants to PWB from the NIH, the Tom Wahlig Foundation, and Drexel University’s CURE program).
P2333

Board Number: B481

The RNA binding protein Zfp106 protects against neurotoxicity caused by C9orf72 GGGGCC repeats.

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Expanded GGGGCC repeats in the first intron of the C9orf72 gene represent the most common cause of familial ALS, but the mechanisms underlying repeat-induced disease remain incompletely resolved. One proposed gain-of-function mechanism is that repeat-containing RNA forms aggregates that sequester RNA binding proteins, leading to altered RNA metabolism in motor neurons.

To identify new proteins associated with GGGGCC repeats, we performed RNA pulldown assays, followed by mass spectrometry and identified Zfp106 as a specific GGGGCC RNA repeat-binding protein. We found that Zfp106 binds directly to GGGGCC RNA repeats, and functionally interacts with the repeats in cultured neuronal cells. To gain additional insight into the molecular function of Zfp106, we used mass spectrometry to identify protein interactors of Zfp106. Remarkably, we found that Zfp106 interacts with multiple other RNA binding proteins, including the ALS-associated factors TDP-43 and FUS. Zfp106 is highly expressed in skeletal muscle and motor neurons, and its human ortholog ZNF106 is located at chromosome 15q15.1, a locus associated with a familial recessive form of ALS. Therefore, we genetically inactivated the Zfp106 gene in mice. Zfp106 knockout mice develop severe motor neuron degeneration, which can be suppressed by transgenic restoration of Zfp106 specifically in motor neurons.

Finally, we used Drosophila as an in vivo gain-of-function model of C9orf72 neurodegeneration to test if the interaction between Zfp106 and GGGGCC repeats had a functional consequence on neurotoxicity. Expression of Zfp106 in glutamatergic neurons suppressed the loss of larval active zones at NMJs and pupal lethality caused by expression of 30 copies of GGGGCC. Moreover, Zfp106 co-expression partially suppressed the adult locomotor defect caused by expression of C9orf72 repeats. Therefore, Zfp106 is a potent suppressor of GGGGCC repeat-mediated neurotoxicity in a Drosophila model of C9orf72 ALS.

P2334

Board Number: B482

SOD1G93A mouse model of ALS presents increased expression of NLRP1 and NLRP3 in cells from spinal cord.

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive weakness, muscle atrophy with eventual paralysis and death as result of a selective loss of motor neurons. The inflammatory mechanisms involved in disease progression have not yet been fully clarified; however, evidence shows that innate immune system receptors from central and peripheral nervous system are involved in this process. Thus, in this work, we evaluate the effects of mutant
superoxide dismutase 1 (SOD1) overexpression on the gene expression of RAGE (receptor for advanced glycation end products) and NLRP1 and NLRP3 inflammasomes in the cells from motor cortex and spinal cord of mutant SOD1 (SOD1\textsuperscript{G93A}) transgenic mouse model of ALS. First, to evaluate the disease progression, we test the animals for motor coordination impairment using the rotarod performance task (once a week) for seven weeks. After four weeks, SOD1\textsuperscript{G93A} transgenic mouse began to present difficult to executing the tasks. These animals progressively presented an impaired rotarod performance when compared with a group of non-mutant SOD1 transgenic mice (Area Under the Curve = 750.20±2.09 vs. 1048.00±13.28, mutant and non-mutant mice, respectively; p<0.01). At eight week, when the SOD1\textsuperscript{G93A} transgenic mice reached its endpoint disease, that is when we observe the functional paralysis of both hind limbs, both groups of animals were euthanized. After, we analyzed the expression of RAGE, NLRP1 and NLRP3 in cells from motor cortex and spinal cord. Our results show that SOD1\textsuperscript{G93A} transgenic mice presented an increased expression of RAGE, NLRP1 and NLRP3 (1.5, 15 and 10-fold-increase, respectively) in cells from spinal cord when compared with non-mutant SOD1 transgenic. However, we do not observed differences between the groups when the same analysis was conducted in the cells from motor cortex. Together, ours results suggests the importance of RAGE, NLRP3 and NLRP1 expression in the spinal cord but not in motor cortex to the progression of murine ALS. In addition, these results highlight the role of NLRP1 as one of the main inflammasomes involved with SOD1 mouse model of ALS.

P2335
Board Number: B483
Autophagolysosome disruption in Drosophila models of ALS/FTD caused by C9orf72 mutations.
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A GGGGCC hexanucleotide repeat expansion (G\textsubscript{6}C\textsubscript{3} HRE) in the first intron of the C9orf72 gene has been identified as the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). One of the pathological hallmarks of C9-ALS is the presence of cytoplasmic protein aggregates colocalized with the autophagy receptor p62/SQSTM1. Interestingly, mutations in p62/SQSTM1 are also a rare genetic cause of ALS through an unclear mechanism. In a Drosophila model of C9-ALS expressing (G\textsubscript{6}C\textsubscript{3})\textsubscript{30}, we have found that p62 is upregulated and forms large aggregates in motor neurons. p62 plays a key role in autophagy by binding ubiquitinated proteins and delivering them to the autophagosome for degradation via the lysosome. Surprisingly, we find that knockdown of p62 rescues degeneration in the fly eye and in motor neurons. Immunofluorescence and western blot analysis of autophagy and lysosome markers demonstrates an expansion of lysosomes and decreased delivery to and digestion of autophagic cargo in the lysosome. Furthermore, we see a decrease in compartments positive for the protease CP1/Cathepsin L by immunostaining, suggesting lysosomal enzyme dysfunction. Using electron microscopy of the Drosophila eye, we observe a remarkable accumulation of expanded multilamellar bodies and autolysosomes that precedes neurodegeneration. We then tested genetic modifiers of autophagosome/lysosome function and find that mTor, Rab7, and TRPML overexpression rescue the neurodegenerative phenotypes in the Drosophila eye. Furthermore, treatment with rapamycin and trehalose, pharmacological inducers of autophagy/lysosome pathway, rescue neurodegeneration in the Drosophila eye as well as the accumulation of p62 positive aggregates. We propose that C9orf72-HRE expression causes dysregulation of protein folding and degradation leading to cytotoxic protein aggregation, and that this is rescued by aggregate clearance through genetic
and pharmacological upregulation of lysosomal function. This study suggests that drugs targeting lysosomal proteostasis pathways may have therapeutic potential for C9orf72-mediated ALS and FTD.

P2336
Board Number: B484
The ataxin-1 interactome reveals direct connection with multiple disrupted nuclear transport pathways.
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The expanded polyglutamine (polyQ) tract form of ataxin-1 drives disease progression in spinocerebellar ataxia type 1 (SCA1). Although polyQ-ataxin-1 is known to form distinctive intranuclear bodies, the cellular pathways and functions it influences remain poorly understood. Here, we identify direct and proximal partners constituting the interactome of ataxin-1[85Q] in Neuro-2a cells. Pathways analyses indicate a significant enrichment of essential nuclear transporters in the interactome, pointing to disruptions in nuclear transport processes in the presence of polyQ-ataxin-1. Our direct assessments of nuclear transporters and their cargos by immunofluorescence imaging and quantitative analyses reinforce these observations, revealing disrupted trafficking often with relocalisation of transporters and/or cargos to ataxin-1[85Q] nuclear bodies. Strikingly, the nucleoporin Nup98, dependent on its GLFG repeats, is recruited into polyQ-ataxin-1 nuclear bodies. Our results highlight a disruption of multiple essential nuclear protein trafficking pathways by polyQ-ataxin-1, a key contribution to furthering understanding of pathogenic mechanisms initiated by polyQ tract proteins.

P2337
Board Number: B485
Sirt1 restores proper cellular homeostasis to achieve neuroprotection in spinocerebellar ataxia type 7.
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Sirtuin 1 (Sirt1) is a NAD+-dependent protein deacetylase with established effects in countering age-related diseases, including neurodegeneration, yet the basis for Sirt1 neuroprotection remains elusive. Spinocerebellar ataxia type 7 (SCA7) is an inherited neurodegenerative disorder in which CAG-polyglutamine repeat expansions in the ataxin-7 gene produce cerebellar degeneration in affected human patients. As transcription dysregulation likely contributes to SCA7 pathogenesis, we performed transcriptome analysis on SCA7 mice, observed down-regulation of genes controlling calcium flux, and documented abnormal calcium-dependent membrane excitability in both SCA7 mouse cerebellum and SCA7 patient-derived neurons. Transcription factor binding site analysis of SCA7 down-regulated genes revealed sites for peroxisome proliferator-activated receptors, which are known Sirt1 targets, and we detected reduced Sirt1 activity in SCA7 mouse cerebellum. We then crossed Sirt1 transgenic mice with two different SCA7 mouse models, and observed amelioration of cerebellar neurodegeneration, calcium flux defects, and membrane excitability in Sirt1-SCA7 bigenic mice. These findings indicate that Sirt1
achieves neuroprotection by promoting proper calcium regulation, and reinforce an emerging view that
cerebellar ataxias exhibit altered calcium homeostasis, due to metabolic dysregulation, suggesting
shared therapy targets.

P2338
Board Number: B486
A neuroprotective agent that inactivates pro-degenerative TrkA and preserves mitochondria.
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Axon degeneration is an early event and pathological in neurodegenerative conditions and nerve
injuries. To discover agents that suppress neuronal death and preceding axonal degeneration, we
performed drug screens on primary rodent neurons, and identified the pan-kinase inhibitor Foretinib
that potently rescued sympathetic, sensory, and motor wt and SOD1 mutant neurons from trophic
factor withdrawal-induced degeneration. By using primary sympathetic neurons grown in mass cultures
and Campenot chambers, we show that Foretinib protected neurons by suppressing both known
degenerative pathways, and a new pathway involving unliganded TrkA and transcriptional regulation
of the pro-apoptotic BH3 family members BimEL, Hirikiri and Puma, culminating in preservation of
mitochondria in the degenerative setting. Foretinib delayed chemotherapy-induced and Wallerian
axonal degeneration in culture by preventing axotomy-induced local energy deficit and preserving
mitochondria, and peripheral Wallerian degeneration in vivo. These findings suggest a new axon
degeneration pathway and identify a potentially clinically-useful therapeutic drug.

P2339
Board Number: B487
Post-Translational Modifications of Dipeptide Repeat Proteins in c9orf72-Associated ALS.
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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are progressive
neurodegenerative diseases associated with protein misfolding and aggregation that lead to neuronal
cell death. Chromosome 9 open reading frame 72 (c9orf72) is the most common genetic mutation
observed both in familial and sporadic cases. C9orf72 contains nucleotide repeat GGGGCC. In c9orf72
ALS/FTD patients, the nucleotide repeat may be expanded to several hundred or thousands of repeats.
By way of unconventional repeat-associated non-ATG translation, the nucleotide repeats are translated
into dipeptide repeat proteins (DPRs). Arginine-rich DPR’s, mainly Gly-Arg (GR100) and Pro-Arg (PR50)
seem to be particularly toxic and aggregation prone. The exact mechanisms linking DPR toxicity to
the development of ALS remain unknown.

Eukaryotic DNA is wrapped around an octameric histone core formed by one H3-H4 tetramer and two
H2A-H2B dimers. The N-terminal tails of histones are subject to many post-translational modifications
(PTMs). PTMs modify the interaction between the histone and DNA, either activating or suppressing
gene transcription. Saccharomyces Cerevisiae has been used as a model system for human
neurodegenerative diseases. At the cellular level, key processes are conserved between yeast and
humans, making yeast an efficient and accessible way of studying human neurodegenerative disease. Cytotoxic DPRs are overexpressed in yeast, and western blotting techniques with antibodies for specific PTMs are used to qualitatively detect changes between control yeast, and yeast overexpressing DPR. Preliminary results show no alterations in the histone modification landscape of cells displaying DPR proteinopathy. We are currently conducting additional trials for other PTMs. Elucidating the epigenetic mechanisms that associate with DPR toxicity will allow us to test compounds that can reverse these PTMs, reverse DPR toxicity and prevent neuronal cell death in ALS.

Neuronal Signal Transduction, Cell-Cell Interactions

P2340
Board Number: B488
Intra-Axonal Translational Control Mechanisms for ER Chaperone Protein mRNAs.
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Transport of neuronal mRNAs into distal nerve terminals and growth cones allows axonal processes to generate proteins autonomously from the cell body. Sensory neurons transport a complex population of mRNAs into their axons, including several encoding ER chaperone proteins. It remains largely unknown how specificity is provided to the localized translational apparatus. For calreticulin mRNA, one of its two 3’ UTR elements can drive its localization to axons but its 5’UTR was shown to confer translational control through a mechanism that requires phosphorylation of eIF2α in axons after stimulation of ER stress with LPA. Interestingly, the proximal 3’UTR localization element in calreticulin mRNA is essential for this phospho-eIF2α-dependent translational control. Thus, 5’ and 3’ UTRs can perform concerted roles to achieve specificity for axonally localized mRNA translation. We show that PERK (protein kinase RNA-like endoplasmic reticulum kinase) is required for the increase of calreticulin translation in response to ER stress. Since PERK has been detected in DRG axons and LPA triggers ER Ca²⁺ release, a Ca²⁺-dependent activation of axonal PERK likely leads to axonal calreticulin translation. Calcium influx in the axoplasm represents a fast signaling event in response to injury that is able to trigger several mechanisms connected to axonal growth. We have seen an increase in the phosphorylation of eIF2α after 20 min of axotomy and axotomized DRG axons undergo a local calreticulin translation through the 3’UTR proximal element. PERK is required for this translational regulation since its inhibition impaired calreticulin translational increase. Similar to LPA results, eIF2α phosphorylation could be a common signaling mechanism mediating axonal calreticulin translational in response to injury. Our static imaging experiments have shown a decrease in the total axonal length of calreticulin depleted DRG neurons suggesting that axonal calreticulin translation could mean a fast triggered response to injury. Further experiments are being performed to understand this mechanism.

P2341
Board Number: B489
The adaptor protein FEZ1 links metabotropic glutamate receptors to the autophagy pathway.
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Group I metabotropic glutamate receptors (mGlu1 and mGlu5) are G protein-coupled receptors (GPCRs) enriched at excitatory synapses throughout the brain where they act both pre- and postsynaptically to
regulate glutamatergic neurotransmission. Signaling by group I mGluRs is critical to formation and maintenance of brain circuitry and activity-dependent synaptic plasticity, a cellular substrate of learning and memory. Recessive mutations in the gene encoding mGlu1 (GRM1) in patients with congenital ataxia and intellectual disability give rise to aberrant mRNA transcripts that produce receptors with abnormal intracellular carboxyl-tails, indicating that this domain is critical to receptor physiopathological functions. Using genetic, biochemical and imaging approaches we found that mGlu1 interacts via its carboxyl-tail with the adaptor protein Fasciculation and Elongation Protein Zeta-1 (FEZ1). FEZ1 was recently shown to participate to the regulation of macroautophagy, a catabolic process critical to neuronal homeostasis and survival throughout organism lifespan. Our findings indicate that mGlu1 receptors function via FEZ1 to regulate autophagy in neurons, a mechanism through which metabotropic signaling may contribute to maintaining neuronal homeostasis.

P2342
Board Number: B490
A pair of E3 ubiquitin ligases coordinate responses of DCC to its ligand, netrin-1.
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Fidelity in axon guidance and axon branching are critical to appropriate wiring of the nervous system. Although the guidance cues and receptors that direct axonal extension have been identified, the complex underlying molecular pathways, and in particular how extracellular guidance cues modulate the receptors that initiate neuronal morphogenesis, have been relatively unexplored. Netrin-1 is a secreted guidance cue, originally considered a soluble, chemotactic cue that functions in axon attraction, axon repulsion, and axon branching. Recently netrin-1 has been found to adhere to the extracellular environment and function as a haptotactic cue. Our work has identified a pair of closely related, brain-enriched E3 ubiquitin ligases, TRIM9 and TRIM67, as critical links between the netrin receptor DCC and the signaling pathways and cytoskeletal responses critical to axonal morphogenesis. In this study, we utilize two purification protocols to separate soluble, non-adhesive netrin-1 and adhesive netrin-1, to determine how responses to these two forms of netrin differ and are modulated by TRIM9 and TRIM67. We use TIRF microscopy to reveal the spatiotemporal clustering of DCC in response to netrin. We have found that low concentrations of soluble netrin-1 promote attractive axonal responses and DCC clustering, whereas high doses of soluble netrin-1 promote repulsive axonal responses without DCC clustering at the cell periphery. Interestingly individual deletion of Trim9 or Trim67 disrupts a unique subset of these responses. We are exploiting a newly-developed FRET-based DCC tension sensor to define how mechanotransduction differs in these netrin regimes in wildtype, Trim9+/−, Trim67+/−, and Trim9−/−/Trim67−/− neurons. Although TRIM9 and TRIM67 share high sequence similarity and binding partners, including DCC, their expression patterns are distinct and the phenotypes associated with their individual deletion or combined deletion are all surprisingly divergent in vitro and in vivo. Published work indicates that TRIM9 is required for attractive growth cone responses to netrin-1. Here we show that TRIM67 is required for both attractive and repulsive responses to netrin-1, and that Trim9−/−/Trim67−/− have additional, unique phenotypes. Our previous work has identified neuroanatomical defects and behavioral deficits in Trim9−/− mice. Here we show distinct neuroanatomical and behavioral deficits occur in the absence of Trim67 in vivo.
P2343  
**Board Number: B491**  
**CHL1 and SLIT1 Colocalize in the Intermediate Zone During Embryonic Development.**  
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Axons are an integral part of a neuron responsible for transmitting electrical signals within the brain. It is important to understand the mechanics of axon growth, including how growth cones function and which proteins are involved in axon migration and how they co-exist with other proteins. This hierarchy of information is essential to combat certain types of cancers and neurological disorders such as Alzheimer’s disease. We currently know of several families of proteins that play major roles in regulating axon guidance. Two such families are neural cell adhesion molecules (NCAMs) of the immunoglobulin superfamily (Maness and Schachner, 2007) and the family of SLIT proteins (Andrews, et al., 2007). Close Homolog of L1 (CHL1), a NCAM, is part of the mammalian L1 family, which is known to play a role in axon growth and migration of developing neurons (Wright, et al., 2007). Roundabout1 (ROBO1), also a member of the immunoglobulin super family, plays a role in axon guidance in the developing forebrain by serving as a receptor for the guidance cue, SLIT1 (Andrews, et al., 2007). Our objective in this study is to investigate a potential interaction between CHL1 and SLIT1. We hypothesize that these two proteins function together to regulate SLIT-mediated axon guidance in developing neurons. The first step in our investigation involved the use of co-immunofluorescence staining to visualize CHL1 and SLIT1 colocalization. We demonstrate here that CHL1 and SLIT1 do colocalize in the intermediate zone of the cerebral cortex during axonal targeting (E16). We also demonstrate that CHL1 and ROBO1, the receptor for SLIT1, are coordinately expressed during this same period but do not colocalize. This data suggests an interaction between CHL1 and SLIT1 and a cooperation between CHL1 and ROBO1 that may help control axon guidance to regulate proper neuronal topographic mapping.

P2344  
**Board Number: B492**  
**Glia and pioneer neurons direct hierarchical assembly of the C. elegans brain.**  
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Faithful assembly of neural circuits requires a complex array of cellular interactions and molecular pathways guiding axon navigation. Circuit assembly begins when early neuronal processes extend over non-neuronal cells to form tracts that guide follower axons. Although axon guidance has been intensely studied for decades, major open questions remain. Among these are the molecular properties of pioneer axons, and the guidance interactions between pioneer axons and glia, which appose pioneer bundles. To determine how neurons and glia interact during CNS formation, we studied assembly of the C. elegans nerve ring (NR), a brain-like neuropil consisting of ~180 axons, and enveloped by four astrocyte-like CEPsh glia.

Using time-lapse embryonic imaging, genetics, protein-interaction, cell ablations and functional studies, we uncovered the early events of NR assembly. We showed that the NR is populated in an orderly manner, with CEPsh glia playing key roles in assembly initiation. We identified a set of ~10 pioneer neurons, with unique cellular, molecular, and growth properties that cooperate with glia to guide follower axons of diverse groups. Importantly, we demonstrate that CEPsh glia regulate pioneer and
follower axon guidance using distinct signals, and identify a network of guidance cues acting in glia and pioneer neurons to drive assembly.

From a genetic screen, we isolated a novel mutant that spares axon-outgrowth initiation, but severely disrupts axon guidance, with more than 70% of axons of different subtypes failing to incorporate into the NR. Two mutations, in a Chimaerin (GTPase regulator) and a Furin (pro-hormone convertase), are causal for the mutant defects. These proteins act non-canonically in glia for pioneer-axon guidance and in both glia and pioneer neurons for follower-axon navigation. Importantly, we show that they together regulate guidance-cue trafficking. Single Chimaerin or Furin mutants, or in other axon guidance genes exhibit only mild defects in NR assembly, suggesting redundancy, a problem that has plagued genetic analysis of axon guidance in vertebrate and invertebrate settings. The double mutant we identified uncovers a genetic bottleneck that allowed us to genetically identify new, redundant axon-guidance genes, several of which appear to be previously unknown.

Taken together, our studies suggest a pivotal role for glia in initiation of CNS assembly, and open the door to uncovering new axon guidance genes. CEPsh glia are reminiscent of vertebrate radial glia, whose molecular biology is not well understood. Moreover, mammalian homologs of some glial genes we identified have axon guidance roles in vertebrates, and are expressed in glia. Our studies, therefore, may reveal conserved glial mechanisms promoting CNS assembly.

P2345

Board Number: B493

PLCb1 escorts Ago2 to stress granules to change the miR population in response to Osmotic Stress.

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Phospholipase C-β1 (PLCb1) is activated by G protein signals initiated by different hormones and neurotransmitters on the plasma membrane to mediate increases in intracellular calcium (Ca2+). We have previously found that PLCb1 localizes to the cytoplasm where it interacts with components that can impact RNA-induced silencing. Using methods including fluorescence imaging, mass spectroscopy, and coimmunoprecipitation, we show that PLCb1 directly binds to one of the key components of the RNA-induced silencing complex, Argonaute 2 (Ago2). However, this association only occurs during specific cell states, and most importantly during cell stress. Specifically, we find that during stress, PLCb1 recruits Ago2 into stress granules (SGs) to help protect mRNA from degradation. This protection is inferred by the inverse correlation of PLCb1 levels with protein synthesis and in miR populations associated with cell survival. Our studies show and important and novel feedback between the external environment and mRNA stability through PLCb1.
Dynamics of Proteins and Organelles in Neurons

P2346
Board Number: B494
The ataxia disease gene VPS13D plays an essential role in mitochondrial morphology and transport in Drosophila neurons.
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The long term maintenance and survival of neurons is highly dependent on the proper functioning of mitochondria. This is evident by the disproportional representation of mitochondrial genes associated with neurodegenerative disorders. Originally discovered in yeast, vacuolar sorting protein 13 (vps13) has multiple variants in metazoans, three in Drosophila melanogaster (A, B, and D) and four in mammals (A through D). Human mutations in VPS13A, B, and C have all been linked to various forms of neurodegenerative diseases. Recent genetic studies in humans have associated mutations in VPS13D with ataxia. In order to understand the cell biology underlying the defects associated with dysfunction of the vsp13d gene in the nervous system, we are using Drosophila melanogaster as a model system. Loss-of-function mutations in vps13d lead to severe defects in mitochondrial morphology in multiple tissues, and early larval lethality. Targeted knockdown of vps13d in the nervous system circumvents this early lethality to allow further analysis, and reveals similar cell-autonomous mitochondrial defects in neurons. In larval motoneurons lacking vps13d, we observed defects in trafficking of mitochondria to axons and synaptic terminals. Loss of vps13d induces the formation of oversized, atypical mitochondria, some of which contain mitochondrial inner membrane proteins but lack markers targeted to the matrix. Most interestingly, targeted knockdown of vps13d in neurons leads to the accumulation of comparably atypical mitochondria in neighboring supportive glial cells. We are currently characterizing the nature of the cell-autonomous mitochondrial defects in neurons, and the origin of the non-cell autonomous effect on mitochondria in glia. An exciting possibility that we are testing is whether there is transfer of atypical mitochondria from neurons to glia. Altogether, these results suggest that vps13d plays an essential role in mitochondrial biology, and is required in neurons for the proper distribution of mitochondria to distal regions. In addition, vps13d disruption in neurons reveals a previously uncharacterized neuron-glia interaction which may be relevant for its roles in neurodegenerative disease.

P2347
Board Number: B495
Fear conditioning affects adenosine2A receptor and glutamate transporter 1 expression during memory consolidation.
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Memory consolidation is a critical stage of memory formation, and is associated with waves of gene expression occurring in the hippocampus four hours after memory acquisition. Yet, an understanding of the genes expressed during memory consolidation remains incomplete. One candidate gene is
ADORA2A, which encodes for the neuronal adenosine2A receptor. This receptor is responsive to the molecule adenosine, an inhibitory neurotransmitter that acts as a central nervous system depressant. It has been shown that deactivation of this receptor results in decreased memory impairment, while activation results in increased memory impairment. Furthermore, there is evidence for an antagonistic relationship between the adenosine2A receptor and glutamate transporter 1 (GLT-1). Astrocytic GLT-1 is responsible for the uptake of glutamate, an excitatory neurotransmitter. It remains unclear if the adenosine2A receptor and GLT-1 are regulated, and if they maintain an inverse relationship, during memory consolidation. Therefore, this study aimed to elucidate changes in mRNA and protein expression of the genes encoding the adenosine2A receptor and GLT-1 during memory consolidation. Mice underwent fear conditioning using a two second foot shock. Four hours later, during memory consolidation, hippocampal tissue was collected and compared with unshocked controls to examine changes in mRNA and protein expression using qPCR analysis and western blotting analysis respectively. Adenosine2A Receptor mRNA expression was significantly decreased (p=0.0467), while GLT-1 mRNA expression was significantly increased (p=0.0255), in the hippocampus four hours after fear conditioning compared to unshocked controls. The significant changes in GLT-1 mRNA expression suggest an astrocytic role in memory consolidation. Notably, these changes were inversely related to the decrease in adenosine2A receptor gene expression levels, supporting the notion that the two may maintain an antagonistic relationship. Interestingly, total protein levels of adenosine2A receptor and GLT-1 was not significantly affected at this time point. These findings highlight that there may be complex temporal interactions between transcriptional and translational events for memory consolidation, which cannot be simplified to single time points.

P2348
Board Number: B496
Neurofilament transport may be regulated by the proximity of neurofilaments to their microtubule tracks.
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Neurofilaments are abundant space-filling cytoskeletal polymers that accumulate in myelinated axons during development to drive the expansion of axon caliber, which is a key determinant of axonal conduction velocity. Neurofilaments are also cargoes of axonal transport that are assembled predominantly in the cell body and move out along axons on microtubule tracks powered by microtubule motor proteins. This dual role of neurofilaments as space-filling structures and cargoes of axonal transport implies a complex relationship between axon caliber, neurofilament influx from the cell body, and neurofilament transport kinetics in the axon. Axonal injury present a good model system to study this complex relationship because of the availability of published morphometric and kinetic data. After injury, retrogradely transported injury signals trigger a transient reduction in neurofilament gene expression in the cell body and a corresponding transient reduction in neurofilament influx into the axon, resulting in a wave of axon thinning that propagates distally at a rate consistent with neurofilament transport. Coincident with these changes, radioisotopic pulse labeling studies have revealed a transient increase the average neurofilament transport velocity. Since axonal neurofilaments normally outnumber microtubules greatly in large myelinated axons, a simple potential explanation for this acceleration of neurofilament transport is that the decrease in neurofilament number due to the decrease in neurofilament influx into the axon results in an increase in the accessibility of neurofilaments to their microtubule tracks, and consequently a greater frequency of movement. To test
this idea, we developed a new computational model for neurofilament transport in which access of
neurofilaments to their microtubule tracks, and their organization in the radial dimension of the axon,
influences their motility. Using this new model, we can explain the time-course of post-injury decrease
in axon caliber and increase in neurofilament transport velocity by a simple reduction of neurofilament
flux, and the subsequent time-course of axonal recovery by a corresponding recovery of neurofilament
flux. These findings demonstrate that proximity of neurofilaments to microtubules may be a key
determinant of their motility. One important prediction of this model is that average neurofilament
transport velocity and axon caliber are sensitive to small changes in neurofilament and microtubule
density and organization. We believe that this simple principle has important implications for the
mechanisms by which neurofilaments accumulate in axons during development, and may also
contribute to the runaway accumulations of neurofilaments observed in neurodegenerative diseases.

P2349
Board Number: B497
Nmnat mitigates sensory dysfunction in a Drosophila model of paclitaxel-induced peripheral
neuropathy.
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Chemotherapy-induced peripheral neuropathy (CIPN) is the major dose-limiting side effect of many
commonly used antimitotic agents, including paclitaxel, for which there is currently no neuroprotective
treatment. Here we established a paradigm for modeling paclitaxel-induced sensory dysfunction in
Drosophila larvae that recapitulates aspects of chemotherapy-induced sensory dysfunction, specifically
hypersensitivity to thermal nociceptive stimuli. Importantly, we showed that nociceptive sensitivity
following paclitaxel treatment is associated with an increase in peripheral dendrite branch density of
larval class IV dendritic arborization (C4da) nociceptors, uncovering a novel mechanism of paclitaxel-
induced sensory dysfunction. Additionally, we were able to show that boosting expression of Nmnat,
extensively studied for its ability to potently delay axon degeneration, cell-autonomously mitigates
paclitaxel-induced nociceptive sensitization. We demonstrated that endogenous Nmnat is crucial for the
maintenance of Drosophila larval C4da sensory neurons and thermal nociceptive behavior. Our work
establishes a robust and tractable model for studying neuronal mechanisms of CIPN and challenges the
conventional sensory axon degeneration hypothesis. Furthermore, our findings advance our
understanding of Nmnat-mediated neuronal protection and maintenance and highlight Nmnat’s
potential role as a modulator of sensory dysfunction in CIPN.

P2350
Board Number: B498
A preliminary association study in Turkish population: Do IL-17 and UCP2 Gene variants
Contribute to The Ethiology of Microtia?
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Microtia is a congenital deformity affecting the outer ear, characterized by a small, abnormally shaped
auricle. External ear canal is commonly narrowed, blocked or absent and middle ear is underdeveloped
because the outer ear and the middle ear have common embryologic origin. The prevalence of microtia
varies between 0.83 and 17.4 per 10,000 births. Recently, findings suggest that uncoupling proteins (UCPs) may play a protective role against reactive oxygen species in neuron and a thermal signaling role for neuron modulating in vestibular nerve. Interleukin-17 (IL-17) can accelerate the release of many pro-inflammatory cytokines. Although many genetic and enviromental factors are investigated the ethiopathogenesis of microtia is still unclear. The aim of this study was to investigate the links between functional promotor variants of IL-17 (-7488 A/G) and UCP2 (-866 A/G) genes and microtia. Fifteen microtia patients and 30 healthy controls were included in the study. We studied the functional promotor variants of IL-17 (rs763780) and UCP2 (659366) gene, using PCR-RFLP method. The frequencies of the alleles and genotypes in patients and controls were compared by the \( \chi^2 \) test No significant difference was found in the distribution of genotypes and alleles frequencies between patients and healthy controls for both IL-17F (rs 763780) and UCP2 (659366) genes (\( P > 0.05 \)). To our knowledge, this is the first study to assess relationship between the IL-17/UCP2 variants and Microtia in a Turkish cohort. This results do not support any major role of IL-17/UCP2 variants in the etiopathogenesis of microtia in Turkish patients. These results need to be replicated in larger series and examined in microtia.

**P2351**

**Board Number: B499**

Alterations in Protein Expression Levels Following Exposure to Mild Traumatic Brain Injury Simulation in 2.5D Culture System.

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Mild traumatic brain injury (mTBI) is a growing diagnosis among returning warfighters and is characterized by a wide array of symptoms lacking a detectable lesion, thus making it challenging to establish a correlation between mTBI and blast exposure. Phenotypic criteria of mTBI includes axonal injury and may include death of neurons and glial cells, but underlying subcellular changes have yet to be discovered. The Naval Research Laboratory (NRL) has developed a two and a half dimensional (2.5D) collagen hydrogel-based culture system with which to study mTBI with the goal of determining and quantifying changes in protein expression after exposure of primary murine cortical neurons to shock tube generated overpressures (simulated blasts). This system has increased physiological relevance as compared with conventional 2D culture systems. Proteins were extracted from the 2.5D cell cultures either with or without exposure to 210 kPa overpressure. Since the cellular responses to stress typically occur relatively quickly after insult, the cell cultures were permitted to recover for 1 hour or 48 hours before protein extraction occurred through a series of incubation/centrifugation steps using collagenase, the enzyme responsible for collagen proteolysis. Expression of caspase-3 increased in lysates exposed to overpressures as compared with control lysates when measured at the 1 hour post-insult time point, but slightly decreased/returned to baseline in exposed lysates when assessed 48 hours after exposure. Expression of Tom-20 showed no substantial changes at either time point. Preliminary evidence shows changes in expression of the intermediate filament neurofilament-medium and the extracellular matrix protein chondroitin sulfate. We will continue our studies on these proteins and report the findings. Additionally, we will assess and report expression levels of cleaved caspase-3 to begin probing the mechanism of death after exposure to overpressures.
P2352  
**Board Number: B500**  
Expression of WIPI2B counteracts age-related decline in autophagosome biogenesis in neurons.  
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Autophagy defects are implicated in multiple late-onset neurodegenerative diseases. Since aging is the most common risk factor in neurodegeneration, we examined autophagy in aged neurons. Autophagosome biogenesis is known to occur preferentially at the distal tips of axons, and autophagosomes mature as they transit to the soma. We compared autophagosome biogenesis in neurons from young adult and aged mice, identifying a significant decrease in the biogenesis of autophagosomes during aging. While nucleation and initiation rates did not change during aging, we observed the frequent production of stalled Atg13-positive, LC3-negative isolation membranes in neurons from aged mice. These stalled structures exhibited aberrant membrane morphology and failed to resolve into LC3-positive autophagosomes. Further, the majority of stalled autophagosomal structures were Atg9-positive, while autophagic vesicles that successfully recruited LC3 did not retain Atg9. To identify the underlying molecular defect, we queried expression levels of autophagy proteins and identified a specific reduction in the PI3P-binding protein WIPI2 in aged mouse brain. WIPI2 depletion in young neurons was sufficient to stall autophagosome biogenesis, phenocopying aged neurons. Importantly, reconstituting WIPI2 expression effectively restored autophagosome biogenesis in aged neurons. We additionally determined that the PI3P and Atg16L1 binding domains of WIPI2 were required for WIPI2-induced restoration of autophagosome biogenesis. Together, these data suggest a novel therapeutic target in age-associated neurodegeneration.

P2353  
**Board Number: B501**  
Autophagy at the synapse: from biogenesis to breakdown.  
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Autophagy removes bulk cytoplasm or organelles from cells by forming double-membrane autophagosomes that fuse to lysosomes for degradation. Regulation of autophagosome formation and trafficking is especially important in neurons, which have long polarized processes distant from the cell body. We and others have observed that autophagosomes form at presynaptic sites and then undergo retrograde transport towards the cell body. However, the regulation of autophagosome formation, trafficking and degradation is only partially understood. To investigate these processes, we examined the ortholog of Atg8/GABARAP, autophagosome marker GFP::LGG-1, in the *C. elegans* interneuron AII. AII is part of the thermotaxis circuit. Using temperature stimuli to control synaptic firing in AII, we observed a correlation between synaptic activity and autophagosome formation. We also observed that autophagosomes undergo Dynactin/DNC-1-dependent retrograde trafficking from the synapse. Via reverse genetic screens, we determined that UNC-16/JIP3, a motor adaptor protein, is required for the retrograde transport of autophagosomes. We performed a forward genetic enhancer screen in *unc-16/jip3* mutant animals and uncovered a novel lesion in autophagy gene *atg-4.2*, one of the two homologues in *C. elegans* for the cysteine protease ATG-4. We determined that *atg-4.2*, but not *atg-4.1* is important for clearance of autophagosomes, and that loss of *atg-4.2* in the *unc-16/jip3* mutants causes an enhancement of autophagosomes in the neurite. These studies suggest that autophagy at the
synapse begins with synaptic activity, followed by retrograde trafficking and ATG-4-dependendent autophagosome breakdown in the cellular soma.

P2354
Board Number: B502
Clarification of the roles of γ-secretases associated with autophagy.
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Accumulation of various types of abnormal/unwanted intercellular substances has been thought to be responsible for the development of neurodegenerative diseases including Alzheimer's disease (AD). Autophagy is responsible for the degradation of these materials using lysosomes. Thus, efforts have been made to identify the underlying cellular pathogenesis of AD by determination of the relationships between AD-related genetic alterations and autophagy. The series of these studies begin with the study on an important familial AD gene, PSEN1, which encodes a subunit of γ-secretase regulating the processing of beta-amyloid (Aβ) from amyloid precursor protein (APP). In 2010, a research group has shown that PSEN1 is required for the maintenance of the acidic lysosome environment by regulating the lysosomal targeting of a V-ATPase subunit, V0a1, in a manner independent of γ-secretase activity. This finding, however, was challenged by studies conducted by two independent research groups. These groups argued that either absence of PSEN does not alter autophagic substrate turnover, or the defects in turnover would be due to abnormal lysosomal calcium homeostasis rather than the impaired function of proton pump. However, these groups mainly used PSEN1/PSEN2 double knockout (KO) cells as an assay system which also could affect the activity of γ-secretase. Therefore, to clarify this conflict issue, we decided to analyze and compare the autophagic activities in the cells with impaired γ-secretase activity and/or expression of PSEs. First, we generated cell lines lacking γ-secretases by CRISPR/CAS9-mediated knockout of PEN2, a subunit of γ-secretase. Intriguingly, we found that the autophagic flux of these cell lines is more active than in normal cells. We will further elucidate the molecular mechanism underlying autophagic regulation of γ-secretase and PSEs at the molecular level.

P2355
Board Number: B503
ER-mitochondria tethering by PDZD8 regulates Ca^{2+} dynamics in mammalian neurons.
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A network of contact sites between the membranes of different organelles are emerging as critical platforms for various forms of intracellular signaling. The interface between ER and mitochondria is of particular interest as a signaling hub because it is thought to play critical physiological functions ranging from Ca^{2+} exchange, to lipid biogenesis and regulation of mitochondria fission. In addition, changes in the number of these contacts have been observed in neurodegenerative mouse models and/or in the brains of patients presenting with Alzheimer’s disease (AD), Parkinson’s disease (PD), and Amyotrophic lateral sclerosis (ALS). However, despite the fact that multiple proteins are enriched at ER-mitochondria contacts sites, the molecular mechanisms underlying ER-mitochondria tethering are still largely unknown in metazoans. Here, we will report the identification of PDZD8 as an ER protein present at ER-
mitochondria contacts (Hirabayashi et al. Science 2017 in press). The SMP domain of PDZD8 is functionally orthologous to the SMP domain found in yeast Mmm1, a member of the four proteins composing the ERMES complex identified in yeast by Peter Walter’s group as essential for ER-mitochondria contact formation in yeast (Kornmann et al. Science 2009). Using 3D FIB-SEM reconstructions, we demonstrate that PDZD8 is required for the formation of ER-mitochondria contacts in mammalian cells. Using a series of functional rescue experiments, we found that PDZD8-dependent ER-mitochondria contacts are required for proper Ca\(^{2+}\) exchange between ER and mitochondria in mammalian cells. In neurons, PDZD8 is required for Ca\(^{2+}\) uptake by mitochondria following synaptically-induced Ca\(^{2+}\)-release from ER and thereby regulated cytoplasmic Ca\(^{2+}\) dynamics. Thus, PDZD8 represents a critical ER-mitochondria tethering protein in metazoans. We suggest that ER-mitochondria coupling is involved in the regulation of dendritic Ca\(^{2+}\) dynamics in mammalian neurons.

This work was supported by grants awarded from the NIH (NS089456) (FP), (GM122589 and GM45735) (LP), an award from the Fondation Roger De Spoelberch (FP), International Research Fellowship of the Japan Society for the Promotion of Science (YH), and JST/PRESTO (JPMJPR16F7) (YH).

P2356

**Board Number: B504**

**A retrograde autophagic filter that removes mitochondria in distal nodes of Ranvier.**

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Neurons have a complex morphology, with processes that can extend beyond a meter in length in larger species. In order to supply distal compartments and synapses, neurons transport mitochondria from the soma in the anterograde direction. Interestingly, significantly less mitochondria return to the soma, where most lysosomal degradation is thought to occur. We investigated whether dysfunctional mitochondria undergo autophagy at distal axonal sites, using the neuromuscular junction (NMJ) as a model. In order to follow the fate of retrogradely moving mitochondria, we employed Thy1-mitoDendra transgenic mice and performed live-imaging following photo-conversion of synapses or intercostal axons. In synapses, we observed about half of mitochondria exiting NMJs are captured before heminodes, i.e. the last node of Ranvier separating axon and synaptic terminal. In contrast, photo-converted mitochondria in the intercostal stem axon were not captured at nodes of Ranvier and were significantly longer than those leaving NMJs. Taken together, we propose a new model, where distal nodes of Ranvier act as checkpoints for retrogradely traveling mitochondria to capture dysfunctional ones, which presumably undergo local mitophagy. We hypothesize that synapses and distal axon branches act as independent ‘mitostatic’ compartments within neurons.

As further evidence pointing towards distal autophagy we found significantly increased densities of lysosomes at distal, but not proximal, nodes of Ranvier. In addition, a cholinergic neuron specific knock-out of the autophagy-related 7 gene (Chat-Cre x Atg7 floxed) led to mitochondrial accumulation and cytoplasmic swelling of synaptic terminals and distal, but not proximal, internodes. Retrograde transport rates decreased in Atg7 knock-out mice, whereas the fraction of captured mitochondria increased, possibly due to accumulation of dysfunctional mitochondria in synaptic terminals. To corroborate the above findings we plan to analyze how captured mitochondria differ in function and morphology using

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transgenic mice expressing functional biosensors in neuronal mitochondria, viral vectors to express mitophagy indicators, as well as volumetric ultrastructural analysis. Taken together, our results suggest the existence of a hitherto unknown autophagic filter at distal nodes of Ranvier.

P2357

Board Number: B505

Preliminary characterization of mitochondrial damage-associated molecular patterns using a three-dimensional microfluidic ex vivo model of the blood-brain barrier/neurovascular unit. A.M. Medina-Lopez, B.S.1,2, I.I. Torres-Vazquez1,2, H. Shinogle-Decker, B.S.1,2, T. Neeland2, N. Martinez-Rivera, Ph.D.2, E. Rosa-Molinar, Ph.D.1,2;

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Interest in mitochondria comes from their role as “potent immunological activators” such as bacteria from which they theoretically evolved. For example, during and following a traumatic brain event, damage to and cell death of brain cells and tissues releases mitochondrial damage-associated molecular patterns (mtDAMPs) into the extracellular space, activating innate immunity and potentially causing a breach in the blood-brain barrier/neurovascular unit (BBB/NVU). To study the role of mtDAMPs in stimulating immunity and potentially breaching the BBB/NVU, we first investigated mitochondrial populations within an intact three-dimensional (3-D) microfluidic ex vivo model of BBB/NVU. We stained mitochondria with cell-permeant MitoTracker™ Red CMXRos (M-7512; Ex 579 nm/Em 599 nm; Thermo Fisher Scientific, Waltham, MA), immunolabeled mitochondrial gap junctions with connexin 43 (Cx43; C6219 [Lot # 045M4882V], Sigma-Aldrich, St. Louis, MO), and visualized them using wide-field epifluorescence microscopy, spinning disk confocal laser microscopy and transmission electron microscopy. In the intact ex vivo model, we noted low numbers of rod-like and circular shaped mitochondria with well-developed cristae were clustered around the nucleus of microvascular endothelial cells and large numbers of rod-like and circular mitochondria with well-developed cristae were randomly distributed throughout the cytoplasm of perivascular astrocytes. Cx43 immunolabeling was unambiguously shown within the mitochondrial membranes of microvascular endothelial cells and perivascular astrocytes. After determining the morphology and numbers of mitochondria, and mitochondrial gap junctions in an intact state, we exposed an intact ex vivo model to mitochondrial DNA to induce a BBB/NVU breach. We are now seeking to identify the location of the breach and to elucidate the role of Cx43 in mtDAMP's release and signaling by using the same microscopies listed above and a fluorescent water-soluble tracer dye (Tracer-653; TR-1001; MTTI, Inc., West Chester, PA) that enables imaging of vascularity, mitochondrial gap junctions, and vascular leakage of the BBB/NVU.

Research partially supported by grants to ER-M from NIH (GM-115042; GM-078441; MH-106245) and NSF (HRD-1137725). AM-L is supported through a fellowship by NIH (GM-008359).
P2358

Board Number: B506

Identification of the NAB2 Nuclear Localization Signal.
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NGFI-A binding protein 2 (NAB2) is a transcriptional coregulator that modulates gene expression through protein-protein interactions with early growth response proteins 1-3 (Egr1-3), which are immediate early genes involved in various cell behaviors. Roles for NAB2 and Egr2 are best characterized in the peripheral nervous system, where they are essential for peripheral nerve myelination. Roles for Egr1 and 3 are better documented in the central nervous system, where they contribute to learning and memory through regulation of genes that mediate synaptic plasticity and long-term potentiation. NAB2 acts largely as a transcriptional repressor, at least in part by recruiting the nucleosome remodeling and deacetylase (NuRD) complex upon binding Egr1-3. However, much remains unknown about the molecular mechanisms that regulate NAB2. This study expands our knowledge of NAB2 by identifying its nuclear localization signal (NLS). More specifically, we generated an expression construct encoding a NAB2-GFP fusion protein, which localized to the nucleus following transfection. Analysis of the NAB2 sequence identified two putative NLS’s, one site spanning amino acids 263-277 that match the bipartite NLS consensus sequence (RXK10KRR) and a second site spanning 363-366 (KKXK). K/R-to-A mutation of the potential bipartite NLS did not disrupt NAB2-GFP nuclear localization; however, K-to-A mutation of the site spanning 363-366 resulted in predominantly cytoplasmic localization, indicating it represents the functional NAB2 NLS. Current experiments are using alternative approaches to further test if amino acids 363-366 function as the bona fide NAB2 NLS. In the first approach, we are generating a series of NAB2-GFP truncation mutants with the prediction that truncations retaining amino acids 363-366 will localize to the nucleus, whereas truncations lacking amino acids 363-366 will localize to the cytoplasm. In the second approach, we are generating fusion constructs in which only amino acids 360-370 of NAB2 are linked to GFP to determine if that is sufficient to drive nuclear localization.

P2359

Board Number: B507

Endogenous alpha-synuclein expression patterns revealed using a novel mouse model.
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Alpha synuclein (aSyn) is involved in synaptic vesicle trafficking and synaptic transmission, but is also strongly linked to Parkinson's disease (PD) and other neurodegenerative disorders. In diseases where aSyn aggregates, or synucleinopathies, it accumulates in and spreads to different brain areas and peripheral organs. Although increased aSyn levels likely underlie familial PD with SNCA mutations thus implicating the protein in disease initiation, most synucleinopathies arise sporadically indicating that the expression of normal levels of wild type aSyn is sufficient for the development of disease. In spite of our increasing knowledge in the field, the physiological function of aSyn and its precise role in disease remain enigmatic urging the development of new tools for further investigations. Here, we report the development and characterization of a new mouse model expressing a GFP-aSyn fusion protein under the control of the endogenous Snca promoter. We describe the expression pattern of the fusion protein in the brain and peripheral organs and characterized its subcellular localization and trafficking in the brain. Primary neurons expressing GFP-aSyn were also successfully derived from this line. In addition, intracerebral injection of aSyn pre-formed fibrils induced formation of GFP-positive inclusions with a
similar distribution pattern to that observed in wild type mice. We anticipate that this new mouse model will facilitate in vitro and in vivo studies that incorporate live imaging and detection of endogenous alpha synuclein, therefore providing new insights into aSyn function in health and disease.

Establishing and Maintaining Organelle Structure 2

P2360
Board Number: B509

Subcellular fractionation of suspension CHO cells producing a monoclonal antibody by a differential and isopicnic centrifugation based protocol.

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A variety of fractionation protocols have been reported before for tissue samples like rat and mouse liver, among others. Since each cell line has a specific cellular organization, each should be evaluated separately. Some protocols have been previously reported for CHO cells, but they consist only of a few steps of purification, without deep demonstration of separation. Hence, we have been interested in developing a subcellular fractionation protocol for Chinese hamster ovary (CHO) cell lines. These cells are highly used for the production of therapeutic recombinant proteins, as is the case of monoclonal antibodies, making them important for the pharmaceutical industry. Thus, the cellular biology study of these cells is demanded. The main goal of the present work is to establish a reproducible and efficient protocol to obtain enriched fractions of the major subcellular organelles of suspension CHO cells. We implemented a combination of differential and isopicnic centrifugation steps. Multiple variables were evaluated like homogenization method, buffers, centrifugation conditions and density gradient mediums. CHO cells producing a monoclonal antibody were cultured in T flasks in agitation and subjected to mechanical disruption. Incubation in a hypotonic medium followed by strokes in a Dounce homogenizer resulted in the best rupture. Nuclear, mitochondrial, and microsomal pellets and cytosol were obtained as a result of differential centrifugation of homogenate. Each fraction was characterized by enrichment of specific protein markers by means of Western blot, ELISA and enzymatic activity, and protein content and distribution in SDS-PAGE. Nuclear pellet and cytosol represented each one around 40% of total proteins. Each pellet obtained was further separated in a discontinuous sucrose gradient. Three main protein peaks were detected in the sucrose gradient of each pellet. Nucleus, Golgi apparatus, endoplasmic reticulum, peroxisomes, lysosomes and mitochondria were identified among all protein peaks. Endoplasmic reticulum and cellular membranes constituted the major contaminants identified in most fractions. Only around 20% of initial total proteins correspond to cellular debris and proteins between peaks. On the other hand, the secreted antibody was fully active by recognition of its specific antigen expressed in E. coli. The present protocol provides a method to obtain enriched fractions of the major intracellular components of suspension CHO cells to conduct future physiological, enzymatic, molecular and proteomic assays. Funding: UNAM PAPPIT-IN-210013, IN-209113, CONACYT-INNOVAPYME 181895, CONACYT 2207955, 178528, 104951-Z, 396822.
P2361
Board Number: B510
Homeostatic remodeling of mammalian membranes in response to dietary lipid perturbations is essential for cellular fitness.
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A major fraction of cellular bioactivity occurs at membranes, with the lipidic matrix constituting a functional, dynamic interface that actively regulates protein activity and cell physiology. Proper membrane functionality requires maintenance of a narrow range of physical properties under challenge from external inputs. The most prominent example of such maintenance is homeostatic adaptation of membrane properties to variations in ambient temperature, a fundamental and ubiquitous design feature of ectothermic organisms termed homeoviscous adaptation. However, rapid and responsive membrane adaptation has not been observed in homeotherms. We report that challenging mammalian membrane homeostasis by dietary lipid inputs leads to robust lipidomic remodeling to maintain membrane physical properties. Specifically, supplementation of a variety of cultured cell lines and primary cells with polyunsaturated fatty acids (PUFAs) leads to rapid and extensive incorporation of the exogenous fats into membrane lipids, inducing a reduction in membrane packing. These effects are rapidly compensated for by upregulation of fully saturated lipids and cholesterol, via activation of the mammalian sterol regulatory machinery, specifically SREBP2. These lipidomic changes result in a compensatory recovery and normalization of membrane fluidity. Inhibition of membrane remodeling results in decreased cellular fitness when membrane homeostasis is challenged by dietary lipids. These results reveal that mammalian cells possess a cell-autonomous mechanism for homeostatic membrane remodeling to maintain functional membrane phenotypes, analogous to homeoviscous adaptation in poikilotherms. These observations suggest that the ubiquitous process of homeoviscous adaptation in simple organisms has been adapted in mammals to respond to membrane perturbations from dietary sources.

P2362
Board Number: B511
Exploring the Proteome of Multilocalizing Proteins.
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Subcellular distribution is one of the main aspects in the characterization of proteins, since the localization to an organelle or structure is strongly connected with the protein’s function. The Cell Atlas, which is an integrated part of the Human Protein Atlas, determined the spatial distribution of the human proteome by indirect immunofluorescence (IF) microscopy using almost 14,000 antibodies. This image-based approach revealed the subcellular localization of 12,003 proteins and mapped them to 32 organelles or subcellular structures.

An intriguing observation was the level of multilocalization among proteins, which led us to define a proteome of multilocalizing proteins (MLPs). More than half of the analyzed proteins (6172) were detected at multiple locations, with around 27% (1653) at three or more locations. The portions of MLPs differ between subproteomes of organelles; e.g. mitochondria mainly contain proteins with a single
location, while cytosol and nucleus contain mainly MLPs. This reflects the nature of organelles, with more autonomous mitochondria and the dynamic exchange between cytosol and nucleus. The reasons behind multilocalization remain unknown for the vast majority of the detected MLPs. MLPs could fulfill the same function at different sites; additional locations are employed as storage site or for controlling the activity; or proteins are moonlighting and perform multiple functions at different sites.

We selected the dual localization of proteins to the nucleus and the Golgi apparatus for further studying MLPs, because these non-neighboring organelles have distinct functions and the number of proteins (173) that are shared by both organelles is overrepresented from what is expected. We are currently performing a siRNA screen to validate the specificity of the antibodies and thereby the detected dual localization. The list of hits contains proteins that are related to specific functions in the nucleus and Golgi apparatus. This indicates that the organelles are closer connected and interact with each other by yet unidentified pathways.

In summary, MLPs are common within the human proteome contributing to cellular complexity, and could help to identify links between distant organelles.

P2363
Board Number: B512
Retrograde Localization of the Contractile Vacuole during Chemotaxis and Cellular Streaming in Dictyostelium discoideum.
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Distinct changes often occur in morphology during the formation of cellular polarity. In the case of directed migration, cells form a distinct front and rear, as they migrate towards or away from migratory cues. In many cell types, the microtubule organizing center and nucleus take up discrete localizations relative to one another in migrating cells. During the directed migration that takes place in cellular aggregation, the early process in fruiting body development, Dictyostelium discoideum cells reorganize many cellular components, including their actin and microtubule cytoskeletons. We have discovered that the contractile vacuole network also polarizes towards the back of the cell during migration.

Observations with light microscopy of retrograde contractile vacuole movement were confirmed by imaging cells expressing the fluorescently tagged contractile vacuole marker dajmin. Interestingly, contractile vacuoles only appear in areas where levels of PI(4,5)P2 appear elevated, and formation of protrusions is inhibited in areas where contractile vacuoles are present. Mutants lacking the huntingtin protein (htt-) were found to lack a detectable contractile vacuole or network. htt- cells have previously been shown to have an inability to regulate their osmotic pressure, and the loss of this dynamic organelle is likely critical to this phenotype. htt- cells made very weak cAMP waves and didn’t stream. Our results demonstrate that posterior redistribution of the contractile vacuole in migrating cells plays an important role in recruiting cells into streams and likely contributes to cAMP secretion/relay. We further demonstrate that htt- cells are incapable of regulating their osmolarity and are non-viable under extreme hypoosmotic conditions because of the absence of the contractile vacuole network.
The Amyloid-β Precursor Protein, a protein relevant to the pathology of Alzheimer’s disease, is a permanent resident of the tubular endoplasmic reticulum.

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The function of the Amyloid-β Precursor Protein (APP), considered at the core of the pathogenesis of Alzheimer’s disease (AD), is poorly understood. Since the intracellular localization of APP could provide insight into its function, we reinvestigated its distribution in a variety of cell types. APP, a type I transmembrane protein, undergoes extensive proteolytic cleavage into smaller polypeptides, which have been implicated in the AD-specific neuronal pathology. In neurons, the cleavage of APP generates N- and C-terminal fragments (NTFs and CTFs), and fragments from the internal region. After cleavage, the NTFs, derived from the ectodomain of APP, are released as soluble, glycosylated polypeptides in the lumen of intracellular membrane-bounded compartments, while the CTFs remain attached to the membrane via their short transmembrane domain. Here we show that, in the brainstem derived neuronal cells, CAD, endogenous APP is cleaved to a large extent in a juxtanuclear compartment of the tubular endoplasmic reticulum (ER). While the CTFs concentrate at the ER exit sites and are subsequently transported into the neuronal processes by TGN-derived vesicles, the NTFs released in the ER lumen never exit the ER, and are being carried to the growth cones within specialized ER tubules where machineries for protein and lipid synthesis are undetectable. The NTFs colocalize with Reticulon 4 (Rtn4), a structural protein of the tubular ER, throughout the neuron, and the NTFs and Rtn4 behave identically under experimental conditions that disrupt the structural integrity of the compartments where they reside. By contrast, the treatment of cells with Brefeldin A, an agent that blocks the anterograde vesicle transport along the traditional secretory route, does not affect the accumulation of NTFs and Rtn4 at the growth cone. Thus, the compartments that contain the NTFs and Rtn4 are identical, and most likely represent a specialized subcompartment of the tubular ER. The presence of NTFs in this ER compartment that extends in the neuronal processes, and the accumulation of NTFs at the growth cone, cannot be simply explained by the diffusion of NTFs from the soma, within the ER lumen. Rather, the NTFs are transported into the processes simultaneously with the extending ER tubules. In addition to CAD cells, we detect similar ER-like distribution of the NTFs in cortical, hippocampal, and DRG neurons, in HEK293 cells, and in all tested endothelial and epithelial cells (HUVEC, BAEC, MDCK). Together, these results suggest that the NTFs are ubiquitous residents of the tubular ER, with functions that remain to be determined. Supported by NIH AG039668 (Z.L.M.), NSF IOS-1347090 (V.M., Z.L.M.), Connecticut Science Fund, and New Jersey Health Foundation (Z.L.M., V.M.).

The interferon inducible human protein “myxovirus resistance protein A” (MxA) is a dynamin-family large GTPase with a broad-spectrum antiviral effect. Human MxA is a cytoplasmic protein which binds to and tubulates lipid membranes. Since 2002, MxA has been often stated to associate with “a
subcompartment of the smooth endoplasmic reticulum” (ER) in uninfected cells, but without evaluation of this co-localization using structural proteins of the ER as markers. In a previous study this inference was evaluated using reticulon-4 (RTN4) and atlastin-3 (ATL3) as structural markers of the standard ER and immunostaining methods. MxA-HA transiently expressed in HEK293T, Cos7 or Huh7/ Huh7.5 cells and in IFN-stimulated Huh7 and endothelial cells was observed in the cytoplasm in a variety of phenotypic forms ranging from small and large endosomes (the MxA endosomes) to larger compact tubuloreticular structures (the MxA reticulum). The previous study showed that by immunofluorescence and immuno-electron microscopy methods, the MxA-positive variably-sized endosomal and larger tubuloreticular structures were largely distinct from RTN4- and ATL3-based standard ER. In the present study, this inference was further tested using an alternative approach based on imaging GFP-tagged MxA in live Huh7 cells expressed using a puromycin-selectable vector. GFP-MxA transiently expressed in Huh7 cells showed association with puncta at the plasma membrane, and with variably-sized endosomes which exhibited spontaneous motility, and which oriented themselves along an intermediate filament meshwork unique to Huh7 cells to generate a reticular pattern. Moreover, in many cells GFP-MxA associated with (a) meshworks of fine tubules with motile segments, and (b) larger reticular structures. Additional imaging data suggested that MxA endosomes likely underwent homotypic fusion to generate elongated tubules that stretched alongside the intermediate filament meshwork to produce a reticulum. Huh7 cells selected for stable GFP-MxA expression using puromycin showed prominent MxA in both endosome and reticular structures. In two-color fluorescence assays these GFP-Mxa structures were distinct from the mCh-RTN4, mCh-KDEL or mCh-CLIMP63-labelled standard ER. The discovery of an alternative membrane reticulum in human cells based on the MxA dynamin-family large GTPase distinct from but lying alongside the standard RTN4-based ER represents a novel advance in mammalian cell biology. Moreover, the data point to the biogenesis of this cytoplasmic MxA reticulum to be from an endosomal compartment and not as a derivative of the ER.

P2366
Board Number: B515
A functional interplay between small GTPase Rab10 and exostosin-1 regulates ER morphology and dynamics.
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Five exostosin (EXT) genes encoding for glycosyltransferases have been identified in mammals, EXT1, EXT2, EXTL1, EXTL2 and EXTL3. The synthesis of heparan sulfate backbone is mediated by them. Loss of function of EXT1, EXT2 and EXTL3 causes developmental defects in different organisms including mouse, fruit fly, zebrafish and nematode. EXT1 and EXT2 proteins are believed to form a golgi-located heterooligomeric complex that is involved in heparan sulfate chain elongation. The putative tumor suppressor function role of EXT1/2 is supported by evidence of inactivating genomic alterations frequently found in patients with an autosomal dominant genetic disorder known as hereditary multiple osteochondromas (HMO). However, the molecular mechanisms governing the developmental roles of EXT genes are not well understood. Using gene silencing strategies in a HeLa cell line we focused in investigating the role of EXT1 role in endomembrane trafficking. We showed that silencing EXT1 can induce ER stress, enhance anterograde vesicles trafficking, change the energy charge and alter the cell growth. We suggest that EXT1 regulates Endoplasmic Reticulum (ER) structure and dynamics through Rab10, an ER specific Rab
GTPase. EXT1 is thus a novel, important player in the secretory pathway controlling normal cell physiology, metabolism and resistance to oncogenic transformation.

P2367  
Board Number: B516  
The role of Myelinophore organelles in myelin sheath formation.  
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Multiple sclerosis (MS) is a neurodegenerative, demyelinating disease with an unknown etiology. The hallmarks of MS pathology involve destruction of oligodendrocytes, the myelinating cells of the central nervous system and neuronal damage. Investigating the molecular mechanisms of myelin production is a critical step for understanding both physiological and pathological myelin sheath formation. Myelinophore organelles (MFOs) are intracellular organelles, found in oligodendrocytes, containing myelin membrane (MyM) tubules. Using transmission electron microscopy and tomography, we have demonstrated the presence of these organelles in ovine oligodendrocyte cultures as well as embryonic avian optic nerves between the ages of embryonic day (E)12 to E21. Immunogold labelling of high pressure frozen avian optic nerve sections, embedded in HM20 resin reveal these MFOs to be positively labelled with galactocerebroside, proteolipid protein and myelin basic protein antibodies indicating the presence of MyMs within. These studies also indicate that MyM tubules are contained within MFOs in the oligodendrocyte soma and transported via oligodendrocyte processes to the site of myelin sheath assembly. As the initial disease trigger in MS may be due to dysfunctional oligodendrocytes, where MyM tubule production or myelin sheath formation has been compromised, defining how these critical cellular processes in oligodendrocytes contribute to MyM production and myelin sheath formation could ultimately lead to the identification of new therapeutic targets for neurodegenerative diseases such as MS.

P2368  
Board Number: B517  
The centriole assembly factor, CPAP, is important for nascent basal body assembly and organization within a multi-ciliary array.  
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Centrioles and basal bodies are microtubule-organizing organelles. Basal bodies nucleate motile cilia and anchor them to resist mechanical forces from ciliary beating. The assembly of new basal bodies occurs adjacent to mature, mother basal bodies, but the dynamics of protein incorporation to build these nascent basal bodies remains poorly understood. Five proteins have been identified as essential for new basal body assembly, including the microtubule binding protein CPAP. Using the ciliate model organism, *Tetrahymena thermophila*, we show that CPAP is enriched at newly assembling basal bodies and that CPAP levels decrease as basal bodies mature. This suggests that there are two protein populations of CPAP at basal bodies: one that is dynamic and transiently associated with daughter basal bodies, and a second that is stably associated with mature basal bodies. These two populations could reflect, respectively, a CPAP population that promotes the elongation of nascent basal bodies, consistent with previous studies, and a CPAP population that stabilizes mature basal bodies to resist cilia-generated forces. In addition, *Tetrahymena* CPAP localizes to basal body-associated structures that are responsible for anchoring basal bodies at the cell cortex. These data suggest that CPAP performs functions beyond
new basal body assembly. Consistent with this hypothesis, genomic knockout of CPAP inhibits new basal body assembly, promotes basal body disassembly, and causes disorganization of the cellular cortical architecture. Together, these data suggest that CPAP plays several distinct roles in assembling, maintaining and organizing basal bodies in multi-ciliated cells.

P2369
Board Number: B518
Identification of Key Rab and Kif Proteins Required for Tether-Dependent Golgi Organization in Epistatic High-Content Screens.
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In mammalian cells, Golgi apparatus organization is dependent on vesicle trafficking. By light microscopy, knockdown of the retrograde, tether protein complexes, ZW10/RINT1 and COG, leads to fragmentation of Golgi ribbon. In epistatic experiments, this can be suppressed by co-depletion of either Rab6 or a small subset of Rab6 motor effectors (BicD, Myosin II and Kif20A) and by overexpression of the truncated BicD C-fragment. Here, we performed epistatic, visual-based suppression screens directed against 20 different Golgi-associated Rab proteins and all 44 human Kifs in order to establish the key Rab and motor proteins involved and through hit validation experiments to test the hypothesis that aborted Golgi proximal vesicle is central to cisternal disruption. We found that co-depletion of a small number of Rab proteins and Kifs epistatically suppressed both ZW10- and COG3-dependent Golgi fragmentation while ZW10-dependent Golgi fragmentation was suppressed selectively by a separate Rab/Kif set. To our surprise, no Rabs or Kifs selectively suppressed COG3-dependent Golgi fragmentation. Under basal conditions, depletion of suppressive Rab proteins or Kifs generally had little-to-no effect on Golgi cisternal organization. By electron microscopy, individual Rab or Kif hits produced a 3-10-fold accumulation of Golgi proximal vesicles suggesting a tight linkage between inhibited vesicle transport and cisternal organization. In our epistatic suppression assay, Golgi cisternal organization was restored and accompanied by a decrease in the number of Golgi proximal vesicles. Rab6 (Rab6α/Rab6α) co-depletion had a particularly strong effect. We propose that our results are most simply interpreted by a model in the absence of rapid tether-dependent capture, Golgi-derived vesicles can “wander” in a Kif-dependent manner. Under these conditions, the Golgi fragments. Funded in part by grants from the NIH (R01 GM092960 and U54 GM105814)

P2370
Board Number: B519
Model of nucleolar assembly in a developing embryo.
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Cells consist of a number of RNA and protein rich bodies that play important roles in gene regulation. Remarkably, these structures maintain a dynamic yet coherent structure even in the absence of membranes. But the mechanisms by which these organelles form and stably persist are not well understood. Examples include germ granules, stress granules and nucleoli. Here we consider the nucleolus, which is composed of proteins and RNA molecules involved in ribosome biogenesis. Recent experiments in C. elegans embryos have implicated the limiting-pool mechanism as one that is

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responsible for controlling the size of the nucleolus. In the limiting pool mechanism, the organelle grows until the pool of its building blocks is depleted to the point where the rates of assembly and disassembly of the organelle are balanced. Here we re-examine recent experimental results on nucleolar assembly to better understand the role of transcription of ribosomal RNA in the assembly process. We show that a simple model that couples transcription with diffusion-limited aggregation of the nucleolar proteins can explain the observed dynamics in a quantitative way. In particular, it reproduces the observed power law scaling of the nucleolus size with time of assembly. Our model makes quantitative predictions that suggest new experiments on nucleolus assembly in developing embryos.

P2371
Board Number: B520
LEM2 recruits CHMP7 for ESCRT-mediated nuclear envelope closure in fission yeast and human cells.
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Endosomal sorting complexes required for transport III (ESCRT-III) proteins have been implicated in sealing the nuclear envelope in mammals, spindle pole body dynamics in fission yeast, and surveillance of defective nuclear pore complexes in budding yeast. Here, we report that Lem2p (LEM2), a member of the LEM (Lap2-Emerin-Man1) family of inner nuclear membrane proteins, and the ESCRT-II/ESCRT-III hybrid protein Cmp7p (CHMP7), work together to recruit additional ESCRT-III proteins to holes in the nuclear membrane. In Schizosaccharomyces pombe, deletion of the ATPase vps4 leads to severe defects in nuclear morphology and integrity. These phenotypes are suppressed by loss-of-function mutations that arise spontaneously in lem2 or cmp7, implying that these proteins may function upstream in the same pathway. Building on these genetic interactions, we explored the role of LEM2 during nuclear envelope reformation in human cells. We found that CHMP7 and LEM2 enrich at the same region of the chromatin disk periphery during this window of cell division and that CHMP7 can bind directly to the C-terminal domain of LEM2 in vitro. We further found that, during nuclear envelope formation, recruitment of the ESCRT factors CHMP7, CHMP2A, and IST1/CHMP8 all depend on LEM2 in human cells. We conclude that Lem2p/LEM2 is a conserved nuclear site-specific adaptor that recruits Cmp7p/CHMP7 and downstream ESCRT factors to the nuclear envelope.

P2372
Board Number: B521
The role of DNA content in regulating cell shapes and sizes during zebrafish development.
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Sizes of cells or intracellular organelles are fundamental properties of biological systems, which are actively regulated in a context specific manner. The phenomenon by which sizes of cells and intracellular organelles are regulated is referred to as biological scaling. During embryonic development, the largest cell is the newly fertilized zygote, which undergoes reductive divisions. However, reductive divisions during early development must be balanced by biological scaling mechanisms to achieve optimum sizes of cells and intracellular organelles during embryogenesis. This is crucial because embryonic development is a dynamic process, which includes germ layer specification, massive co-ordinated
reorganization of germ layers during gastrulation and fate commitments to tissue and organ progenitors. Our research attempts to elucidate the role of DNA content in dictating the sizes and shapes of cells and intracellular organelles during early development in zebrafish embryos. In animals, deviations from diploidy are incompatible with embryonic viability. In zebrafish, deviations from diploidy at fertilization do not elicit early embryonic lethality, but such embryos do not survive beyond 4-6 days post fertilization (dpf). The actual cause of lethality remains unknown and as in other species is generically attributed to dosage compensation errors. Dosage compensation errors can manifest only after the zygotic genome becomes transcriptionally active. Our analysis of the early cell biology in haploid and tetraploid zebrafish embryos reveal that prior to zygotic genome activation (ZGA), specific aspects of the mitotic spindle machinery are altered from the first few cytokinesis cycles onwards. These changes later manifest as altered cell shapes and sizes in haploids and tetraploids prior to initiation of germ layer specification and gastrulation. Analysis of cell behaviors reveals that haploids and tetraploids have defects in gastrulation due to changes in cell shapes and sizes, which culminate in patterning defects. Such early defects in the fundamental nature of the cell biology due to alteration in DNA content is surprising since death of non-diploid zebrafish embryos occur only by 4-6 dpf. Our work reveals that early cell biological defects in addition to dosage compensation errors (perhaps due to faulty ZGA) may exacerbate a faulty developmental program that culminates in eventual lethality of non-diploid embryos. Fundamentally, our work also sheds light on the ability of DNA content to dictate sizes of cells and intracellular organelles in a dynamically evolving system such as a developing vertebrate embryo.

P2373

**Board Number: B522**

**Inhibition of MEK1/2 and MLK3 impairs plasma membrane repair responses to bacterial pore-forming toxins.**

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Diseases like necrotizing fasciitis and gas gangrene are caused by bacterial pathogens like *Streptococcus pyogenes* and *Clostridium perfringens*. These pathogens secrete pore-forming toxins (PFTs) like *Streptolysin O* (SLO) and *Perfringolysin O* (PFO), which form large transmembrane pores on host mammalian cells. Nucleated mammalian cells eliminate these pores using a cohort of poorly-defined membrane repair responses. One response is generation of ceramide, which can signal through downstream mitogen activated protein kinase (MAPK) pathways, including Mixed-Lineage Protein Kinase 3 (MLK3). We tested whether MLK3 and potential downstream kinases Mitogen-Activated Protein Kinase Kinase 1/2 (MEK1/2) and Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) protect cells from PFTs. We treated mammalian cell lines (HeLa, HEK293) or primary cells (murine bone marrow derived macrophages) with either MEK1/2 inhibitor U0126, MLK3 inhibitor URMCO99 or both and challenged cells with SLO or PFO. We found that MEK1/2 or MLK3 inhibition made cells about two fold more sensitive to killing by toxins. We confirmed the role of MEK1/2 in repair using siRNA knockdown. Interestingly, ERK1/2 siRNA did not alter cell sensitivity to toxin challenge. These results suggest a MEK1/2 dependent but ERK1/2 independent mechanism of membrane repair against PFTs. These findings provide a potential link between lipid modification and protein-mediated membrane repair mechanisms. A deeper understanding of membrane repair responses could provide new insight into how SLO and PFO act as virulence factors during diseases like necrotizing fasciitis and gas gangrene.

P2374
Board Number: B523

ESCRT membrane scission revealed by optical tweezers.
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ESCRT proteins catalyze the function of membrane budding and scission from the inside of the cytosol to the outside. This process is fundamental in cell biology, comprising multivesicular endosome biogenesis, cytokinesis, viral budding (e.g. HIV, Ebola, Dengue) and other pathways. The mechanism underlying ESCRT-III-mediated membrane budding and scission remains elusive. We have encapsulated within giant unilamellar vesicles (GUVs) a minimal ESCRT module consisting of ESCRT-III subunits and the AAA+ ATPase Vps4. Using optical tweezers, membrane nanotubes reflecting the correct topology of scission can be pulled from these GUVs. Upon photo-uncaging of ATP, surprisingly large forces in the tens of piconewtons were recorded and tube scission could be observed. ESCRT subunit composition and concentration alter force generation and scission behavior. In combining confocal fluorescence microscopy and optical tweezers, the scission events can be observed in both force and fluorescence and studied in detail. For the first time, the biophysics of ESCRT membrane budding and scission are revealed.

P2375

Board Number: B524

The Mechanism of the Membrane Binding of the F-BAR Domain Protein GAS7.
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Protein-lipid interactions play important roles in cell signaling, lipid metabolism, membrane trafficking and so on. Bin-Amphiphysin-Rvs (BAR) domains are highly conserved and play major roles in membrane curvature formation. Extended Fes-CIP4 homology (EFC)/FCH-BAR (F-BAR) domain is one of the subfamilies in the BAR domain. The BAR domains are thought to bind to membrane as homodimers, which then assemble into ordered oligomer on the membrane. Growth-Arrest-Specific-Protein-7 (GAS7) consists of the SH3 domain, the WW domain, and the F-BAR domain. It is the only protein in the F-BAR family, with its F-BAR located at the C-terminal. GAS7 is highly expressed in brain, which includes cerebral cortex, hippocampus and cerebellum and also in spleen, lung and heart. GAS7 is shown to be involved in neurite outgrowth, neuronal differentiation and is also involved in cytoskeleton. There are splicing isoforms of GAS7, which are GAS7c (SH3-WW-F-BAR), GAS7b (WW-F-BAR) and GAS7a (only F-BAR domain). In this study, we examined GAS7 and its isoforms, for their ability to bind to the liposomes made of bovine Folch fraction. The binding of the protein to the liposome was examined by liposome cosedimentation assay and the shape of liposome were observed by electron microscope. We have found that all these three isoforms have binding ability under the same conditions. We also introduced mutations to determine the mechanism for their membrane binding ability. We found that characteristic amino-acid residues of GAS7 compared to the other F-BAR domains plays an important role for membrane binding as oligomer.
P2376
Board Number: B525
AMPK Regulates Peroxisomal Cargo Proteins Import via PEX5 Phosphorylation.
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Peroxisomes are highly metabolic, autonomously replicating organelles. While their function as metabolic organelles is well known, their place in the world of cell signaling is only just beginning to emerge. AMP-activated protein kinase (AMPK) is a major regulator of cellular energy homeostasis, but has yet to be linked to peroxisome biology. In exploring targets for AMPK phosphorylation, we identified the peroxisome import receptor PEX5, which delivers peroxisome proteins to this organelle to regulate peroxisome function and homeostasis, as a target for this kinase. An optimal AMPK substrate motif used to analyze all PEX proteins in Peroxisome DB 2.0 identified a potential AMPK phosphorylation motif in PEX5 at serine 279 (S279). Using an AMPK-substrate-specific antibody, in vitro kinase assays, and a phospho-specific antibody generated by our group (anti-S279 on PEX5), we identified S279 as a bona fide site for PEX5 phosphorylation by AMPK. Furthermore, we found that AMPK phosphorylation of PEX5 regulated translocation of cargo proteins with a peroxisome targeting sequence (PTS1) recognized by PEX5 to the peroxisome. These data provide a model where under conditions of nutrient/energy stress, activation of AMPK increases the import of proteins essential for peroxisome functions such as beta-oxidation. Thus, we have uncovered a previously unappreciated linkage between the AMPK signaling pathway and the peroxisome, which opens new horizons for understanding peroxisomal homeostasis and its role in cellular metabolism.

P2377
Board Number: B526
The peroxisomal AAA-ATPase Pex1/Pex6 unfolds substrates by processive threading.
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The AAA-ATPases Pex1 and Pex6 are essential for peroxisome stability and peroxisomal matrix-protein import in eukaryotes. While mutations in Pex1 and Pex6 cause the majority of peroxisome biogenesis disorders in humans, their exact function is unclear. Together, Pex1 and Pex6 form a heterohexameric motor that is recruited to the peroxisomal membrane by the tail-anchored protein Pex15. At the peroxisome, Pex1/Pex6 is thought to extract the matrix protein receptor Pex5 for repeated rounds of import. Here we determined that in vitro Pex1/Pex6 from S. cerevisiae is a protein translocase that unfolds Pex15 in a pore-loop and ATP-hydrolysis dependent manner. Our structural studies of Pex15 in isolation and in complex with Pex1/Pex6 illustrate that Pex15 binds the N-terminal domains of Pex6 and presents its C-terminal disordered region for engagement by the pore loops of the AAA motor, which then processively threads Pex15 through the central pore. In vitro, Pex5, the expected substrate of Pex1/Pex6, was not engaged by the motor. Instead, we found that Pex15 binds directly to the cargo receptor Pex5 and thereby links Pex1/Pex6 to other components of the peroxisomal import machinery.
Our results thus support a role of Pex1/Pex6 in mechanical unfolding of peroxin proteins and their extraction from the peroxisomal membrane during matrix-protein import, but raise questions as to the true Pex1/Pex6 substrate in vivo.

P2378
Board Number: B527
Diurnal regulation of peroxisome function in RPE cells.
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The retinal pigment epithelium (RPE) of the eye is a single layer of supportive epithelium overlying the neural retina. Each day the phagocytic RPE ingests the shed lipid-rich tips of the photoreceptor outer segments (OS) as new material is added basally. Metabolism of OS lipids is an essential function of the RPE, and these lipids are abundant in very long chain fatty acids (VLCFA) that require oxidation within peroxisomes. The committed step in peroxisomal β-oxidation of VLCFA, catalyzed by Acyl CoA Oxidase 1 (ACOX1), produces H2O2 that is reduced by antioxidant enzymes (e.g. catalase). Despite their important role in lipid metabolism, regulation of the biogenesis, activity, and turnover of peroxisomes in RPE is not well characterized. The purpose of this study is twofold; we investigated whether peroxisome function and turnover depends on time of day. We also tested the hypothesis that RPE peroxisomes are degraded by selective autophagy by examining peroxisome number and function in RPE of mice lacking ATG5 or LC3B.

Catalase activity was measured in RPE lysates of Atg5ΔRPE, LC3BΔ and WT (C57Bl6/J) mice at various times relative to light onset using a fluorescence-based assay and normalized either to total protein or to total catalase (specific activity) as assessed by dot blot. The expression of peroxisome-specific membrane proteins Pex14 and PMP70 was assessed by immunohistochemistry (IHC) and Western blot analysis (WB) in RPE from WT, LC3BΔ and Atg5ΔRPE mice. Pex14 mediates the import of peroxisomal enzymes, and PMP70 is an ATP binding cassette transporter for long chain fatty acyl CoA intermediates. Dysregulated autophagy was determined by comparing expression of p62, LC3I, and LC3II in Atg5ΔRPE by WB.

In WT RPE, catalase activity varied diurnally, with the highest at 8 AM and lowest at 3 PM. This was opposed by a 50% increase in catalase concentration in RPE in the afternoon vs. morning. Catalase specific activity had a similar diurnal pattern with maximal activity in the morning. Overall catalase activity did not vary diurnally in LC3BΔ mice. However, catalase specific activity was double in the morning vs afternoon. Pex14 expression did not vary with time of day. Pex14 immunoreactivity was increased by 60% and 70% in ATG5ΔRPE and LC3BΔ mice vs. controls. PMP70 expression was similarly elevated. Additionally, p62 immunoreactivity was increased ~6 fold in Western blots of Atg5ΔRPE vs. controls.

Our data suggest that RPE peroxisomes rely on selective autophagy for degradation that does not vary with time of day. In contrast, activity of the peroxisomal antioxidant catalase varies diurnally through a predominantly post-translational mechanism.
**P2379**

**Board Number: B528**

How to build a granule: distinct roles for globular and intrinsically-disordered domains in P granule assembly.

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RNA granules are assemblies of RNA and proteins that form without membranes in the cytoplasm or nucleoplasm of cells. In vitro experiments have suggested that RNA granules assemble by a liquid-liquid phase separation (LLPS) driven by low-affinity interactions between intrinsically-disordered regions (IDRs) in RNA-binding proteins. LLPS is a spontaneous process that allows molecules to distribute passively between a high-concentration phase (granule) and a low-concentration phase (cytoplasm). To examine RNA granule assembly *in vivo*, we are studying the P granules of *C. elegans*. In zygotes, P granules assembly requires the granule scaffold MEG-3 (1). MEG-3 is related to the GCNA family of intrinsically-disordered proteins that contain long IDRs at their N-termini and shorter globular domains at their C-termini (2). Surprisingly, we found that the MEG-3 globular domain is primarily responsible for granule assembly in vivo. Genome editing experiments at the meg-3 locus revealed that the MEG-3 C-terminus is necessary and sufficient to form assemblies that recruit other P granule proteins. The IDR, in contrast, is not sufficient to assemble granules, but is required to concentrate MEG-3 in the posterior cytoplasm. The IDR binds RNA and responds to changes in RNA availability *in vivo*. *In vitro*, full-length MEG-3 forms gel-like condensates that concentrate RNA and P granule proteins. Consistent with a gel-like scaffold, P granules are stable under diluted conditions *ex vivo*. Together, these results suggest that LLPS driven by IDRs is not sufficient to support granule assembly *in vivo*. Our current working model is that P granules assemble around a gel-like polymer that uses MEG-3’s disordered and globular protein domains to recruit RNAs and proteins, respectively.


**P2380**

**Board Number: B529**

Nano-scale size holes in ER sheets provide an alternative to tubules for highly-curved membranes.

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The endoplasmic reticulum (ER) is a continuous membrane network partitioned into the nuclear envelope and peripheral ER domains. Unlike the large sheets of the nuclear envelope, the peripheral ER is composed of interconnected sheets and tubules. Recent work using super-resolution microscopy revealed that highly dynamic, densely packed ER tubules exist in the peripheral ER, highlighting the importance of revisiting classical views of ER structure with high spatial resolution in living cells. Here, we use live-cell Stimulated Emission Depletion (STED) microscopy to show that highly dynamic, subdiffraction-sized holes are prominent features of flat ER sheets. These fenestrated sheets coexist...
with uniform sheet regions and are clearly distinct from tubular ER regions. The curvature-stabilizing reticulon protein Rtn4 localizes to a subset of these holes. Overexpressing Clmp63 in the ER lumen to stabilize sheets constricts the diameter of holes and decreases their mobility. Analytical modeling demonstrates that holes in ER sheets are a feasible storage location for curvature-stabilizing proteins, suggesting that they serve as reservoirs to support rapid ER tubule extension and retraction. This mechanism does not rely on protein or lipid synthesis and thus provides a new explanation for how the ER locally alters its morphology on fast time scales.

P2381
Board Number: B530
The ribosome preservation factor Stm1 regulates ribosome abundance under starvation.
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Proliferation requires that growing cells adjust their protein biosynthetic capacity in response to nutrient availability. This response involves coordinated changes both in the rate of translational initiation as well as in the abundance of the protein synthesis machinery itself, especially ribosomes. Once cells exhaust their nutrients, they enter a resting state known as quiescence. Under such unfavorable conditions cells catabolize macromolecules and organelles including their ribosomes, reducing to the minimal amount required. Yet, some ribosomes need to be preserved during quiescence for the anticipated recovery from nutrient deprivation. Recent evidence suggests that the ribosome-associated protein Stm1 could act as a ribosome preservation factor, clamping 40S and 60S subunit together. Based on the binding of Stm1 on the ribosome, Stm1 may stabilize non-translating 80S ribosomes, thereby preventing ribosome degradation under nutrient deprivation. Functionally, loss of Stm1 leads to decrease in ribosome number under starvation. However, in contrast to this direct preservation model of Stm1, we propose a transcriptionally-regulated response depending on Stm1 that maintains a critical number of ribosomes under starvation. We are currently investigating the molecular mechanism for how Stm1 regulates ribosome abundance.

Mitochondrial Metabolism and Physiology

P2382
Board Number: B531
Inter-ethnic variations of mitochondrial DNA polymerase (POLG1) in two large American populations and their functional analysis.
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Purpose: Large fraction of genetic diversity in ethnic population is contributed by germline variants which helps explain the differences in genetic predisposition and susceptibility from population to another. Our previous study revealed that mitochondrial DNA (mtDNA) content is low in African
American (AA) when compared to Caucasian American (CA). Since catalytic subunit of DNA polymerase gamma (POLG1) performs critical function in mtDNA replication and repair, perturbations in POLG1 function can impair integrity of mtDNA causing reduction of mtDNA. We were thus interested to analyze if the differences in allelic frequencies of mutated POLG1 between the two American populations affect cellular state and mtDNA content.

Methods: We conducted comprehensive race based bioinformatics analysis of POLG1 gene in 33,000 European-Americans and 5,000 African-Americans and discovered several unique germline variants specifically present in one population but absent in another and common variants with relative different mutation frequency in two American populations with different ancestry. Human POLG1 at and around 1143, 251 and 587 amino acid position is evolutionary conserved; we therefore envisioned that these variations might be associated with altered mitochondrial functions. We generated these germline variants by site directed mutagenesis and cloned human wild-type (WT) POLG1, E1143G, PT2511, P587L and double mutant T2511/P587L in p426ADH yeast constitutive expression vector and transformed these constructs in BY4741 yeast strain individually and allowed them grew in YPD media for clone formation. Another set of constructs were generated in inducible mammalian expression vector, pTRE-Tight-BI-AcGFP1 using above-mentioned germline variants along with WT-POLG1.

Results: Yeast petite formation assay with POLG1 mutations (T2511, P587L and E1143G) increased petite clone formation indicative of mitochondrial respiratory chain defects.

Conclusion: We demonstrate that POLG1 genetic variants contribute to disruption of mitochondrial function. Other functional assays i.e stability of OXPHOS supercomplexes, mitochondrial complex activity, cellular respiration including analysis of mtDNA content with constructs employing mammalian expression vector pTRE-Tight-BI-AcGFP1 are under investigation.

P2383

Board Number: B532

Mitochondrial Calcium Uniporter controls AMPK activity and lipid metabolism.

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Mitochondrial calcium dynamics promote the buildup of reducing equivalents that fuel oxidative phosphorylation and ATP production for cellular metabolism. Mitochondria sequester cytosolic calcium into the matrix through the Mitochondrial Calcium Uniporter (MCU). Although MCU plays an integral role in regulating mitochondrial bioenergetics, its function in in-vivo energy homeostasis remains elusive. We found that the tissue-specific deletion of the Mcu gene in mouse liver exhibited loss of mitochondrial calcium uptake, depletes matrix calcium, reduced glucose and fatty acid coupled mitochondrial oxygen consumption and leads to the accumulation of lipids. Loss of MCU results in hepatic lipidome remodeling with increased diglyceride and triglyceride lipid species in the mouse model. MCU-knockout Danio rerio generated by targeted disruption of Mcu gene using CRISPR/Cas9 approach, recapitulate the lipid accumulation phenotype. Mouse hepatic lipid accumulation is associated with decreased phosphorylation of AMPK. This indicates that besides beta-oxidation of fatty acids, AMPK phosphorylation regulated lipid synthesis is also controlled by MCU. Deletion of MCU in liver promoted extramitochondrial calcium-dependent protein phosphatase-4 (PP4) activity that dephosphorylates AMPK. Treating the hepatic MCU-knockout mice with Metformin restored AMPK-dependent lipid clearance in the liver by an MCU-independent mechanism. MCU appears to be key in a
regulatory circuit involving mitochondrial calcium-dependent activation of AMPK to control hepatic lipid metabolism.

P2384
Board Number: B533
Deciphering the function of CLYBL, a missing human gene and a mitochondrial orphan metabolic enzyme.
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CLYBL is a "missing" human gene that is absent in approximately 2.7% of human chromosomes and whose homozygous loss is associated with reduced circulating vitamin B12 levels. CLYBL encodes an orphan mitochondrial matrix enzyme whose enzymatic activity, pathway assignment, and link to B12 are unknown. Here, we demonstrate that CLYBL loss leads to a cell autonomous defect in the mitochondrial B12 metabolism. By combining enzymology, structural biology and activity-based metabolite profiling we discover that CLYBL operates as a citramalyl-CoA lyase. Cells lacking CLYBL accumulate citramalyl-CoA, an intermediate in metabolism of the C5-dicarboxylates, including itaconate, a recently identified human antimicrobial metabolite and immunomodulator. We find that itaconyl-CoA is a substrate analogue, cofactor-inactivating inhibitor of the mitochondrial B12-dependent methylmalonyl-CoA mutase (MUT). Our work de-orphants the activity of CLYBL, explains why its loss leads to B12 deficiency, and reveals a novel mechanism of B12 poisoning by the CoA ester of itaconate, an immunomodulatory metabolite.

P2385
Board Number: B534
Sulfotransferase 1C2 (SULT1C2) post-translationally increases mitochondria respiration.
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Sulfotransferases are enzymes responsible for xenobiotic detoxification. Mechanistically, these enzymes add a sulfate group to xenobiotics which increases their water solubility and urinary excretion. We identified SULT1C2 in a proteomic screen of mitochondria isolated from ischemic preconditioned kidneys. Using hydrodynamic gene delivery, we show that SULT1C2 protects against subsequent ischemia one week after gene delivery. To determine SULT1C2’s mechanism of action, we assayed mitochondria function with and without human recombinant SULT1C2 and its substrate, 3’ Phosphoadenosine-5’-phosphosulfate (PAPS). Mitochondria respiration was assayed using an Oroboros Oxygraph O2K. We found that that PAPS and SULT1C2 incubated with mitochondria increase mitochondria state 3 respiration (add O2 consumption data), following succinate and rotenone addition, 3-fold compared to mitochondria (P<0.05). The increase in SULT1C2/PAPS dependent respiration was inhibitable with antimycin A but not rotenone. Mitochondria incubated with SULT1C2/PAPS have increased cholesterol sulfate as determined by thin layer chromatography assays and incubating isolated mitochondrial with cholesterol sulfate increases state 3 respiration. In conclusion SULT1C2 and PAPS increase the efficiency of state 3 respiration by modifying cholesterol in mitochondria membranes and
potentially increasing oxidative phosphorylation. This is a novel new function for an enzyme that heretofore was considered to be solely involved in detoxifying xenobiotics.

**P2386**
**Board Number: B535**
**Characterization of mitochondrial metabolic oscillations in live rodents.**
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Mitochondria are specialized cellular compartments that function in energy production and calcium homeostasis. Using Intravital Subcellular Microscopy (ISMic) we previously found that mitochondrial metabolic activity in the salivary glands of live rodents exhibits rapid and periodic oscillations under basal physiological conditions. The mitochondrial oscillations are also synchronized within the salivary epithelium through the activity of gap junctions. While mitochondrial oscillations may exert tissues specific biological functions, their physiological roles and how they are orchestrated particularly at the whole organismal level is still not exactly clear. Moreover, increasing reports have suggested dysfunctional mitochondrial oscillations are associated with many metabolic diseases including obesity, cardiovascular disease, developmental defects and cancers, suggesting a need to better understand the metabolic oscillations in vivo. Here, by using selected transgenic mouse models we have extended our previous work and characterized mitochondrial metabolic oscillations in several tissues under both physiological and pathological conditions, thus providing for the first time: 1) a detailed quantitative analysis of their characteristics and spatio-temporal coordination, and 2) a correlation with other signaling pathways (e.g. ROS, Ca\(^{2+}\)).

**P2387**
**Board Number: B536**
**S6 kinase 1 plays a key role in mitochondrial morphology and cellular energy flow.**
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Mitochondrial morphology, which is associated with changes in metabolism, cell cycle, cell development and cell death, is tightly regulated by the balance between fission and fusion. In this study, we found that S6 kinase 1 (S6K1) contributes to mitochondrial dynamics, homeostasis and function. Mouse embryo fibroblasts lacking S6K1 (S6K1-KO) MEFs exhibited more fragmented mitochondria and a higher level of Dynamin related protein 1 (Drp1) and active Drp1 (pS616) in both whole cell extracts and mitochondrial fraction. In addition, there was no evidence for autophagy and mitophagy induction in S6K1 depleted cells. Glycolysis and mitochondrial respiratory activity was higher in S6K1-KO MEFs, whereas OxPhos ATP production was not altered. However, inhibition of Drp1 by Mdivi1 (Drp1 inhibitor) resulted in higher OxPhos ATP production and lower mitochondrial membrane potential. Taken together the depletion of S6K1 increased Drp1-mediated fission, leading to the enhancement of glycolysis. The fission form of mitochondria resulted in lower yield for OxPhos ATP production as well as in higher
mitochondrial membrane potential. Thus, these results have suggested a potential role of S6K1 in energy metabolism by modulating mitochondrial respiratory capacity and mitochondrial morphology.

**P2388**

**Board Number: B537**

Deep mutational scanning reveals characteristics important for mitochondrial targeting of a tail-anchored protein.

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Proteins localized to mitochondria by a carboxyl-terminal tail anchor (TA) play key roles in apoptosis, mitochondrial dynamics, and mitochondrial protein import. In order to reveal structural characteristics of TAs that may be important for mitochondrial targeting, we focused our attention upon the TA of the *Saccharomyces cerevisiae* Fis1 protein. We generated a library of Fis1p TA variants fused to the transcription factor Gal4p, and by selecting for mutations within the TA that permit Gal4p to translocate to the nucleus and activate transcription, we were able to enrich for TA variants within our mutant pool which led to decreased membrane insertion. Next-generation sequencing allowed quantification of each TA variant in our mutant library before and after selection, and high-throughput results were confirmed by microscopy-based and functional analysis of individual, reconstructed Fis1p TA mutants. Consistent with the prediction that the Fis1p TA is alpha-helical, prolines within the TA generally prevented membrane insertion. Moreover, our data affirm previous results indicating that the charged carboxyl-terminus of Fis1p is important for specific localization and insertion at the mitochondrial outer membrane. In further experiments, we found that lengthening or shortening the Fis1p TA by up to three amino acids did not alter its behavior or localization, arguing against a model in which TA length directs insertion into specific organelles. Most prominently, while charged residues within the hydrophobic core of the Fis1p TA were also able to perturb membrane insertion, we found that positively charged residues were much more acceptable at several positions within the Fis1p TA than negatively charged residues. These results provide strong, *in vivo* evidence that lysine and arginine can “snorkel,” or partition the nonpolar portion of their side chains into the hydrophobic region of the lipid bilayer while placing the terminal charge near the polar interface of the membrane.

**P2389**

**Board Number: B538**

Sengers syndrome associated mitochondrial acylglycerol kinase, is a subunit of the human TIM22 protein import complex.

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Mitochondria are crucial players in cell metabolism, which requires tight regulation of ion exchange between the cytosol and mitochondrial matrix. This exchange is mediated by a class of multi-membrane spanning proteins localised to the mitochondrial inner membrane, known as the mitochondrial carrier proteins. Mitochondrial carrier proteins are inserted into the inner membrane by a protein import complex known the Translocase of the Inner Membrane 22 (TIM22 complex). Despite the tight conservation of protein import subunits from yeast to humans, the human TIM22 complex has a distinct subunit composition to its yeast counterparts. In this study, we discovered the presence of a novel subunit, Acylglycerol kinase (AGK), which was previously described to be a mitochondrial lipid kinase. Mutations in AGK cause Sengers syndrome, a mitochondrial disorder characterised by lactic acidosis, hypertrophic cardiomyopathy, skeletal myopathy and congenital cataracts. We generated a CRISPR knock-out of AGK and established that the protein has a kinase-independent function at the TIM22 complex, where it mediates the assembly of TIM22 complex and the import of mitochondrial carrier proteins. We observed similar protein import defects and destabilisation of the TIM22 complex in mitochondria isolated from Sengers syndrome patient cells. Consistent with this phenotype, loss of AGK also affects cellular metabolism via perturbation of TCA cycle flux. This data uncovers the important and unexpected relationship between the TIM22 complex, mitochondrial protein import and Sengers syndrome.

P2390
Board Number: B539
Functional analysis of mitochondria subpopulations by novel nanoscale flow cytometry platform.
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Mitochondria play an essential role in the function of numerous cellular processes, principally bioenergetics. To support these diverse activities, mitochondria adapt in size and morphology to serve cell-specific and tissue-specific functions, resulting in a high-degree of mitochondrial heterogeneity. New tools are needed to understand mitochondria at the scale of individual organelles, as well as the role of mitochondria subpopulation dynamics within cells. Herein we sought to precisely evaluate the functional differences in mitochondria subpopulations, using a novel nanoscale flow cytometry platform.

All analyses were conducted using a custom-built BD FACSaria III, calibrated using nanoparticle calibration beads (0.22- 2.0µm). With our platform, we directly isolated mitochondria populations from whole cell lysates prepared from dissected mouse tissues, using the mitochondria specific fluorescent dye, Mitotracker GreenFM (MTG). We subjected our sorted samples to several validation techniques to confirm the identity, purity, and functionality of the isolated events. Sorted MTG⁺ events were enriched in mitochondria DNA (mtDNA) over nuclear DNA via PCR (n=3), and sorted samples processed for scanning-electron microscopy displayed conserved mitochondrial morphology. Isolated mitochondria were labeled with stable isotope TMT tags, and subjected to nLC-MS/MS analysis. Pathway enrichment analysis confirmed mitochondrial identity, and quantitative and differential expression analysis revealed differentially expressed proteins between tissues (p<0.01 cutoff). Ultimately we sought to determine whether sorted mitochondria retained functional competence. Mitochondria sorted from cultured cells as well as primary cells isolated from mouse liver tissue were capable of generating quantifiable levels of ATP when provided an ADP substrate (Cells: 15.12 nM ATP; Liver: 19.27 nM, n=3). Pretreatment of the isolated mitochondria with the membrane potential uncoupler FCCP completely inhibited this ability.
(P<0.01, n=3). Furthermore, subpopulations of mitochondria were successfully sorted based on the incorporation of the membrane potential probe, JC-1. Actively respiring mitochondria more readily incorporate JC-1, resulting in a shift in fluorescence emission from 525 nm to 590 nm. Co-labeling cells with MTG and JC-1 allowed the collection of MTG⁺JC-1₅₂⁵ and MTG⁺JC-1₅₉₀ populations, hypothesized to be low respiring and high respiring, respectively, based on membrane potential. This hypothesis was supported by a confirmatory ATP generation assay (MTG⁺JC-1₅₂⁵: 9.39 nM; MTG⁺JC-1₅₉₀: 36.92, n=3), with only the MTG⁺JC-1₅₉₀ population exhibiting ATP production following pretreatment with FCCP (7.56 nM, n=3), highlighting the dynamic heterogeneity of mitochondria subpopulations.

P2391

Board Number: B540

Mitochondrial subpopulations exhibit differential dynamic responses to support increased energy demand during exocytosis.

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Mitochondria are highly dynamic organelles that undergo fission, fusion, and translocation in support of cellular metabolic needs. Although the mechanisms regulating these processes have been extensively studied in cultured cells, little is known about mitochondrial dynamics and their overall distribution in cells within tissues. We applied Intravital Microscopy (IVM) to study the spatio-temporal regulation of mitochondrial dynamics in the salivary glands of anesthetized mice. We found that 60-70% of mitochondria are positioned within 2 microns of the basolateral plasma membrane while 30-40% are scattered in the cytosol. A similar distribution pattern is conserved in other exocrine glands including the pancreas, lacrimal and parotid glands. Interestingly, by using a transgenic mouse expressing a mitochondrial-targeted photoswitchable probe (i.e. Dendra2) we found that under basal conditions: 1) basolateral mitochondria are static, whereas central mitochondria exhibit a microtubule-dependent motility; 2) both populations exhibit a low rate of fusion and fission; and 3) both populations do not mix over the course of 4 hours of observation. Since the differential distribution, dynamic properties, and segregation of these two subpopulations suggest distinct functions, we investigated their behavior under conditions of increased energy demand namely, during stimulated protein or water secretion, which are regulated by the b-adrenergic and the muscarinic receptors, respectively. We found that central mitochondria motility significantly increases during protein exocytosis, but not water secretion. In contrast to non-stimulated conditions, this increase in motility correlated with fusion and elongation of central mitochondria that consequently lead to mixing with the basolateral mitochondria. In support of the increased fusion, b-adrenergic stimulation triggered Protein Kinase A (PKA)-dependent phosphorylation of the fission protein Drp1 on the residue S637, resulting in inhibition of its fission activity. Finally, under these conditions the central mitochondria increased the frequency of interactions with lipid droplets. Current experiments explore the possibility of free fatty acids transfer, as a mean to provide substrates for ATP production. Taken together, our results define for the first time two distinct mitochondria populations in the secretory epithelia that undergo acute dynamic remodeling in response to increase in tissue energy demand.
P2392
Board Number: B541
Nutrient-regulated destruction of mitochondrial metabolite carriers by the MDC pathway.
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Mitochondrial dysfunction is a hallmark of aging, and underlies the development of many age-associated and metabolic disorders. Cells maintain mitochondrial health through a number of quality control systems that can detect dysfunctional mitochondria and repair or eliminate problematic organelles. We have now discovered a new mitochondrial quality control system conserved from yeast to humans, the Mitochondrial-Derived Compartment (MDC) pathway, which eliminates proteins from dysfunctional mitochondria by autophagy. Unlike many common mitochondrial autophagy pathways, this system selectively sorts and removes a subset of membrane proteins from the mitochondrial inner and outer membranes, while leaving the remainder of the organelle intact. Selective removal of preexisting proteins is achieved by membrane remodeling and sorting of proteins into a newly discovered mitochondrial-derived compartment, or MDC, followed by release through mitochondrial fission and elimination by autophagy. We have screened the mitochondrial proteome to catalog the substrates of this pathway, and found that the primary target of this system is a large class of inner membrane nutrient transporters called the SLC25A nutrient carrier protein family, which promote all nutrient exchange across the mitochondrial inner membrane. Based on this unique substrate selectivity, combined with our results that MDC formation is triggered by high levels of cytoplasmic amino acids, we propose that the MDC pathway acts as a mechanism to control levels of mitochondrial nutrient transporters to protect mitochondrial from metabolic stress. We are now working to understanding how cells form MDCs and selectively sort proteins into them, and how nutrient signals are relayed to the MDC activation machinery.

P2393
Board Number: B542
From Dictyostelium to Human Airway Epithelium: Adenine Nucleotide Translocase as a Protector Against Cigarette Smoke.
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Chronic obstructive pulmonary disease (COPD), the 3rd leading cause of death in the U.S., is primarily caused by cigarette smoke. Despite ongoing research, treatments for COPD have been stagnant due to the difficulty of conducting studies in the complex tissue of the human lung. Hence, we utilized a simpler model organism, the social amoeba Dictyostelium discoideum, as a tool to identify new genetic targets that associate with causal pathways. Adenine nucleotide translocase (ANT) was discovered to be protective in Dictyostelium and human bronchial epithelial cells (HBE) exposed to cigarette smoke extract (CSE). By examining ANT’s protective functions, we can elucidate its potential as a therapeutic target for COPD.

ANT resides at the inner mitochondrial membrane and functions as an ATP4/ADP3 exchanger. Given the role of ANT in mitochondria, we examined the effects of ANT1 and ANT2 expression on mitochondrial...
metabolism in the context of cigarette smoke using the Seahorse XF96e FluxAnalyzer. ANT2 overexpression increases oxidative phosphorylation and ATP flux in HBEs. Interestingly, ANT overexpression also abrogates the decrease in these parameters caused by CSE exposure. Utilizing MitoSOX staining and high-throughput imaging to assess mitochondrial reactive oxygen species (ROS) production, we see a reduction in ROS production with ANT overexpression when HBEs are acutely exposed to CSE. Surprisingly, while examining ANT localization in human lung tissue, we identified that the canonical mitochondrial protein also resides at the plasma membrane of ciliated airway epithelium. We find novel functions of ANT, particularly ANT2, on ciliary function in primary normal human bronchial epithelial cells (NHBEs). Confocal images of the airway surface liquid (ASL) stained with Texas Red dextran reveal that ANT2 overexpression increases ASL height compared to control, confirmed using various ANT inhibitors which abrogate this effect on ASL. ANT2 overexpression also maintains a normal ciliary beat frequency (CBF) in NHBEs when exposed to cigarette smoke, a noxious stimuli that decreases CBF in control cells. From our results, we conclude that ANT has protective functions against cigarette smoke in the mitochondria and unexpectedly, in the cilia of the airway. ANT improves cell viability, cell metabolism, and ciliary function. Further studies will evaluate the mechanisms behind ANT’s functions in the airway including whether ANT is responsible for ATP transport into the ASL. This will provide insight on a key regulatory step in airway surface hydration, which is essential for many airway diseases including COPD and cystic fibrosis.

P2394
Board Number: B543
Tracking global changes in acetylated mitochondria by immunofluorescence provides new insight into HDAC class I and class III crosstalk.
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Lysine acetylation is a critical post-translational modification that has been shown to play a fundamental role in epigenetic histone regulation. Recent acetylome studies indicate that acetyl-lysine regulation expands well beyond histones, and can target thousands of proteins and many different cellular processes. Many of these acetylome studies often link changes in global acetylation to disease progression. Of interest, changes in the acetylated mitochondrial protein profiles are linked to neurologic, oncogenic, cardiac, and diabetic pathologies. A recent study by Horton et al. specifically identified hyperacetylation of mitochondrial proteins in patients with heart failure. As hyperacetylation of mitochondrial proteins appear to be a critical marker of metabolic disease, having tools to quickly access the acetylated mitochondrial state may be beneficial for diagnosis or treatment. A new, pan-acetyl-lysine antibody was developed that can identify acetylated mitochondrial proteins by immunofluorescence. Importantly, this antibody was tested in combination with mitotracker, and co-localized with the mitochondria marker; conversely, the acetyl-lysine antibody did not co-localize with the LAMP 1 lysosomal marker. This acetyl-lysine antibody identified acetylated mitochondrial proteins in fibroblast, epithelial, and fibroblast-like cell lines highlighting its range of utility. Metabolic-regulating drugs, as well as class I and class III HDAC inhibitors were used to examine the antibodies ability to detect changes in the acetylated mitochondria profile. Global acetylated mitochondrial protein changes in response to these various drug treatments were detected with the acetyl-lysine antibody. Suprisingly, class I HDAC inhibitor, TSA, promoted significant down regulation of global acetylated mitochondria signal, which was reversed with class III HDAC inhibitor treatment. This result supports previous
publications showing opposing regulation between class I and class III HDACs; however, this is the first report implicating global acetylation of mitochondrial proteins as a target of this crosstalk. In summary, these data highlight the ability of this novel antibody to detect and track acetylation in the mitochondria, and may be a critical tool to gain a better mechanistic understanding of the role of HDACs in disease, and ultimately, may be an important diagnostic tool for dysfunctional mitochondrial related diseases.

P2395
Board Number: B544
Posttranslational Arginylation Enzyme Ate1 Controls Mitochondrial Functions and Cellular Warburg Effects.
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The Warburg effect is the preference of glycolysis over mitochondrial respiration in the presence of oxygen. This phenotype is commonly seen in cancer cells but its cause still remains less clear. Here we report that the down-regulation of an evolutionarily conserved enzyme, arginyltransferase 1 (Ate1), is sufficient to cause the Warburg effect at the cellular level.

As a central component of the N-end rule, Ate1 is the sole enzyme in mammalian cells mediating posttranslational addition of an extra arginine in a process called arginylation. In this study, by measuring the rates of glucose intake and lactate production, and by measuring glycolytic profiles with the Seahorse metabolic analyzer, we found that a knockout (KO) or a down-regulation of Ate1 is sufficient to up-regulate glycolysis. Consistently, by using glycolysis inhibitors 2-DG and 2-FDG, we found that the portion of glycolysis derived ATP was increased by the KO or down-regulation of Ate1, and such a phenotype can be rescued when recombinant Ate1 is expressed in the Ate1-KO cells. Therefore, our data indicates that a down-regulation of Ate1 increases glycolysis. In contrast, the KO of Ate1 appears to impair the function and morphology of mitochondria. By using Western blot on whole cells and isolated mitochondria separated on denaturing or native PAGE, we found that the Ate1-KO, while not affecting the quantity of mitochondria in the cell, changed its quality with defects in the formation of respiration chain complexes and super complexes. Particularly, Ate1-KO appears to compromise the formation and the function of succinate dehydrogenase (complex II) by impairing the import and modification of several subunits. Importantly, we found that the level of hypoxia-inducible factor 1a (HIF1a) is increased in Ate1-KO cells and the knockdown of HIF1a was able to suppress the glycolytic phenotype in these cells.

We also found that the increase of HIF1a in Ate1-KO cells are mediated by two pathways, the arginylation-dependent degradation of this protein by the N-end rule, and the increase of succinate level as expected by the compromise of succinate dehydrogenase. Finally, by confocal microscopy and by biochemical assays, we found that a portion of Ate1 is located inside mitochondria, and this may be explained by the evolutionary origin of Ate1 as a gene transferred from the mitochondrial ancestors. Our study shows for the first time that Ate1 is tightly connected to mitochondrial function, established HIF1a as a previously unknown substrate of the N-end rule, and discovered a novel pathway in mediating the cancer Warburg effect by an evolutionarily posttranslational modification enzyme.
P2396
Board Number: B545
Mitochondrial protein transport in Trypanosoma brucei: The divergent machinery for conserved function.
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A vast majority of mitochondrial proteins are nucleus-encoded and are imported into mitochondria via translocases of the mitochondrial outer and inner membrane, TOM and TIMs, respectively. These multi-protein complexes have been studied mostly in eukaryotic supergroups, opisthoknots (i.e. fungi and animals) and in archaeplastida (i.e. plants). There are two major types of mitochondrial targeting signals (MTS) located either at the N-terminal or internal of the substrate proteins, which are recognized by different subsets of Tom and Tim proteins during translocation. In spite of similarities in basic characteristics of the MTSs and evidences of multiple cross-species protein translocation into mitochondria, the TOM and TIM complexes in trypanosomatid parasites that belong to the eukaryotic supergroup excavatae, are uniquely divergent. Trypanosoma brucei, a unicellular parasitic protozoan and the infectious agent for African trypanosomiasis, possesses a non-canonical TOM complex, known as ATOM consisting of Atom40 and several other trypanosome-specific Tom proteins. In spite of two TIM complexes in fungi and animals, trypanosomatids most likely possess a single TIM capable of importing different substrate proteins. One of the major components of the TbTIM complex is TbTim17, the single member of the Tim17/Tim23/Tim22 family proteins found in other eukaryotes. We found that TbTim17 is associated with at least two other novel trypanosome-specific proteins, TbTim62 and TbTim54, and three small TbTims, TbTim9, TbTim10, and TbTim8/13, and also as a relatively conserved protein TbTim50. Using both in vitro and in vivo import assays we determined the substrate specificities of these TbTims. Our results show that TbTim17 is involved in the import of both the N-terminal and internal MTS-containing proteins. TbTim62 and TbTim50 are needed primarily for translocation of the N-terminal MTS-containing proteins, whereas TbTim54 and small TbTims are required for import of internal signal-containing proteins. TbTim62, TbTim50 and TbTim17 are membrane integral components, while small TbTims and TbTim54 are peripherally associated with TbTim17 and located in the intermembrane space. TbTim62 and small TbTims are essential for the stability and assembly of the TbTim17 complex. Together, the data suggest that TbTim62, TbTim17, and small TbTims are the core components of the TbTIM complex whereas TbTim54 and TbTim50 are accessory components required to be associated with the complex for translocation of different substrate proteins. Supported by NIH grants 1RO1AI125662 and 2SC1GM081146

P2397
Board Number: B546
Investigation of the role of ubiquitination in mitochondrial dynamics and mitophagy.
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It is well known that defects in the proper targeting and regulation of ubiquitination of mitochondria can have disastrous consequences for a cell and an organism as a whole. A classic example being the deregulation of the ubiquitin ligase Parkin, which of is associated with the in neurodegenerative disease Parkinson’s. We sought to characterize the role deubiquitinating enzymes (DUBs) play in the morphology, dynamics, and degradation of mitochondria in Saccharomyces cerevisiae. First, localization of the DUBs was determined during conditions of exponential growth condition, oxidative stress, and

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nitrogen starvation. DUBs that were found to have localization to the mitochondria, were then screened for their role in mitochondrial homeostasis. Yeast strains deficient in the known DUBs were screened by microscopy and immunoblot for defects in mitochondrial network morphology, membrane potential, dynamics, and mitophagy. Interestingly, a previously uncharacterized DUB was found to have exclusive localization to the mitochondria, be a negative regulator of mitochondrial network morphology and dynamics but not affect membrane potential.

P2398
Board Number: B547
Improvement of Cell Metabolism through Direct Mitochondrial Transfer.
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Mitochondria are essential cellular organelles involved in the maintenance of cell growth and function, and have been investigated as therapeutic targets in various diseases. Recent studies have demonstrated that direct mitochondrial transfer can restore cellular functions of cells with inherited or acquired mitochondrial dysfunction. However, previous mitochondrial transfer methods are inefficient and time-consuming. Here, we developed a simple and easy mitochondrial transfer method based on centrifugation that is cell type independent and that does not require further cultivation. After functional characterization of isolated mitochondria they were transferred into target cells using centrifugation. The high mitochondrial transfer efficiency was unaffected by the amounts of mitochondria being transferred. We first established that exogenous mitochondria could be transferred into target cells via centrifugation without causing intracellular damage, which led to increase in ATP content and improved metabolic activity. Then, we investigated whether transferring intact mitochondria into mitochondrial DNA-deleted Rho0 cells and dexamethasone-induced atrophic muscle cells could restore their metabolic functions. The transfer of intact mitochondria normalized impaired mitochondrial functions such as ATP production, membrane potential, mitochondrial ROS, and oxygen consumption rate. We also demonstrated that intact mitochondria delivered to atrophied muscle cells blocked the AMPK/FoxO3/Atrogenes pathway leading to muscle atrophy. In conclusion, it is expected that simple and rapid mitochondrial transfer can be used as a useful method for further mitochondria-related investigations into the rescue of atrophy and the restoration of muscular function.

P2399
Board Number: B548
Loss-of-function mutations in the SIGMAR1 gene cause distal hereditary motor neuropathy by impairing ER-mitochondria tethering and Ca2+ signaling.
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Distal Hereditary Motor Neuropathies (dHMN) are clinically and genetically heterogeneous neurological conditions characterized by degeneration of the lower motor neurons. To date, 19 dHMN genes have been identified but 80% of dHMN cases remain without genetic description. By a combination of autozygosity mapping, identity-by-descent segment detection and whole-exome sequencing approaches we identified two novel homozygous mutations in the SIGMAR1 gene (p.E138Q and p.E150K) in two
distinct Italian families affected by an autosomal recessive form of HMN. Sigma non-opioid intracellular receptor 1 (sigma-1R) is a 28 kDa chaperone protein of the endoplasmic reticulum (ER) that localizes at the mitochondria-associated ER membrane (MAM). It is involved in several aspects of cellular homeostasis in the nervous system, including regulation of ion channels, neurite growth and Ca2+ signalling. Functional analyses in several neuronal cell models strongly support the pathogenicity of the sigma-1R mutations and provide insights into the underlying pathomechanisms involving the regulation of ER-mitochondria tethering, Ca2+ homeostasis and autophagy. Indeed, we demonstrated that, in vitro, both sigma-1R mutations behave as “loss-of-function” mutations reducing cell viability, leading to the formation of abnormal protein aggregates preventing the correct targeting of sigma-1R protein to the MAM and thus impinging on the intracellular Ca2+ handling and triggering autophagy. In line with this, primary skin fibroblasts derived from patients with homozygous E150K sigma-1R mutation showed marked alterations of MAM number and distribution, and an increased level of basal autophagy compared to controls. Our data definitively point out the role of sigma-1R in motor neuron survival and homeostasis by correlating, for the first time in the Caucasian population, mutations in SIGMAR1 gene to distal motor disease highlighting the importance of this protein in MAM establishment and maintenance and in Ca2+ signaling modulation as a critical aspect of motor neuron degeneration in dHMN pathology.

P2400
Board Number: B549
Kinetic model of free radical generation in complex II of mitochondria.
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For the sites of free radical generation from mitochondria, mainly complex I and III have been studied in electron transport chains of mitochondrial respiration. However recently the roles of complex II are revealed in disease models such as ischemia-reperfusion, inflammatory response, and neurodegeneration. We hypothesize that complex II, involving both in electron transport chain and tricarboxylic acid (TCA) cycle plays an important role in the physiological development of cardiac differentiation. To characterize the conditions for the reactive oxygen species (ROS) generation, for the first step, we developed a kinetic model of ROS generation with multiple states of electron occupancy. The complex protein of complex II is constituted with flavin adenine dinucleotide (FAD) and three iron-sulfur clusters. The forward and backward transition rates are thermodynamically constrained with specific midpotentials from E0 to E6 states. The hydrogen peroxide is supposed to be generated with competitive binding by reduction of ubiquinone (Q) to ubiquinol (QH2) in Qp site, oxidation of oxygen to H2O2 or oxidation of succinate to fumarate in FAD site catalyzed by succinate dehydrogenase and fumarate reductase. The superoxide is assumed to be generated while electrons are shuttled through the iron-sulfur clusters as well as via the intermediate chemicals of FAD semiquinone and semiquinone. In the steady state of transition, we obtained the distribution of electron occupancy and fluxes of free radical generation in terms of ratio of succinate and fumarate concentrations, Q-pool, and oxygenation. The computational model predicts that the ROS generation is highly elevated in low succinate to fumarate ratio and low oxygen levels. The model will be validated with experiments and integrated with the reactions in complex I and III.

P2401
Board Number: B550
The new functions of the Nek family in mitochondria context.

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NIMA-related kinases (NEKS) are involved in cell cycle control, DDR and more recently mitochondrial functions. In humans, eleven members were identified, named Nek1 to Nek11. Despite the conserved kinase domains, the selectivity and diversity of partners is due to heterogeneity of the regulatory domain. We investigate the interacting partners of Nek1 (Hanchuk et al, 2017), Nek3, Nek5 and Nek10 by immunoprecipitation followed by Mass Spectrometry. Most of the identified partners are mitochondrial proteins involved in biological process such as apoptosis, metabolism and DNA repair. Using cell fractionation followed by Western blot, we identified for the first time Nek5 and Nek10 in mitochondria. We showed that stable cells expressing Nek5 regulate cell death by decreasing the level of Reactive Oxygen Species. Additionally, Real-Time PCR in Hela and Hela Nek10-silenced cells evaluated the total and mtDNA content. Nek10 depletion causes the mtDNA increase, suggesting that Nek10 is controlling mtDNA replication. Using OROBOROS Oxygraph-2k the mitochondrial respiration rates were analyzed in Nek5 and Nek10 knockdown cells. Nek5 silenced cells presented 1.7 fold increased basal rates of respiration, especially at the electrons transfer steps from TMPD to cytochrome c and at the complex II (Hanchuk et al, 2015). On the other hand, in Nek10 depleted cells it was observed a decrease in oxygen consumption in response to rotenone, a complex I inhibitor. Taken together these results showed that Nek5 and Nek10 have important roles in cell respiration, which could be regulated by its interactors. The roles Neks during the cell cycle are clear, but now, those findings raise a new role of the family associated to mitochondrial cell death and respiration. In this context, the function of each member and its partners need to be re-evaluated.


P2402
Board Number: B551
Mitochondrial electron transport chain Complex I and II in the modulation of Ca\(^{2+}\) homeostasis in breast cancer cells.
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Mammalian cells use calcium ions (Ca\(^{2+}\)) as signaling agents to regulate metabolism and other cellular functions. The flow of calcium into mitochondria, the chief producers of energy-rich ATP molecules in cells, allows them to get enough reductive equivalents and building blocks for surviving and proliferation. Krebs cycle, into the mitochondrial matrix, relies on Ca\(^{2+}\) supply for the proper functioning of at least three dehydrogenases which are essential for the generation of reduced equivalent NADH, which is used by the electron transport chain (ETC). ETC consists of four protein complexes embedded in the internal mitochondrial membrane, and they transport electrons from NADH through Complex I (CI)
to Complex IV, bombing protons from the matrix out to the inter-membrane space, generating a proton gradient used by ATP synthase to generate ATP. The main entrance of electrons to the ETC is CI. This complex has at least 40 subunits, and its assembly is a intricate process which involves several assembly factors and intermediaries. On the other hand, a secondary electron entrance is succinate dehydrogenase (SDH), also called Complex II (CII). SDH oxidizes succinate to fumarate and reduces ubiquinone. Together, complex I and II deliver most of the electrons used by ETC.

Until now, the role of CI and CII in the regulation of mitochondrial Ca$^{2+}$ homeostasis has not been studied. To assess this, we used lentiviral particles to generate stable MCF7 cells expressing a shRNA against the assembly factor of CI, NDUFAF3 and CII subunit, SDHA. Knockdown (KD) levels of 75% for NDUFAF3 and 80% for SDHA generate a severe reduction of respiratory parameters. The reduction in respiration induces a bioenergetic crisis that is accompanied by AMPK phosphorylation. Although the ETC was defective in these cells, the mitochondrial membrane potential was unchanged in both NDUFAF3 and SDHA KD cells, probably by the reverse function of the ATP synthase. Considering that the dominant driving force for Ca$^{2+}$ accumulation is the membrane potential no changes were expected. Surprisingly, we found that extramitochondrial Ca$^{2+}$ uptake by mitochondria was decreased in both NDUFAF3 and SDHA KD cells, as well as the Ca$^{2+}$ efflux.

Also, we observed an increased resistance to cell death induced by mitochondrial stressors in KD cells, suggesting that cells with CI or CII downregulated have less calcium available to be released, and this has implications in cell death. Surprisingly, we did not observe an effect on cell proliferation, although a diminution in cell migration in CII KD cells was observed. In summary, we show that NDUFAF3 and SDHA are essential for mitochondrial respiration and mitochondrial Ca$^{2+}$ uptake and efflux, and CII downregulation has an impact on migration of cancer cells.

P2403
Board Number: B552
Examining the role of ubiquitination in mitochondrial morphology, membrane potential, and mitophagy in Saccharomyces cerevisiae.

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Ubiquitination plays a vital role in mitochondrial homeostasis through regulating and targeting proteins for degradation. Defects in the ubiquitination mechanism are known to cause destructive consequences for the cell and organism, as seen with certain neurodegenerative diseases such as Parkinson’s disease. Deubiquitinating enzymes (DUBs) reverse the effects of ubiquitination and are integral in regulation. We investigated the role of DUBs in mitochondrial structure, membrane potential, dynamics, and mitophagy in baker’s yeast (Saccharomyces cerevisiae). Our primary subject for analysis is UBP11p, a previously uncharacterized DUB that we localized to the mitochondria. *ubp11Δ* and wildtype cells were imaged via epifluorescence microscopy with a mitochondrial-localized GFP (mtGFP, Westermann and Neupert 2000) in order quantify morphological changes. Morphological network data was obtained using Mitograph (Viana et al., 2015), an ImageJ Z-stack processing software, for structural analysis. Preliminary data suggests the *ubp11Δ* cells have larger mitochondrial networks with greater surface and skeletal volume. Mitochondrial membrane potential was examined with MitoLoc (Vowinckel et al., 2015), an indicator of mitochondrial functional deficiency, and then compared in wildtype and *ubp11Δ* cells with data suggesting no qualitative defects. However, the UBP11p does appear to have a role in mitophagy, as determined by the quenching of the pH sensitive GFP fused with a pH stable RFP, Rosella-m, a biosensor of mitophagy function (Rosado et al., 2008). Together, these data suggest a role for the
uncharacterized DUB UBP11p in the regulation of mitochondria.

P2404
Board Number: B553
Bioinformatic and functional analysis of uncharacterized open reading frames YPL247C and YGR021W in *Saccharomyces cerevisiae*.
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Approximately 10% of open reading frames (ORF) in the Saccharomyces cerevisiae genome are considered uncharacterized for the gene ontology (GO) terms molecular functions, biological processes, and cellular components. Combined, “¼ of the ORFs are considered to have an unknown function in at least one of these GO terms. Specifically, we sought to characterize ORFs associated with mitochondrial function. The ORFs YPL247C and YGR021W are uncharacterized genes (ORFans) in Saccharomyces cerevisiae with known DNA sequences but unknown functions for all three GO terms. Through the bioinformatics-based workflow established by the Yeast ORFan Gene Project (Bowling et al., 2015), we analyzed existing data using conserved domain database (CDD) analysis, multiple sequence alignment, protein family sequencing, and cellular localization. Previous data indicated that both ORFs localize to the mitochondria with increased localization of YPL247C under autophagy inducing conditions (rapamycin). Additionally, it was found that YPL247C is homologous to the mammalian DDB1- and CUL4-associated factor 7 (DCAF7), which contains multiple WD40 repeats that facilitate protein-protein interactions and enable the assembly of multiprotein complexes. Combined with phenotypic data indicating that overexpression of YPL247C causes cell cycle arrest, we hypothesised a role of YPL247C in regulating mitochondrial fission during cell division. The localization of YPL247C to the mitochondria was confirmed by microscopy of GFP-tagged alleles of this ORFan. Phenotypic assays in ypl247Δ cells are currently being performed to define the function of YPL247C. Furthermore, YGR021W was found to have homology to TACO1, which is a translational activator of cytochrome c oxidase I and has many known interactions with the mitochondrial translation machinery. We hypothesized that YGR021W is involved in mitochondrial translation. Again, the localization of YGR021W to the mitochondria was confirmed with GFP-tagged alleles, and phenotypic assays are ongoing to determine the role of YGR021W in the mitochondrial function.

P2405
Board Number: B554
Bioinformatic and functional characterization of the ORFs YPR117W and YHL018W in *Saccharomyces cerevisiae*.
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Yeast is an ideal model organism for modern cell biology research due to its relatively low price, homology with other eukaryotes including humans, and the relative ease of genetic manipulation. Most of the genome of Saccharomyces cerevisiae (Baker’s or Brewer’s yeast) has been studied and characterized for function. Still, around 10% of open reading frames in the genome (ORFs) are categorized as unknown for the gene ontology terms (GO terms) cellular component, molecular function and biological process. Using the bioinformatic workflow of the Yeast Gene ORFan project, the uncharacterized genes YPR117W (BSH1) and YHL018W (MCO14) were studied. First, genetic interactions, multiple sequence alignment, cellular localization data, gene deletion phenotypes and
homology protein homology were studied. BSH1 was found to contain several conserved domains: a Golgi-body localization protein domain at the C-terminus, three domains from the Fmp27 family involved in RNA pol II promoter, one N-terminal domain highly conserved in fungi but of unknown function. In addition, BSH1 was predicted to localize to the mitochondria and interacts with several proteins with the GO term of organelle organization and cytoskeleton organization. We then hypothesized that BSH1 would localize to the Golgi and have a role in transcriptional regulation and/or cytoskeleton dynamics. Current efforts are underway to determine the cellular localization of BSH1 using GFP-tagged proteins as well as phenotypic assays to determine the biological function. In contrast, MCO14 was found to exclusively to localize to mitochondria and have a predicted function as a regulator of NF1a and be a dehydratase of pterin-4a-carbinolamine. We hypothesized that MCO14 to regulate gene transcription in the mitochondria. Currently, phenotypic assays examining mitochondrial dynamics, morphology, and membrane potential are underway.

P2406
Board Number: B555
MICU1 Restricts Spatial Crosstalk Between InsP₃R and MCU Channels by Regulating Threshold and Gain of MICU1-Mediated Inhibition and Activation of MCU. R. Payne¹, H. Hoff¹, A. Roskowski¹, J.K. Foskett¹,²; ¹Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, ²Cell and Developmental Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Ca²⁺ entry into mitochondria is mediated by the Ca²⁺ uniporter channel complex containing MCU, the Ca²⁺-selective pore, and associated regulatory proteins. The roles of MICU proteins are controversial. MICU1 was proposed to be necessary for MCU activity whereas subsequent studies suggested it inhibits the channel in the low-cytoplasmic Ca²⁺ ([Ca²⁺]ₜ) regime, a mechanism referred to as “gatekeeping”, that imposed a [Ca²⁺]ₜ threshold for channel activation at ~1-3 micromolar. Here we quantitated MCU activity over a wide range of quantitatively-controlled and recorded [Ca²⁺]ₜ. MICU1 alone can mediate gatekeeping as well as highly-cooperative activation of MCU activity, whereas the fundamental role of MICU2 is to regulate the threshold and gain of MICU1-mediated inhibition and activation of MCU. Our results provide a unifying model for the roles of the MICU1/2 hetero-dimer in MCU-channel regulation and suggest an evolutionary role for MICU2 in spatially-restricting Ca²⁺ crosstalk between single InsP₃R and MCU channels.

Cellular Lipid Metabolism and Membrane Dynamics

P2407
Board Number: B556
Phosphatidylinositol synthesis is controlled at the level of substrate availability. N. Sengupta¹, D.J. Tóth¹, J. Pemberton¹, Y. Kim¹, T. Balla¹; ¹NICHD, NIH, Bethesda, MD

Phosphatidylinositol (PI) is an important lipid having roles as key membrane component as well as precursor of phosphoinositides, the tiny lipids that control essential cellular functions in both plants and animals. Phosphatidylinositol synthase (PIS) enzyme catalyzes the biosynthesis of PI from CTP-activated diacylglycerol produced from phosphatidic acid (PA). PI synthesis is accelerated during stimulation by agonists that activate phospholipase C (PLC), but beyond that little is known about the mode of regulation of the PIS enzyme. Here we investigated the substrate-product relationship of PI synthesis.
both in intact cells and membrane preparations from cells expressing the PIS enzyme. In crude membranes we found that PIS can work both in forward and reverse modes depending on the relative amounts of products and precursors. Overexpression of PIS had no major impact on the level or synthetic rate of PI in intact cell and CRISPR/Cas9-mediated gene targeting indicated that while the enzyme is essential, haploid cells showed no major abnormalities. These results suggested that PI synthesis is not controlled at the level of the amount of PIS enzyme. Using intact cells and short-term labeling with [3H]-inositol to evaluate PI synthetic rate we found that PI synthesis is highly dependent on the availability of PA, especially when PI turnover is increased by stimulation of PLC enzymes. We also found that a significant amount of PA is derived from de novo synthesis from glycerol-3-phosphate, in addition to recycling the PLC hydrolytic product, diacylglycerol. Accordingly, inhibition of de novo PA synthesis inhibited PI synthesis both under basal and angiotensin stimulated condition. Taken together our results indicated that PI levels are controlled by the availability of PA produced by either the de novo biosynthetic pathway or by diacylglycerol kinase. More importantly, these studies also pointed to the importance of PI removal as part of the control of PI synthesis, a process presumably mediated by PI lipid transport pathways.

P2408
Board Number: B557
Acute control of plasma membrane PtdIns(4,5)P2.
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The inner leaflet of the plasma membrane (PM) is a bustling hub for cellular proteins that facilitate membrane traffic, signal transduction, cytoskeletal assembly and ion flux at the cell surface. Many of these proteins, including ones executing each of the previous functions, engage the PM lipid phosphatidylinositol(4,5)-bisphosphate [PtdIns(4,5)P₂]; binding to this lipid recruits and/or a activates these proteins. Therefore, harmonious PM function is contingent on tightly regulated control of PtdIns(4,5)P₂ levels - and interrogation of these functions is greatly informed by the ability to acutely manipulate PtdIns(4,5)P₂ levels. Whereas approaches to acutely deplete PtdIns(4,5)P₂ have been available for over a decade, tools that permit acute increases in the lipid have been lacking. We found that expression of constitutive or acutely PM-recruited PI4P 5-kinases (PIPSK) lead to basally elevated PtdIns(4,5)P₂ levels as well as depletion of the substrate for this enzyme, PtdIns4P. These alterations were present in the case of the recruitable PIP5K even prior to PM recruitment. We therefore developed a new acutely recruitable PIP5K, based on a mutant kinase domain unable to homodimerize with endogenous PIPSKs. This enzyme does not effect basal lipid levels, but does produce acute (~minutes) elevations in PM PtdIns(4,5)P₂ and concomitant depletion of PM PtdIns4P. We are using this tool to evaluate how dynamic PM functions such as clathrin mediated endocytosis, PLC activation, PI3K-signaling and cortical actin assembly are influenced by acute changes in these lipids. There results provide insights into how homeostasis of one crucial lipid impacts multiple aspects of cellular function.
Uncovering a novel and Ca\textsuperscript{2+} dependent mechanism that regulates phosphatidylinositol 4-phosphate production at the plasma membrane.

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Phosphoinositides (PtdIns) are low-abundance membrane phospholipids with major roles in diverse cellular processes including membrane trafficking and signal transduction. Distribution of specific PtdIns to distinct cellular membranes establishes the membrane identity for each organelle and plays regulatory roles in their activities. At the plasma membrane (PM), phosphatidylinositol 4-phosphate (PI4P), the most abundant PtdIns at the PM, and its downstream metabolite P(4,5)P\textsubscript{2} not only establish an ionic gradient which regulates the activity and targeting of PM-associated proteins, but are also critical signaling lipids that ensure ligand-induced Ca\textsuperscript{2+} signaling from G-protein coupled receptors (GPCR). The synthesis of PI4P at the PM requires the recruitment of the PI4-kinase (PI4KIII\textalpha) from the cytosol to the PM. This is mediated by an evolutionary conserved complex comprised of the PM anchor of the complex, EFR3B, and the cytosolic shuttling proteins, TTC7B and FAM126A. In yeast, complex formation and function requires the complex to be dephosphorylated, however such a regulatory mechanism has not been shown in higher eukaryotes. We show that the Ca\textsuperscript{2+}/calmodulin-activated serine/threonine protein phosphatase, calcineurin (CN) associates with the PI4-kinase complex at the PM. Specifically, the PI4-kinase complex interacts preferentially with an isoform of CN, CnA\textbeta, which has distinct physiological and regulatory properties from other, better characterized CN isozymes. We characterize CnA\textbeta1 to be membrane-associated, in part through palmitoylation of its C-terminus and that it co-localizes with the PI4P. Furthermore, we identify a CN-binding motif in FAM126A, the auxiliary component of the PI4-kinase complex that evolved in higher eukaryotes, that is necessary and sufficient for CnA\textbeta1 binding both in vivo and in vitro. We find that a FAM126A CN-binding mutant is hyper-phosphorylated and forms a complex with PI4-kinase and TT7CB in the cytosol. However, this complex has significantly reduced PM association, suggesting that CnA\textbeta1 promotes the stable association of the PI4-kinase complex at the PM. Overall our findings propose a mechanism wherein the regulation of PI4P synthesis at the PM is mediated via CnA\textbeta1-dependent dephosphorylation of the PI4-kinase complex. We hypothesize that this Ca\textsuperscript{2+} dependent mechanism would replenish PI4P levels consumed during ligand activated PLC-coupled GPCRs to promote sustained Ca\textsuperscript{2+} signaling under high stimulatory conditions.

Novel biosensors for an enigmatic phosphoinositide.

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Phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P\textsubscript{2}) functions at the nexus of plasma membrane (PM) signaling and trafficking. Specifically PtdIns(3,4)P\textsubscript{2} is produced from PtdIns4P during endocytosis or from PtdInsP\textsubscript{3} during signaling. Elucidating the relative roles of these pathways requires the capacity to image this lipid’s production with real-time live-cell microscopy. However, the biosensors currently employed either recognize both PtdIns(3,4)P\textsubscript{2} and PtdInsP\textsubscript{3}, or have insufficient affinity to image these low abundance lipids. We therefore set out to identify PtdIns(3,4)P\textsubscript{2} and PtdInsP\textsubscript{3} probes that have high sensitivity and fidelity. Pleckstrin homology (PH) domains from Akt, Btk, ARNO, and GRP1 have been
shown to recognize and bind PtdInsP3, while the C-terminal PH domain from TAPP1 has been reported to interact with PtdIns(3,4)P2. To increase their efficacy as probes, we cloned a fluorescent protein in tandem with these PH domains as monomers, dimers, or trimers. Using TIRF and confocal microscopy, we demonstrate that our trimer TAPP1-PH and dimer ARNO-PH-2G constructs are specific and sufficient for detecting PtdIns(3,4)P2 and PtdInsP3, respectively. In addition, we developed several chemical- and opto-genetic tools to specifically manipulate P(3,4)P2 in living cells. A rapamycin inducible system was used by conjugating FKBP to P(3)K C2a and INPP4B (PtdIns(3,4)P2-specific 4-phosphatase), and a photoactivatable PtdIns3 P 4-kinase derived from Legionella pneumophila (LepB) was also used to manipulate PtdIns(3,4)P2. Collectively, the tools produced in this study provide powerful new means of understanding the spatiotemporal control of PtdIns(3,4)P2 and PtdInsP3 in signaling and membrane traffic.

P2411
Board Number: B560
Diffusion of lipids and GPI-anchored proteins in actin-free plasma membrane vesicles measured by STED-FCS.
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Diffusion and interaction dynamics of molecules at the plasma membrane play an important role in cellular signalling, and they are suggested to be strongly associated with the actin cytoskeleton. Here, we utilize super-resolution STED microscopy combined with fluorescence correlation spectroscopy (STED-FCS) to access and compare the diffusion characteristics of fluorescent lipid analogues and GPI-anchored proteins (GPI-APs) in the live cell plasma membrane and in actin cytoskeleton-free cell-derived giant plasma membrane vesicles (GPMVs). Hindered diffusion of phospholipids and sphingolipids is abolished in the GPMVs while transient nanodomain incorporation of ganglioside lipid GM1 is apparent both in the live cell membrane and in GPMVs. For GPI-APs, we detect two molecular pools in living cells; one pool showing high mobility with transient incorporation into nanodomains, and the other pool forming immobile clusters, both of which disappear in GPMVs. Our data underline the crucial role of the actin cortex in maintaining hindered diffusion modes of many but not all of the membrane molecules, and highlight a powerful experimental approach to decipher specific influences on molecular plasma membrane dynamics.


P2412
Board Number: B561
Membrane scission activity of Endophilin A2 depending on phospholipid composition.
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The shape of the plasma membrane changes during various cellular processes. The dynamic changes of membrane shapes associate with the fission and fusion of membrane vesicles. Endophilin A2 with the Bin-Amphiphysin-Rvs167 (BAR) domain which determines the formation of the lipid membrane curvature were found to have the membrane scission activity. Endophilin A2 is involved in receptor endocytosis, regulating the amount of growth factor receptor localized at the cell membrane. Here we
focused on a reported mutation of endophilin A2 at the tip of the putative structure. We examined the interaction between the lipid membrane and the endophilin A2 mutant by liposome co-sedimentation method using ultracentrifugation, with fluorescence lipid as sedimentation marker. When the size of liposomes was changed to smaller one, the sedimentation efficiency was reduced and fluorescence was detected in the supernatant after ultracentrifugation. Liposomes were prepared from three kinds of natural organ sources. However, both wild type and the mutant had different scission activities dependently on the source of lipids, and the mutant had stronger membrane scission activity than the wild type when the liposome was made of kidney derived lipids. Then, we examined the lipid compositions by thin layer chromatography, and found that lipid composition differs among three lipid different sources. Phospholipids are classified by the hydrophilic head groups, but they have two hydrophobic fatty acids that have great variety. Based on the lipid composition of kidney, reconstituted membrane was prepared by synthetic phospholipids of defined fatty acid composition. We found that endophilin A2 had scission activity only when the liposomes were made of phospholipids that have certain types of unsaturated fatty acids in kidney phospholipid composition, but not with the other compositions. In addition, the mutant had stronger membrane scission activity than wild type. Therefore, membrane scission activity of endophilin A2 is dependent on the fatty acid composition as well as types of phospholipids.

P2413
Board Number: B562
Rab1 is essential of lipid droplet biogenesis.
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Lipid droplets (LD) are regulated dynamic intracellular lipid storage organelles. Abnormal lipid storage regulation is associated with various pathologies. LD biogenesis occurs at the endoplasmic reticulum (ER) following accumulation of synthesized lipids. In spite of extensive research, many steps in this process are still unclear. Several proteins have been described to be involved in LD biogenesis. However, depletion of these proteins, while affecting LD size or distribution, did not abolish LD formation. Here we report that Rab1, a small GTPase previously linked to ER-to-Golgi trafficking, is an LD associated protein. Overexpression of its dominant-negative (DN) form abolished all steady state LDs. Inhibition of ER-to-Golgi trafficking using brefeldin A, an ADP ribosylation factor guanine exchange factor (Arf-GEF) inhibitor did not affect LD formation. Loss of function mutations in TBC1D20, a Rab1 GTPase-activating protein (GAP), were found to be associated with Warburg Micro syndrome (WARBM) a severe genetic disorder. Interestingly, fibroblasts from WRBM patents and mouse fibroblasts with similar mutations, show enlarged LDs upon induction of LD formation, further linking Rab1 to LD biogenesis. Transfection of TBC1D20 into these mouse fibroblasts rescued LDs size. Furthermore, Rab1-GFP and Diacylglycerol O-Acyltransferase 2 (DGAT2), an LD associated triglyceride synthesis enzyme accumulated around these large LDs under similar conditions. This accumulation was inhibited by expression DN-Rab1. These results support a model in which Rab1 controls ER budding and connectivity and thus the allocation of biogenesis essential factors and establishes Rab1 as an essential LD biogenesis mediator.
Board Number: B563
Rapid sterol transfer in vitro by a StAR-kin domain from Lam4p, a Lipid transfer protein Anchored at a Membrane contact site (LAM) in yeast.
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Sterol traffic inside cells does not require vesicular traffic. Using processes that are completely independent of vesicles passing through the exocytic and endocytic pathways, sterol moves between all membrane bound organelles rapidly and reversibly. Such processes have not been described at the molecular level, but they have been widely ascribed to various lipid transfer proteins that can solubilise sterol, typically by taking it inside a hydrophobic pocket. Such sterol-specific lipid transfer proteins might be able to pick up sterol from donors and deliver it to acceptors with some specificity, and the proteins may even apply some directionality to the lipid traffic. But much remains to be described, including whether the currently known group of sterol-specific lipid transfer proteins has the capacity to meet cellular demand for sterol traffic. Even this parameter is not completely mapped out, but cellular capacity for sterol traffic between the endoplasmic reticulum (where it is made) and the plasma membrane (where it mostly resides) is typically estimated as 4-10 fold higher than needed for cell growth alone.

In 2015 we published the discovery of a large family of Lipid transfer proteins Anchored at Membrane contact sites (called LAMs) common to most eukaryotes. We showed that in budding yeast Lam4p and its parologue Ysp2p have two domains each that solubilise the major species of sterol: cholesterol from mammals, and ergosterol from fungi. Here we have carried out detailed lipid transfer experiments in vitro with the second StAR-kin domain of Lam4p (Lam4S2). Our main finding is that the Lam4S2 in solution, without any accessory domains, transfers sterol at a fast rate compared to most other lipid transfer proteins. By designing symmetric lipid transfer assays, where donor and acceptor are essentially identical, we calculate rate constants for individual steps in more complex transfer reactions. This allows us to dissect each separate step within an entire round of lipid transfer to a greater extent than is usually reported.

Board Number: B564
Recycling of lysosome membranes in neurons is regulated by lysosomal ganglioside levels.
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During autophagy, functional lysosomes are consumed by their fusion with autophagosomes, leading to formation of autolysosomes. The pool of functional lysosomes is maintained by a process named...
autophagic lysosome reformation (ALR). Lysosomal membrane components are extruded from autolysosomes to form new lysosomes. Most proteins involved in ALR are encoded by genes responsible for neurodegenerative diseases when mutated. This suggest that ALR is important for neuron survival. However, its mechanisms have only been investigated in non-neuronal models so far. Here, we investigated the role of one key player of ALR in neurons, spatacsin that is encoded by SPG11, a gene responsible for a severe form of hereditary spastic paraplegia when mutated. Loss of function of spatacsin impairs ALR leading to autolysosome accumulation and free lysosomes depletion as well as lipids accumulation in lysosomes. We showed that loss of function of spatacsin led to the accumulation of simple gangliosides in autolysosomes in a knockout mouse model, primary cultures of neurons and neurons of cortical organoids derived from induced pluripotent stem cells of SPG11 patients. By preventing gangliosides synthesis in Spg11 knockout cells or by inducing their accumulation in lysosomes in wild-type cells, we demonstrated that elevated concentrations of gangliosides in lysosomes blocked their recycling and promoted the accumulation of autolysosomes. Our results suggest that altered ALR in absence of spatacsin is a consequence of the accumulation of gangliosides rather than a direct role of the protein in lysosome recycling.

P2416
Board Number: B565
Lipid-laden macrophages downregulate Akt phosphorylation and metabolize lipid droplets via autophagy.
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Macrophages contribute to plaque formation in atherosclerosis. Macrophages take up modified low-density lipoproteins and store excess cholesterol and triglycerides in lipid droplet organelles. Evidence of lipid-laden macrophages or “foam cells” is apparent on histology sections of diseased arteries, and this lipid-laden appearance can be recreated in cell culture upon exposure of primary macrophages to modified LDLS. Previous work in a variety of cell types suggests that under nutrient stress, neutral lipids in lipid droplets are hydrolyzed by lipolysis, autophagy, or both. However, these processes are not well understood in macrophages. We created lipid-laden macrophages by 24-h exposure to acetylated LDL or oxidized LDL and analyzed dynamics of lipid droplet degradation following removal of LDL from cell culture medium. We found that lipid droplets in these macrophages are cleared within 24 h of LDL removal from the medium. During lipid droplet clearance, there is no evidence of fatty acid delivery to the mitochondria suggesting these lipid droplets are not used as an energy source. Electron microscopy visualization of lipid droplets during formation and degradation showed the presence of double membrane structures surrounding lipid droplets at early stages of degradation. This may indicate the involvement of autophagy in lipid droplet disappearance from macrophages. Inhibition of autophagosome formation by 3-methyl adenine prevented lipid droplet degradation. Immunostaining of phosphorylated Akt showed decreased Akt activation in lipid laden macrophages during lipid droplet degradation. Altogether, these data indicate lipid droplets formed by exposure of macrophages to modified LDLS are degraded by an autophagic process that could be signaled via disruptions in growth factor signaling. Studying the mechanisms of lipid droplet degradation will allow us to understand how lipid droplets could be cleared from foam cells residing in atherosclerotic plaques and identify potential therapeutic targets for treating atherosclerosis.
P2417  
**Board Number: B566**  
*Mechanisms of selective death of tumor cells after COPI complex depletion.*  
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The coatomer protein complex 1 (COPI) is a multisubunit complex that coats intracellular vesicles and is involved in intracellular protein trafficking. Recently we and others found that depletion of COPI complex subunits zeta (COPZ1) and delta (ARCN1) preferentially kills tumor cells relative to normal cells. Here we studied the cellular effects of COPI complex depletion in tumor cells. We showed that this depletion led to the inhibition of mitochondrial oxidative phosphorylation and elevation of ROS production, followed by accumulation of lipid droplets and autophagy associated proteins LC3-II and SQSTM1. At a later stage, the COPI complex depletion caused classical apoptosis of the tumor cells. Inhibition of ROS level in COPI-depleted cells with the mitochondrial-specific quencher mitoquinone (MitoQ) attenuated this apoptosis and drastically decreased both size and number of lipid droplets. Similar results were obtained after treatment of COPI-depleted cells with SP600, an inhibitor of JNK kinase which is known to inhibit mitochondrial ROS production. In COPI depleted cells LC3-II and SQSTM1 colocalized with lipid droplets, suggesting that these droplets may be associated with autophagosomes. However, electron microscopy of COPI depleted cells showed that these droplets are not surrounded by double membranes indicative of autophagic degradation of the lipids. Moreover, no excessive autophagic vacuole formation was seen in COPI-depleted cells. Our findings suggest a sequence of cellular events triggered by COPI-depletion, starting with inhibition of oxidative phosphorylation, followed by ROS activation and accumulation of lipid droplets, which in turn further elevate the level of ROS and triggers apoptosis. This mechanism is highly promising in the development of novel cancer therapy by targeting tumor cells with the diminished level of COPI proteins.

P2418  
**Board Number: B567**  
*The GOLPH3 oncogene controls the intra-Golgi recycling of sphingolipid glycosylating enzymes to promote proliferation.*  
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The GOLPH3 oncogene is located on a human chromosome region, which is frequently amplified in solid tumours. GOLPH3 encodes a Golgi complex associated protein involved in membrane trafficking and Golgi structure maintenance. While GOLPH3 role in the stimulation of cell proliferation pathways is widely accepted, how GOLPH3 gain of function mediates oncogenesis is not completely understood. Here we show that GOLPH3 regulates proliferation by promoting sphingolipid glycosylation. Specifically,
we found that GOLPH3 interacts with sphingolipid glycosylating enzymes at the Golgi complex and controls their intra-Golgi recycling during cisternal progression. Through this mechanism GOLPH3 counteracts the physiological leakage sphingolipid enzymes to lysosomes and their consequent degradation thus resulting in increased enzyme levels. Alterations in proteostasis of glycosylating enzymes and in sphingolipid metabolism induced by increased GOLPH3 levels impact on growth signalling pathways, thus promoting uncontrolled proliferation. Importantly, GOLPH3 overexpression/amplification tightly correlates with the sensitivity of tumour cells to pharmacological inhibition of sphingolipid glycosylation suggesting that inhibition of sphingolipid metabolism represents a valuable therapeutic option for patients affected by GOLPH3 dependent tumours.

P2419
Board Number: B568
Molecular mechanisms of Scavenger Receptor SR-BI regulation: linking HDL binding to cholesterol transport.
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Cholesterol is delivered to cells by LDL (low-density lipoprotein) and removed from them by HDL (high-density lipoprotein). Excess cholesterol removed from peripheral tissues by HDL is transported to the liver for conversion into bile acids that are excreted with gastrointestinal contents, a process known as reverse cholesterol transport. HDL also delivers cholesterol to adrenocortical cells for steroid hormone synthesis. Delivery of cholesterol from HDL to these tissues is mediated by the scavenger receptor SR-BI. To better understand the molecular mechanism whereby SR-BI removes cholesterol from HDL before releasing the depleted lipoprotein, we analyzed the subcellular distribution of the receptor, its mobility in the membrane and the dynamics of its internalization. To this end, we developed a receptor-specific short-chain antibody (ScFv). Immunostaining using ScFv covalently labeled with fluorescent probes confirmed that SR-BI is highly expressed on the surface of adrenocortical and liver cells. Monitoring SR-BI in live cells revealed that, strikingly, this receptor was retained at the surface of cells for extended periods, while other receptors and the bulk of the PM were being constantly and rapidly internalized. Failure to internalize did not result from attachment of SR-BI to cytoskeletal structures, since single-particle tracking indicated that the receptors move freely in the plane of the membrane. Accordingly, removal of the PDZ-binding domain proposed to anchor SR-BI to the cytoskeleton did not affect its retention on the membrane. Quantitative analysis of ScFv binding showed that SR-BI exists on the surface of adrenocortical cells as large multimers consisting of an average 10 receptor molecules. These large clusters fail to enter clathrin coated pits, caveolae or GEEC/CLICs, accounting for the inordinate retention of the receptors at the surface. Importantly, SR-BI was able to remove cholesterol from HDL while at the cell surface, without requiring internalization. Cholesterol transported from the lipoprotein to the PM via SR-BI was delivered to the endoplasmic reticulum and eventually to lipid droplets. In this regard, HDL-associated cholesterol is handled very differently from that bound to LDL, which was rapidly internalized and routed through the endocytic pathway. We suggest that the surface retention of SR-BI is attributable to its ability to multimerize and that this feature plays an important role in its function.
P2420

Board Number: B569

FUSEXINS, a family of sexual, somatic and viral cell fusion proteins.
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Cell-cell fusion is essential for fertilization and sculpting of bones, muscles, epidermis and placenta in different organisms. However, the proteins that mediate membrane fusion between cells have not yet been well-characterized and in some cases are still unknown. HAP2(GCS1) are membrane glycoproteins essential for gamete fusion in plants, Chlamydomonas, Plasmodium, Tetrahymena, and Dictyostelium. To investigate whether HAP2 is not only essential but also sufficient for cell-cell fusion, we expressed the Arabidopsis sperm HAP2 in cultured baby hamster kidney cells that normally do not fuse. We found that HAP2 expression in these heterologous cells results in the formation of multinucleate cells by cell-to-cell fusion. Genetic and cell biological analyses showed that HAP2 has to be present in only one of the fusion partners (usually the male gamete) to fuse. However, we found that when expressed in mammalian cells, Arabidopsis HAP2 is sufficient to fuse them only when expressed in both fusing cells. Thus, HAP2-mediated plasma membrane merger occurs via a bilateral zipper mechanism reminiscent to intracellular fusions mediated by SNAREs, atlastins and mitofusins. Furthermore, expression of HAP2 on the surface of pseudotyped vesicular stomatitis virus results in virus-cell fusion only to cells that also express HAP2. Thus, we propose that, in addition to sperm HAP2, a HAP2-like protein is needed in the egg for gamete fusion. Structural modeling of the HAP2 protein family predicts that it is homologous to class II viral fusion proteins (e.g. Zika, rubella and dengue) and the somatic cell fusogen EFF-1 from C. elegans. Moreover, the recently solved crystal structure of Chlamydomonas’ HAP2 demonstrates structural homology with EFF-1 and class II viral fusion proteins. We name this superfamilly FUSEXINS: Fusexins proteins essential for sexual reproduction and EXplasmic merger of plasma membranes. Fusexins mediate enveloped virus entry into cells, sexual reproduction, and somatic cell fusion. These proteins share the same structure and function but use distinct mechanisms to merge membranes. We hypothesize that modern fusexins have existed since the dawn of eukaryotic cells.

P2421

Board Number: B570

Short Chain Ceramides Disrupt Segregation of Liquid-ordered from Liquid-disordered Components in the Plasma Membrane.
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Perturbation of plasma membrane structure by lipid derivatives could provide a general method for assessing functional roles for ordered membrane regions in cell biology. We previously reported that short chain ceramides with either C2 or C6 acyl chains reduce the fluidity of giant plasma membrane vesicles (GPMVs) derived from RBL mast cells; at similar micromolar concentrations, these ceramides
effectively inhibit antigen-stimulated Ca\(^{2+}\) mobilization in these cells (Gidwani et al., J Cell Sci., 2003). We now find that these ceramide analogues inhibit liquid order-liquid disorder phase separation in these GPMVs that normally occurs at low temperatures. Furthermore, they are effective inhibitors of antigen-stimulated tyrosine phosphorylation, as well as thapsigargin-stimulated store-operated Ca\(^{2+}\) entry. The latter points to a role for liquid ordered-like membrane structure in Orai1-mediated Ca\(^{2+}\) entry. In Jurkat T cells, C2-ceramide is also effective at inhibiting Ca\(^{2+}\) mobilization stimulated by either anti-TCR or thapsigargin, consistent with the view that these short chain ceramides effectively interfere with functional responses that depend on ordered membrane structure.

P2422
Board Number: B571

Pattern formation and stochastic geometry sensing in a lipid kinase-phosphatase competitive reaction.
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Symmetry breaking and the establishment of spatial patterns of molecules in a dynamic, fluid environment is a hallmark of cellular organization in living systems. Central to many of these cellular signal pathways are phosphorylation reactions, driven by competing lipid kinases and phosphatases. Here, we describe the biochemical reconstitution of a lipid kinase-phosphatase reaction system that drives the interconversion of PI(4)P and PI(4,5)P2 lipids on planar membrane surfaces. This system can be tuned to exhibit bistability and spontaneous pattern formation. Using micropatterned membranes to spatially confine the competitive reaction, we discover that final reaction outcome can be modulated by the geometric size and shape of the membrane environment. The physical mechanism of this process, which we call stochastic geometry sensing, is based on the system’s asymmetrical response to stochastic compositional fluctuations in enzyme copy number and the corresponding local changes lipid density. The fundamental finding is that stochastic geometry sensing provides a relatively simple route for a biologically relevant chemical reaction to form patterns and differentially respond to the geometric constraints of the reaction environment.

P2423
Board Number: B572

Ca\(^{2+}\) releases E-Syt1 autoinhibition to couple ER-plasma membrane tethering with lipid transport.
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The extended synaptotagmins (E-Syts) are endoplasmic reticulum (ER) proteins that bind the plasma membrane (PM) via C2 domains and transport lipids between them via SMP domains. E-Syt1 tethers and transports lipids in a Ca\(^{2+}\)-dependent manner, but the role of Ca\(^{2+}\) in this regulation is unclear. Of the

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five C2 domains of E-Syt1 only C2A and C2C contain Ca2+-binding sites. Using liposomes-based assays, we show that Ca2+ binding to C2C promotes E-Syt1-mediated membrane tethering by releasing an inhibition that prevents C2E from interacting with the Pl(4,5)P2-rich membrane, as reported in semipermeabilized cells. Importantly, Ca2+ binding to C2A enables lipid transport by releasing a charge-based autoinhibitory interaction between this domain and the SMP domain. Supporting these results, E-Syt1 constructs defective in Ca2+ binding in either C2A or C2C failed to rescue two defects in PM lipid homeostasis observed in E-Syts KO cells, delayed diacylglycerol clearance from the PM and impaired Ca2+-triggered phosphatidylserine scrambling. Thus, a main effect of Ca2+ on E-Syt1 is to reverse an autoinhibited state and to couple membrane tethering with lipid transport.

**Kinases and Phosphatases 2**

**P2425**  
**Board Number: B575**

The β4-subunit of the voltage-gated calcium channel down-regulates Wnt/β-catenin signaling and cell proliferation.

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The voltage-gated calcium channel is a heteromeric protein complex comprised of a main pore-forming α1-subunit and the auxiliary subunits β, α2δ and γ. The β-subunits (CACNB) (β1 to β4) are structurally related cytosolic proteins with varying tissue expression patterns that regulate trafficking of the pore-forming α1-subunit to the cell membrane and channel biophysical properties. Mutations in the neuronal β4-subunit (CACNB4) are linked to neuropsychiatric disorders in humans including epilepsy and intellectual disabilities; however, whether the pathogenic mechanism involves channel dysfunction or other cellular processes affected by β4-subunit mutations has not been elucidated. In addition to interacting with the pore-forming α1-subunit, the β4-subunit binds to B56β (a regulatory subunit of the protein phosphatase 2A), translocates to the cell nucleus and regulates gene transcription and cell proliferation, suggesting that the β4-subunit partakes in signaling pathways involved in cell cycle progression. This study investigated whether the β4-subunit regulates the canonical Wnt/β-catenin signaling pathway and affects cell proliferation. We found that co-expression of the β4-subunit with actors of the canonical Wnt/β-catenin pathway in a hepatoma cell line inhibits Wnt responsive gene transcription and decreases the rate of cell division. Treatment with LiCl, which increases β-catenin stability and Wnt signaling by inhibiting glycogen synthase kinase 3 (GSK3), has no effect on β4-subunit-mediated inhibition of Wnt signaling, indicating that the β4-subunit acts downstream GSK3. In contrast, expression β4-subunits mutants unable to translocate to the cell nucleus does not affect Wnt signaling, indicating that nuclear translocation of β4-subunit is necessary to inhibit the Wnt signaling pathway. Co-immunoprecipitation assays show that β4-subunit interacts with the Transcription Factor 4 (TCF4) and overexpression of TCF4 reverses the inhibitory effect of β4-subunit on Wnt signaling. We therefore...
hypothesize that β4-subunit interaction with TCF4 in the cell nucleus interferes with TCF4 binding to β-catenin and inhibits TCF4-mediated gene transcription, suggesting that β4-subunit is a member of the TCF4 repressor family. Regulation of the cell cycle in neuronal precursors is critical for determining the cell number and composition of the cerebral cortex; therefore, these findings suggest that expression and subcellular localization of the β4-subunit regulates neural development and that CACNB4 mutations may affect the physiology of the mature central nervous system by interfering with Wnt signaling in neuronal progenitors.

P2426

Board Number: BS76

Divergence in the temporal dynamics of Extracellular-signal regulated kinase (ERK) activity between subcellular compartments.

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The ERK1/2 pathway plays critical roles in eukaryotic biology by transducing extracellular signals into cell-fate decisions. One conundrum is in understanding how disparate signals induce specific responses through a common, ERK-dependent kinase cascade. One major mechanism to control ERK-signaling to induce proper cellular responses is through precise regulation over the temporal dynamics of ERK activity. For example, in the pheochromocytoma PC-12 cell line, ERK exhibits transient activity in response to Epidermal Growth Factor (EGF), ultimately resulting in proliferation. On the other hand, PC-12 cells respond to Nerve growth factor (NGF) with sustained ERK activity and eventual differentiation into neuronal-like cells. An additional mechanism of control over ERK is through spatial regulation of activity; however, it has been difficult to accurately examine differences in activity among specific subcellular locations. To address this issue, we have expanded the toolbox of FRET-based ERK biosensors by creating a series of improved biosensors that are specifically targeted to various subcellular regions. Using these sensors, we have tested the hypothesis that a single extracellular signal can induce different temporal dynamics between subcellular compartments in PC-12 cells. We report that EGF induces transient ERK activity in the cytosol and nucleus, as expected based on previous reports. However, we have discovered that EGF simultaneously induces sustained ERK activity at the plasma membrane of PC-12 cells. Furthermore, we report that PKA and cAMP play an integral role in the differential regulation of ERK at the plasma membrane and the cytosol. Specifically, we observe that increasing basal cAMP induces a more transient activity at the plasma membrane without changing the temporal nature of ERK activity within the cytosol. Inhibiting PKA slows and minimizes the ERK response in the cytosol without affecting ERK activity at the plasma membrane. These spatially disparate results indicate that subpopulations of ERK are differentially regulated in a spatiotemporal manner and that crosstalk with other signaling pathways play an integral role in the regulation of these subpopulations, furthering our fundamental understanding of how this central kinase is regulated in cells.
P2427
Board Number: B577
Transient activation of fission yeast AMPK is required for cell proliferation during osmotic stress.
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The heterotrimeric kinase AMPK acts as an energy sensor to coordinate cell metabolism with environmental status in species from yeast through humans. Low intracellular ATP leads to AMPK activation through phosphorylation of the activation loop within the catalytic subunit. Other environmental stresses also activate AMPK, but it is unclear whether cellular energy status affects AMPK activation under these conditions. Fission yeast AMPK catalytic subunit Ssp2 is phosphorylated at Thr-189 by the upstream kinase Ssp1 in low-glucose conditions, similar to other systems. Here we find that hyperosmotic stress induces strong phosphorylation of Ssp2-T189 by Ssp1. Ssp2-pT189 during osmotic stress is transient and leads to transient regulation of AMPK targets, unlike sustained activation by low glucose. Cells lacking this activation mechanism fail to proliferate after hyperosmotic stress. Activation during osmotic stress requires energy sensing by AMPK heterotrimer, and osmotic stress leads to decreased intracellular ATP levels. We observed mitochondrial fission during osmotic stress, but blocking fission did not affect AMPK activation. Stress-activated kinases Sty1 and Pmk1 did not promote AMPK activation but contributed to subsequent inactivation. Our results show that osmotic stress induces transient energy stress, and AMPK activation allows cells to manage this energy stress for proliferation in new osmotic states.

P2428
Board Number: B578
Signal transduction of human Fc gamma RIIIb for neutrophil extracellular trap (NET) formation.
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Neutrophils are considered the first line of defense during inflammation and infections. Neutrophils migrate from the circulation to sites of infection where they are responsible for antimicrobial functions, including phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs). Many microorganisms, including bacteria, fungi, and parasites, induce NET formation. Some pharmacological stimuli, such as phorbol 12-myristate 13-acetate (PMA), can also induce NETs. Antigen–antibody complexes are also capable of inducing NET formation. Only, FcgammaR and FcgammaRIIIb have been reported as receptors for NET formation, similarly to PMA stimulation. Direct cross-linking of FcgammaRIIA or integrins did not promote NET formation. FcgammaRIIIb-induced NET formation presented different kinetics from PMA-induced NET formation, suggesting differences in signaling. We explored the signaling pathway activated by FcgammaRIIIb leading to NET formation. FcgammaRIIIb was stimulated by cross-linking it with specific monoclonal antibodies, and NET formation was evaluated in the presence or absence of pharmacological inhibitors. The antibiotic LL Z1640-2, a selective inhibitor of TAK1 prevented FcgammaRIIIb-induced, but not PMA-induced NET formation. Both PMA and FcgammaRIIIb cross-linking induced phosphorylation of ERK. But, LL Z1640-2 only inhibited the FcgammaRIIIb-mediated activation of ERK. Also, only FcgammaRIIIb, similarly to transforming growth factor-beta, induced TAK1 phosphorylation. A MEK (ERK kinase)-specific inhibitor was able to prevent ERK phosphorylation induced by both PMA and FcgammaRIIIb. These data show that FcgammaRIIIb
cross-linking activates TAK1, and that this kinase is required for triggering the MEK/ERK signaling pathway to NETosis.

P2429
Board Number: B579
Testing a model of CK1 autoinhibition.
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CK1 enzymes signal in a variety of important cellular pathways, including DNA damage repair, mitotic checkpoint signaling, circadian rhythm, Wnt signaling, and neurodegenerative disease progression. Like other multifunctional kinases, CK1 must be regulated in space and time in order to target specific subsets of its substrates in each of the pathways it participates in. However, CK1 is generally regarded as a “rogue” kinase, which is constitutively active, ubiquitous throughout cells and tissues, and unregulated except by autoinhibition.

The current model of autoinhibition holds that CK1 autophosphorylates its flexible carboxy-terminal tail in cis, which causes the C-terminus to interact intermolecularly with the catalytic domain and inhibit kinase activity by acting as a pseudosubstrate. It has been shown that autophosphorylation is a conserved property of CK1 enzymes, that it primarily occurs on the C-terminal tail, and that it results in decreased kinase activity, but we are unaware of evidence demonstrating that the phosphorylated tail actually binds the kinase domain. Therefore, our laboratory is evaluating this proposed mechanism of autoinhibition by identifying autophosphorylation sites on CK1 and biochemically testing the interaction between the C-terminus and the kinase domain. Because CK1 is nonessential in Schizosaccharomyces pombe, we can then investigate the significance of autophosphorylation in vivo.

Using a combination of phosphopeptide mapping and mass spectrometry, we have identified six autophosphorylation sites on the C-terminus of S. pombe Hhp1 and are testing candidate sites on Hhp2 as well as the human homologues CK1d/e. We have produced the C-terminal fragments of Hhp1, Hhp2, CK1d, and CK1e recombinantly and are comparing their abilities to bind the kinase domains when phosphorylated or dephosphorylated. Hhp1 mutants with single autophosphorylation sites mutated to alanine or glutamate have a wildtype phenotype in vivo; now, we are concurrently mutating all of the sites in the C-terminus to determine the functional consequences of autophosphorylation. With this work, we hope to elucidate conserved mechanisms of regulation for the CK1 family of enzymes, which are apical mediators of cell signaling.

P2430
Board Number: B580
ASK1 Activation in Platelets by Oxidized LDL is Independent of CD36.
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Oxidized low-density lipoprotein (oxLDL) is a major contributor to platelet hyper-activation in hyperlipidemic conditions. OxLDL induces a prothrombotic phenotype by sensitizing the platelets for activation. Several receptors have been identified for oxLDL on platelets, but majority of the studies were focused on CD36. OxLDL binding to CD36 results in activation of multiple signaling molecules including Src family kinases (SFKs), Vav 1&3, JNK2, ERK5, and reactive oxygen species (ROS). The mechanism(s) by which oxLDL sensitizes platelets for activation is poorly understood. We have recently

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identified Apoptosis Signal Regulating Kinase 1 (ASK1), a MAP kinase kinase kinase, in platelets. Here, we show that in human platelets ASK1 is activated by oxLDL as determined by its activation-specific phosphorylation on T845. ASK1 activation was evident with oxLDL concentrations as low as 10μg/ml, with maximal activation observed with 100μg/ml. In contrast, native LDL (nLDL, 10-200μg/ml) failed to stimulate ASK1 activation. The activation of ASK1 was rapid peaking at 1 minute and returning to baseline by 5 minutes. To determine if ROS is responsible for the activation of ASK1, we pretreated platelets with MnTMPyP, a ROS scavenger. MnTMPyP failed to inhibit the activation of ASK1 induced by oxLDL. To determine the mechanism by which oxLDL induces ASK1 activation, we pretreated platelets with 10μM each of pharmacological inhibitors, PP2 for SFKs, Y27632 for Rho kinase, bisindolylmaleimide for PKC, LY294002 for PI3 Kinase, U73122 for PLC, and 25μM of BAPTA-AM to chelate calcium. We found that platelets pretreated with U73122 and BAPTA-AM abolished oxLDL induced ASK1 activation, whereas others had no effect suggesting that ASK1 is activated in a calcium-dependent manner. To investigate the role of CD36 in oxLDL induced ASK1 activation, we used FA6-152, a function-blocking antibody for human CD36, and murine CD36 null platelets. Interestingly, either blocking or lack of CD36 had no effect on oxLDL-induced ASK1/p38 activation suggesting that CD36 is not involved in oxLDL induced ASK1 activation. To determine if ASK1 plays a role in oxLDL-induced sensitization of platelets for activation, we treated platelets with GS-4997, a specific ASK1 inhibitor, before stimulating them with ADP and oxLDL. ADP (10μM) or oxLDL (100μg/ml), showed no aggregation, but combination of oxLDL and ADP showed robust aggregation of vehicle-treated platelets. However, pretreatment of platelets with GS-4997 significantly inhibited oxLDL-induced potentiation of ADP-induced platelet aggregation suggesting that ASK1 plays a role in platelet activation under hyperlipidemic conditions.

P2431
Board Number: B581
ARL11/ARLTS1 is a novel regulator of ERK signaling in macrophages.
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Although there are more than 20 members of ADP-ribosylation factor (Arf)-like (Arf) family of small GTPases in mammals, function of most Arls remain unknown. Arl11 is a cancer-predisposing gene that has remained functionally uncharacterized to date, except its ectopic expression in cancer cell lines is known to induce apoptosis and inhibit tumor growth. In this study, we report that mammalian Arl11 is expressed in macrophages and its expression is increased upon LPS stimulation. Notably, depletion of Arl11 in both primary macrophages and macrophage cell lines impaired secretion of pro-inflammatory cytokines and phagocytic function of macrophages (including killing of intracellular bacteria). Analysis of LPS-mediated signaling events revealed that ERK1/2 phosphorylation was substantially compromised in Arl11-silenced macrophages. In contrast, increased expression of Arl11 in macrophages led to constitutive ERK1/2 phosphorylation, resulting in macrophage exhaustion and a failure to respond to subsequent LPS stimulation. These findings suggest that Arl11 is both essential and sufficient for MAPK signaling, which in turn regulates pro-inflammatory functions of macrophages. Finally, we found that Arl11 colocalizes and interacts specifically with the phosphorylated form of ERK1/2 associated with cortical actin structures. Taken together, our findings establish Arl11 as one of the target genes upregulated upon pro-inflammatory signaling in macrophages that in turn promotes ERK signaling required for macrophage activation and their immune function.
P2432  
**Board Number: B582**  
Cdk2 dependent activation of β-cell metabolism is eclipsed by its inhibitory effect on plasma membrane excitability and insulin secretion.  
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The loss of insulin secretion is a hallmark of diabetes, and it is becoming clear that cell cycle regulators control insulin secretion as well as proliferation. We recently reported that embryonic deletion of cyclin-dependent kinase 2 (Cdk2) in β-cells (Pdx1-Cre:Cdk2−/−) results in impaired glucose tolerance due to defective insulin secretion (Kim et al., J Biol Chem, 2017), revealing a developmental or perinatal requirement of Cdk2. Here, we show that tamoxifen-inducible deletion of Cdk2 in adult β-cells (MIP-CreER:Cdk2−/−) results in improved glucose tolerance and enhanced insulin secretion. Despite these differences, both models of Cdk2 depletion exhibited a strong increase in the glucose dependence of β-cell calcium oscillations. This effect is likely due to the loss of ATP-sensitive K+ (KATP) channels, since kir6.2 transcript was reduced in Cdk2−/−null β-cells, and pharmacological inhibition of Cdk2 correspondingly reduced KATP channel conductance. These results reveal that Cdk2 inhibits insulin secretion by limiting plasma membrane excitability via KATP. In adult β-cells lacking Cdk2, we also observed a 45% reduction in depolarization-induced exocytosis, indicating that Cdk2 is required for the metabolic amplification of insulin secretion. Within the intermediary metabolic pathways, Cdk2-null β-cells exhibited reductions in glucose-dependent lactate accumulation, mitochondrial membrane potential, and mitochondrial NADH utilization. Together, these studies reveal that Cdk2 transcriptionally limits β-cell membrane excitability and the insulin secretory response, and is required to maintain proper metabolic function.

P2433  
**Board Number: B583**  
PTP activity for IRS-1 regulated by the interaction of C1-Ten with PIP3 via SH2 domain.  
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The termination of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) is essential for the regulation of insulin signaling. C1-Ten has previously been implicated in the regulation of IRS-1 as a relevant protein-tyrosine phosphatase (PTP). However, the molecular basis for its regulatory mechanism is not fully understood. Here, we report the characterization of lipid binding property of C1-Ten SH2 domain as a novel character and the role of its SH2 domain in the PTP activity of C1-Ten. C1-Ten SH2 domain has strong preference and high affinity for PtdIns(3,4,5)P3, this allows C1-Ten to function as PTP in cell. Lipid-binding deficient mutant of C1-Ten completely abrogates PTP activity that even though it has activity in vitro. Thus, PTP activity for IRS-1 is regulated by the interaction of C1-Ten with PtdIns(3,4,5)P3 through SH2 domain. These findings have important implications for negative feedback regulation of IRS-1 tyrosine phosphorylation through C1-Ten mediated by PtdIns(3,4,5)P3.
P2434
Board Number: B584
Role of ERK Pathway Inhibition and Retinoic Acid on the Neuronal Differentiation of Mouse ES Cells. Author: Sri Kona, PI: Dr. Eduardo Martinez Ceballos, Southern University and A and M College, Btr LA.
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Embryonic stem cells derived from the blastocyst of mouse are pluripotent in nature. These cells can give rise to one of the three germ layers i.e. Ectoderm, Mesoderm or Endoderm. Retinoic acid (RA), a morphogen can induce Hoxa1 through the activation of retinoic acid receptors (RARs). Binding of RA to the RARy receptor may lead to ectodermal (neuronal) differentiation, while binding to RARβ may induce endodermal differentiation and suppression of ectodermal signaling. We have found that the ERK cell signaling pathway plays a major role in ES endodermal differentiation. Inhibition of ERK using the pharmacological inhibitor PD98059 (PD) promotes neurogenesis, which confirms the role of this cell signaling pathway in germ layer specification. In the present study, we have investigated the effect of cell signaling pathways on neuronal vs endodermal differentiation. For this purpose, we treated mouse ES cells with 1μM RA, 10μM PD, 40μM PD and the combination of RA and PD for about 48hrs followed by culturing them in neurobasal medium supplemented with B27. The inhibitor PD and RA increased the generation of neuronal precursors. This was assessed by analyzing the gene expression profiles of various ectodermal, endodermal and mesodermal genes were tested using RT PCR. Upon treatment with 1μM PD, real time PCR analysis showed a drastic increase in expression levels of Nestin and beta tubulin III, which are ectodermal markers. The ectodermal gene expression was also tested at protein level using immunofluorescence. Neurons were generated in the cells treated with PD and RA plus PD. Hence PD treatment could possibly lead to generation of more neurons in 2D cultures. This study might help in tissue engineering and neurodegeneration treatment in long run and also may lead to cost effective regenerative medicine to treat brain diseases.

P2435
Board Number: B585
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It has been generally recognized that acidic extracellular pH (pHe) is an important feature of solid tumors that induces tumor invasion and metastasis. We investigate whether acidic pHe induce epithelial mesenchymal transition (EMT) using breast cancer cell line MCF-7. MCF-7 cells normally showed tightly-packed clusters, characteristic of epithelial cells. The cells cultured in acidic medium (pH 6.6) for 2 days showed loss of cell-cell adhesion and delocalization of E-cadherin and β-catenin from cell-cell contact. These changes were also observed in other breast cancer cell line, T-47D. The absolute amounts of E-cadherin and β-catenin were not changed after EMT in total lysates. N-cadherin, vimentin or transcriptional factor that regulate EMT were not upregulated in 2 days, indicating that the change is not complete EMT. Phosphorylation of p38MAP kinase at T180/Y182 was elevated up to approximately three folds after culturing with acidic medium for 3 hours, while Either ERK1/2 or SAPK/JNK did not change in acidic condition. Phosphorylation of SRC at Y416 and FAK at Y925 of SRC substrate site was enhanced after acidic treatments for 30 minutes and 3 hours, respectively. Furthermore,
phosphorylation of cortactin at Y421 and Y466, known as tyrosine residue of SRC substrate, was also enhanced, and the phosphorylated cortactin was co-localized with vinculin at the focal adhesions. SRC kinase inhibitor (PP2) inhibited EMT-like change induced by acidosis, whereas p38MAP kinase inhibitor (SB203580) did not. In conclusion, lowered pHe could induce EMT-like change showing loss of intercellular adhesion. SRC/FAK activation in acidosis might evoke phosphorylation of cortactin and the dislocation from intercellular junctions to the focal adhesions, suggesting promotion of cell motility.

P2436
Board Number: B586
Mechanisms of cross pathway regulation in Saccharomyces cerevisiae.
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Crosstalk between signaling pathways is a complex process for which the mechanism is poorly understood. For example, in the model organism Saccharomyces cerevisiae, crosstalk occurs between the high osmolarity glycerol (HOG) and pheromone mating pathways. Although these pathways share several protein components, S. cerevisiae maintains the capacity to initiate distinct responses to the appropriate environmental stimulus. These responses include activation of the terminal MAPKs in the pheromone pathway, Fus3 and Kss1, which are homologous to ERK1/2. The MAPK in the osmotic stress pathway, Hog1, is homologous to p38. Here we describe two mechanisms of cross-pathway inhibition, one by branched chain amino acid second messengers and the second by accumulation of a monophosphorylated and inhibitory form of the MAPK. By conducting a metabolomic screen and genetic studies we found three branched chain amino acids derivatives (HIV, HIC, HMVA) that accumulate in response to salt stimulation. These derivatives dampen the pheromone response in part by phosphorylation of the G protein Gpa1, mediated by the AMPK kinase Elm1. Under the same conditions, we observe accumulation of a monophosphorylated, inhibitory Fus3 species which dampens and delays the mating response. Finally, an important and unexpected consequence of crosstalk is increased cell-to-cell variability. We proposed that output noise in the pheromone response represents a bet hedging strategy that confers a selective advantage to the population in response to stress. We are determining the mechanism by which distinct responders arise by combining transcriptional fluorescent reporters, genetic approaches and mathematical models. Altogether, we are able to propose various mechanisms for cross pathway regulation that offer the potential for more targeted therapies.

P2437
Board Number: B587
The role of MAPK and SCF in the destruction of Med13 in cyclin C mediated cell death.
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In response to stress, the yeast and mammalian cyclin C translocate from the nucleus to the cytoplasm, where it associates with the GTPase Drp1/Dnm1 to drive mitochondrial fragmentation and apoptosis. Therefore, the decision to release cyclin C represents a key life or death decision. In unstressed cells, the cyclin C-Cdk8 kinase regulates transcription by associating with the Mediator of RNA polymerase II. We previously reported that the Mediator component Med13 anchors cyclin C in the nucleus. Loss of Med13 function leads to constitutive cytoplasmic localization of cyclin C, resulting in fragmented mitochondria, hypersensitivity to stress and mitochondrial dysfunction due to
loss of mtDNA. Recently we showed that this molecular switch operates in a two step process. First, efficient cyclin C nuclear release requires its ROS-induced phosphorylation by the MAP kinase Slt2 in a carboxyl terminal region of cyclin C, which includes a putative Med13 interaction site. The second step involves ROS-induced Med13 destruction by the SCFGrp1 ubiquitin ligase. Med13 associates with Grp1 in two-hybrid assays, and SCF mediated degradation of Med13 requires active cyclin C-Cdk8. However, phosphorylation of Med13 by cyclin C-Cdk8 does not trigger Med13 destruction. This suggests a model in which this kinase primes the Med13 degron for SCFGrp1 and either Slt2, or an as yet unidentified kinase, triggers its destruction. Taken together, these results are consistent with a model in which cyclin C phosphorylation by Slt2 permits its disassociation from Med13, and that Med13 destruction allows full cyclin C release and prevents re-accumulation of the cyclin in the nucleus.


Grant support: W.W. Smith Charitable Trust (CO 604) and NIH R15-113196 to K.F.C and NIH (CA099003 and GM086788) to R.S.

P2438
Board Number: B588
Snf1 dependent destruction of Med13 is required for programmed cell death following oxidative stress in yeast.
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All eukaryotic cells, when faced with unfavorable environmental conditions have to decide whether to mount a survival or cell death response. The conserved cyclin C and its kinase partner Cdk8 play a key role in this decision. Both are members of the Cdk8 kinase module that, along with Med12 and Med13, associate with the core mediator complex of RNA polymerase II. In S. cerevisiae, oxidative stress triggers Med13 destruction, which thereafter releases cyclin C into the cytoplasm. Cytoplasmic cyclin C associates with mitochondria where it induces hyper-fragmentation and programmed cell death. This suggests a model in which oxidative stress mediated destruction of Med13 represents a key molecular switch which commits the cell to programmed cell death. Thus it is important to decipher the precise molecular mechanisms that control Med13 destruction following exposure to oxidative stress. Previous studies have revealed that both cyclin C/Cdk8 and Slt2, the MAPK of the cell wall integrity pathway, are required for the SCFGrp1 mediated destruction of Med13 following oxidative stress. Here we show that the conserved AMP kinase Snf1 is also required for this event. Deletion of Snf1 results in stable Med13 protein levels and predominantly reticular mitochondria morphology following H2O2 treatment. Consistent with this, deletion of Sak1, a Snf1 activating kinase or using the inactive snf1K84R mutant as the only source of Snf1 results in the same phenotypes. Deletion analysis has revealed that the Snf1 degron lies adjacent to the Slt2 degron and contains a potential Snf1 phosphorylation site. Taken together, these results suggest that ubiquitin mediated destruction of Med13 is very tightly controlled, requiring the action of 3 different kinases. As Med13 destruction results in cyclin C nuclear translocation these results support a model in which Med13 degradation plays a key role in controlling a molecular switch that dictates cell fate following exposure to adverse environments.


Grant support: W.W. Smith Charitable Trust (CO 604) and NIH R15-113196 to K.F.C
P2439

Board Number: B589

Cdk1, by Stimulating Mitochondrial Respiration, Restricts the Metabolic Amplifying Pathways of Insulin Secretion.

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Type 2 diabetes is linked genetically to cyclin-dependent kinase (Cdk) signaling, however the significance of Cdk1/Cyclin B1 signaling in adult ß-cells remains largely unknown. Here, we show that one of the key mitotic functions of Cdk1/Cyclin B1 – the activation of mitochondrial respiratory Complex I – is active in adult ß-cells and hyperactive in ß-cells from obese (ob/ob) mice. In wild-type islets, an analysis of TCA cycle and electron transport chain (ETC) fluxes with NAD(P)H FLIM and respirometry revealed that 65 ± 13% (P = 0.0106) of Complex I flux and 49 ± 12% (P < 0.05) of State 3 respiration is sensitive to Cdk1 inhibition. Islets from ob/ob mice express more Cyclin B1 and exhibit an even higher sensitivity to Cdk1 blockade, which reduced Complex I flux by 76 ± 11% (P < 0.001) and State 3 respiration by 79 ± 6% (P < 0.001). While Cdk1 signaling and ETC flux were correlated with the activity of glucose-stimulated Ca²⁺ oscillations, insulin secretion increased following Cdk1 inhibition as carbon shifted to the ATP-independent metabolic amplifying pathways. These results reveal that ETC activity is inversely proportional to insulin secretion. Our results further demonstrate that Cdk1 and Complex I are control points for ß-cell metabolism which, in the setting of obesity, rebalance the insulin secretory pathway.

P2440

Board Number: B590

Involvement of specific Akt isoforms in the decidualization mechanisms of the mouse uterus.

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Canadian couples find it increasingly difficult to conceive; unsurprisingly, infertility is a rising problem that currently touches 16% of Canadians trying to conceive. Similar statistics are observed in the USA. Many factors can contribute to this condition, which is thought to mainly arise from dysregulation in the maternal-embryonal communication mechanisms. Successful implantation necessitates an extraordinarily well-coordinated interaction between both the embryonic and maternal cells. In order to achieve this, the endometrium is required to be fully prepared and receptive for implantation; this "window of implantation" is dependent on the process of decidualization. Under the effect of progesterone, estrogen and cyclic AMP, the endometrial stromal cells undergo fundamental phenotypical changes, transitioning from fibroblasts to epithelial, secretory, glycogen-filled cells. Little is known on the signaling pathways involved in the intricate regulation of the decidualization process; the role of multiple crucial pathways, involved in proliferation and apoptosis, remain largely obscure. It has been demonstrated previously that Akt is involved in cell survival and in glycogen synthesis. Three isoforms of Akt have been identified and it is well recognized that they have distinct physiological and pathological roles. We hypothesize that this is also the case during decidualization and the pregnancy onset. The aim of this study is to investigate the regulation of PI3K/Akt pathway during the decidualization process of endometrial stromal cells. Previous in vitro results obtained in our lab using Human

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Immortalized Endometrial Stromal Cells suggest that the decrease of specific Akt isoforms and the downregulation of the Akt pathway is necessary for the decidualization process to successfully occur. Therefore, we developed a novel endometrial-targeted mouse model with simple and combine KO of each Akt isoforms. By artificially inducing mouse uterine decidualization, the specific cellular localization, the expression and the activation of each Akt isoforms was investigated during this process in order to evaluate the regulation of PI3-K/Akt pathway. Our results suggest that there is a specific regulation of each Akt isoforms during decidualization that could reveal distinct role during this biological process. Moreover, differences in uterus morphological aspects and average mouse litter number as been observed depending of the KO genotype. Thus, it is clear that the PI3-K/Akt pathway has an important role in fertility. Further studies will allow us to understand the precise signaling mechanisms by which this pathway is regulated and will lead to a better understanding of the cellular and molecular aspects of infertility.

**Signaling Receptors (RTKs and GPCRs)**

**P2441**

**Board Number: BS91**

*Analysis of receptor tyrosine kinase and G-protein coupled receptor signaling dynamics on micro-structured surfaces.*

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Membrane-localized proteins are essential to transmit signals into the cell. An important issue is the interaction of these proteins with cytosolic proteins. To quantify such often short-lived interactions we have introduced a method based on the combination of micro-structured surfaces and TIRF microscopy.(1,2) We have used the assay to validate the efficacy of medically relevant receptor tyrosine kinase (RTK) modulators.(3) Bait epidermal growth factor (EGF) receptor molecules were forced into microscopic domains, while monitoring co-recruitment of fluorescent intracellular prey Grb2 molecules. Pretreatment with pharmacologically active ingredients used for the treatment of human cancers significantly reduced this interaction. A similar approach was used for the quantitative analysis of the interaction between different insulin receptor substrate (IRS) proteins and the insulin/IGF-I receptor.(4) The micro-patterning technique enabled the measurement of equilibrium associations and interaction dynamics of these molecules with high specificity. We revealed that several domains of IRS critically determine the turnover rate of the receptors. Furthermore, we found significant differences among IRS proteins in the strength and kinetic stability of the interaction with the receptors, which could account for the diverse IRS functions. Finally, we studied the interaction of the GPCR ß2-adrenoceptor (ß2AR) with arrestin-3.(5) By measuring arrestin-3 recruitment and the stability of arrestin-3-receptor complexes in real time using FRAP analysis on micro-patterned surfaces, we could demonstrate that arrestin-3 dissociates quickly and almost completely from the ß2AR. Recently, we have implemented micro-structured and functionalized multi-well plates. This development step sets a milestone in terms of throughput rates of our methodology.

References:
G-protein coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors in eukaryotes, and detects a wide array of physiological cues in the human body. We describe a new molecular device that couples CRISPR-Cas9 programmed genome regulation to natural and synthetic extracellular signals via GPCRs. The design of our synthetic device, named CRISPR ChaCha, displays superior performance over an architecture proposed by the previously reported Tango system. Using a parsimonious mathematical model and gene-reporter assays, we find that CRISPR ChaCha can recruit and activate multiple Cas9 molecules for each GPCR molecule. We also characterize key molecular features that modulate CRISPR ChaCha performance. We adopt the design to diverse GPCRs that sense synthetic and natural ligands including chemokines, mitogens, and fatty acids, and observe efficient conversion of signals to customizable genetic programs in mammalian cells, including regulation of endogenous genes. The new class of CRISPR-coupled GPCRs provides a robust and efficient platform for engineering cells with novel behaviors in response to the diverse GPCR ligand repertoire.

Developing platforms to interrogate membrane protein oligomerization and its functional impact.
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Despite the dramatic increase in high resolution integral membrane protein structures and growing evidence with respect to their oligomerization properties over the last decade, the native oligomeric state of GPCRs, ion channel and transporters is uncertain. Moreover the physiological role of oligomerization remains largely elusive. Given the potential impact on pharmacological modulation, resolving this ambiguity provides a potential path to the design of more ‘relevant’ screening assays to identify novel chemical matter. Utilizing GLP-1R and TRPC6 as case studies, here we describe experiments aiming to probe oligomeric state in recombinant and physiologically relevant cells using either genetic or pharmacologic tools. Covalent modification of oligomeric test-pairs with donor or acceptor fluorescent proteins allows an assessment of proximity-based resonance energy transfer. Notably, the approach indicates that when expressed in recombinant cells, GLP-1R as well as TRPC6 forms homo-oligomers. In addition, TRPC6 forms hetero-oligomers with TRPC3, but not TRPC5. Utilizing a complementary approach, fluorescent ligand based TR-FRET, we’ve been working to develop a system enabling the translation of recombinant cell observations to physiologically relevant cells (PRC’s). Preliminary observations provide orthogonal support for the GLP-1R homo-oligomers observed with
fluorescent proteins in recombinant cells, and serve as a starting point to evaluate oligomeric state in PRC’s. By utilizing these approaches in conjunction to CRISPR we aim to develop an ‘oligomerization toolbox’ to provide additional texture into membrane protein biology and modulation strategies.

P2444
Board Number: B594
Manipulating and quantifying cAMP *in vivo* in *Caenorhabditis elegans.*

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The cyclic adenosine monophosphate (cAMP)/Protein Kinase A (PKA) pathway promotes arousal in nematodes, fruit flies and mammals. *Caenorhabditis elegans* displays unique forms of sleep: developmentally timed sleep (DTS) and stress-induced sleep (SIS). The cAMP/PKA pathway is downregulated during both DTS and SIS, however where in the nervous system this is occurring is unknown. *C. elegans* provides a unique avenue for studying the ubiquitous cAMP/PKA pathway with single cell resolution in a living animal. To do this, we have introduced a red light activated adenyl cyclase (AC) called ilac22 and a Förster resonance energy transfer (FRET)-based cAMP biosensor called epac1-camps, into the *C. elegans* system. We have found that activation of IlaC in the DVA interneuron is sufficient to disrupt DTS, SIS and non-physiological quiescence induced by over-expression of neuropeptides. Next, we expressed epac1-camps in the DVA neuron. We predict that during sleep, cAMP will be reduced in the DVA relative to other neurons, and we are currently testing this hypothesis. PKA phosphorylation of the metabolic regulator adenosine monophosphate-activated protein kinase (AMPK) inhibits its activity. To determine if AMPK is functioning downstream of cAMP/PKA we are quantifying AMPK-phosphorylation during SIS.

P2445
Board Number: B595
Localization of MCHR1 to a transient primary cilium in differentiating pre-adipocytes alters MCH signaling.

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The cellular context within which G protein-coupled receptors reside ultimately dictates their overall function. This is particularly important to consider when studying signaling pathways during tissue development. Our laboratory explores pathways that dictate differentiation of adipocytes as well as those that are significantly impacted by this transformation from pre- to post-adipocyte. Of particular interest is the melanin-concentrating hormone (MCH) signaling pathway, which plays a critical role in the physiology of feeding, sleep-wake cycles, mood, metabolism and energy expenditure in higher mammals through its binding to neuronal and/or peripherally localized G protein-coupled receptors, MCHR1 and MCHR2. MCHR1 is localized to the plasma membrane of pre- and post-adipocytes and physiological responses to MCH seem to vary depending on dose, but in an unusual way such that responses are more pronounced with less hormone; this seems to be the case for influence on lipid droplet formation, cell size and cell senescence. We’ve also discovered that MCHR1 translocates to a transient primary cilium on the surface of differentiating 3T3-L1 pre-adipocytes and we hypothesize that this translocation significantly impacts MCH signaling. Furthermore, we hypothesize that the activity of MCHR1 influences 3T3-L1 cell differentiation. We performed RNA-Seq analysis to compare transcriptomes of preadipocytes under four conditions: +/- MCH and +/- primary cilia. We were able to
confirm that MCH significantly alters gene expression profiles and that ciliated cells have an altered transcriptional response to MCH when compared to non-ciliated cells. Quantitative PCR was subsequently used to verify changes in individual transcripts. We have identified gene expression changes in those responsible for lipolysis, lipid droplet formation, circadian rhythm, and the inflammatory response. Future directions include verifying protein responses and expanding the study to include fully differentiated adipocytes.

P2446
Board Number: B596
To Investigate the Roles of Lysophosphatidic Acid Type 2 Receptor in Cell Senescence.
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Cellular senescence is the state of cell ceases to replicate and has been considered as tumor-suppressive mechanism. Senescent cells reduced rate of cell proliferation, enhanced accumulation of reactive oxygen species (ROS), cell apoptosis and positive signal of senescence activated-β-galactosidase (SA-β-gal) staining. Lysophosphatidic acid (LPA) is a growth factor-like lipid mediator that regulates multiple physiological functions via activation of six LPA GPCR. Moreover, LPA2 was reported to be a protective factor against radiation caused cell damage. However, the roles of LPA and LPA2 in cell senescence still remain unclear. Therefore, we aim to investigate the physiological function of LPA2 in cellular senescence. HEK-293 cell was treated by selective LPA2 agonist compound GR1977143 (GRI) or shRNA to knockdown Lpa2r transcript. Activation of LPA2 in HEK-293 induced accumulation of reactive oxygen species (ROS) and caused high percentage of SA-β-gal+ cells. In addition, activation of LPA2 enhanced expression level of p53 mRNA and led to higher percentage of apoptotic cells in Annexin V/PI flow-cytometry assay. Taken together, our results demonstrated that activation of LPA2 correlates with the accumulation of ROS, enhanced cell apoptosis, and further promotes cell senescence in HEK-293 cells.

P2447
Board Number: B597
Coagulation factor VIIa-mediated protease-activated receptor 2 activation leads to enhanced topoisomerase1 level and contributes to breast cancer progression.
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Thrombotic complications have significant impact on the mortality and morbidity of cancer patients, therefore understanding of the molecular interrelation between coagulation factors and tumour development mechanism is vital for thromboprophylaxis. Our finding deals with the intriguing relationship between coagulation activation and cancer development. Role of blood clotting proteins TF-FVIIa in tumour progression have been suggested in various studies. Expression level of tissue factor and its enhanced activity after binding with FVIIa has an augmentative effect on tumour metastasis and cancer associated thrombosis. This binary complex leads to activation of GPCR transmembrane protein protease activated receptor 2 (PAR2), which leads to activation of various cancer signalling pathways. Never before, it has been suggested that coagulatory proteins directly affect the DNA topology. Our finding first time suggests that TF-FVIIa interaction leads to enhanced replicatory and transcriptional progression in cancer cells by relaxing the negatively supercoiled DNA of cancer cells. In-vitro experiments in breast cancer cells showed enhanced transcript and protein level of topoisomerase 1 (TOP1) due to TF-FVIIa interaction by activating the NFκB pathway. Our findings suggest that enhanced
level of cytokines critical for tumour progression are regulated by TF-FVIIa interaction via TOP1 enhanced activity. Coagulation factor VIIa treatment enhances the sensitivity of cancer cells towards camptothecin (TOP1 inhibitor) by several folds. Our observations clearly suggest that the combined effect of FVIIa and camptothecin critically leads to accumulation of potentially lethal DNA lesions in cancer cells leading to its enhanced apoptosis. Our results provide a novel platform to modulate the targeted chemo-therapy against breast cancer cells.

**P2448**

**Board Number: B598**

**Molecular Signatures of Opioid Receptors in Response to Ethanol.**

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Alcohol (i.e., ethanol) is one of the most widely used and abused psychoactive substances, with a substantial negative effect on health and public safety. Despite considerable scientific and industrial efforts, there are few drugs available for an effective treatment of alcohol dependence. This is partially explained by the fact that ethanol, unlike most psychoactive drugs, does not act on a specific receptor system. Instead, ethanol modulates, directly or indirectly, a variety of important central nervous system functions, including neurotransmission. Interestingly, naltrexone (NTX) – a general pharmacological antagonist of opioid receptor (OR) function – has been reported to reduce relapse in heroin and alcohol abuse. While mechanisms of NTX action on opiate (heroin) abuse are well understood, little is known about the mechanisms underlying its effect on non-opiates (ethanol). To elucidate molecular mechanisms of ethanol action, we are utilizing quantitative super-resolution microscopy techniques. In particular, we are employing pair-correlation analysis of photoactivated localization microscopy data (PC-PALM). Our results indicate that within cells, pharmacologically relevant ethanol concentrations alter the nano-domain distributions of mu- and kappa-opioid receptors (MOR, KOR), actin, and, the lipid raft marker glycosylphosphatidylinositol (GPI). Specifically, ethanol 1) affects short- and long-range organization of ORs in a concentration dependent manner, 2) induces actin polymerization, and 3) increases GPI. We have found that pretreatment of cells with NTX protects against ethanol-induced reorganization of MOR and the lipid raft marker GPI. Unexpectedly, treatment with NTX significantly affects KOR organization, inducing formation of larger and more occupied nano-domains.

**P2449**

**Board Number: B599**

**Activation of Airway T2R Bitter Taste Receptors by Pseudomonas aeruginosa Quinolones.**

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In recent years, taste receptors have become a potential therapeutic target for many diseases because functional bitter taste receptors have been discovered in a vast array of tissues outside of the oral cavity. We previously showed that cells in the airway express a variety of bitter taste receptors (T2Rs). These T2Rs are responsible for innate immune responses in the airway, which are therapeutically
important for ailments such as Chronic Rhinosinusitus and infections. Therefore, it is imperative to understand T2Rs full range of functions in the airway. T2Rs are G protein coupled receptors (GPCRs) that couple to G beta-gamma (Gβγ) (which in turn facilitates IP3 production and calcium release) and Ga-gustducin (a Gai that decreases cAMP). We previously reported that T2Rs found on airway cilia increase ciliary beating and nitric oxide (NO) production when activated by bitter taste receptor agonists. The increased ciliary beating of these cells promotes the movement of mucus and the secreted NO is bactericidal. These actions provide an innate immune response that helps clear the airway of bacteria. One category of bacterial secretions called acyl-homoserine lactone quorum sensing molecules activates T2Rs and elicits an immune response from primary airway cells. However, another type of bacterial quorum sensing molecule, quinolones, had yet to be explored. Non-bacterial quinolones such as quinine and chloroquine are bitter and activate T2Rs. To elucidate whether or not bacterial quinolones are T2R agonists and if they elicit an immune response, calcium and cAMP responses from transfected HEK293T cells and cultured and primary airway cells were analyzed. Various T2Rs were expressed heterologously in HEK293T cells and exposed to bacterial quinolones, 2-heptyl-3-hydroxy-4-quinolone (PQS), 2,4-dihydroxyquinoline (DHQ), and 4-hydroxy-2-heptylquinoline (HHQ). In the transfected HEK293T cells, PQS activated T2R4, 16, 38, and 39 and HHQ activated T214, while DHQ appeared to have no effect. PQS also activated calcium responses in cultured and primary airway cells, and decreased cAMP concentrations in cultured airway cells. In primary airway cells, PQS activated bactericidal NO synthesis and release. Ultimately, we found that T2Rs recognize some bacterial quinolones to activate an innate immune response to these bacterial secreted products.

**P2450**
**Board Number: B600**
LAT-associated molecules show distinct kinetic and spatial recruitment during TCR signaling in T cells.
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Engagement of the T cell antigen receptor (TCR) by a peptide-bound major histocompatibility complex molecule (pMHC) results in the rapid formation of microclusters at the site of activation in T cells. Microclusters contain numerous molecules involved in the TCR signaling cascade and are the basic signaling units required for immunological synapse formation and T cell activation. Although TCR microclusters have been studied extensively using confocal microscopy, the stoichiometry and spatial relationships of their signaling components, the kinetics of their recruitment to the microcluster, and the fate of these structures have not been well characterized due to limits in image resolution and acquisition speed. Using TIRF-SIM to examine the kinetics of microcluster formation in Jurkat T cells, and by altering activation conditions, we observed surprising delays between the sequential recruitment of TCR, ZAP70, and LAT-associated signaling proteins. These kinetic delays were affected by activation of co-stimulatory and inhibitory pathways. In addition, TIR-SIM images showed spatially distinct patterns of localization for TCR and LAT-associated signaling molecules. These results reveal novel insights into the spatial and kinetic regulation of TCR microcluster formation and T cell activation.
P2451
Board Number: B601
Basigin (CD147) associates with toll-like receptor 4 (TLR4) via its transmembrane domain.
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It is known that inflammatory signaling pathways are used by metastatic cancer cells for proliferation and the ability to alter cell interactions. Basigin variant-2, or CD147, is a ubiquitously-expressed member of the immunoglobulin superfamily that is found on tumors and serves as a cell surface marker for the identification of some cancer cells. In addition to being implicated in the Wnt/beta-catenin pathway, CD147 activation also leads to signal transduction pathways using MyD88 and TRIF, despite the lack of consensus amino acid binding sequences for those signaling molecules. Toll-like receptors (TLRs) are a major group of pattern recognition receptors that recognize molecular patterns associated with all classes of pathogenic microorganisms. TLR4 recognizes the lipopolysaccharide component of Gram-negative bacterial cell walls and is the only TLR known to induce signaling through both the MyD88 and TRIF pathways. The purpose of the present study was to determine if CD147 uses TLR4 for signal transduction. It was hypothesized that CD147 interacts with TLR4 using its transmembrane domain, and that the glutamate residue positioned at the center of the otherwise hydrophobic domain plays a role in the interaction. Enzyme-linked immunosorbent binding assays were performed using endogenous TLR4 and recombinant CD147 proteins. The analyses demonstrated that the transmembrane domain of CD147 does interact with TLR4 using the glutamate residue, as well as hydrophobic amino acids within the transmembrane domain. The data suggest that CD147 interacts with TLR4 to influence signaling cascades using MyD88 and TRIF and may be involved in inflammatory signaling cascades used in metastatic cancer cells.

P2452
Board Number: B602
Optogenetic control of TrkA signaling.
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Nerve growth factor/tropomyosin receptor kinase A (NGF/TrkA) signaling plays a key role in neuronal development, function, survival, and growth. TrkA is also implicated in diverse neurodegenerative diseases including Alzheimer’s disease, pain disorders, inflammations and cancers. NGF binds the extracellular domain of TrkA, leading to receptor dimerization and auto-phosphorylation of its intracellular kinase domain. The signaling cascade initiated by NGF/TrkA binding is highly dynamic and complex, and dissection of finer spatial and temporal aspects of TrkA signaling has proven difficult using traditional biochemical methods. We have developed light-inducible strategies that allow for optical control of TrkA activity based on A. thaliana cryptochrome 2 (CRY2) and its binding partner CIB1. We have been able to recapitulate native NGF/TrkA function by optically inducing the plasma membrane recruitment and homo-interaction of the intracellular domain of TrkA. This method successfully initiates PI3K/Akt and Raf/ERK signaling, promotes neurite growth in PC12 cells, and supports the survival of DRG neurons in the absence of NGF. The ability to activate TrkA using light bestows high spatial and temporal resolution for future investigation of dynamic NGF/TrkA signaling.

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P2453
Board Number: B603
The Na,K-ATPase β2-subunit modulates epidermal growth factor signaling through NF2/Merlin.
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The Na,K-ATPase is a ubiquitously expressed integral membrane protein that carries out the extrusion and uptake of Na\(^+\) and K\(^+\) ions across the plasma membrane of most animal cells. The pump consists of a catalytic α-subunit and a regulatory β-subunit, both of which have multiple isoforms. Previous studies from our laboratories and others have established additional roles for Na,K-ATPase as a signaling scaffold and a cell adhesion molecule, independent of its pump activity. While the β1-subunit of Na,K-ATPase is found in most animal cells, the expression of the β2-subunit is mostly restricted to brain and muscle, suggesting tissue-specific functions. To distinguish between isoform specific-functions of the β1- and β2-subunits we chose to use cerebellar granule precursor (CGP) cells, which, unlike most other cell types, express both the β1- and β2-subunit isoforms. In CGP cells, mRNA and protein levels of both the β1- and β2-subunits increased during post-natal maturation. Nevertheless, during this differentiation period, the transcription of the β1- and β2-subunits were regulated by distinct mechanisms, further suggesting that the two isoforms may have separate functions. Knockdown studies of the β1- and β2-subunits in medulloblastoma cells, which originate from CGP cells, and studies in primary mouse CGP cultures revealed a role for the β2-subunit, but not the β1-subunit, in Epidermal Growth Factor (EGF) induced reorganization of the actin cytoskeleton. Our studies further showed that knockdown of the β2-subunit, but not the β1-subunit, resulted in increased Merlin/NF2 levels, accompanied by Merlin-mediated changes in the kinetics of Epidermal Growth Factor Receptor (EGFR) signaling. Thus, our studies for the first time provide a functional link between the Na,K-ATPase β2-subunit and Merlin/NF2, a member of the ERM family of proteins, suggesting a role for the β2-subunit in the organization of actin-dependent structures during neuronal differentiation.

P2454
Board Number: B604
Single-molecule study of VEGFR2 spatiotemporal organization on the endothelial cell surface.
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Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) is a receptor tyrosine kinase that plays an essential role in the formation of blood vessels in mammals. Activation of VEGFR-2 in endothelial cells (ECs) leads to critical changes in cell behavior such as cell sprouting, polarization, migration and proliferation. The classic model of VEGFR-2 activation involves binding of the growth factor VEGF, followed by dimerization of VEGFR-2 leading to autophosphorylation, which activates complex downstream signaling pathways. Alternative models predict that VEGFR-2 can be in a pre-dimerized state without VEGF. Yet, little is known about the spatial distribution, dynamics, and oligomerization status of VEGFR-2 in its native membrane environment in live endothelial cells. To fill this gap in our knowledge, we have undertaken a quantitative single-molecule study of VEGFR-2 in live microvascular ECs. Specifically, we labeled endogenous VEGFR-2 using Fab fragments of a neutral anti-VEGFR-2 monoclonal antibody, and imaged using total internal reflection fluorescence microscopy. The imaged molecules were then detected and tracked, allowing us to characterize VEGFR-2 movement as well as...
possible interactions as captured by trajectory merging and splitting events. These studies revealed a mix of freely diffusing, confined and immobile VEGFR-2 molecules, with mobility slowing down upon VEGF addition. They also revealed VEGFR-2 trajectory merging and splitting events both in the absence and presence of VEGF. While there were more merging and splitting events in the presence of VEGF, their existence in its absence provides evidence in live cells for the model of pre-dimerization. We hypothesized that pre-dimerization primes the cell to respond to VEGF, as previous studies indicate that the affinity of VEGF to dimeric VEGFR-2 is higher than to monomeric VEGFR-2. We tested this hypothesis in two ways. First, we expressed in CHO cells genetically modified VEGFR-2 constructs that have an increased or decreased tendency for pre-dimerization, and measured VEGF binding via single-cell imaging. We found that, for the same level of VEGFR-2 surface expression, VEGF binding was indeed proportional to the degree of pre-dimerization tendency. Second, we grew ECs long-term in the almost complete absence of VEGF, and monitored the behavior of VEGFR-2 on the cell surface. These experiments revealed that under these conditions ECs presented more VEGFR-2 on their surface, with higher mobility when compared to cells grown in VEGF-containing medium. Together, these experiments and analyses suggest that ECs indeed might modulate the spatiotemporal organization of VEGFR-2 on the cell surface to modulate their sensitivity and response to VEGF. Supported by: CPRIT R1216 and NIH/NIGMS R35 GM1

P2455
Board Number: B605
A 3D culture system identifies a new mode of cetuximab resistance in colorectal cancer and provides means to overcome cetuximab resistance.
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A human colorectal cancer (CRC) cell line, HCA-7, when cultured in 3D in type-1 collagen forms two kinds of colonies; either hollow single-layered polarized cysts or solid spiky masses. Individual colonies of both morphologies were isolated and propagated; the lines thus derived retained their cystic and spiky morphologies and were termed CC and SC, respectively. In 3D cultures, CC are sensitive to the EGF receptor (EGFR) neutralizing antibody, cetuximab, while SC are resistant (PNAS 114: E2852-61, 2017). Additionally, when injected subcutaneously in nude mice, CC form well-differentiated tumors, while SC form poorly-differentiated, invasive tumors. Interestingly, in SC, MAPK pathway members were found to be upregulated. We further show that inhibition of MAPK signaling with MEK inhibitor, U0126, restores cetuximab sensitivity in SC. In an RTK array we found upregulation of MET and RON phosphorylation in SC compared to CC. A small molecular inhibitor, crizotinib targeting both MET and RON restored cetuximab sensitivity in SC. Thus, with this 3D system, we have identified a new mode of cetuximab resistance and provided means to overcome cetuximab resistance. We are currently studying the effect of cetuximab/crizotinib combination on inhibition of proliferation, colony morphology, and apoptosis induction in 3D CC and SC cultures. We are also investing other CRC cell lines in 3D for their dependence on this mechanism of cetuximab resistance. As such, this represents a potentially powerful system to identify additional therapeutic sensitivities and disease-relevant genes for CRC.
P2456
Board Number: B606
Alternative Dopamine Signaling in Macrophages.
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Throughout the HIV epidemic, drug abuse has been an important comorbidity in HIV infection. All drugs of abuse significantly elevate CNS dopamine levels, and dopamine has been shown to play a significant role in modulating immune cell function. Previous data from our lab have shown that in macrophages, elevated dopamine concentrations significantly increase HIV infection of these cells. These increases are mediated by activation of both D1-like and D2-like dopamine receptors present on the macrophage surface. However, most studies of dopamine receptor signaling have been done in neurons, and the signaling pathways mediated by dopamine receptors in macrophages remain unclear. To define more precisely the signaling pathways mediating the dopamine-induced increase in HIV infection, we examined the dopamine-mediated cAMP production pathway in primary human macrophages. We detected transcripts of all five dopamine receptor subtypes with qRT-PCR, showing D1-like receptors expressed at a significantly higher level than D2-like receptors in this cell type. To determine if D1-like receptors signaled through the Gas pathway as previously described in neurons, we utilized a sensitive cAMP assay to assess the ability of dopamine to stimulate adenylate cyclase in primary human macrophages. We were unable to observe any increase in cAMP in our cells after 5, 10, 15, or 30 minute treatments with dopamine or the D1-specific agonists SKF-38393 and A68,930. In contrast, isoproterenol increased cAMP in a dose-dependent manner, indicating adenyl cyclase was active in these cells. We looked downstream of cAMP activation and found dopamine treatment does not increase levels of phosphorylated PKA, confirming this pathway is inactive. However, dopamine treatment increased levels of phosphorylated PKC. Taken with our previous data, these findings suggest dopamine signaling in macrophages occurs primarily through an alternative signaling pathway, potentially mediated by Gsα/11 or Gβγ. Significantly, these studies suggest a novel signaling pathway associated with the dopamine mediated increase in HIV infection in macrophages. Improving our understanding this signaling mechanism, and how it interacts with the HIV infection process, may indicate new targets or therapeutic strategies to more effectively ameliorate the effects of HIV in the CNS of HIV-infected drug abusers.

P2457
Board Number: B607
The phosphatase PTPRG inactivates AXL sequestered in membrane lipid domains by the tumor suppressor OPCML.
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In ovarian cancer, the pro-metastatic RTK AXL promotes motility, invasion and poor prognosis. Here we observed that the reduced survival caused by AXL overexpression could be moderated by the expression of the GPI-anchored tumor suppressor OPCML. We subsequently discovered that AXL interacts with OPCML, preferentially so when AXL is activated by its ligand Gas6. As a consequence, AXL accumulates in cholesterol-rich lipid domains, where OPCML resides. Here phospho-AXL is brought in proximity to the lipid domain restricted phosphatase PTPRG, which dephosphorylates the RTK/ligand complex. This prevents AXL-mediated transactivation of other RTKs (cMET and EGFR), thereby inhibiting sustained
phospho-ERK signaling, induction of the EMT transcription factor Slug, cell migration and invasion. From a clinical perspective, we show that OPCML enhances the effect of the phase II AXL inhibitor R428 in vitro and in vivo. We thus unveil a novel mechanism by which two spatially restricted tumour suppressors, OPCML and PTPRG, coordinate to repress AXL onco-genic signalling.

P2458

Board Number: B608

Automated imaging-based technique for simultaneous monitoring of Ca\(^{2+}\) and DAG biosensors enables detailed characterization of GPCR signaling pathways.

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G protein coupled receptor (GPCR)-mediated pathways are critical for cells to respond to intercellular and environmental cues and are a major focus of drug discovery efforts. The molecules that activate GPCRs, and the resulting signaling cascades triggered by associated G proteins, are diverse. Activation of the G\(_q\) sub-family of G proteins increases phospholipase C (PLC) activity, converting phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG), both of which function as second messengers. IP\(_3\) triggers release of intracellular Ca\(^{2+}\) stores which regulate calcium-dependent proteins, while membrane-bound DAG activates the signal cascade via protein kinase C.

Here we describe an automated imaging-based approach to characterize the IP\(_3\)/DAG signaling pathway using multiplexed R-GECO Ca\(^{2+}\) and DAG fluorescent biosensors from Montana Molecular. This method produces detailed kinetic profiles of Ca\(^{2+}\) flux and DAG levels following activation of G\(_q\)-coupled receptors within the same population of cells. Background subtraction and image analysis tools enable detection of changes in Ca\(^{2+}\) and DAG levels that is up to 8 times more sensitive than techniques relying on bulk fluorescence measurements.

Carbachol induced stimulation of G\(_q\)-coupled hM1 receptors in HEK-293 and HeLa cells expressing DAG and R-GECO biosensors was used to demonstrate the ability of this system to detect and quantify changes in DAG levels and IP\(_3\)-induced Ca\(^{2+}\) flux. Addition of 30 \(\mu\)M carbachol to HEK-293 resulted in a 9-fold increase in R-GECO fluorescence over baseline. Maximum intracellular Ca\(^{2+}\) concentration was reached within one second of carbachol addition and returned to baseline after approximately 60 seconds. DAG fluorescence levels in the same population increased 2.6-fold over 11 seconds before falling back to baseline over a period of 3 minutes.

HeLa cells exhibited a similar Ca\(^{2+}\) flux profile in response to 30 \(\mu\)M carbachol compared to HEK-293, with a 10-fold increase in R-GECO fluorescence. However, a much smaller increase in DAG levels was detected, with levels increasing 1.2-fold over baseline. Analysis of single cell kinetics revealed that HeLa cells exhibit multiple Ca\(^{2+}\) spikes following stimulation by carbachol that decrease in intensity, with an average interval of 20 seconds. In contrast, stimulated HEK-293 exhibit a single sustained spike in intracellular Ca\(^{2+}\) levels.
Rho-Family GTPases

P2459

Board Number: B609

The RhoGAP SPV-1 acts through CDC-42 to regulate calcium signaling in the C. elegans spermatheca during embryo transits.

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Biological tubes are essential components of many animal body systems, and the proper regulation of contractility in these tubes is vital for many life processes. The spermatheca, a 28-cell contractile tube and valve in the reproductive system of the nematode C. elegans, provides an ideal model for detailed studies of such tubes. During embryo transits the spermatheca is stretched significantly by oocyte entry, remains distended for a regulated period of time while the eggshell forms, and finally initiates coordinated contraction of the cells, opening the valve and expelling the egg. Spermathecal contractility is controlled by calcium signaling and Rho signaling, and these two signaling pathways control cellular contractility in many other biological contexts, suggesting that mechanisms at work in the spermatheca potentially represent fundamental biological responses to cell and tissue level deformations in biological tubes.

The RhoGAP SPV-1 was previously identified as a key regulator of contractility in the spermatheca, acting through RHO-1. When spv-1 is lost the spermatheca contracts immediately upon oocyte entry, leading to faster embryo transits. While it is known that calcium signaling is necessary for spermathecal contraction, it was unknown if SPV-1 affects calcium signaling, so we examined this using the genetically encoded calcium sensor GCaMP. We find that the loss of spv-1 results in a more rapid onset of calcium signaling upon oocyte entry, and elevated calcium levels throughout embryo transits. Overexpressing spv-1 results in the opposite calcium phenotype, with delayed onset of calcium signaling and decreased calcium levels throughout embryo transits. Studies using a mutated spv-1 which lacks RhoGAP activity exhibit calcium signaling similar to the loss of spv-1, indicating that SPV-1 controls calcium signaling primarily through GTPase activity. SPV-1 has significant GAP activity toward RHO-1 and CDC-42, so we used constitutively active versions of these GTPases to further explore how SPV-1 regulates calcium signaling. We find that increasing RHO-1 activity alters the timing of embryo transits without recapitulating spv-1 mutant calcium signaling, while increasing CDC-42 activity approaches spv-1 mutant calcium signaling without significantly altering the timing of embryo transits. These results reveal a previously unknown role for CDC-42 in the spermatheca, and show that in addition to regulating contractility, SPV-1 in the spermatheca also coordinates the activities of multiple GTPases and regulates calcium signaling. Ongoing work seeks to identify effectors that CDC-42 uses to regulate calcium signaling and to understand how the calcium signaling of individual cells is coordinated to generate tissue level responses.
Flares of active Rho and F-actin locally reinforce the tight junction barrier in response to mechanical stress.

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The epithelial barrier is important for generating specialized compartments in multicellular organisms. The barrier property of vertebrate epithelia is dependent on tight junctions, which restrict the flow of ions, water, and small molecules between epithelial cells. Epithelia are subject to a number of cell- and tissue-scale forces, such as cell divisions, extrusions, wound healing, and morphogenetic events. During these events, cell-cell junctions must remodel to accommodate changes in cell shape and mechanical force. However, very little is known about how tight junctions are able to maintain barrier function during these events. In order to examine this question, we developed a highly sensitive barrier assay compatible with live imaging. When we applied this barrier assay to gastrula-stage *Xenopus laevis* embryos, we found that the epithelial barrier is not uniform across space and time. Instead, small barrier breaches occur—often around dividing cells—and persist for minutes before barrier function is restored.

We have previously reported that “flares” of active Rho accumulate at cell-cell junctions, particularly in situations where the cell-cell junctions are compromised. Because Rho flakes occur on similar time scales and at similar locations as barrier breaches, we investigated whether the two may be correlated in space and time. Indeed, we found that local barrier breaches are followed by local increases in Rho activity. As Rho returns to baseline levels, the barrier function is restored. Thus, we hypothesized that Rho may be involved in restoring barrier function on subcellular scales. To further investigate this possibility, we examined fluorescently-tagged tight junction proteins ZO-1 and Occludin with respect to Rho.

Intriguingly, we observed local discontinuities in ZO-1 and Occludin prior to the flare, and both proteins remained locally increased over baseline, or reinforced, following the flare. In order to investigate how active Rho contributes to reinstatement of the barrier and reinforcement of ZO-1, we perturbed actin polymerization and junction contraction using pharmacological tools. Both junction contraction and actin polymerization appear to contribute to ZO-1 reinforcement, as disruption of either results in only partial ZO-1 reinforcement. When both junction contraction and actin polymerization are lost, ZO-1 fails to be reinforced, and the barrier function is not reinstated. Taken together, these data indicate that transient breaches of the tight junction barrier arise in response to mechanical force, and Rho flakes serve to rapidly repair these breaches, preserving the overall barrier function of the epithelium.

Cytokines augment GEF-H1 expression through a cytoskeleton-dependent self-regulatory cycle in the tubular epithelium.

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Transforming Growth Factor β1 (TGFβ1) is a major pro-fibrotic cytokine that along with tissue injury promotes epithelial phenotype shifts such as Epithelial-Mesenchymal Transition (EMT). While EMT supports wound healing and cell migration, thereby promoting tissue regeneration, it also plays a
central role in the development of organ fibrosis and cancer metastasis. Cytoskeletal remodeling mediated by Rho family small GTPases is a central event in EMT-associated cell reprogramming, in large part due to its effects on Myocardin Related Transcription Factor (MRTF), a coactivator of Serum Response Factor (SRF). Thus, the Rho- Rho kinase (ROK)-MRTF axis has a major role in pathologies such as inflammation, fibrosis and cancer. However, the context-dependent control of Rho regulators, including Guanine Nucleotide Exchange Factors (GEFs) during EMT remains less explored. We previously found that in tubular cells proinflammatory and profibrotic stimuli activate RhoA through the exchange factor GEF-H1. Here we show that prolonged stimulation of LLC-PK1 tubular cells with TGFβ1, or mechanical stress (cell stretch) or the proinflammatory cytokine Tumor Necrosis Factor-α (TNFα) elevates expression of GEF-H1. Tubular GEF-H1 expression was also increased in unilateral ureteral obstruction (UOO), a mouse model of kidney fibrosis, as shown using laser capture microdissection and micro-PCR. Silencing or inhibiting RhoA, ROK or MRTF reduced basal GEF-H1 expression in LLC-PK1 cells and prevented the TGFβ1- and TNFα-induced elevation. Conversely, Jasplakinolide that induces actin polymerization and activation of MRTF elevated GEF-H1 expression. TNFα and TGFβ activated a luciferase coupled GEF-H1 promoter construct through ROK, and MRTF silencing reduced this effect. Interestingly, silencing of endogenous GEF-H1 also reduced the activity of the promoter construct, suggesting a feedback regulation. Finally, TNFα augmented TGFβ-induced expression of the EMT marker smooth muscle actin (SMA) in a GEF-H1- dependent manner. In summary, our study shows that GEF-H1 expression is elevated by pro-fibrotic stimuli through cytoskeleton remodeling and the RhoA/Rho kinase/MRTF pathway, and this can further augment EMT. Such a self-regulatory positive feed-back cycle might play a key role in the further progression of kidney fibrosis.

P2462
Board Number: B612
RhoGDI Mediates Spatiotemporal Patterning of GTPase Activity During Cell Wound Repair.
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The RhoGTPases experience constant flux as they move through the GTPase cycle. GTPases associated with membrane lipids are activated by GEFs and inactivated by GAPs. A third regulator, RhoGDI, is believed to extract inactive GTPase from the membrane and drive it into a soluble form within the cytoplasm. To better understand RhoGDI function in vivo, we have employed the Xenopus laevis system in which cell damage elicits rapid formation of concentric zones of Rho and Cdc42 activity around wounds. Overexpression of RhoGDI negatively regulates Rho and Cdc42 activity. Simultaneous overexpression of RhoGDI with PKCβ, previously shown to positively regulate Cdc42 activity during cell wound repair, rescues Cdc42 activity but not Rho activity. However, in the presence of non-phosphorylatable S90A RhoGDI, PKCβ is unable to rescue Cdc42 activity. These results suggest that the phosphorylation of RhoGDI by PKCβ promotes localized Cdc42 activity during cell wound repair. To better understand how RhoGDI contributes to the spatiotemporal patterning of GTPases, I generated a RhoGDI mutant designed to bind but be unable to extract GTPases from the membrane. The mutant RhoGDI localizes more strongly to wounds than wild-type and, remarkably, decreases Rho but increases Cdc42 activity. In contrast to the textbook model of RhoGDI function, these results suggest that RhoGDI extracts both active and inactive Cdc42 from membranes. If this is indeed true, RhoGDI can directly control GTPase patterning via extraction of active GTPases.
P2463
Board Number: B613
Identifying signaling connections in cancer cell motility using partial correlation analysis of simultaneously-imaged Rho GTPase and RhoGEF activities.
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RhoGEFs directly activate Rho family GTPases leading to the regulation of cell shape through alterations in cytoskeletal dynamics. Consequently, cellular morphodynamics requires the exquisite spatiotemporal activation of diverse RhoGEFs acting in concert. Here, we describe a general strategy to produce RhoGEF biosensors. A pair of fluorescent proteins was inserted in a region of the RhoGEF where relief of autoinhibition altered FRET between the fluorescent pair. The design was optimized using high-content screening to produce Vav2 and Asef biosensors where activation causes changes in FRET emission ratios of 210% and 110%, respectively. The optimized Vav2 biosensor is responsive to Src kinase and epidermal growth factor receptor (EGFR), while the Asef biosensor reports activation by APC. The design strategy works for diverse RhoGEFs, including: LARG, b-Pix, Tim and Tiam1.

We used the RhoGEF biosensors together with updated versions of our Rho family GTPase biosensors to examine the relative dynamics of GTPases and RhoGEFs during constitutive migration of MDA-MB-231 breast cancer cells. The RhoGEFs Asef and Vav2, and the GTPases Rac1, Cdc42 and RhoA were all active at the edge of protruding/retracting regions of the cell. Asef, Vav2, Rac1 and Cdc42 had activation patterns that positively correlated with edge velocity, while RhoA showed a negative correlation. We modified our GTPase biosensors to enable simultaneous imaging of RhoGEF and GTPase activity. This revealed that the activity of Asef and Vav2 are closely correlated with Rac1 at the cell edge. Partial correlation analysis of simultaneous imaging data was used to dissect the role of Asef and Vav2 in regulating Rac1 activity. Rac1 activation relevant to protrusion initiation was specifically dependent on Asef. This was confirmed using shRNA knockdown of Asef. This study demonstrates that partial correlation analysis of multiple biosensors can be used to reveal how upstream molecules regulate different target proteins without disruption of normal biological processes.

P2464
Board Number: B614
Co-regulation of Rac and Rho Signalling in Cell Motility by a Scaffold RhoGAP BPGAP1.
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Cancer metastasis comprises multiple steps of cell migration that requires active remodeling of the cytoskeleton for cells to invade the surrounding tissues. Rho small GTPases are key molecular switches that control such processes and are known to contribute to cancer progression. Among them, RhoA,
Rac1 and Cdc42 specifically promote actomyosin contractility, lamellipodia and filopodia formation, respectively. They are activated by guanine exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs). Mutations in Rho are frequent only in a few cancer types, but deregulation of Rho signaling is often associated with tumorigenesis at the level of their gene expression or activation through their regulators or downstream effectors. Intriguingly, RhoA and Rac1 often act antagonistically and have distinct spatiotemporal activity profiles in lamellipodia. However, it is unclear whether the distinct spatiotemporal activity of Rho/Rac can be synchronized by a single protein that is common to both GTPases, which would help in facilitating a more efficient control in cell dynamics. We hypothesize that such an integrator for Rho/Rac signaling could exist by targeting and regulating both RhoA and Rac1 in close proximity.

Expression of the BPGAP1 (or ARHGAP8) is often elevated in primary colorectal tumors, invasive cervical and breast cancer. It is a multi-domain RhoGAP that inactivates RhoA and induces cell protrusions and cell migration via the interplay of its BH domain, Proline-Rich Region and RhoGAP domain. Consistent with its pro-metastatic potential, BPGAP1 translocates the actin regulator, cortactin, to lamellipodia where BPGAP1 also interacts with the inactive form of Rac1, raising the possibility that BPGAP1 could coordinate and integrate Rac1 and RhoA signaling in cell motility. Mechanistically, stimulation by growth factor EGF releases BPGAP1 autoinhibition, exposing its BH domain to recruit the RacGEF, Vav, to activate Rac1 and promote polarized cell motility, cell spreading, invadopodia formation and cancer extravasation. Importantly, BPGAP1 controls local Rho activity that influences Rac1 binding to BPGAP1 and its subsequent activation by Vav1. BPGAP1 therefore acts as a dual-functional scaffold that recruits Vav to activate Rac1 while inactivating RhoA to synchronize both Rho and Rac signaling in cell motility. As EGF receptor, Vav, Rho, Rac and BPGAP1 are all associated with cancer metastasis, BPGAP1 offers a crucial checkpoint for EGFR-BPGAP1-Vav-Rac-Rho signaling axis and possible regimes in cancer intervention.

P2465
Board Number: B615
Rac3 GTPase regulates breast cancer invasion and metastasis by controlling adhesion and matrix degradation.
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Metastasis is responsible for 90 percent of cancer related deaths. One of the first steps in metastasis is the local invasion of tumor cells into the surrounding tissue. Invadopodia are actin-based protrusions that mediate the matrix degradation necessary for the invasion, dissemination and metastasis of tumor cells. Here, we demonstrate that Rac3 GTPase is critical for integrating the adhesion of invadopodia to the extracellular matrix (ECM) with their ability to degrade the ECM in breast tumor cells. We identify two signaling pathways important for the regulation of integrin activation at invadopodia and the delivery of matrix metalloproteinase to invadopodia, through the upstream recruiter Calcium and integrin binding protein 1 (CIB1) and the downstream effector G-protein coupled receptor kinase interactor 1 (GIT1). We then show that the activation dynamics of Rac3 at and surrounding the invadopodia core are controlled by two upstream GEFs, Vav2 and BPIX. These GEFs play key roles in
regulating the spatiotemporal dynamics of Rac3 activity, impacting GIT1 localization. Furthermore, we show that the GTPase activating function of GIT1 toward the vesicular trafficking regulator Arf6 GTPase is required for invadopodia matrix degradation. Importantly, Rac3 also regulates the ability of breast adenocarcinoma cells to invade, disseminate and metastasize in vivo. The Rac3-dependent mechanisms that we show here are critical for balancing proteolytic activity and adhesive activity, and to achieve a maximally invasive and metastatic phenotype.

P2466
Board Number: B616
Cdc42 Regulates Dynein and Actin in Terminal Differentiation of Human Erythroblasts.

Mammalian erythroblasts undergo enucleation through a process thought to be similar to cytokinesis. We have reported that dynein and microtubule-organizing centers (MTOCs), that are known to be crucial for proper cytokinesis, mediate organization of the mitotic spindle apparatus that separates the chromosomes during mitosis in erythrocyte progenitors. We showed that dynein inhibition impairs nuclear polarization, thereby blocking enucleation (Kobayashi I. et al. Exp. Hematol. (2016) 44: 247). It is well known that the nucleus is actively extruded by actomyosin in contractile ring. These results suggested to us that an upstream effector in the signal transduction pathway responsible for regulation of dynein, such as the small GTPase Cdc42, may play an important role during terminal differentiation of human erythroblasts. We therefore investigated the effect of the Cdc42 inhibitor, CASIN, 2-[(2,3,4,9-Tetrahydro-6-phenyl-1H-carbazol-1-yl) amino]ethanol, on cytokinesis and enucleation of human colony-forming unit-erythroblasts (CFU-Es, Day7) and mature erythroblasts (Day10) derived from purified CD34+ cells. We assessed the effect of this Cdc42 inhibitor on proliferation and enucleation ratio of CFU-Es and on the distribution of dynein and actin filaments during terminal differentiation of human erythroblasts. CASIN inhibited proliferation of CFU-Es and blocked their enucleation in a dose-dependent manner (0 – 25 μM). Whereas dynein adopted an island-like distribution in the cytoplasm of non-treated CFU-Es, in contrast, in CASIN treated cells, dynein was concentrated near the nucleus as a dot and co-localized with γ-tubulin. The cell number in cell cycle G2/M phase was decreased by CASIN treatment in CFU-Es but not in Day10 cells. Phalloidin staining revealed that formation of actin filaments was inhibited by CASIN in both CFU-Es and Day10 cells. In the CASIN-treated Day10 cells, formation of the actomyosin contractile ring was also inhibited. Notably, the expression of CD71 and glycoporphin A (GPA) in both CFU-Es and Day10 cells and the morphology of erythroblasts were not significantly affected by CASIN treatment. Our data confirm that Cdc42 plays an essential role in both cytokinesis and cell polarization through regulation of dynein and actin filament organization during terminal differentiation of human erythroblasts.
This work was supported by JSPS KAKENHI Grants 15K09448 (WN), 15K19540 (KU), 17K16177 (YG) and 15K09516 (HW) and by a private donation from Dr. Ken Satoh, Satoh Naika Clinic, Sakata 998-0013, Japan.
MAPK pathways play diverse roles in cell differentiation, proliferation, and the response to stress. Different MAPK pathways induce different responses yet can be regulated by shared components. Misregulation of MAPK signaling by inappropriate cross talk has been linked to cancer and other diseases. In the model system budding yeast, three MAPK pathways (filamentous growth or fMAPK, mating and HOG) share components such as the polarity control Rho GTPase Cdc42, p21 activated (PAK) kinase Ste20 and MAPKKK Ste11. We developed methods to directly compare yeast MAPK pathways that share components. Using this system, we showed that each pathway has a unique modality. In particular, the fMAPK pathway was a ‘low and slow’ pathway that sensitized the cell to a variety of inputs, including positional cues and the integrity of the mother bud neck. Testing a library of CDC42 alleles in this system identified pathway-specific versions of Cdc42. Pathway-specific inputs came in part from scaffold-type adaptors (Bem1 and Bem4) that interact with Cdc42. Bem1 and Bem4 failed to interact with versions of Cdc42 specifically compromised for fMAPK signaling. Bem4 preferentially associated with versions of Cdc42 that mimic the closed or GDP-locked conformation that also are sequestered by the guanine nucleotide exchange factor (GEF) Cdc24. Thus, Bem4 may stabilize the interaction between GDP-Cdc42 and Cdc24 to promote GTPase activation. Bem1 associates preferentially with versions of Cdc42 that mimic the GTP-locked or active conformation. Thus, Bem1 may promote subsequent steps in MAPK activation, such as the interaction of GTP-Cdc42 with effector proteins. In support of this idea, a version of Bem1 that was unable to associate with the PAK kinase Ste20 was defective for fMAPK signaling. Bem4 also interacts with the MAPKKK Ste11. Thus, the Bem1-Ste20 and Bem4-Ste11 interactions may promote fMAPK signaling and account for their unique roles in fMAPK regulation. Another control point was PM recruitment of Cdc42 and fMAPK specific components. Bem4 promoted the plasma membrane (PM) localization of Cdc42, and overexpression of the Rho GDP dissociation inhibitor Rdi1 dampened fMAPK activity. Our results indicate that one aspect of MAPK regulation is to boost and sustain Cdc42 activity. Consistent with these results, MAPK activation rescued the temperature sensitivity of a subset of cdc42 alleles. In conclusion, differential regulation of Cdc42 by different scaffold-type adaptors contributes to the unique modality of the fMAPK pathway and may provide a model for how other GTPases are directed to specific pathways to perform specific functions.
by guanine nucleotide exchange factors (GEFs), it is still unclear whether and how RhoA is activated by GEFs at the bleb membrane. Here, we report that a GEF termed MYOGEF can promote bleb retraction through activating RhoA at the bleb membrane. Our results further demonstrate that ezrin, a key regulator of bleb retraction, binds and recruits MYOGEF to retracting bleb membranes. CRISPR/Cas9- or RNAi-mediated depletion of ezrin disrupted the localization of MYOGEF at the bleb membrane. Results from yeast two-hybrid and immunoprecipitation assays indicate that amino acid residues 640-752 in MYOGEF are required for interactions with the amino-terminal region of ezrin that contains a FERM domain. Consistently, a truncated MYOGEF fragment lacking the ezrin-binding region failed to localize to the bleb membrane. In addition, ectopic expression of the MYOGEF fragment lacking the ezrin-binding region slowed down bleb retraction and induced the formation of extended blebs even though ezrin was still recruited to the bleb membrane, suggesting that the ezrin-MYOGEF interaction plays a critical role in promoting bleb retraction. Importantly, CRISPR/Cas9-mediated depletion of MYOGEF or ezrin resulted in deficiency in bleb retraction as well as interfered with RhoA activation and actomyosin cortex reassembly at the bleb membrane. Overall, our findings suggest that, by interacting with ezrin, MYOGEF is recruited to the retracting bleb membrane, where MYOGEF activates RhoA and stimulates the reassembly of the cortical actomyosin network at the bleb membrane, thus promoting bleb retraction.

P2469
Board Number: B619
Complex Colony Morphology as a Multicellular Behavior with Macroscopic Benefits.
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Individuals can work together in many settings to provide a benefit to the population. How individuals work together to accomplish biological tasks is not clear. One rudimentary cooperative behavior that occurs across microbial species is biofilm/mat formation. During this process, cells remain connected to each other by adhesion molecules and/or an extracellular matrix. In pathogens, biofilm/mat formation is required for virulence. One aspect of biofilm/mat formation is the development of complex colony morphology (CCM), which impacts the surface architecture of a biofilm. In the budding yeast S. cerevisiae, CCM involves the development of ruffled ridges across the colony surface. It is not known how cells form this structure or what its function(s) might be. To begin to address this question, CCM was examined in wild-type cells and various mutants by fluorescence microscopy and scanning electron microscopy (SEM). Fluorescence microscopy revealed that ruffles are composites of multiple groups of cells. SEM showed that individual cells within a ruffle align to the axis of the ruffle. Time-lapse photography of CCM development showed how surface architecture changes over time and the role of adhesion during development. The Rho GTPase Cdc42p and its cognate MAP kinase pathway regulated the development of CCM through the mucin Flo11p. Cdc42p’s regulation of apical growth also appeared to play a role. Comparative RNaseq analysis of biofilms with exaggerated CCM and biofilms that fail to make CCM was explored. CCM benefitted cells in a mock predator-prey experiment. Thus, our results provide an example of a role for Rho GTPases, like Cdc42p, in coordinating collective behaviors to produce macroscopic structures that can provide a protective benefit.
P2470
Board Number: B620
Breaking and entering: a pore-ensics analysis.
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Gaining entry into a host cell provides a survival advantage to any capable pathogen and this process contributes to the progression of infection. The typical mechanisms of gaining entry include passive phagocytosis by professional phagocytes or active entry into host cells via receptor-mediated endocytosis or introduction of effector molecules into the host cell to modulate endocytic processes. Interestingly, recent evidence indicates that pathogens can also induce host plasma membrane damage as a means to gain entry. Adenovirus HAdV-C2 and eukaryotic parasite Trypanosoma cruzi, the etiologic agents of acute respiratory disease and Chagas disease, have been shown to induce host plasma membrane damage via membrane lytic protein VI or through mechanical disruption, respectively, to take advantage of the subsequent host membrane repair machineries in order to gain entry into the target cells. Our lab recently evidenced that the intracellular pathogen Listeria monocytogenes can use its pore forming toxin listeriolysin O (LLO), a virulence factor thought to only be used to promote bacterial escape from the endocytic vacuole, to disrupt the host plasma membrane and mediate entry. This study was directed at characterizing the signaling cascade downstream of LLO-mediated membrane damage that leads to bacterial entry. Using siRNA, chemical inhibitors, and a live-cell fluorescence resonance energy transfer (FRET) imaging approach, we were able to dissect the spatiotemporal activities of key signaling molecules involved in LLO-mediated entry. Our findings indicate that pore-formation on the plasma membrane leads to an influx of Ca2+, which induces the translocation and activation of a conventional protein kinase C at the plasma membrane and activation of the central F-actin regulator, the Rho GTPase Rac1. Transduction of this signal results in the activation of Arp2/3 and formation of F-actin-mediated membrane protrusions that internalize the bacterium. Using a high-throughput membrane repair assay recently developed in our lab, we showed that the entry signaling pathway was dispensable for membrane repair indicating that although membrane damage may act as an initial step to entry, this novel pathway is characteristically distinct from membrane repair.

P2471
Board Number: B621
 Snf4 promotes Snf1/AMPK GEF activity for Arf3 activation during glucose starvation.
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Upon nutrient deprivation, several fungi, including S cerevisiae, undergo invasive growth depicting a developmental switch to penetrate into agar for the exploration of sufficient nutrients. Our previous study indicated that Arf3 is required for yeast invasive growth and that Arf3 is highly activated in response to glucose depletion. We further identified a serine/threonine protein kinase, Snf1/AMPK, acts as a guanine nucleotide exchange factor (GEF) for Arf3p activation upon glucose deprivation. Snf1/AMPK has been identified as an obligate heterotrimer, containing a catalytic α subunit and two regulatory subunits (β and γ). In yeast, the kinase activity of Snf1 (α subunit) is tightly regulated by its activating γ subunit Snf4 and regulatory β subunits Sip1, Sip2 and Gal83. The regulation of Snf1 kinase activity is
much understood; however, how Snf1 acts as GEF to activate Arf3 is unknown. In this study, we address
the regulation of Snf1 GEF activity on Arf3 activation. We show that γ subunit Snf4, but not β subunits,
associated with inactive Arf3 and Snf1 in response to glucose depletion. We further demonstrate that
Snf4 is required for Arf3 activation by directly interacting Snf1 C-terminal GEF domain to efficiently
promote Snf1 GEF activity. These results reveal the regulatory role of Snf4 for Snf1 functions in releasing
its N-terminal kinase domain and enhancing C-terminal GEF activity during glucose starvation.

P2472
Board Number: B622
Deregulation of Rho GTPase Family Members in Myelodysplastic Syndromes and Acute Myeloid
Leukemia.
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The Rho GTPase family is composed of twenty proteins, of which RhoA, Cdc42 and Rac1 are the most
studied. In hematopoietic cells, Rho GTPases have been reported to participate in self-renewal,
differentiation and stem cell interaction with the bone marrow niche. In recent years, abnormal gene
expression of Rho GTPases has been found in many cancers, but most of the family members have been
poorly studied in myeloid malignancies. We aimed to characterize the expression of Rho GTPases in
myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Gene expression of ten Rho
GTPases (RhoA, Cdc42, Rac1, RhoB, RhoC, Rac2, Rac3, RhoG, RhoQ and RhoH) was analyzed in six
myeloid cell lines and in bone marrow samples by quantitative PCR using TaqMan assays. Bone marrow
aspirates were collected from patients with MDS (n=35), AML with myelodysplasia-related changes
(AML-MRC) (n=9), de novo AML (n=30) and from healthy donors (n=13). Comparisons were performed
with Mann-Whitney U test and the data is showed as median [max-min]. The study was approved by the
Local Ethical Committee Board. All the Rho GTPases evaluated were expressed in the myeloid cell lines.
A similar pattern of expression was observed between genes from the same subfamily: Rho subfamily,
Cdc42 subfamily and Rac subfamily. When analyzing the expression of Rho GTPases in primary cells, we
observed a reduction of Cdc42 transcripts in MDS patients (0.48 [2.47-0.08]) in comparison with healthy
donors (0.97 [7.08-0.17], P=0.02). Cdc42 showed a tendency to decrease in MDS patients with less than
5% blast cells in the bone marrow (0.53 [1.19-0.08]), P= 0.058 and showed a significant reduction in
MDS patients with higher blast number (0.45 [2.47-0.08], P=0.045). RhoB expression was increased in
AML-MRC (7.78 [23.00-1.07]), compared to healthy donors (2.25 [15.25-0.94], P=0.03). RhoC was
upregulated in AML-MRC (23.45 [84.87-1.41]) and in de novo AML (12.28 [99.22-0.80]) compared with
healthy donors (1.53 [34.66-0.59]), P=0.008, P=0.002, respectively. The other Rho GTPases evaluated
were not altered in MDS or in AML. We also analyzed the correlation of Rho GTPases in patient samples.
Rac2 strongly correlated with RhoA (r=0.70, P<0.0001) and RhoG (r=0.83, P<0.0001). Rho GTPases
appear to have a dual role in cancer and may execute both oncogenic and tumor suppressor activities.
Therefore, Rho GTPase expression is cancer type-specific. Herein we provide first evidence of
downregulation of Cdc42 in MDS, and upregulation of RhoB and RhoC in AML. Since these proteins are
involved in critical hematopoietic cell functions, further studies will be important to elucidate the
relation of their deregulation to the physiopathology of myeloid malignancies. Supported by FAPESP,
CNPq and CAPES.
P2473
Board Number: B623
Investigation of RhoA binding proteins using BioID system.
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RhoA belongs to the Rho family of small GTPases and acts as a molecular switch to convert extracellular stimulation by growth factors into cellular signals. Many effector and regulatory proteins of RhoA are known, but, due to rapid activation and deactivation cycle of RhoA, it is difficult to comprehensively analyze the activation state specific binding proteins of small GTPases. To identify cell-type specific signaling molecules via activation of RhoA, we have employed a proximity-dependent biotin identification (BioID) method to compare RhoA (WT) with its active mutant RhoA (G14V). BirA-fused RhoA proteins were ectopically expressed in 293T cells, and biotinylated proteins were purified with Tamavidin 2-REV beads and analyzed by nanoLC/MS/MS. In this study, 147 biotinylated proteins were detected in BirA-RhoA (WT) and 212 proteins in BirA- RhoA (G14V). The emPAI score estimating protein abundance from peptide counts in mass spectrometry were analyzed with OPLS-DA by multivariate analysis to compare the profiles. In this analysis, Ste20-like kinase, SLK was calculated with a higher emPAI score than other proteins, and its biotinylination was confirmed at a lysine residue in the C-terminal region of SLK. In a previous report, SLK was reported to phosphorylate Ser188 of RhoA in vascular smooth muscle cells. Therefore, we verified the role of the interaction between RhoA and SLK using a muscle hypertrophy model of C2C12 cells, carried out in C2C12 myotubes during IGF-I treatment for 48 hours. In the C2C12 myotubes, the level of SLK proteins was increased with differentiation, and IGF-I-treatment enhanced SLK phosphorylation at Ser189, which is an autophosphorylation site related to kinase activity of SLK. Overexpression of RhoA (G14V) enhanced, while inhibition of RhoA activation by dominant-negative mutant RhoA (T19N) suppressed the IGF-I-dependent SLK phosphorylation. Pretreatment with the Rho kinase (ROCK) inhibitor Y-27632 also suppressed the IGF-I-dependent SLK phosphorylation. On the other hand, an increase in phosphorylation of RhoA Ser188 was not observed in the C2C12 myotubes. Furthermore, pretreatment with the SLK inhibitor (Erlotinib) inhibited the IGF-I-stimulated increase in myotube diameter and the expression of myosin heavy chain. Our results indicate that a RhoA-SLK pathway may be involved in regulating muscle hypertrophy in C2C12 cells.

P2474
Board Number: B624
Paclitaxel induces post-translational modifications of RhoGDI alpha as a mechanism to regulate RhoA activity.
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Communication between the microtubule and microfilament systems have been shown previously, and are thought to be critical for coordinated cellular function. Studies utilizing stabilizing and depolymerizing microtubule agents have been shown to regulate stress fiber formation in part through RhoA dependent mechanisms. A recent study showed low doses of paclitaxel inhibits RhoA function;
however, the mechanism by which microtubule stabilization or destabilization regulates RhoA activity is not fully understood. RhoA activity is regulated by several mechanisms including RhoGDI alpha interaction, which inhibits RhoA activity. RhoGDI alpha is regulated by post-translational modifications such as SUMOylation (SUMO) and acetylation (Ac). Ac of RhoGDI alpha has been shown to inhibit binding to RhoA; thus, promoting RhoA activity. Conversely, SUMO-1 modification of RhoGDI alpha enhances RhoGDI alpha-RhoA complex formation and inhibits RhoA activity. Interestingly, paclitaxel has been shown to alter the SUMO 2/3 state of RhoGDI alpha in mitosis. Thus, we asked whether paclitaxel regulates RhoGDI alpha PTMs as a mechanism to alter RhoA activity. Physiologic and endogenous SUMO 2/3 modified RhoGDI alpha was detected in response to 6 hr paclitaxel treatment. The RhoGDI alpha SUMO 2/3 modification was validated with desumoylase inhibitor, as well as inhibition of the SUMO ligase with 2-D08. RhoA activity was measured over a timecourse of paclitaxel treatment, and surprisingly, there was a rapid increase in RhoA activity at early time points prior to the decrease observed at 6 hr of paclitaxel treatment. The significant decrease in RhoA activity observed at 6 hr correlated with the increase in SUMO 2/3 RhoGDI alpha modification, and suggests that SUMO 2/3 modification of RhoGDI alpha may have a similar role to SUMO-1 modification of RhoGDI alpha. As RhoGDI alpha SUMO 2/3 levels were not detectable at early time points, this PTM could not be responsible for the early change in RhoA activity. However, a paclitaxel induced increase in Ac of RhoGDI alpha did correlate with the increased RhoA activity. Of particular interest, the Ac RhoGDI alpha signal disappeared as the SUMO 2/3 signal appeared, which supports previous in vitro findings of competition between these two types of PTMs on RhoGDI alpha. Importantly, data shown here suggests that crosstalk between RhoGDI alpha Ac and SUMO 2/3 modification occur physiologically in response to paclitaxel, and trapping RhoGDI alpha in an Ac state with TSA treatment prevented SUMO 2/3 modification of RhoGDI alpha in response to paclitaxel. Collectively, these data identify additional regulatory mechanisms between the microtubule and microfilament system, which functions through regulating RhoGDI alpha's PTM state.

**P2475**

**Board Number: B625**

**Intermolecular steric inhibition of Ephexin4 is relieved by Elmo1.**

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Ephexin4, a guanine nucleotide-exchange factor for RhoG, promotes engulfment of apoptotic cells and cancer cell migration in a RhoG-dependent manner, which is synergistically augmented by Elmo1, an Ephexin4-interacting protein. However, the underlying molecular mechanism remains elusive. Here, we report a mechanism by which Elmo1 cooperates with Ephexin4 to activate RhoG. We found that Ephexin4 activity was increased by elimination of its SH3 domain which intermolecularly interacts with the N20 region of Ephexin4. This interaction prevented RhoG from binding to Ephexin4 and thus inhibited RhoG activation. Moreover, we also found that Elmo1 associated with the SH3 domain as well as the N20 region and competed with the SH3 domain for binding to the N20 region, interrupting the interaction of the SH3 domain with the N20 region and thereby promoting RhoG binding to Ephexin4. In addition, the activity of Ephexin4 lacking the SH3 domain was comparable to that of Ephexin4 with
Elmo1. Taken together, the data suggest that Elmo1 relieves the steric hindrance of Ephexin4 generated by the intermolecular interaction of the SH3 domain and makes Ephexin4 more accessible to RhoG.

P2476  
Board Number: B626  
GTPase Steering by an Enzymatic Corral.  
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Dynamic arrays of actin filaments and myosin-2 ("actomyosin") drive a broad variety of dynamic biological processes ranging from cell division to wound repair. Such arrays are controlled by the Rho GTPases, proteins that exert their effects on actomyosin by stimulating "effector" proteins when in their active (GTP-bound) state. Traditionally, information flow from the Rho GTPases to the cytoskeleton has been viewed as linear, with GTPase activators (GEFs) stimulating a given GTPase, which then activates effectors which, in turn, modify actomyosin. Subsequently, the process is terminated by inactivation of GTPases by inhibitor proteins (GAPs). However, it is becoming apparent that control of actomyosin arrays entails rapid flux of GTPases from the active to inactive stages that is somehow subject to continual modulation via feedback from the actomyosin itself. Here we test a feedback model in which circular, ring-like waves of Rho activity that direct cell wound repair in the Xenopus oocyte model are driven forward by a self-organizing "enzymatic corral" that forms at their trailing edge. Preliminary results indicate that 1) trailing edge Rho inactivation depends on an F-actin binding protein known as Cortactin and 2) Cortactin exerts its effects on Rho activity by serving as a binding site for two GAPs (RG1 and RG8, RG1/8). Both Cortactin and RG1/8 localize behind the Rho zone, perfectly situated to facilitate the trailing-edge inactivation. As predicted, Cortactin and RG1/8 functionally affect Rho levels. Over-expression of RG1/8 dramatically diminishes the Rho zone. Expression of a Cortactin truncation missing its SH3 domain (implicated in RG1/8 recruitment) causes increased Rho levels, and expression of the SH3 domain in isolation not only causes an outward spreading of Rho activity, but imparts gaps in the usually contiguous ring of RG1/8. F-actin assembly and turnover likely play a crucial role in cortical formation as well. Inhibition of the Arp2/3 complex with the small-molecule inhibitor CK-666 significantly depletes Cortactin from behind the Rho zone, and pharmacological stabilization of F-actin with Jasplakinolide delocalizes Cortactin resulting in an accompanied failure of RG1/8 localization

P2477  
Board Number: B627  
The dual Rho GEF/Rac GAP protein Bcr regulates p38 MAPK signaling and cell proliferation.  
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The Rho GTPase family of proteins (including Rho and Rac) control many cell functions including cytoskeleton rearrangement, cell growth, cell to cell adhesion, and cell migration. These proteins cycle between active GTP bound and inactive GDP bound states. Guanine nucleotide exchange factors (GEFs) activate GTPase signaling by exchanging GDP for GTP. GTPase activating proteins (GAPs) inhibit the activity of the G-proteins by hydrolyzing GTP to GDP. Mutations or deregulated signaling have been linked to multiple phenotypes of tumorigenesis for both the Rho and Rac signaling pathways. Though the roles of Rho and Rac in tumorigenesis are extensively studied, less is known about which GEFs or GAPs regulate tumorigenic signaling. The proteins Bcr and Abr are of interest as they contain both a Rho GEF (DH/PH) and a Rac GAP domain, giving them the dual ability to both activate Rho and inhibit Rac
signaling. Bcr has recently been shown to regulate the terminal differentiation of keratinocytes via control of Rho GTPase signaling. Interestingly, we have observed an increase in p38 MAPK activity in a range of skin and breast cancer cells in response to Bcr knockdown. p38 mitogen-activated protein kinase (MAPK) is a transcription factor which controls many biological functions including cell growth and apoptosis. We therefore hypothesize that Bcr-mediated Rho GTPase signaling controls cell proliferation via coordination of p38 MAPK activity. In this study, we show that knockdown of Bcr results in a significant decrease in Raf-mediated anchorage-independent growth in soft agar. Our preliminary evidence using pharmacological modulators of Rho and Rac activity suggest that these proteins are involved in mediating p38 MAPK signaling via Bcr. Future studies will determine if regulation of p38 MAPK signaling by Bcr is responsible for its effect on Raf-mediated anchorage-independent growth. Taken together, these data have highlighted a novel link between the Rho GEF/Rac GAP Bcr and coordination of cell proliferation.

P2478
Board Number: B628
An artificial cell-like system linking cell size and GTPase signaling pathways.
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Signaling pathways in human cells are organized in the cytoplasm and have a pivotal importance in defining cell shape and leading tissue morphology. Rho-GTPases signaling proteins are involved in the formation of actin-based structures, such as filopodia and lamellipodia, which are required for cell motility and polarity, and acto myosin filaments that mediate cell division. The interplay of activation and inactivation of GTPases signaling is controlled by Exchange Factors (GEFs) and GTPase activating proteins (GAPs). GEFs and GAPs form patterns of activation by diffusing, oscillating and concentrating in specific subcellular positions. In this study, we test the hypothesis that GTPase activation is patterned by cellular geometry. Our approach is to overcome the complexity of living cells by encapsulating minimal GTPase networks in artificial cell-like systems. Here, I describe my progress in building this system, including the 1) the reconstitution and the in vitro characterization of pathways regulating Cdc42, RhoA, and Rac1 GTPases, 2) the encapsulation and spatial patterning of GTPase regulators in water in oil emulsions. The latter will enable us to determine how pathway organization and cell geometry control the timing and the pattern of Cdc42 activation. Furthermore I propose a model for feedback can lead to pattern of Cdc42 activation.

Cytoskeleton-Membrane Interactions: Septins

P2479
Board Number: B630
Oligomerization properties and structural plasticity of shs1 capped-rod in interaction with membranes.
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Saccaromyces Cerevisiae express five mitotics septins (Cdc10, Cdc3, Cdc12, Cdc11 and Shs1) leading to the formation of two octamers capped by shs1 or/and cdc11 with different structural properties1,2. During cytokinesis, both of them assemble into a filamentous collar at the mother-bud neck in contact with the plasma membrane. This structure is essential for normal morphogenesis and a proper
cytokinesis. Furthermore, Cdc10 is known to interact specifically with phosphatidylinositol-4,5-bisphosphate PI(4,5)P2 in vitro, promoting then filament polymerization and organization on monolayers of one of the octamer.

As cdc11 rods organization and properties are well characterized both in solution and lipid monolayer, we decided to focus on shs1 rods characterization in presence of membrane mimetics studied by (cryo) electron microscopy and cryo-tomography. We highlighted a direct interaction between shs1 subunit and some phosphoinositides, including PI(4,5)P2. This interaction is not as selective as the one characterized for cdc11 octamer. This result led us to dock phosphoinositides polar heads with modeled shs1/cdc11 gtpase domain. The resulting models brought new features concerning shs1/cdc11 interaction with lipids, especially regarding the selectivity of phosphoinositides by septins. Moreover, it appears that presence of membrane stabilizes the NC-interface between shs1 subunits, promoting then the self-assembly of the octamers into paired-filaments and sheets of filaments unseen in solution. The interaction between shs1 rods and the membrane leads to a distortion and a flattening of vesicles, probably in a dynamic way. As a matter of fact, different steps of distortion as well as different organization of shs1 octamers towards these local distortions were observed by cryo-tomography. Finally, it has been proposed that both shs1 and GTP could orchestrate the assembly of cdc11 octamers into the hourglass structure observed during cytokinesis in Saccharomyces Cerevisae. We bring here some clues comforting this hypothesis in vitro, in presence of membrane.


P2480
Board Number: B631
Membrane reshaping by curvature sensitive septin filaments.
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Septins are cytoskeletal proteins that assemble into a variety of supramolecular organizations from paired filaments to bundles, ring like structures or gauzes of orthogonal filaments. Septins are bound to the inner plasma membrane through specific interactions with phosphoinositides. Septins are essential for cytokinesis, participate in the formation of diffusion barrier and might be involved in membrane deformation and rigidity.

Both positive and negative micron-scale curvatures are present at the cleavage furrow (Gaussian “horse saddle” curvatures). To mimic specific curvatures and geometries, we have used PDMS substrates covered with a supported lipid bilayer doped with PI(4,5)P2. “Wavy” PDMS patterned substrates displaying both positive (“bumps”) and negative (“valleys”) curvatures were utilized. Using Scanning electron Microscopy, we have seen that Septin filaments have a preference for negative micrometric size curvatures. On positively curved geometries, septins spontaneously align on the top of the bumps and orient towards null curvature. Besides, this curvature preference is closely related to the ability of septins to reshape and deform membranes. Indeed, bound to Giant Unilamellar Vesicles, septins induce...
striking deformations with regular spikes and hollow micrometric deformations at the surface of liposomes, as visualized by fluorescence microscopy. Smaller vesicles (LUVs of 100-300 nm in diameter), highly positively curved, are flattened by Septin filaments as shown in 3D by cryo-electron tomography. By cryo-EM, we visualize both the septin filaments and the deformed vesicles. In addition, on supported lipid bilayers, septins induce a dramatic reorganization of the lipids in the membrane and deformation of the bilayers as visualized by Atomic Force Microscopy coupled to fluorescence microscopy. Finally, a theoretical model has been generated and accounts for both the visualized deformations and curvature sensitivity which are relevant to describe cytokinesis in vivo.


P2481
Board Number: B632
Dynamic exchange of septin complexes in plasma membrane filaments.
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Septins are a family of cytoskeletal GTP-binding proteins which are highly conserved in eukaryotes. They play a key role in a number of essential biological processes such as cytokinesis, cell polarity and exocytosis. Septin molecules form linear non-polar hetero-oligomeric complexes, which further coalesce into filaments, rings and other higher-order structures. Defects in septin organization and expression have been linked with various pathological conditions, including neurodegenerative diseases and multiple types of cancer. However, despite the growing number of studies, the exact architecture and mechanism of septin filament formation in cells remains unknown. Here we created a genome-edited cell line in which septin 2 is quantitatively coupled to EGFP to investigate septin filaments in vivo and with single molecule microscopy techniques.

We introduce a rat cell line in which all SEPT2 is endogenously tagged with EGFP. We characterize it using live-cell imaging, RT-PCR, and migration and proliferation assays. We visualize a network of ~500 nm-long septin filaments at the plasma membrane by Single-Molecule Localization Microscopy via GFP-specific nanobodies and resolve a systematic ca. 35nm spacing between consecutive SEPT2-EGFP localizations, suggesting the direct assembly of filaments from complexes. Combined with our immunoprecipitation, immunofluorescence and mass spectroscopy data, we identify the septin complex as an octamer consisting of septins 2, 8 or 11, 7 and 9. Using dual color super-resolution imaging we demonstrate that septins 2 and 7 are incorporated into the same filaments and their positions alternate along a filament in a regular fashion confirming an end-to-end arrangement of septin complexes.

We have found that bleached septin filaments recover their fluorescence in vivo and postulate that septins can dynamically exchange within their higher-order structures. To determine the basic unit of septin exchange we have performed stepwise-photobleaching during the turnover of septin structures in the generated cells. We present that by counting the number of EGFP bleaching steps we can identify the newly-recruited septin structures to the plasma membrane. Furthermore, by following the EGFP
fluorescence we resolve septin filaments during their formation in cells. Based on the obtained data we suggest that in filaments septins exchange as single complexes and there is no preferential addition of new septin subunits to the filament ends.

Here we have established a new genome-edited mammalian system for biochemical and microscopy studies of septins. In this cell line, septin complexes assemble into actin-independent filaments in a dynamic fashion. Our data present unprecedented insight into the assembly of septins in live mammalian cells.

P2482
Board Number: B633
Deciphering the regulatory role of Septin9 N-terminus in mammalian septin polymerization.
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Septins are GTP-binding cytoskeletal proteins conserved from yeast to man. In humans there are 13 genes, many of which undergo alternative splicing, giving rise to a large number of isoforms, although the significance of these isoforms remains unknown. The 13 genes fall into 4 subfamilies and it is thought that two copies of a member of each family assemble in an ordered array into hexamers or octamers that serve as the building blocks for larger filaments. In the case of octamers, SEPT9 is located at the terminal positions, indicating that SEPT9-SEPT9 interactions are critical for polymerization. However, the mechanisms that control septin polymerization are not understood. Moreover, SEPT9 undergoes alternative splicing to produce 5 N-terminal splice variants. Importantly, mutations in SEPT9 N termini have been linked to hereditary neuralgic amyotrophy and changes in the relative expression of SEPT9 splice variants have been associated with cancer progression. Since the N termini constitute the interaction surface involved in octameric septin polymerization, it is possible that different isoforms may have different polymerization properties.
To begin to examine the factors regulating septin polymerization, we developed in vitro polymerization assays using septin complexes purified from mammalian cells. Stable mammalian cell lines were generated expressing GFP-FLAG-His6-tagged versions of SEPT9i1, SEPT9i3, SEPT9i4 or SEPT9i5 under the control of an inducible promoter. Ectopic expression of each isoform caused a reduction in the levels of endogenous isoforms as they compete to cap the limited numbers of octameric complexes. Blue native PAGE revealed that the expressed GFP-FLAG-His6 tagged version of Sept9 capped the hexamers to form octamers that are detectable with GFP antibody. Immunopurification using anti-FLAG antibody conjugated to beads allowed isolation of the mammalian septin complexes from these cell lines, as confirmed by Coomassie blue staining after SDS-PAGE and Western blot. Isolated complexes were imaged using fluorescence microscopy and electron microscopy. The majority of septin complexes isolated from this cell line were rod shaped, 32-35nm long and 4-5nm in diameter, consistent with the predicted size for septin octamers. In-vitro polymerization was promoted by dialysis against low salt buffer and micron-long septin filaments and higher order structures were visualized with EM and fluorescence microscopy. Now that we have established a reliable system to produce functional mammalian septin octamers for in-vitro polymerization, we will isolate mammalian septin octamers that are distinguished by different SEPT9 isoforms to determine the importance of isoform specificity on the formation of filaments and higher ordered structures.
Mechanisms controlling micron-scale membrane curvature recognition by septins.
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Shape is an important feature of cells and can be described in terms of membrane curvature. It is relatively well understood how cells generate different shapes, but comparatively little is known about how cells recognize their own shape and use information about their geometry. In yeast and mammalian cells the septin cytoskeleton localizes to sites of micron-scale membrane curvature. How septins recognize micron-scale membrane curvature are not understood. Septins can differentiate between different curvatures in vitro in a minimal system composed of supported lipid bilayers on beads and rods of different diameters, allowing dissection of the mechanisms of septin curvature sensing. We predict that the basis for curvature sensing involves a combination of membrane binding affinity, filament polymerization rates and higher-order structure formation that are tuned by association with different curvatures. There is some support for a polybasic patch that septins use to bind lipid membranes, however what surfaces of septins are capable of membrane binding are not established. Primary sequence analysis revealed an amphipathic helix at the C-terminus Cdc12. Amphipathic helices are used by many proteins that sense curvature on the nanometer scale because of their ability to insert into membranes. We investigated the role of this amphipathic helix in membrane binding and curvature sensing by mutating either the polar or hydrophobic face of the helix, followed by measuring septin adsorption onto lipid-coated beads. In both cases, we found septin adsorption was significantly reduced indicating that this region of the protein can modulate association with curved membranes. Previous work identified a role for septin polymerization in robust curvature sensing. Saturation binding curves on beads of optimum curvatures show that septin adsorption is a highly cooperative process. This cooperativity in assembly on curved surfaces could emerge from polymerization and/or higher-order assembly of filaments. Using scanning electron microscopy imaging of septins assembled on rods of different diameters, we found that septin filament orientation is dependent on the local curvature. On rods, filaments arrange in parallel arrays orthogonal to the positive curvature axis at low radii, at a variety of angles at intermediate radii and parallel to the curvature axis at larger radii. This indicates that curvature influences the arrangement and orientation of septin filaments within a higher-order structure and supports that cooperativity could exist at multiple stages of the curvature-dependent assembly process. This work provides insight into how the septin cytoskeleton binds membranes and uses cooperative assembly to distinguish micron-scale membrane curvatures.

Analysis of septin assembly using optogenetics and in vitro reconstitution.
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Cells build micron-scaled structures from relatively small, nanometer-scaled proteins. The septin cytoskeleton is particularly adaptable in its ability to form micron-scale structures in a variety of shapes, sizes, and functions. Septins are conserved from fungi to mammals and self-assemble into heterooligomeric, rod-shaped complexes 17-32 nanometers in length. These “rods” can polymerize into filaments (hundreds of nanometers long) at the plasma membrane or associated with other cytoskeletal proteins. Septin filaments can be arranged into higher-order assemblies such as laterally associated...
bundles, crosslinked lattices, wrapped gauzes, and curved rings. Higher-order assemblies serve as platforms for protein localization including the polymerization of other cytoskeleton proteins, scaffolds for cell signaling events during morphogenesis, and possibly even act as protein diffusion barriers. How are septin filaments arranged into higher-order assemblies? And how are different assemblies positioned at distinct cortical locations? Genetic studies in multiple model systems have identified a substantial network of factors proposed to regulate septins, including kinases and cell polarity proteins. However, little is known mechanistically about how these septin regulators help form higher-order septin assemblies at the molecular level. To investigate which candidate regulators might position and shape septin assemblies, we used optogenetics in both budding yeast and the filamentous fungus Ashbya gossypii. Optogenetics enable tunable subcellular targeting of a protein of interest to a specified cortical site using focused light. Our results suggest that polarity proteins, such as Cdc42, can both position and shape septin assemblies. In parallel experiments, we reconstitute septin assembly on supported lipid bilayers using purified, recombinant septin complexes co-incubated with recombinant regulators. These combined approaches are revealing how septin filaments can be shaped and patterned into different geometries to build distinct higher-order septin assemblies.

Mechanotransduction 1

P2485
Board Number: B636
The role of focal adhesion-localized calcium sparks in the sensing of extracellular matrix mechanical properties.
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The ability of individual cells to sense and integrate multiple extracellular cues underlies many types of cellular behavior including proliferation, differentiation, and migration. Recently, it has been established that mechanical cues such as extracellular matrix (ECM) stiffness are sensed by cells to direct both normal and pathological phenotypes. Several cellular structures, including integrin-based focal adhesions (FAs), have been shown to be essential for ECM stiffness sensing. Nevertheless, the exact molecular mechanism of ECM stiffness sensing remains elusive. Here we show that mouse embryo fibroblasts increase cell area as substrate stiffness increases and that calcium is necessary for this mechanosensitive response, particularly at high ECM stiffness. Furthermore, fibroblasts plated on stiff substrates display local fluctuations of extracellular calcium entry within FAs, i.e. calcium sparks, indicating that calcium sparks may mediate the detection of stiff ECM. Chemical perturbations showed that these calcium sparks occur via FA-localized stretch-activated ion channels under the control of cellular actomyosin contractility, indicating an intimacy between these mechanosensing-critical calcium sparks and the mechanical activity of the cytoskeleton. Analyzing the properties of calcium sparks at both the small scale along individual FAs and at the large scale across the whole cell clarified the relationship between calcium spark dynamics, FA turnover, and cell migration. Critically, chemical and genetic manipulation allowed us to narrow down the stretch-activated ion channel responsible for sparks and critical for ECM stiffness detection. Thus, we present FA-localized calcium sparks as a means for cells to detect ECM stiffness by converting the mechanical activity of the cell against the ECM to a discrete intracellular biochemical signalling event.
P2486
Board Number: B637
Substratum stiffness modulates proliferation downstream of Wnt3a by regulating integrin-linked kinase and frizzled-1.
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The Wnt/β-catenin signaling pathway controls a variety of cellular behaviors, aberrant activation of which is closely associated with tumor progression in several types of cancer. The same cellular behaviors are also affected by the mechanical properties of the extracellular matrix (ECM), which induces signaling through integrins and integrin-linked kinase (ILK). However, it remains unclear how cells integrate mechanical signals such as ECM stiffness with biochemical signals such as Wnt. Here, we investigated the mechanisms by which cell proliferation is regulated by ECM stiffness and Wnt signaling. We found that treatment with Wnt3a increased the proliferation of cells cultured on stiff substrata, with compliances characteristic of breast tumors, but not on soft substrata, with compliances comparable to that of normal mammary tissue. Depleting ILK rendered cells unresponsive to Wnt3a on both substrata. Ectopic expression of ILK permitted Wnt3a to induce proliferation of cells on both soft and stiff substrata. These results suggest that ILK is required for proliferation downstream of Wnt3a on stiff substrata and sufficient to permit proliferation downstream of Wnt3a on soft substrata. We further showed ILK regulates expression of the Wnt receptor Frizzled-1 (Fzd1), suggesting the presence of a positive feedback loop between Wnt3a, ILK, and Fzd1. These findings suggest that tissue mechanics regulates the cellular response to Wnt under physiological and pathological microenvironmental conditions.

P2487
Board Number: B638
Stopping Transformed Growth with Rigidity Sensing Modules: Turning a Devil into an Angel.
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The growth of non-transformed cells depends upon rigid matrices (anchorage-dependent growth). In contrast, a hallmark of cancer cells is their ability to grow on soft surfaces (transformed phenotype), i.e. on soft agar. One plausible explanation for transformation is that it results from depletion of the rigidity-sensing apparatus. Recent findings reveal that rigidity sensing depends upon a modular complex of cytoskeletal proteins and tyrosine kinases that produce sarcomere-like local contractions. Depletion of any one of several cytoskeletal proteins in the sensing module blocks local contractions and rigidity sensing. It also promotes growth on soft surfaces. To test if this is relevant to cancer, we analyzed different transformed cancer cell lines and discovered that they all lacked rigidity sensing. Further, different cytoskeletal proteins involved in mechanosensing were depleted in each cancer. Restoration of normal cytoskeletal protein levels, either of myosin-IIA in Cos7 cells (Cos7-IIA) or of tropomyosin 2.1 (Tpm2.1) in MDA-MB-231 cells (231-Tpm) restored normal anchorage-dependent growth and rigidity sensing. Importantly, reciprocal depletion of Tpm2.1 and myosin-IIA from the Cos7-IIA and 231-Tpm cells, respectively, blocked rigidity sensing and enabled transformed anchorage-independent growth.

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Thus, depletion of any one of multiple cytoskeletal components of rigidity-sensing modules enables transformed growth in metastatic and non-metastatic cells.

P2488
Board Number: B639
Myofibroblast Differentiation of Fetal Fibroblasts is Inhibited in Response to ECM Rigidity and TGF-b1.
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During dermal wound healing, extracellular matrix (ECM) rigidity and transforming growth factor-b1 (TGF-b1) induce fibroblasts to differentiate into myofibroblasts in the wound bed. Myofibroblasts generate large contractile forces using stress fibers rich in a-smooth muscle actin (a-SMA) that are transduced via focal adhesions to excessively contract and remodel the ECM leading to scarring and fibrosis. In contrast, injured fetal skin heals scarlessly without myofibroblast activity suggesting that fetal fibroblasts exert smaller contractile forces due to their unique wound environment. However, fetal wounds have less TGF-b1 and are more compliant than adult wounds. Therefore, it remains unclear whether the lack of myofibroblast differentiation is a result of inherent properties of fetal fibroblasts or biochemical and biomechanical differences in fetal wounds. In this study, we tested whether physiologic wound rigidities and TGF-b1 can mediate actomyosin contractility in fetal fibroblasts and promote myofibroblast differentiation. Using traction force microscopy and polyacrylamide gels (PAAs) that mimic the biomechanical stages of healing wounds, we found that traction forces and focal adhesion formation by fetal fibroblasts were impaired on rigid PAAs that mimic late stage granulation tissue when compared to their adult counterparts. On rigid PAAs with TGF-b1 stimulation, we found that fetal fibroblasts exhibited no differences in traction forces, focal adhesions, or a-SMA while all three increased for adult fibroblasts indicating myofibroblast differentiation. Overall, our data indicate that fetal fibroblasts exhibit a unique contractile phenotype that prevents myofibroblast differentiation due to altered mechanical responses to ECM rigidity and TGF-b1.

P2489
Board Number: B640
Plasma Membrane and Cell Surface Mechanics in Embryonic Stem Cells.
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Embryonic Stem Cells (ESCs) are able to generate any tissue in a given organism; this ability is called pluripotency. Membrane mechanical properties, such as tension, have been shown to influence cell behavior and differentiation in many systems. However, very little is known about plasma membrane dynamics and mechanics in ESCs. Here, we investigate the link between the mechanical properties of the plasma membrane and the molecular factors that lead to exit from pluripotency. Using optical tweezers, we show that naïve mouse ESCs have a higher effective membrane tension compared to cells exiting pluripotency. We show that naïve cells have a higher expression and level of activation of protein regulating membrane-to-cortex attachment and that affecting these proteins results in defects in exiting
pluripotency. We also observe an increase in expression and differential localization of caveolae components as ESCs exit pluripotency. Caveolae are membrane tension sensing lipid raft and have been suggested to play an important role in other stem cell types, such as human mesenchymal stem cells, and are sensitive to change in membrane tension. Together, our data show that membrane organization and tension change during exit from naïve pluripotency in mouse ESCs. We are currently investigating the impact of these changes on cellular fate transitions.

P2490
**Board Number: B641**
**Fibrillar Force Generation by Fibroblasts Depends on Microenvironmental Stiffness.**

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Fibroblasts in connective tissue interact with a fibrillar extra-cellular matrix (ECM) that often restricts their shape along one-dimension (1D, along the fiber). At the same time, the fibroblast responds to and affects the mechanical nature of its microenvironment which consists of the inter-woven fibrillar ECM, other matrix components and cells. To simultaneously constrain a fibroblast along 1D and still let it mechanically interact with an extended microenvironment of defined stiffness, we plated NIH 3T3 fibroblasts on micropatterned 1.5 \(\mu\)m-wide fibronectin lines on polyacrylamide gels of stiffness (Young’s modulus) 4, 7 or 15 kPa. The cells adhered to the fibronectin lines, exhibiting 1D cell-matrix adhesions marked by paxillin and vinculin. We observed that cell lengths only modestly increased with hydrogel stiffness, at a rate of 3 \(\mu\)m/kPa. We then used traction force microscopy to quantify the cellular traction force transmitted to and deforming the substrate and the associated strain energy. We found that the strain energy stored in the substrate increased with substrate stiffness, more than doubling between the lower (4 kPa) and higher substrate stiffness (15 kPa) considered here. Finally, we assessed whether Arp2/3, which plays a key role in fibrillary cell migration, also affected force generation in this context. Pharmacological inhibition of Arp 2/3 modestly increased cell length but decreased the strain energy associated with traction force exertion. Thus, our data show that fibrillar force generation by fibroblasts is dependent on the stiffness of the microenvironment and on nucleation promoting factors such as Arp2/3.

P2491
**Board Number: B642**
**Force triggers YAP nuclear entry by mechanically regulating transport across nuclear pores.**

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YAP is a mechanosensitive transcriptional activator with a critical role in cancer, regeneration, and organ size control. Here we show that force applied to the nucleus directly drives YAP nuclear translocation by decreasing the mechanical restriction of nuclear pores to molecular transport. We demonstrate that the
nucleus only connects mechanically to the cytoskeleton above a threshold in substrate rigidity, allowing forces exerted through focal adhesions to reach the nucleus. This leads to nuclear flattening, which increases YAP nuclear import by decreasing the mechanical restriction of nuclear pores to molecular transport. This restriction is further regulated by the mechanical stability of the transported protein. Control of YAP translocation by nuclear force is independent of focal adhesions, the actin cytoskeleton, substrate rigidity, cell-cell adhesion, and the Hippo pathway. Our results unveil a mechanosensing mechanism mediated directly by nuclear pores, demonstrated for YAP but with potential general applicability in transcriptional regulation.

P2492
Board Number: B643
Modulation of T cell Priming by Dendritic Cell Stiffness.
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Priming of T-cell responses by dendritic cells (DCs) is essential for protective immunity to pathogen invasion and cancer. T cell activation requires intimate cell-cell interactions at a site termed the immunological synapse (IS) and mounting evidence indicates that this process involves mechanotransduction. In response to inflammatory stimuli, DCs undergo a maturation process during which their phenotype changes from one specialized for pathogen surveillance to one optimized for T cell priming. During maturation, T cell stimulatory ligands are upregulated, and cytoskeletal proteins are reprogrammed to downregulate antigen uptake and facilitate migration to lymphoid tissues. We hypothesized that maturation also alters the biophysical properties of the DC cortex, and that these properties represent an unexplored control point for T cell priming. Using atomic force microscopy, we show that upon LPS-induced maturation, DC stiffness increases from \textasciitilde2kPa to \textasciitilde4kPa in an actin-cytoskeleton dependent process. Using inhibitors and DCs from KO mice, we identify several actin regulatory pathways downstream of Rho GTPases involved in modulating DC stiffness. Interestingly, activating T-cells with agonist-coated acrylamide hydrogels of different compliance reveals a threshold for T cell activation within the range of 1-4 kPa, similar to the range over which the DC stiffness changes. The specific stiffness at which T cells are activated depends on the concentration of the stimulating molecules, indicating that mechanical cues are integrated with other stimulatory and co-stimulatory signals. Finally, by engineering DCs with altered stiffness, we show that the stiffness of mature DCs directly correlates with their ability to prime ex-vivo T-cells. Taken together, these findings indicate that the stiffness of the DC cortex provides a novel form of co-stimulation that has yet been considered.

P2493
Board Number: B644
Force Dynamics During T Cell Activation.
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T cell activation is an essential step in the adaptive immune response. The binding of the T cell receptor (TCR) with antigen triggers signaling cascades and cell spreading. Physical forces exerted on the TCR by the cytoskeleton have been shown to induce signaling events. While cellular forces are known to depend on the mechanical properties of the cytoskeleton, the biophysical mechanisms underlying force induced activation of TCR-antigen interactions unknown. Here, we use traction force microscopy to
measure the force dynamics of activated Jurkat T cells. The movements of beads embedded in an elastic gel serve as a non-invasive reporter of cytoskeletal rheological properties and molecular motor dynamics. We examined the statistical structure of the force profiles throughout the cell during signaling activation. We found two spatially distinct active regimes of force generation characterized by different time scales and rheological properties of the cytoskeleton. Typically, the interior of the cells was found to be more active than the periphery. Inhibition of myosin motor activity altered the correlation time of the bead displacements indicating additional sources of stochastic force generation. Our results indicate a complex interaction between myosin activity and actin polymerization dynamics in producing cellular forces in immune cells.

P2494

Board Number: B645

B cell mechanosensing: is it a myth?

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Mechanosensing capability of lymphocytes plays an important role in shaping the initiation of an immune response. Although it is evident that B lymphocytes use such capabilities to discriminate the stiffness features of the substrate presenting antigens, but the underlying molecular mechanism remains unexplored. Here, through a combination of molecule imaging, genetic ablation and pharmacological approaches, we demonstrated that B cells with genetic ablation of each of the early BCR signaling molecules, Lyn, Syk, PLCγ2, Btk, BLNK or PKCβ, lost the capability to discriminate substrate stiffness during initiation of B cell activation, while exogenous addition of each molecule rescued the defects, suggesting B cell discrimination of substrate stiffness is dependent on the BCR signaling. In marked contrast, BCR signaling-independent BCR accumulation in the initiation of B cell activation is insensitive to substrate stiffness. Mechanistically, we showed that PMA induced activation of PKCβ can bypass the requirements on Btk and PLCγ2 for the substrate stiffness discrimination ability of B cells. Importantly, we excluded the contribution of PKCβ mediated NF-κB activation to the discrimination capabilities of B cells, instead we provided evidence for a model that PKCβ-dependent activation of focal adhesion kinase (FAK) is required in these events by the FAK-mediated potentiation of B cell spreading and adhesion responses. FAK inactivation or deficiency impaired B cell discrimination against substrate stiffness. As supporting evidence for this model, we showed that the presence of adhesion molecules, ICAM-1 or VCAM-1, greatly enhanced B cell’s capability to discriminate substrate stiffness. In contrast, integrin inactivation, drastically impaired the capability of B cell to discriminate substrate stiffness. Lastly, rheumatoid arthritis (RA) patient B cells strikingly exhibited disordered capability to discriminate substrate stiffness in contrast with B cells from healthy controls. All these data shed light on the precise molecular mechanism of how B cells discriminate substrate stiffness through a BCR signaling and PKCβ mediated FAK activation dependent manner in the process of B cell activation, improving our understanding of the sophisticated mechanosensing capability of B cells.
P2495
Board Number: B646
The lamellipodium is a myosin independent mechanosensor.
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Numerous cellular processes have been shown to be sensitive to changes in the mechanical properties of their extracellular environments. Here we show that cell spreading is acutely impacted by the stiffness of the substrate and displays a biphasic behavior with a transition stiffness between ~5-8 kPa. While this has long been thought to depend on cells' ability to generate tension, here we demonstrate that this behavior is independent of myosin activity. Cells on soft substrates actively try to spread, but display impaired nascent adhesion formation. Addition of Mn2+, surprisingly, recovers the ability of cells to spread on soft substrates, in addition to the other morphological characteristics of cells on rigid surfaces. Using a computational model to simulate integrin catch-bond kinetics with the extracellular matrix, we find that changes to both the unloaded and peak force lifetimes by addition of Mn2+ regulate the fraction of bound integrins. Together these data suggest that actin retrograde flow driven forces in the lamellipodium disrupt integrin binding on soft substrates, thereby impairing clustering required to form nascent adhesions and inhibiting cell spreading. These results illustrate that nascent adhesion formation can act as a myosin independent mechanism to control cell spreading, and thereby potentially other stiffness sensing processes.

P2496
Board Number: B647
Control of cell morphology, stiffness, and differentiation by substrates with independently tunable elasticity and viscous dissipation.
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The mechanical properties of extracellular matrices control the function of cells during development, homeostasis, and wound healing. Studies of cellular responses to biomimetic soft materials have been largely restricted to soft hydrogels and elastomers that have stiffness values independent of time and extent of deformation, so the effective stiffness can be unambiguously related to its effect on cells. Real tissues, however, often have loss moduli that are 10 to 20% of their elastic moduli and therefore behave as viscoelastic solids on a timescale relevant to mechanical sensing. The response of cells to a time-dependent viscous loss is largely uncharacterized because appropriate viscoelastic materials are lacking for quantitative studies. Here we report the synthesis of soft viscoelastic solids in which the elastic and viscous moduli can be independently tuned to produce gels with viscoelastic properties that closely resemble those of soft tissues. Systematic alteration of the viscous and elastic components in viscoelastic substrates demonstrated the time dependence of cellular mechanical sensing and the influence of viscous dissipation on the mechanical signals to which cells respond.
Microtubule-based control of motor-clutch system mechanics in glioma cell migration.
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Microtubules are attractive chemotherapy targets for microtubule-targeting agents (MTAs), which disrupt cell migration in nanomolar doses. Cell migration mechanisms have been largely studied in the context of coordinated actomyosin self-assembly and force generation, and cell-substrate adhesion dynamics, while the mechanisms by which MTAs block cell migration are poorly understood. Focusing on MTAs that bind to two clinically relevant binding sites on tubulin – the taxane site and vinca site – we show that both types of MTAs disrupt stiffness sensitive glioma cell polarization and random motility on compliant hydrogels. Measuring traction force, actin retrograde flow, and microtubule dynamics, we identify dose-dependent mechanisms for each class of MTA: paclitaxel (a taxane site binding MTA) promotes microtubule polymer assembly and decreases strain energy, while vinblastine (a vinca site binding MTA) disassembles polymer and increases actin retrograde flow and strain energy. Results from a stochastic motor-clutch model suggest that MTA site-specific mechanisms are achieved by disrupting a balance between actomyosin contraction and adhesion, rather than a direct mechanical role of microtubules in traction force generation. Finally, we employ a cell migration simulator that incorporates actin protrusion dynamics, force balances, and mass conservation principles to investigate other cell systems-level effects of MTAs. Interestingly, slowing the extension rate of individual protrusions suppresses stiffness sensitivity of simulated cell polarization and random motility, as observed in experiments, providing a systems-level theoretical basis for the actions of MTAs in controlling cell migration.

Sea anemone as a model to study inner ear hair bundle mechanotransduction and Usher proteins interactions.
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Cnidarians use a very specialized structure to capture their preys and protect themself : the nematocyst. Nematocyt discharge is controlled by a mechanosensitive actin made structure located on the tentacles : the hair bundle. Hair bundles of sea anemones are structurally close ancestors of vertebrate hair bundles and may constitute a valuable model to study inner ear vertebrate hair bundle functions. It has been previously shown that some molecules crucial for vertebrate hair bundles shaping and function are present in sea anemone. In the inner ear of superior vertebrates, including human, USH1 and USH2 proteins (defective in the Usher syndrome type 1 and type 2, respectively associating deafness and blindness) are localized in the auditory hair bundle, the mechanosensitive structure receptive to sound stimulation. The presence of a homolog of cadherin 23, i.e. the USH1D protein in vertebrates, has been
demonstrated some years ago in sea anemone. Here we show that other USH1-protein homologues could also be observed by in situ immunohistochemistry within sea anemone *Anemonea viridis* hair bundles, by using antibodies designed against specific vertebrates USH1 proteins. In parallel, by using scanning electron microscopy approach, we have done a morphological comparison between the different types of sea anemone hair bundles structures and frog and mouse inner ear mechanoreceptor neurons hair bundles.

**P2499**

**Board Number: B650**

*Magnetic Bead Pulling Forces on Soft Substrates Calibrated Using Traction Force Microscopy.*

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Localized application of exogenous forces on soft biomaterials and cells is the essential first step in studying their response to mechanical perturbations. Here, we demonstrate the in situ determination of magnetic bead pulling forces on a soft substrate using traction force microscopy. A magnetically permeable pencil-shaped probe was machined and its tip electro polished. An adapter was used to mount the needle, along with permanent neodymium magnets, to a micromanipulator. Magnetic beads were coated with Protein A and allowed to bind to an Fc coated 100 Pa polyacrylamide gel. We found that magnetic beads bound to the polyacrylamide gels (via protein A to Fc bonds) and moved, but did not detach from the gel when approached by the probe within a magnetic bead-probe distance of tens of microns. We could thus determine the force-distance plot showing the force exerted on the magnetic bead by the magnet probe – and thus exerted by the magnetic bead on the soft polyacrylamide substrate – as a function of the distance between the probe tip and the magnetic bead. We have thus devised a set-up where localized traction forces can be applied on soft materials like cells using a magnet probe. We demonstrated that forces of 1-10 nN can be locally exerted – a range ideal for cellular mechanobiology studies. Application of this method for the in situ measurement of localized exogenous forces exerted on cells can enable dissection of cellular force transmission and transduction pathways.

**P2500**

**Board Number: B651**

*Normal extracellular matrix restricts cancer cell proliferation via mechanosensitive epigenetic reprograming.*

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Interactions between cells and the extracellular matrix (ECM) are integral for tissue regulation and homeostasis. While cancer stroma plays a well-characterized role in tumor progression, emerging evidence is now starting to point to a specific contribution of the ECM itself. Moreover, the tumor-suppressive properties of normal, non-cancerous stromata remain poorly understood. We show that the ECMs generated by normal (NF) and cancer-associated fibroblasts (CAF) are physically distinct and regulate cancer cell proliferation. Normal, soft ECM triggers the downregulation and nuclear exit of histone demethylase JMJD1a, leading to localized H3K9 hypermethylation and persistent growth

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inhibition in carcinoma cells. Mechanoresponsive targets of JMJD1a include Hippo pathway transcriptional coactivators YAP/TAZ, wherein active JMJD1a directly binds and demethylates the WWTR1 (TAZ) promoter to drive TAZ expression. JMJD1a localization is unperturbed by the actomyosin targeting drugs cytochalasin D and blebbistatin, indicating that JMJD1a activity is independent of intracellular contractility and inside-out mechanosignaling. Finally, we explore upstream mechanotransduction pathways and coregulatory transcription factors that may help modulate the JMJD1a-mediated mechanosensitive transcriptional regulation. Taken together, the results show that soft ECM, like normal stroma, restricts cancer cell proliferation through stiffness-dependent epigenetic modulation, underlining the significance of biophysical tumor-stroma interplay.

P2501
Board Number: B652
Using proximity based biotin identification (BioID) to identify mechano-sensitive interactions surrounding α-catenin.
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Protein-protein interactions are the molecular basis of cell signaling. Recently, proximity based biotin identification (BioID) has emerged as an alternative approach to traditional co-immunoprecipitation. In this protocol, a mutant biotin ligase promiscuously labels proximal binding partners with biotin, and resulting biotinylated proteins are purified using streptavidin conjugated beads. This technique does not require preservation of protein complexes in vitro, making it an ideal approach to identify transient or weak protein complexes. Using MDCK epithelial cells expressing BirA* tagged α-catenin, we analyzed the biotinylation of proteins surrounding α-catenin, a component of the E-cadherin complex at cell-cell contacts. To effectively release biotinylated proteins bound to streptavidin conjugated beads, we designed a series of experiments to determine optimal binding and elution conditions. Interestingly, the presence of SDS and IGEPAL-CA630 during the incubation with streptavidin conjugated beads was the key to how effectively biotinylated proteins eluted from the beads with excess biotin and heating. High SDS concentration during binding of biotinylated proteins to streptavidin conjugated beads promoted efficient release of the proteins from the beads, whereas high IGEPAL-CA630 concentration prevented efficient release. Using mass spectrometry analysis along with this protocol, we identified potential force sensitive binding partners of α-catenin. This proximal biotinylation analysis is applicable to a wide range of proteins to identify their force sensitive binding partners and ultimately uncover the force sensitive interactome.

P2502
Board Number: B653
The epithelial circumferential actin belt regulates YAP/TAZ through nucleocytoplasmic shuttling of Merlin.
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Circumferential actin belts underlying the adherens junctions of columnar epithelial cell monolayers control intercellular surface tension and cell shape to maintain tissue integrity. Yes-associated protein (YAP) and its parologue TAZ are proliferation-activating transcriptional coactivators that shuttle between the nucleus and cytoplasm in a context-dependent manner. In most cases, nuclear localizing YAP/TAZ promotes cell proliferation through activating transcription factor TEAD. Previous studies suggest the
importance of stress fibers, one of the major actin cytoskeleton, on the regulation of YAP nuclear localization; however, the effect of the circumferential actin belt, the epithelial cell-specific actin cytoskeleton, on YAP localization remains to be clarified. In low-density cultured columnar epithelial cells, where proliferation is promoted by nuclear YAP/TAZ, circumferential actin belts are poorly organized. On the other hand, in high-density cultured columnar epithelial cells, where proliferation is suppressed, nuclear localization of YAP/TAZ is inhibited, and circumferential actin belts are well developed. By manipulating actin fiber tension in columnar epithelial cell monolayers, we demonstrate that circumferential actin belt tension suppresses YAP/TAZ nuclear localization and their activities at high cell density. In addition, we revealed that this suppression of YAP/TAZ nuclear localization requires Merlin, an F-actin binding protein associated with adherens junctions. Overexpression of wild-type Merlin suppressed YAP/TAZ nuclear localization, however, a Merlin mutant lacking NESs failed to suppress YAP/TAZ nuclear localization, suggesting that Merlin nuclear export sequences (NESs) are critical for its function. Merlin physically interacts with YAP/TAZ, and nuclear export sequences of Merlin are required for the suppression. Together with the observation that the association between E-cadherin and Merlin was diminished by tension in circumferential actin belts, our results support a novel mechanism whereby contraction of the circumferential actin belt promotes Merlin release from E-cadherin, released Merlin undergoes nucleocyttoplasmic shuttling and mediates nuclear export of YAP/TAZ through formation of the Merlin-YAP/TAZ complex.

P2503
Board Number: B654
Mechanosensing in endothelial cells involves novel heparin receptor transmembrane protein 184A.
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Endothelial cells (ECs) make up the blood vessel interface and provide a semi-permeable barrier between blood and tissues. Blood flow through the vessels exerts dynamic physical forces, such as fluid shear stress (FSS), that dictate vascular patterning during embryonic development, regulate vessel size and integrity throughout adulthood, and maintain EC function at the molecular level. Several proteins act as mechanosensors to transduce information about the intensity, duration, and direction of FSS. Mechanotransduction is a coordinated process that leads to changes in gene expression and ultimately promotes decreased proliferative signaling, cytoskeletal changes, and realignment in the direction of flow. PECAM-1, VE-cadherin, and VEGFRs expressed at adherens junctions form a complex that senses shear and initiates many signaling pathways that lead to actin stress fiber formation. Luminal glycosylated proteoglycans such as syndecans (SDC) and glypicans interact directly with growth factors and plasma proteins in the blood to transduce force directly to the cytoskeleton. Although much is known about the complex primary mechanosensing systems required to sense and respond to FSS, knowledge of mechanisms underlying the integration of these systems and the involvement of potential cofactors is incomplete. Our laboratory has identified a novel receptor, transmembrane protein 184A (TMEM184A), that is expressed in vascular cells and binds specifically to heparin. TMEM184A is required for heparin-induced effects on proliferative signaling, inflammation, and cytoskeletal dynamics in vitro. We hypothesize that TMEM184A is also involved in mechanosensing through interactions with endogenous heparan sulfate proteoglycans (HSPGs). Using immunofluorescence microscopy and immunoprecipitation, we show that TMEM184A interacts with VE-cadherin, VEGFR2, and HSPGs in primary bovine and rat aortic ECs. Upon onset of laminar FSS (5 min – 2 hours), TMEM184A traffics to the surface and clusters similarly to HSPGs. Overall surface TMEM184A expression changes to evenly

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distributed punctate clusters in cells acclimated to FSS (up to 24 hours). ECs that overexpress TMEM184A align normally and have enhanced VE-cadherin expression at adherens junctions. Degradation of endogenous HS with heparinase results in decelerated clustering of TMEM184A and impaired cell elongation and alignment. These data support TMEM184A functioning as a modulator of mechanotransduction. Further investigation is needed to confirm this potential role for TMEM184A during EC responses to physiological FSS.

P2504  
**Board Number: B655**  
**Mechanical behaviors of catch bonds under varied loads.**  
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Within the framework of two-state models, we construct two types of catch bonds that have a similar force-lifetime profile upon a constant force-clamp load. However, when a single catch bond of either type is subjected to varied forces, we find that they can behave very differently. We further find that a cluster of catch bonds of either type generally becomes unstable when subjected to a periodically oscillating force. These results suggest that it is necessary to further differentiate those bonds that are all phenomenologically referred to as “Catch bonds”.

P2505  
**Board Number: B656**  
**Mechanosensitivity in LIM domain proteins.**  
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The actin cytoskeleton is used to build major load-bearing structures in the cell including stress fibers, muscle sarcomeres and the cytokinetic ring. These structures generate and respond to mechanical forces while maintaining tension homeostasis. The LIM (Lin11, Isl-1 & Mec-3) domain superfamily is heavily associated with these load-bearing structures and a many LIM domain proteins have been implicated in various studies probing mechanoresponsive elements of the mammalian cell. Zyxin and Paxillin are well-studied members of this family and are known to rapidly localize to strain sites in these stress-bearing elements where they aid in tension homeostasis. Zyxin LIM domains are necessary and sufficient for localization to stress fiber strain sites in mammalian cells. Surprisingly, the LIM domains from fission yeast Paxillin-like 1 (Pxl1) are also sufficient for its localization to strain sites in mammalian cells as well as the contractile ring in fission yeast. This suggests that LIM recognition of strain sites existed in the common ancestor of yeast and humans and is a conserved LIM domain function. To detect sequence signatures in LIM and determine when mechanosensitivity arose in evolution, we are screening for this function in mammalian LIMs as well as LIM domains across the phyllogenetic tree. To study the molecular mechanism of LIM mechanosensitivity, we have reconstituted LIM domain localization to stressed actin networks containing purified alpha-actinin and Myosin II. We observe that LIM localization requires Myosin II activity. We hypothesize that Pxl1 and Zyxin LIM domains recognize a stress-induced deformation of either Actin or Alpha-actinin.

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Intermediate Filaments

P2506

Board Number: B657
ADP-ribosylation of vimentin induces changes in morphology and motility of microglia through enhancing phosphorylation of vimentin at Ser56 and disassembly of vimentin filaments.
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Microglia are the immune effector cells in the central nervous system (CNS). Pathological stimuli such as neuronal injury induce transformation/activation of resting microglia with ramified morphology into a motile amoeboid form. It has been shown that ADP-ribosylation by poly (ADP-ribose) polymerase (PARP) is required for TNF-α-induced microglial activation and our goal is to get mechanistic insights how ADP-ribosylation contributes to dramatic changes in morphology and motility of microglia. Through the use of highly specific AF1521 macrodomain beads that can bind to ADP-ribose, poly-ADP-ribosylated (PARylated) proteins were pulled down from lysates of resting or activated microglia. SDS-PAGE showed a few prominent bands eluted only from activated microglia, and these proteins were identified by Mass Spectroscopy. Surprisingly, the most prominent PARylated band was vimentin which is a type III intermediate filament (IF) protein. We found that ADP-ribosylation of vimentin is required for the disassembly of vimentin filaments and the change of morphology upon activation of microglia with LPS. Vimentin gets phosphorylated at, at least, five different serine/threonine residues and vimentin assembly/disassembly have been reported to be affected by these phosphorylations. We found that veliparib (ABT-888), a PARP inhibitor, blocks the increase of phosphorylation of vimentin at Ser56, suggesting that ADP-ribosylation is required for Ser56 phosphorylation. Further investigation revealed that U0126, an ERK1/2 inhibitor, significantly reduced the ADP-ribosylation of vimentin, indicating that ERK1/2 activation upon microglia activation plays a role in the regulation of PARP activity. These results suggest that ADP-ribosylation of vimentin takes an important part in the regulation of changes in the morphology and motility of microglia upon activation.

P2507

Board Number: B658
The role of vimentin intermediate filaments in confined cell migration.
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The ability of cells to deform and migrate through tight constricting spaces in tissues is important for biological processes, such as embryogenesis, wound healing, and cancer metastasis, and depends on the mechanical properties of the cell cytoskeleton. Migratory and invasive cells often express and upregulate the intermediate filament protein vimentin. Yet, little is known about the role of vimentin in three-dimensional (3D) cell motility. To this end, we fabricate micro-fluidic channels of varying size and ligand surface coatings and use time-lapse imaging to visualize cell migration in a knocked-out model of vimentin in mouse embryo fibroblasts (mEFs). Unlike two-dimensional flat surfaces where vimentin-null mEFs exhibit impaired migratory behavior, their migration in micro-channels is enhanced, outpacing wild-type mEFs. In particular, vimentin-null cells enter channels more quickly and move at faster speeds compared to wild-type cells in collagen type 1 coated channels. The impeding effects of vimentin on cell
motility is strongest in small channels where cell deformations are highest; however, the use of fibronectin surface coating equalizes the 3D motility of the two cell types. Live nuclear staining reveals that the cell nucleus in vimentin-null cells undergoes higher deformations and rates of damage than wild-type cells. Our results demonstrate that vimentin hinders cell motility through small pores and suggest that vimentin networks serve a protective role during migration by providing mechanical resistance to large strains that can cause nuclear damage. This work may be of significant importance in understanding the links between vimentin expression and invasive cell behavior.

**P2508**

**Board Number: B659**

**A role for 14-3-3 in recruitment of keratin filaments to mechanically sensitive cadherin junctions.**

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Intermediate filament cytoskeleton forms vital networks that are increasingly being shown to be subject to dynamic change, despite early characterization as static elements in cells. Dramatic remodeling of the keratin intermediate filament network has been described as an essential process required for collective cellular movements that occur during early developmental morphogenesis of the frog *Xenopus laevis*¹. Transduction of mechanical forces across cell junctions in these tissues results in rapid reorganization of cytosolic keratins to the junctions through a mechanism that is at present not understood. Although previous research has demonstrated that 14-3-3 proteins facilitate dynamic protein exchange within keratin filaments², it remains to be seen whether these proteins have a role in network reorganization across cellular compartments.

In this work we propose that 14-3-3 proteins regulate keratin reorganization dynamics in *Xenopus* mesendoderm. Utilizing immunofluorescence, we found that 14-3-3 proteins display different expression and sub-cellular localization across the tissues of the gastrulating embryo and co-localize with keratins at cell-cell junctions in migrating mesendoderm. Employing LC/MS-MS, we identified Keratin 19 as a novel intermediate filament target of 14-3-3 proteins in the whole embryo and more specifically mesendoderm tissue. We subsequently used co-immunoprecipitation of endogenous proteins to demonstrate that 14-3-3 proteins associate with Keratin 19 as well as C-cadherin. To gain insight into the relationship between 14-3-3 proteins and keratin reorganization, we induced expression of a well-described ‘R18’ 14-3-3 inhibitor³. Performing FRAP experiments with Keratin 19-GFP as the target in migrating mesendoderm explants, we found that inhibition of 14-3-3 results in reduced recruitment of keratin proteins to bleach zones. Additionally, dissociation and reassembly of explanted mesendoderm tissue demonstrated that R18-mCherry expressing cells fail to recruit keratin filaments to newly assembled cell-cell junctions. Taken together, these findings indicate that 14-3-3 acts on keratin intermediate filaments and is involved in their reorganization to sites of cell adhesion. These results imply the possibility of a novel role for 14-3-3 as a transmitter of mechanosensitivity to cytoskeletal scaffolds at cellular sites that experience tension.

**P2509**

**Board Number: B660**

*Influence of nebulin on the assembly mechanics of desmin intermediate filaments.*

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Desmin intermediate filaments bind to nebulin, an integral actin-binding sarcomere protein, forming a direct physical link between the intermediate filament cytoskeletal network and the Z-discs of myofibrils in muscle cells. As the main intermediate filament protein in myocytes, desmin maintains the structural organization of sarcomeres by providing their alignment and integrating their function to that of the mitochondria. Our research investigated how nebulin impacts the assembly of desmin and desminopathy–associated desmin mutants in vitro. Three dimensional electron-microscopy in vitreous ice shows that nebulin appears to organize desmin filament bundles into networks. Atomic force images taken 10 minutes after initiation of assembly showed that the mean length of mutant desmin E245D was shorter with or without nebulin as compared to that of WT desmin. Force displacement measurements showed that nebulin binding dramatically decreased the elasticity of mutant desmin networks. Networks assembled from equal amounts of wildtype and mutant desmin filaments and nebulin were significantly more fluid as compared to wildtype desmin-nebulin networks. Together, our results indicate that nebulin binding to desmin has a significant influence on the assembly properties of desmin. This influence likely is to efficiently coordinate sarcomere function within the myofibril during its changing physiology.

**P2510**

**Board Number: B661**

*Adaptive Multiple Orientation Analysis for the Segmentation of Intermediate Filament Networks.*

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Intermediate filaments (IF) typically form complex overlapping cytoplasmic and nucleoplasmic networks and meshworks in mammalian cells. In order to accurately detect and analyze these networks, we propose a computational image analysis method that can detect ridge-like structures with variable orientation resolution. IF often join together in junctions that involve two or more directions. These junctions may be physical in nature or due to two dimensional projections of three dimensional structures. Multiple orientations can complicate the segmentation and detection of these cellular structures. Many existing methods cannot distinguish between two structures that meet at small acute angles. Methods such as Canny-based ridge detection or anisotropic diffusion are limited both in terms of the number of orientations that can be detected at a point in an image and their orientation resolution. Since the number of structures and their orientations may not be known a priori, we propose a method where the orientation resolution can be directly selected as a parameter such that an arbitrary number of joining structures can be detected. Furthermore, the method can adaptively select the minimum orientation resolution needed. By doing so, we improve the spatial localization of the detected orientations. Our method is based on steerable wavelets and applies concepts from the analysis of scale-space to the orientation dimension to form a variable resolution orientation space. This

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orientation space analysis is orthogonal to scale analysis and thus can also be combined with existing methods that evaluate scale. As a test, we apply our method to light microscopy images of IF including elongated structures formed by vimentin and keratin as well as meshwork-like structures formed by nuclear lamins. The method is able to accurately detect junctions in these structures directly while providing multiple orientation information that can be used in post-processing and analysis. In summary, we have developed a method that can be used to analyze images where an arbitrary number of IF structures intersect by employing an adaptive algorithm for orientation resolution. Our method can distinguish intersecting structures and yields multiple orientation information along with the orientation resolution required. The resulting analyses are being used to determine IF functions, especially their roles in cellular mechanics and motility.

This work was supported in part by NIGMS (2R01GM106023-05) to Yixian Zheng and Robert Goldman, CPRIT recruitment award (R1216) to Khuloud Jaqaman, and training funds from NICHD (5U54HD087351) and NCI (3T32CA080621) provided to Mark Kittisopikul.

P2511
Board Number: B662
The Effects of Vimentin Serine 72 phosphorylation in OxLDL uptake mechanism in macrophage.
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Oxidized low density lipoprotein (oxLDL) is a form of modified LDL which causes atherosclerosis, a chronic inflammatory disorder of arterial wall. Atherosclerosis is initiated by lipid-laden macrophages called foam cells. Although foam cell formation is a critical initial stage of atherosclerosis, the mechanism by which macrophages uptake oxLDL is not clearly defined. It is recently reported that vimentin, a type of intermediate filament protein in macrophages, is a component of intracellular lipid droplet in adipocytes and involves in vesicular trafficking. In this study, we aimed to reveal the function of vimentin in foam cell formation and atherosclerosis.

We generated Apoe-/- mice lacking vimentin and Ldlr -/- mice reconstituted with Vim-/- bone marrow and fed western diet for 15weeks. Apoe-/-Vim-/- mice showed less atherosclerotic lesion formation than Apoe-/- mice. In accordance, Ldlr -/- mice with Vim -/- bone marrow showed 15% less lesion formation than Ldlr -/- mice with Vim +/+ bone marrow. We demonstrated that Vim -/- mouse peritoneal macrophages uptake less oxLDL and foam cell formation than Vim +/+ mouse peritoneal macrophages, which was proven by Dil-oxLDL uptake assay and oil-red O staining of macrophages incubated with oxLDL. Despite less uptake of oxLDL in Vim -/- macrophages, there was no difference in the expression of CD36 which is known to mediate oxLDL uptake between Vim +/+ and Vim -/- macrophages. However, there was significant difference in CD36 localized in plasma membrane between Vim +/+ and Vim -/- macrophages. CD36 localized in plasma membrane measured by flow cytometry and subcellular fractionation assay was 50% less in Vim -/- macrophages than in Vim +/+ macrophage.
We also found that oxLDL induced vimentin (Ser72) phosphorylation. Vimentin (Ser72) phosphorylation was dependent on protein kinase A (PKA). H-89, a specific PKA inhibitor, decreased vimentin phosphorylation and oxLDL uptake in macrophages. Moreover, CD36 null macrophages didn’t exhibit phosphorylation ser72 on vimentin induced by oxLDL. To evaluate the role of vimentin phosphorylation at Serine 72, we generated phosho-mimetic vimentin and transfected into Vim -/- bone marrow-derived macrophages and performed Dil-oxLDL uptake assay. We observed that mimicking phosphorylation at serine 72 of vimentin increased oxLDL uptake and membrane CD36 localization. We revealed that vimentin involves in oxLDL uptake of macrophages and vimentin (Ser72) phosphorylation via PKA induced by oxLDL mediates this process. Our study suggests a mechanism of macrophage foam cell formation which may aid to establish a new therapeutic strategy.
P2512

**Board Number: B663**

Keratin intermediate filament recruitment to cell-cell contacts is dependent on proper actin localization and function.

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Cell-cell adhesion assembly and maturation within epithelial cells is a highly dynamic process involving several adhesive complexes and their respective cytoskeletal anchors. Of these, adherens junctions, associated with the actin cytoskeleton, are responsible for adhesion initiation while desmosomal junctions, tethered by keratin intermediate filaments, are the final complex to assemble. Although these structures were once thought to be distinct, recent literature suggests that the interaction between actin and keratin cytoskeletal systems, as well as their adhesion complexes, is required for proper cell-cell adhesion. However, the extent of these associations is still not fully understood. It remains unknown whether keratin intermediate filaments are recruited during adherens junction assembly or as a result of desmosomal junction assembly. To answer this question, we characterized the temporal sequence of keratin intermediate filament recruitment during cell-cell adhesion assembly. To examine the relationship between both cytoskeletal systems and the adherens junction complex, we disrupted actin cytoskeletal translation at cell-cell contact sites during adhesion assembly and show that keratin intermediate filament recruitment is reduced at the cell periphery in correlation with E-cadherin. Additionally, we demonstrate that inhibition of acto-myosin tension increases keratin intermediate filament cytoplasmic localization and disrupts keratin cytoskeletal organization. Thus, these data indicate that keratin intermediate filament recruitment and organization is dependent on the proper function and translation of the actin cytoskeleton in steady state and during adherens junction assembly, providing stronger evidence for extensive association between these complex structures.

P2513

**Board Number: B664**

Optogenetic perturbation of intermediate filament networks.

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Cytoplasmic intermediate filaments play important roles in simultaneously supporting mechanical integrity and influencing signal transduction pathways. The significance of cytoplasmic intermediate filament proteins has previously been examined largely through various genetic approaches, including knockdown, knockout and transgenic overexpression. However, few studies to date have attempted to examine the role of specifically the filamentous intermediate filament network in orchestrating various cell functions. To directly assess the role of the filamentous keratin network in regulation of cellular behavior, we created genetically-encoded photoactivatable (PA-) and constitutively active (CA-) versions of a peptide disruptor of keratin intermediate filaments (PA-dIF and CA-dIF, respectively). This genetically encoded construct consists of a peptide derived from the 2B2 region of keratin 8 fused to the photosensitive LOV2 domain from *Avena sativa* phototropin-1. To validate the ability of this construct to disrupt intermediate filament networks, we expressed PA-dIF and CA-dIF in multiple cell lines and tissues. Across multiple species and cell types, CA-dIF induces disruption of keratin networks. Upon 457nm photoradiation, PA-dIF disrupts keratin intermediate filaments and induces dynamic changes in cell shape and protrusive behavior. Marked remodeling of the keratin intermediate filament network
accompanieds collective cellular morphogenetic movements that occur during gastrulation and neurulation in the *Xenopus laevis* frog embryo. Disruption of keratin intermediate filaments by expression of CA-dIF in presumptive neuroectoderm results in a dramatic failure of neural tube closure. Expression of CA-dIF in presumptive mesendoderm causes marked reduction in head structures. Altogether our data show a fundamental requirement for keratin intermediate filaments in orchestrating morphogenetic movements during development that have yet to be revealed in other model systems. Moreover, our data validate the utility of a new genetically encoded photoactivatable tool for the disruption and examination of intermediate filaments.

**Cell-cell Junctions 2**

**P2514**

**Board Number: B666**

The gap junction Nexus controls localization and mobility of neural proteins.

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Gap junctions connect astrocytes and oligodendrocytes, providing bidirectional exchange of nutrients, metabolites and intracellular signaling molecules. Each of these glial cell types expresses a distinct set of gap junction (GJ) proteins (astrocytes: Cx43, Cx30, & Cx26 oligodendrocytes: Cx32, Cx47 & Cx29) and each connexin forms a supramolecular complex (the GJ Nexus) made up of GJ channels and molecules that interact with GJs. Nexus composition is also determined by the posttranslational modification state of the Cx proteins and cell physiological state. GJs also have important non-channel functions including cell-cell adhesion and control of autophagy. We recently used live cell confocal microscopy and Fluorescence Recovery After Photobleaching (FRAP) to show that cysteine (Cys) residues within the cytoplasmic carboxyl-termini (CT) of Connexin 43 and Connexin 32 (Cx43 and Cx32) produce stably arranged orthogonal GJ channel arrays (GJ plaques) in the membranes of cells joined by those connexin isoforms whereas Cx30 & 26 (which have no Cys residues in the CT) form highly fluid GJ plaques. Here we report for the first time that Cx47 (contains 3 CT Cys residues) forms stably arranged GJs- thereby completing exploration of macroglial GJ stability characteristics (leaving the postulated xenotypic Cx29::Kv channel for continuing studies). Our discovery that mutation of Cys residues to alanine in the CT of Cx43 and Cx32 produces a switch from stable GJ Nexuses to a fluid structures allowed us to employ this as a new tool to test how Nexus stability affects mobility and localization of other membrane proteins- since known protein interaction sites are preserved in the Cys mutants. Here, we used live cell microscopy for four dimensional and multi-color FRAP to test how the GJ Nexus controls subcellular morphology near the GJ plaque and Cx43 mobility affects mobility of other Nexus components. We found that GJs excluded many membrane proteins from the GJ plaque membrane area. Some membrane proteins did infiltrate the Nexus and localization of the tight junction protein occludin (Ocludin) to the GJ plaque area was higher than other membrane areas. Cx43-CT mediated Nexus stability had a substantial effect on mobility of some membrane proteins (Cx30 and Ocludin) but a minor effect on other Nexus components. We examined localization and mobility of other proteins at the Nexus that are critical for function of specialized cellular compartments in astrocytes- where GJs are preferentially localized- such as peri-synaptic astrocyte processes (EAAT2b) and the perivascular endfeet (AQP4, Nectin-2, TJP1). We continue to explore ways the GJ Nexus acts as intercellular channels and membrane organizing nodes to determine astrocyte and oligodendrocyte cellular physiology.
P2515
Board Number: B667
Molecular machinery of gap junction turnover.
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Gap junctions, one of four principle cell-cell junction types, provide direct cell-to-cell communication by forming clusters of membrane channels that bridge the plasma membranes of neighboring cells. Gap junctions and cell-to-cell communication are widespread in the animal kingdom and are a hallmark of multi-cellular life. Interestingly, gap junctions turn over with a short half life of only a few hours, however the reason for this short and for membrane channels untypical turnover kinetics is not known. One possibility is that channels, once accrued to a gap junction plaque permanently close, become non-functional and thus need to be replaced by newly synthesized functional channels. Similarly, to allow cell migration, cells will need to physically uncouple from their neighbors, and this uncoupling requires the coordinated, rapid removal of all cell-cell junction types, including gap junctions. Regulated turnover is important for gap junction function, as abnormal turnover characteristics can lead to severe pathological conditions. We have characterized the turnover of the major gap junction protein, connexin 43 (Cx43), on a molecular basis and characterized a sequence of posttranslational modifications that occur in sequence on Cx43 in gap junctions: (1) binding and release of the scaffolding protein ZO-1, (2) earlier and later phosphorylation/de-phosphorylation events on several regulatory serine residues located in the Cx43-C-terminal domain, and (3) Cx43 K63-poly-ubiquitination. These hierarchical regulatory events transition functional into non-functional gap junction channels that then can interact with clathrin/clathrin-adaptors to internalize and recycle gap junctions. These novel and exciting findings will be summarized in this presentation.

P2516
Board Number: B668
Desmoplakin bears tension under externally applied load but not during epithelial monolayer growth and homeostasis.
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Desmosomes are intercellular adhesion complexes that link the intermediate filament cytoskeletons of neighboring cells. In humans, mutations in desmosomal proteins lead to cardiomyopathies and skin blistering diseases, suggesting that desmosomes play an important role in defining the mechanical properties of multicellular tissues. However, how and whether desmosomes bear mechanical load in living tissues was unclear due to limitations inherent in existing measurement techniques. In this study, we inserted a Förster resonance energy transfer (FRET)-based tension sensing module (TSMod) into desmoplakin-I (DPI), a scaffolding protein that links desmosomal adherins to intermediate filaments. In a model epithelial system consisting of Madin-Darby canine kidney (MDCK) cells, live-cell FRET measurements are consistent with negligible mechanical tension transmitted through DPI-TSMod in confluent monolayers, sparse cell colonies, and at the edge of expanding monolayers. However, we observed a significant decrease in FRET efficiency, consistent with mechanical tension transmitted through DPI-TSMod, in epithelial monolayers subjected to external mechanical stretch, particularly at cell-cell junctions oriented perpendicular to the axis of applied strain. These data support a model in which desmosomes bear mechanical loads from external deformations, but bear negligible mechanical loads arising from local, cell-generated forces. We suggest that, in simple epithelia, desmosomes have
evolved to protect tissues from mechanical disruption while still allowing the cellular movements and shape changes that are essential to tissue homeostasis.

P2517
Board Number: B669
Proximity Labeling Proteomics of Desmosomes Reveals Novel Components Essential for Epidermal Integrity.
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Desmosomes are cell-cell adhesion structures that provide mechanical robustness to the epidermis and heart. Perturbation of desmosomes that occurs in genetic, autoimmune and infectious pathologies produce phenotypes that range from skin blistering diseases to cardiomyopathies. While the core components in desmosomes have been identified decades ago, more recent work has uncovered additional desmosome-associated proteins that have either regulatory and/or non-canonical functions. Here we used a combination of proximity biotinylation and quantitative mass spectrometry to identify novel desmosome-associated proteins. We performed separate analyses to enrich for proteins at the outer and inner dense plaques regions within desmosomes and identified many candidates with potential functionality within each region. Additionally, we found a significant overlap with proteins normally associated with both adherens junction and tight junction complexes. We show that this is due, in part, to differential promiscuity in the association of some proteins with adherens junctions, tight junctions, and desmosomes in different tissues. Lastly, we demonstrate that a subset of the identified proteins co-localize with core desmosome components and require these proteins for their localization. Finally, we performed functional evaluation of the role of signal adapter molecules, Crk and Crkl, two homologous proteins that were identified in our analysis. Loss of Crk/Crkl in cultured keratinocytes and in the mouse epidermis resulted in defects in keratin organization, impaired adhesion, and neonatal lethality. These data position Crk/Crkl as crucial regulators of the desmosome:keratin interaction which is essential for mechanical integrity of the skin and highlights the utility of the approach to identify novel regulators of the desmosome.

P2518
Board Number: B670
Increased cardiac arrhythmogenesis associated with gap junction remodeling with upregulation of RNA binding protein FXR1.
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Gap junction remodeling is well established as a consistent feature of human heart disease involving spontaneous ventricular arrhythmia. The mechanisms responsible for gap junction remodeling are still debated. Studies reveal that multiple transcriptional and post-transcriptional regulatory pathways are triggered in response to cardiac disease, such as those involving RNA-binding proteins. The expression levels of Fragile X mental retardation autosomal homolog 1 (FXR1), an RNA-binding protein, are critical to maintain proper cardiac muscle function; however, the connection between FXR1 and disease is not

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clear. To identify the mechanisms regulating gap junction remodeling in cardiac disease, we sought to identify: 1) the functional properties of FXR1 expression, 2) direct targets of FXR1 in human dilated cardiomyopathy (DCM) biopsy samples and mouse models of DCM, and 3) how FXR1 regulates its targets, and the functional consequences of altering the levels of this important RNA binding protein. We discovered that FXR1 expression is significantly increased in tissue samples from human and mouse models of DCM. FXR1 associates with intercalated discs. The integral gap junction proteins Cx43, Cx45 and ZO-1 were identified as novel mRNA targets of FXR1 using a BioID proximity assay and RNA immunoprecipitation. Our findings show FXR1 is a multifunctional protein involved in translational regulation and stabilization of its mRNA targets in heart muscle. Additionally, introduction of 3xMyc-FXR1 via adeno-associated virus into mice leads to redistribution of gap junctions and promotes ventricular tachycardia showing functional significance of FXR1 upregulation observed in DCM. Furthermore, we have demonstrated that knocking out FXR1 in the heart leads to DCM. However, Cx43 expression is not changed in cardiac-specific FXR1 KO mice as observed in other DCM models, consistent with our observation that FXR1 directly regulates gap junction remodeling in DCM. Together this study provides a novel function of FXR1 namely that it directly regulates major gap junction components, contributing to proper cell-cell communication in the heart.

P2519
Board Number: B671
Dynamic Equilibrium of Endothelial Cell Junctions is Required for Vessel Morphogenesis.
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Intercellular junctions are maintained by complexes of cytoplasmic and transmembrane proteins stabilized by scaffold proteins. Multi PDZ (MPDZ, also known as MUPP1), which contains 13 PDZ modular domains and one L27 domain, is a major scaffold proteins localized at the endothelial cell (EC) junctions. It is thought to function as a paralog of Pals1-associated tight junction protein (PATJ) in the Crumbs polarity complex. Though the apical basal polarity complexes Crumbs and PAR are involved in the regulation of cell junction assembly during development, it is unknown which protein complex, if any, regulates EC junctional dynamics in response to physiological factors that alter vessel permeability, such as vascular endothelial growth factor (VEGF). We suggest that MPDZ serves as the backbone permeability, such as junctional protein complex which mediates the translocation of junction proteins to and from the junctions. We found that either depletion or absence of MUPP1 in mouse or human endothelial cells (ECs), respectively, resulted in acute loss of adherens as well as tight junction transmembrane and membrane-proximal proteins. Surprisingly, however, the in vitro permeability of confluent Mpdz-/-/6NJEC monolayers to fluorescently-conjugated dextran was lower than that of Mpdz+/+6NJ monolayers either in the presence or absence of VEGF. This finding suggests that MPDZ is required for conferring vascular plasticity rather than for maintaining EC junction integrity. In vivo, we observed that: (1) the typical radial symmetry of the major retina arteries and veins of the wild type mouse (Mpdz+/+6NJ) was lost in the Mpdz-/-/6NJ retina; (2) the diameter of the major vessels was twice larger in the Mpdz-/-/6NJ retina; (3) their branching frequency was substantially higher. The Mpdz+/+6NJ and Mpdz-/-/6NJ vessels differed also at the cellular level: EC pattern along Mpdz-/-/6NJ veins, but not along arteries, was irregular and their junctions were jagged. In agreement with the in vitro result, the in permeability of Mpdz-/-/6NJ skin vessels in response to VEGF was lower than that of Mpdz+/+6NJ vessels. VEGF treatment of Mpdz+/+6NJ EC monolayers caused a reduction in MPDZ abundance at the junctions, whereas the number and size of MPDZ in recycling endosomes and the trans Golginetwork (TGN) increased substantially. We detected by mass-spectrometry novel MPDZ-associated proteins, including TBC1D2, a Rab7 inhibitor that is likely to impede MPDZ trafficking to the
lysosomes. MPDZ was shown by others to associate with retromer and sorting nexin 3 (SNX3), which sort endocytosed cargo proteins and recruit them to the trans Golgi, respectively. Our findings indicate that EC junctions are maintained in a dynamic equilibrium by recycling between the plasma membrane and the TGN.

P2520
Board Number: B672
Miniaturized Permeability Assay for Whole Genome Screening.
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Maintenance of vessel wall integrity is the primary function of endothelial cells. Unlike other frequently assayed cell activities such as proliferation or migration, permeability is a cell-group behavior. Current whole genome screening techniques can be readily applied to identify genes involved activities phenotyped by measuring cell proliferation rates, such as drug resistance. In contrast, a screen to identify genes involved in regulating endothelial cell junctions would require as many samples as the number of annotated genes in the genome, multiplied by the numbers of probes and technical replicates. This is so because screens, such as those using shRNA probes, require targeting several loci in each gene to achieve effective transcriptional inhibition. Currently, multi-well Transwell plates provide the largest number of monolayer permeability assay per plate (96 wells). To produce statistically valid results, each experimental group would have to contain as many as 106 samples – an impractical number for most if not all labs. To solve this problem, we have designed a permeability assay based on microcarrier beads averaging 150 μm in diameter. While microcarriers had been used to measure endothelial cell (EC) permeability in a chromatographic format (Haselton et al., 1989), we use each bead as a single assay. ECs are grown on these beads to confluence and are then challenged by any compound of interest. In one assay version, we treated cells grown on gelatin-coated commercially-available beads by thrombin, a protease that disrupts cell-cell junctions. To distinguish between microspheres coated by cells that respond to thrombin from others that do not, we added to the medium fluorescently-conjugated collagen-binding fibronectin fragment (fFN) that attaches to gelatin with high affinity. The fFN probe bound to the newly-exposed gelatin surface between adjacent thrombin-treated cells. On the other hand, only a low amount bound to the surface of beads that were not treated by thrombin. The difference in fluorescence intensity between the thrombin-treated and untreated cell samples is large enough to facilitate separation by fluorescence-assisted sorting between beads carrying thrombin-responsive versus non-responsive cells grown in the same volume. The relevant information can then be produced by sequencing the genomic DNA or the mRNA of each cell group.
Desmosomal Regulation of Gap Junctions via Ras: Implications for Cardiocutaneous Disease.

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The mammalian heart is a vital organ comprising specialized muscle cells, or cardiomyocytes (CMs) that adhere to each other via intercalated discs, specialized junctions made up of elements from desmosomes, adherens junctions, and gap junctions. These junctions are crucial for integrating mechanical, chemical, and electrical signals to coordinate cardiac rhythmic contraction. Desmoplakin (DP) is an essential component of desmosomes, that tethers intermediate filaments to sites of cell adhesion. DP is a frequent target for mutation in cardiocutaneous disorders. In particular, DP mutations cause arrhythmogenic cardiomyopathy (AC), an inherited disorder characterized by replacement of healthy myocardium by fibro-fatty deposits that presents with or without cutaneous defects. During AC pathogenesis, a loss of electrical conduction between CMs can occur prior to fibro-fatty infiltration, an event that has been dubbed the “concealed phase”. Providing a possible explanation for the conduction defect, DP loss has been linked to decreased levels of the gap junction protein Connexin-43 (Cx43). However, the mechanism by which loss of DP decreases Cx43 expression is unknown.

We utilized *in vitro* and *in vivo* models of DP deficiency in heart and skin to elucidate the mechanism by which DP regulates Cx43 protein levels. Depletion of DP in rat CMs led to lowered Cx43 protein levels, consistent with prior *in vivo* studies. Gap junction dynamics are regulated by a number of phosphorylation sites on the Cx43 C-terminus. Systematic analysis of known Cx43 phospho-sites in DP-deficient CMs revealed a specific increase in the MAPK sites S279/282, previously shown to signal internalization and degradation of Cx43. Elevated phosphorylation was also conserved in cardiac and epidermal tissues from mice with conditional ablation of DP. Furthermore, elevated p-Cx43 to total Cx43 signal ratio is observed in a cardiac patient sample harboring a pathogenic AC mutation. DP depletion in CMs accelerated loss of Cx43, which was prevented by inhibitors of lysosomal, but not proteosomal, degradation.

RNA-sequencing analysis revealed Ras-GTPases as candidates for DP-dependent activation of the ERK1/2 MAPK pathway. DP loss led to elevation of K-ras transcript, protein, and activity. We utilized a novel reagent derived from the bacterium Vibrio vulnificus termed RRSP that is able to cleave Ras isoforms to test if dampening of Ras activity could ameliorate DP-dependent Cx defects. Cx43 levels were restored in CMs treated with RRSP, supporting a mechanistic connection between ERK activation and Cx43 expression. Collectively, our results reveal a novel mechanism for the regulation of Cx43 levels in cardiac disease caused by loss of DP and highlight a potential novel therapeutic for AC patients.
**P2522**

**Board Number: B674**

**Connexin 43 loss induces proliferation and invasion pathways in non-neoplastic breast epithelium.**


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The loss of connexin 43 (Cx43) expression has been reported in breast cancer cell lines, in-vivo models and tissue biopsies, suggesting a tumor suppressive role for Cx43 in the breast. Our earlier studies demonstrated disruption of apical polarity and mitotic spindle orientation in 3-dimensional (3-D) culture of Cx43-silenced HMT-3522 S1 cells, non-neoplastic human mammary epithelial cells (S1 cells). The current study aims to investigate the role of Cx43 in proliferation and invasion pathways in non-neoplastic breast epithelium. Cx43 shRNA-transfected and control S1 cells were cultured under 2-D and 3-D conditions. Proliferation and cell cycle progression were monitored by cell counting (2-D) or measurement of acinus size (3-D) and flow cytometry. Motility and invasion were assessed by time-lapse imaging and transwell cell invasion assays. Western blotting, co-immunoprecipitation, immunofluorescence, gelatin zymography and pulldown assays were used to decipher the mechanism of Cx43 signaling. Cells in 2-D cultures were assayed on days 4, 6, 9, and/or 11, while those in 3-D cultures on day 11. Silencing Cx43 enhanced proliferation of S1 cells in 2-D and 3-D cultures, as shown by the increased cell counts and acinar size, respectively and throughout the culture period. Cell cycle entry was concomitantly enhanced, as indicated by the increased percentages of cells in S and G2/M phases and the reduced percentage of cells in G0/G1 phase. c-Myc and cyclin D1, but not β-catenin, were consistently upregulated in 2-D and 3-D (day 11) cultures of Cx43-silenced S1 cells. Cx43 associated with β-catenin in 2-D and 3-D cultures of S1 cells. β-catenin colocalized with Cx43 in 3-D cultures of S1 cells, and exhibited mislocalization in Cx43-silenced cells, suggesting involvement of Cx43 in the Wnt/β-catenin pathway. Furthermore, silencing Cx43 enhanced motility in 2-D cultures of S1 cells and distorted the characteristic spheroid morphology in 3-D cultures, indicating cell migration. In addition, matrigel-invasion and MMP-9 activity were enhanced. The expression and activity of Rho GTPases (RhoA, Rac1 and Cdc42) were upregulated in 2-D and 3-D cultures of Cx43-silenced S1 cells, suggesting implication of Cx43 in non-canonical Wnt signaling. We propose a role for Cx43 loss in triggering proliferation and invasion events in non-neoplastic breast epithelium downstream of disrupted Wnt signaling.

**P2523**

**Board Number: B675**

**Dynasore disrupts gap junction-mediated cell-cell communication.**

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Dynamin, a mechanoenzyme, has been established to be a key player in scissoring vesicles from the plasma membrane. Further, gap junction plaque internalization is dependent on dynamin for detachment of gap junction buds into the cytoplasm. A dynamin GTPase inhibitor, dynasore, has been extensively used to study the role of dynamin in membrane scissoring and it has been demonstrated to decrease the scissoring of invaginated gap junction plaque membrane from the cell surface. To investigate if gap junction-mediated cell communication was altered following dynasore treatment,
fluorescence recovery after photobleaching (FRAP) techniques were used. In addition, live-cell imaging, immunocytochemistry and transmission electron microscopy (TEM) were used to demonstrate gap junction plaque location and morphology. With live-cell imaging, fewer annular gap junction vesicles were observed to be released into the cytoplasm in the dynasore treated populations compared to diluent treated controls. This corresponded to an increase in the number and size (area) of gap junction “buds” suspended from the gap junction plaques. Gap junction plaque invaginations were observed to remain on the cell surface and to undergo dynamic changes but not to be internalized to form annular gap junction vesicles in the dynasore treated cultures. Ultrastructural analysis revealed large numbers of invaginated gap junction structures with atypical morphology, including those with separations greater than the typical 2-4 nm space found at the gap junction plaque membrane. This would be consistent with undocking of the gap junction channels that compose the gap junction plaque and thus the loss of cell-cell communication. When dye communication was evaluated by FRAP analysis, control cells rapidly recovered fluorescence after photobleaching, while dynasore treated cells did not recover. The failure of dynasore treated cells to recover fluorescence compared to controls is consistent with the atypical gap junction morphology seen with TEM and the loss of cell communication. Cell communication was restored when dynasore was washed from cell cultures. Based on these findings, dynasore treatment, in addition to altering the scissoring and release of buds into the cytoplasm, impaired the capacity for gap junction-mediated dye communication. The disruptive effects of dynasore on cell communication may be important considerations needed in interpreting outcomes in studies that utilize dynasore to disrupt dynamin and study changes. This research was supported by NSF grant #MCB-1408986

P2524

Board Number: B676

Using concatemerization as a tool to investigate the dominant effect of the mutation N188T of Connexin46.

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The mutation N188T of Connexin46 (Cx46) is associated with an autosomal dominant congenital nuclear pulverulent cataract. The aim of the project was to analyze how the N188T mutant might affect the formation of Cx46 gap junctions when concomitantly expressed with the wild type Cx46. As membrane proteins connexins are embedded in the membrane of the ER during synthesis. They oligomerize along the secretory pathway on the way to the plasma membrane to form hexamers called connexons or hemichannels, which are built into the plasma membrane of the cell. Connexons of adjacent cells dock with each other forming gap junction channels, which might be assembled in gap junction plaques. To study how Cx46N188T might affect the formation of gap junctions by Cx46, generated eGFP labelled Cx46-Cx46, Cx46N188T-Cx46N188T, Cx46-Cx46N188T and Cx46N188T-Cx46 concatemers were expressed in gap junction communication deficient HeLa cells. The concatemers were compared with the Cx46 and Cx46N188T monomers with respect to trafficking and formation of gap junction plaques using confocal laser scanning microscopy. First, ethidium bromide dye uptake experiments demonstrated that all constructs formed functional hemichannels in the membrane. To study the formation of gap junction plaques, randomly taken confocal laser scanning micrographs of cells expressing the eGFP-tagged variants were analyzed by ImageJ. The quantification revealed a three-step model that shows the highest number of gap junction plaques per cell pair in cells expressing the monomer and the homodimer Cx46, suggesting that the concatemerization per se did not alter the

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formation of gap junction plaques. Cells expressing the Cx46N188T monomer and the Cx46N188T-Cx46N188T homodimer showed the lowest number of gap junction plaques per cell pair. For cells expressing Cx46-Cx46N188T or Cx46N188T-Cx46 heterodimer a number of plaques in between the pre-mentioned extremes was found. With respect to trafficking, retention of the protein in the ER was observed for the concatameric variants in which Cx46N188T was present, either in a homodimeric or in a heterodimeric variant. Although the monomeric variants and the homodimeric Cx46 did not significantly differ, a trend to retention in ER was perceived for Cx46N188T monomer. Taken together, the results show that the presence of Cx46N188T affected the trafficking as well as the formation of gap junction plaques by Cx46.

P2525
**Board Number: B677**
Understanding the Molecular Mechanisms Regulating Gap Junction Turnover and its Relevance to Disease in an Animal Model.
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Cell to cell communication is a crucial cellular function of multicellular organisms. This cellular communication is established by gap junction channels. Gap Junctions are intercellular connections that directly link the cytoplasm of two adjacent cells. This provides a direct means of diffusion for various molecules, ions and electrical impulses between cells. Previous research supports several models explaining the mechanism of gap junction related pathologies. Specific mutations in gap junction proteins (connexins) have been mapped genetically and correlated to disease phenotypes. However, our understanding if and how gap junction turnover mechanisms may also lead to disease are still largely unknown. The Falk lab and others have previously characterized the gap junction turnover pathway and characterized a set of posttranslational modifications that include phosphorylation/dephosphorylation, ubiquitination, and binding/release of a scaffolding protein) that occur on key amino acid residues on connexin43 that regulate turnover. However, their implications in disease are still unclear. To explore how gap junction turnover is associated with disease phenotypes, an animal model must be utilized. The purpose of this research is to determine the molecular mechanisms regulating gap junction turnover and its relevance in disease in a zebrafish model system. To accomplish this, the CRISPR/Cas9 technology is employed to generate the proper mutations to define the molecular source of disease phenotypes. Currently, the CRISPR/Cas9 system has been established in our laboratory for this study, and a mutation that deletes a region of connexin43 that contains several of the key residues involved in gap junction turnover has been generated and injected into zebrafish embryos. The next steps will be to generate mutations that specifically target key phosphorylation residues and ubiquitination residues to investigate their potential relevance to disease.

P2526
**Board Number: B678**
Connexin43 (Cx43) and Zebrafish Fin Regeneration.
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I am interested in the mechanisms responsible for the control of bone growth in Zebrafish caudal fins. Zebrafish have the remarkable ability to re-grow missing caudal fin tissue upon amputation, making them an ideal model to study bone growth. There are several fin length mutants where bone growth is...
affected, including the short fin mutant (sof). Mutations in the gap junction gene connexin43 (cx43) cause sof mutants to develop short fins due to defects in segment length. Gap junctions serve as intercellular pathways for the exchange of small molecules between neighboring cells, particularly, the exchange of signals between bone cells (i.e. osteoblasts) that coordinate bone growth. Therefore, gap junctional intercellular communication (GJIC) may be responsible for the regulation of bony segment length. We know that sof mutants exhibit fins that are half the length of wild-type fins, have reduced levels of cx43 mRNA and a reduced amount of cell proliferation in the mesenchyme of regenerating fins. We have examined both functional (dye and ionic coupling, morpholino knockdown) as well as cellular processes (cell proliferation, cell differentiation, cell structure and protein expression) in an attempt to identify the cellular defect in the zebrafish sof mutant (Hoptak-Solga et al., 2008). Using a novel technique of targeted gene knockdown of cx43 in regenerating fins, we were able to recapitulate both the segment length as well as the cell proliferation defect of the sof mutants. We hypothesize that the level of GJIC within the population of dividing cells in the regenerating fin may regulate bone growth by regulating the level of cell proliferation. It is not known how a reduction in cell proliferation can lead to short fins, however, the level of cell-cell coupling may directly regulate the number of mitotic cells in the blastema which in turn controls bone growth. This research establishes a key role for Cx43 cell-cell coupling in the regulation of a proliferative cell population that is responsible for the regulation of bone growth (Hoptak-Solga et al., 2008). Understanding the role Cx43 plays in cell-cell communication in the regenerating fin will serve as a model to examine how cells can coordinate a response to re-grow missing tissue. It is hypothesized that defects in Cx43 may lead to aberrant channel structure in sof mutants. Future projects include an electron microscopic study of gap junction structure in both wild-type and sof mutants to determine if structure affects the function of channels and thus GJIC between cells.

P2527
Board Number: B679
Desmoplakin promotes cell migration via coordinated control of p38 MAPK and Rho GTPase signaling.
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Desmosomes are cell-cell adhesion complexes crucial for maintaining the integrity of tissues, especially those under high mechanical stress. One of the key components of desmosomes is desmoplakin (DSP), a plakin protein that links intermediate filaments to other proteins of the desmosome complex. Due to the vital nature of this connection for mechanical stability of the desmosome, DSP has been a frequent protein of interest for studying the stability of cell-cell adhesion. Although DSP also affects other types of cellular behavior (such as cell-matrix adhesion and cell migration), little is known about the mechanism by which this occurs. Our preliminary findings have shown that loss of DSP results in a dramatic increase in the rate of cell migration, as measured by performing wound healing assays (scratching a monolayer of cells and measuring the rate of wound closure over time). Some studies have indicated that DSP can modulate cellular signaling pathways known to affect cell migration, such as p38 MAPK and Rho GTPase signaling. We therefore hypothesize that DSP regulates the process of cell migration via coordinated effects on p38 MAPK and Rho/Rac activity in epithelial cells. Western blots demonstrated that DSP knockdown results in elevation of p38 MAPK activity (phosphorylation) in A431 epithelial cells, which supports similar findings previously shown in cardiomyocytes. Interestingly, both inhibition of p38 MAPK and an upstream activator, TAK1, were found to rescue the elevated rate of migration of DSP knockdown cells. Rac1 inhibition also rescued migration, suggesting a positive role for
Rac in promoting DSP-mediated migration. Preliminary evidence using GST-pulldown assays for GTP-bound Rac indicates that loss of DSP results in increased Rac activity, providing further support for this hypothesis. Conversely, inhibition of the Rho/ROCK pathway increased migration while activation of the same pathway rescued migration, suggesting that Rho signaling can abrogate DP-mediated migration. Further, we have characterized significant changes in actin cytoskeleton morphology (specifically increases in filopodia number and length) that are associated with the loss of DSP. Altogether, these findings indicate that DSP is responsible for coordinated control of p38 MAPK and Rho GTPase signaling pathways involved in cell migration. This study therefore sheds light on the mechanisms via which desmosomal proteins can induce changes in migratory properties via control of cellular signaling.

P2528

Board Number: B680

The MAL/SRF pathway regulates desmosomal gene expression and protein localization in cancer cells.

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The desmosome (DSM) is a cell-cell adhesion complex required for the mechanical stability of tissues and regulation of other biological processes, such as proliferation and migration. Proteins from three families make up the complex: Transmembrane cadherins connect adjacent cells, plaque proteins stabilize attachment on the intracellular face, and desmoplakin (DSP) anchors the DSM complex to the IF cytoskeleton. Many studies have reported diverse mechanisms by which cancer progression alters DSM gene expression, but the signaling pathways that govern the expression and localization of DSM constituent proteins remain elusive. Recent work has identified the transcription factor serum response factor (SRF) as a regulator of mRNA levels and localization of DSM proteins such as the cadherin Desmoglein-1. Here, we treated several cancer cell lines with siRNA and a pharmacological inhibitor (CCG-1423) of SRF and its cofactor, MAL, to broadly investigate the role of these transcription factors in regulating DSM gene expression and protein localization. Our results demonstrate that abrogation of MAL/SRF signaling through both siRNA and pharmacological inhibition results in a decrease in DSP transcription, and a concomitant decrease in DSP protein levels. In addition, localization of DSP to borders is also reduced upon inhibition of MAL/SRF signaling. We observed similar changes for the plaque proteins Plakophilin-2 and Plakoglobin, but not for desmosomal cadherins. As DSP is required to maintain the strength of DSM attachments, we also investigated the role of MAL/SRF signaling on the adhesion strength between cells. Our data highlight a novel link between MAL/SRF signaling and DSP expression, and contribute to a growing understanding of the variety of signaling pathways involved in mediating DSM gene expression.

P2529

Board Number: B681

Investigating the Role of Connexin 32 in Cell Cycle, Cell Viability and Epithelial to Mesenchymal Transition of Normal Breast and Breast Cancer Cells.

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Breast cancer is one of the most prominent cancer-related deaths among females. Among many molecules, connexins and gap junctional communication were shown to have role in breast cancer. Gap
junctions, formed from hemichannels of six connexins (Cx), facilitate intercellular communication between adjacent cells. Connexins, including Cx26, Cx32 and Cx43, were shown to be expressed during different stages of breast cancer. Cx32 was observed both in normal pre-menopausal breast tissue and patient tumor samples. In lymph node metastases, elevated levels of Cx32 was observed compared to primary breast cancer. However, the role of Cx32 in breast cancer is not known but elevation of Cx32 in lymph node metastasis may indicate its diverse functions in different stages of breast cancer. To verify this, in normal breast MCF10A and breast cancer MDA-MB-231 cell lines, connexin32 was overexpressed. The localization of the protein was compared with immunostaining method after transient Cx32 overexpression. In untransfected MDA-MB-231 cells, Cx32 localized in the nucleus and the cytoplasm, while it was mostly observed in the cytoplasm in MDA-MB-231 Cx32-EGFP cells. In untransfected MCF10A cells Cx32 localized in the nucleus, whereas Cx32 formed gap junctional plaques between the adjacent MCF10A Cx32-EGFP cells. Scrape loading assay for gap junction activity and dye uptake assay for hemichannel functionality were performed with stable cell lines. When Cx32 was overexpressed, the gap junction coupling significantly increased in MCF10A cells whereas it did not change in MDA-MB-231 cells. In both cell lines, the hemichannel activity did not alter as a result of Cx32 overexpression. The effects of Cx32 expression on cell viability and cell cycle profile were examined. Cx32 overexpression led to a significant decrease in cell viability in MCF10A cells but did not affect the viability of MDA-MB-231. Cell cycle analysis experiments indicated that the percentage of cells in G1 decreased and in G2 increased in Cx32 overexpressing MDA-MB-231, which may indicate that increase in Cx32 may lead to a G2 arrest in MDA-MB-231 cells. However, the overexpression did not alter cell cycle profile of MCF10A significantly. In addition, Cx32 overexpression in MDA-MB-231 cells caused upregulation of vimentin and SNAI2; downregulation of E-cadherin, leading to a shift towards epithelial to mesenchymal phenotype. Assessment of Cx32 role in migration and invasion would further support its importance in this process. Finally, determination of the differential role of Cx32 in breast cancer initiation and progression may help us to understand its diagnostic and/or therapeutic potential.

P2530
Board Number: B682
The cell-cell adhesion component PLEKHA7 regulates the pro-tumorigenic MIR17HG long non-coding RNA in colon epithelial cells.
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The adherens junctions (AJs) are essential architectural elements of epithelial tissues. Compromised junctional integrity is frequently observed in colon cancer, one of the most predominant forms of cancer. Recently, we identified a novel mechanism whereby the AJs of non-transformed colon cells suppress aberrant cell behavior by recruiting the RNAi machinery, mRNAs, and miRNAs, via the AJs component PLEKHA7. Our current data reveal widespread dysregulation of PLEKHA7 and of this mechanism in colon cancer cell lines and patient tissues. Interestingly, RNA-CLIP and subsequent RNA-Seq identified association of PLEKHA7 with numerous long non-coding RNAs (lncRNAs). LncRNAs interact with the RNAi machinery in multiple ways. While a number of lncRNAs have been associated with tumorigenesis, the underlying mechanisms of their regulation during tumor progression are still unclear. We hypothesize that the AJs regulate the levels and function of lncRNAs via PLEKHA7 and its associated RNAi mechanism to suppress pro-tumorigenic colon cell behavior. Comprehensive examination of the junction-associated lncRNAs by RNA-seq identified several whose expression levels are indeed regulated by PLEKHA7. From this set, the top upregulated lncRNA upon PLEKHA7 depletion is MIR17HG (miR-17-92; Oncomir-1), an oncogenic polycistronic host transcript of a set of miRNAs that includes miR-17, miR-
18a, miR-19a, miR-19b, miR-20a, miR-92a. Notably, the mature forms of these miRNAs also co-precipitate with PLEKHA7. Although PLEKHA7 knockdown results in increased levels of MIR17HG, this is followed by increased expression of only a subset of its hosted miRNAs, namely miR-19a, miR-19b and miR-20a. Our data suggest that two PLEKHA7-associated miRNAs, miR-203a and miR-372, mediate suppression of this lncRNA by PLEKHA7 and by its associated RNAi machinery. Re-expression of PLEKHA7 in aggressive colon cancer cells that lack PLEKHA7 suppressed expression of MIR17HG, as well as anchorage independent growth of these cells. Our present data point towards a novel mechanism of lncRNA regulation that tethers epithelial tissue integrity with pro-tumorigenic cell transformation.

P2531
Board Number: B683
The role of calcium in Rho-dependent remodeling of epithelial tight junctions.
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In order for an epithelium to serve as an effective barrier, cell-cell junctions must be maintained during events that cause cell shape changes, such as morphogenetic movements and cytokinesis. During cytokinesis, the dividing cell and its adjacent cells undergo cell shape changes, thus generating tension changes at the junctions. Using live imaging in the epithelium of gastrula-stage *Xenopus laevis* embryos, we show that local loss of tight junction proteins (ZO-1 and Occludin) occur with increased frequency around dividing cells and their neighbors, resulting in a leaky barrier. These discontinuities are rapidly repaired by localized, transient activations of RhoA, which we call “Rho flares”. However, the mechanism underlying activation of Rho flares is unknown. Here, we show that transient influx of calcium (Ca²⁺) precedes activation of Rho flares. Because the frequency of Rho flares increases around cells undergoing cytokinesis, we hypothesized that a mechanical trigger may initiate Rho flares. One way Ca²⁺ may enter the cells at these sites is through a mechanosensitive cation channel (MSC) called Piezo1. Piezo1 can localize to the plasma membrane and opens in response to changes in membrane tension to allow Ca²⁺ influx. In ongoing work, we are testing whether Piezo1 is responsible for the Ca²⁺ influx at Rho flare sites. We find that embryos treated with the MSC blockers Gadolinium chloride (Gd³⁺) or GsMtx4 exhibit reduced Rho flares. Identifying the mechanism by which cells sense and respond to challenges to cell-cell junction integrity will help us gain a better understanding of diseases like Crohn’s Disease and Inflammatory Bowel Disease where barrier function is impaired.

Integrins and Cell-ECM Interactions 1

P2532
Board Number: B684
Myosin-X filopodia during cancer cell invasion.
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The formation of metastases is responsible for 90% of deaths in patients with solid tumours. Consequently, there is a pressing need to develop therapeutic strategies that block the ability of cancer cell to disseminate throughout the body. We and others have made an intriguing discovery that cancer metastasis is associated with the development of specialized cellular protrusions called filopodia. In migrating cells, filopodia are “antenna-like” protrusions, which contain cell-surface adhesion receptors,
such as integrins, responsible for constantly probing the cellular environment. At filopodia, integrins modulate signalling pathways that support cell migration, survival and proliferation. Integrins are transported to filopodia via a motor protein called Myosin-X, a regulator of filopodia formation. We discovered that myosin-X contribute to cancer cell metastases in vitro and in vivo models and that myosin-X is highly expressed in patient samples (including breast, pancreatic, colorectal, glioma and lung carcinoma) where it correlates with poor prognosis. Mechanistically, we found that myosin-X-mediated transport of integrins, together with integrin signalling in filopodia are two important prerequisites for cancer metastasis. In particular, we identified a signalling pathway, downstream of integrin signalling, involving Src, L-type calcium channels, and calpain-1 that regulates filopodia stability and directional migration. We are now studying the role of myosin-X filopodia during the different steps of the metastatic cascade, in vivo, using both intravital and extravital microscopy strategies. Intravital microscopy, using zebrafish embryo, revealed that cancer cells use filopodia extensively in vivo during both local invasion and extravasation. In addition, ex-vivo live cell imaging of whole tumors (subcutaneous tumours dissected from mice) revealed that myosin-x regulate cell protrusions at the invading edges but may also contribute to stromal ECM organization.

P2533
Board Number: B685
Actin retrograde flow actively aligns and orients ligand-engaged integrins in focal adhesions. V. Swaminathan¹, J.K. Mathew², S. Mehta³, P. Nordenfelt⁴, T.I. Moore⁵, N. KOGA⁶, D. Baker⁷, R. Oldenbourg³, T. Tani³, S. Mayor⁷, T.A. Springer⁵, C.M. Waterman¹; ¹Cell Biology and Physiology Center, National Heart Lung and Blood Institute, Bethesda, MD, ²National Centre for Biological Sciences, Bangalore, India, ³Eugene Bell Center, Marine Biological Laboratory, Woods Hole, MA, ⁴Division of Infection Medicine, Lund University, Lund, Sweden, ⁵Program in Cellular and Molecular Medicine, Harvard Medical School, Boston, MD, ⁶Institute for Molecular Science, Okazaki, Japan, ⁷Department of Biochemistry, University of Washington, Seattle, WA

Integrins are transmembrane receptors that, upon activation, bind extracellular matrix (ECM) or cell surface ligands and link them to the actin cytoskeleton to mediate cell adhesion, mechanosensing and migration. How integrin activation and organization in adhesions mediate the cells ability to sense directional mechanical cues is unknown. One model for the structural transitions mediating integrin activation termed “the cytoskeletal force hypothesis” posits that force transmitted from the cytoskeleton to ligand-bound integrins acts as an allosteric stabilizer of the extended-open, high-affinity state. Since cytoskeletal forces in migrating cells are generated by centripetal “retrograde flow” of F-actin from the cell leading edge, where integrin-based adhesions are initiated, this model predicts that F-actin flow should align and orient activated, ligand-bound integrins in integrin-based adhesions. Here, polarization-sensitive fluorescence microscopy of GFP-αV 3 integrin chimeras in migrating fibroblasts shows that integrins are co-aligned with respect to the axis of FAs and the direction of F-actin flow in a specific orientation, and this alignment requires binding immobilized ligand and talin-mediated linkage to a flowing cytoskeleton. Polarization imaging and Rosetta modelling of chimeras engineered to orient GFP differentially with respect to the integrin headpiece suggest that ligand-bound αVβ3 integrin may be markedly tilted by the force of F-actin flow. These results show that actin cytoskeletal forces actively sculpt an anisotropic molecular scaffold in FAs that may underlie the ability of cells to sense directional ECM and physical cues.
P2534
Board Number: B686
Increase in the protein levels of an anti-sprouting factor and integrin receptor, Thy-1, with age in the supraoptic nucleus: implications for a role in collateral axonal sprouting.
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It has been demonstrated that a young brain can overcome injury by axonal sprouting; however, it is well understood that the mature brain has a reduced capacity for functional or structural reorganization following injury. To this point, following injury, uninjured axons from the supraoptic nucleus (SON) undergo collateral axonal sprouting in the 35-day-old rat, but not in 125-day-old rats. Therefore, it appears that within the SON there are age-related changes that preclude the older rat from recovering following injury. Cell adhesion molecules have been previously demonstrated to play a role in axonal sprouting, both in a stimulatory and inhibitory manner. Thus, we compared protein levels of the integrin family of cell adhesion molecules and the integrin receptor, Thy-1, in 35 and 125-day-old SON using Western blot analysis. Our results demonstrated that in the 125-day-old SON, there was a significant increase in Thy-1 protein levels, which is an anti-sprouting factor that can interact with integrins. Conversely, there were no changes in the protein levels of alpha v, beta 3, or beta 5 integrin when comparing the sprouting (35-day-old rat) and non-sprouting SON (125-day-old rat). Additionally, there were no changes in protein levels of Thy-1 or the integrin proteins in the neural lobe (posterior pituitary), which is the site of axonal sprouting. Our results suggest that the observed increase in Thy-1 protein levels in the SON with age may contribute to an environment that prevents the collateral axonal sprouting in the SON of an older rat.

P2535
Board Number: B687
Spatiotemporal regulation of focal adhesions disassembly at the G2/M transition of the cell cycle.
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A hallmark of metazoan cell division is the drastic shape change occurring during mitotic cell rounding. Recent studies have shown that such shape change is required for proper chromosome segregation as well as spindle positioning, orientation and stability. For a dividing cell to round up, it must reduce its contact area with the extracellular matrix (ECM). This can be achieved by increasing cell cortical tension beyond the cell-ECM adhesion strength or reducing cell-ECM adhesion, or both. Here, we tested the hypothesis that mitotic cell rounding may be mediated by reducing cell-ECM adhesion through focal adhesion (FA) disassembly prior to mitosis. Using TIRF-based live cell imaging of FA, nuclear and cell cycle marker, we showed that FA loss is a prophase event that starts before nuclear envelope breakdown and coincides with cyclin B1 nuclear translocation. Analysis of FA dynamics showed that during mitosis, the number of assembling FA decreased while the FA disassembly rate increased. Traction force microscopy showed that this loss of cell adhesion was accompanied by a rapid drop in traction forces right at the onset of cell detachment with no apparent pre-deadhesion peak, suggesting that FAs are not disassembled simply by increasing cell contraction beyond the adhesion strength. Inhibition of mitotic kinases showed that at mitotic onset, PLK1 activity is required for inhibition of FA assembly, while CDK1 activation drives FA disassembly. To examine the possible pathways governing FA
disassembly downstream of CDK1, we manipulated regulators of FA disassembly known from studies of cell migration, including myosin II, FAK, calpain and its cleavage target talin, and integrins. This showed that neither calpain, myosin II nor FAK activity are required for cell de-adhesion during mitosis. To determine the requirement for integrin inactivation in FA disassembly, we locked integrins in their active/high affinity ECM-binding conformation using either manganese or conformation specific antibodies. As expected, we found that integrin inactivation was required for mitotic deadhesion, but quite unexpectedly, we found that blocking integrin inactivation caused failure of cells to complete mitosis. Inhibiting integrin inactivation in cells expressing GFP-PCNA as a marker of cell cycle stage showed that integrin inactivation and subsequent FA disassembly inhibited entry into mitosis, with the GFP-PCNA localization pattern indicating a G2 block. Taken together, our data suggest that mitotic cells use a specific mechanism, distinct from the ones used by migrating cells but dependent on Cdk1 and Plk1 activity, to de-adhere at mitosis. Our data further suggest that integrin inactivation and FA disassembly are required for cell cycle progression in adherent cells.

**P2536**

**Board Number: B688**

**Extracellular Matrix Substrates Affect Focal Adhesion Kinase (FAK) Distribution in Prostatic Smooth Muscle Cells.**

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Smooth muscle cells (SMCs) shift between the contractile and the synthetic phenotype in response to different environmental cues. The extracellular matrix (ECM) triggers specific signaling pathways during development, homeostasis in adults and in disease. External stimuli from the ECM translate through the transmembrane receptors integrins, which bind specific ECM proteins. This allows the cell to recognize specific clues and respond to the surrounding environment. The integrin cytoplasmic domain interacts with several proteins in a complex called focal adhesion. FAK is one such 125-KDa protein-tyrosine kinase protein, responsible for transducing the ECM-integrin-cytoskeleton signaling. The aim of this work was to identify how collagen, laminin-rich matrigel and fibrin substrates regulate SMC phenotype and to map how these ECM substrates affects FAK subcellular distribution in these cells. SMCs were obtained by culturing ventral prostate explants from male Wistar rats, in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 10 nM dihydrotestosterone, and 5 μg/mL insulin. Subsequently, SMCs in the fourth-to-seventh passages were seeded on plastic, type I collagen, matrigel or fibrin substrates. After 48 hours in culture, the SMCs were fixed and then incubated with antibodies against smooth muscle alpha-actin (alpha-SMA) and FAK A17. Images were acquired using a confocal LSM780 microscope (Carl Zeiss). ECM substrates did not affect α-SMA expression though cell morphology differed markedly. SMCs seeded on plastic or type I collagen were well spread, and FAK was mainly located at the focal adhesion and in the cell nucleus. Cells seeded on fibrin or matrigel were elongated and had many cell processes, while FAK was found predominantly in discrete cytoplasmic dots. In contrast to the cells on plastic or collagen, most cells on fibrin and matrigel lacked nuclear staining for FAK. These observations suggest that ECM components regulate SMC behavior and that FAK might be involved in the maintenance of the SMC phenotype in the prostate, irrespective of the expression of alpha-SMA.
P2537
Board Number: B689
Alternative splicing of tenascin-C modulates cell-matrix interactions during inflammation and disease.
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The extracellular matrix (ECM) is a complex network of proteins secreted by cells, which is essential for providing structural support and facilitating cell processes including adhesion, migration and survival. Tenascin-C is an immunomodulatory ECM protein that exhibits limited expression in healthy tissues, but is transiently elevated at sites of tissue injury, and is persistently expressed in chronic inflammatory diseases and tumours. Alternative splicing of tenascin-C’s 9 fibronectin type III-like domains (FnIII- A1, A2, A3, A4, B, AD2, AD1, C and D) generates enormous diversity in form; yielding 511 possible isoforms. Post-transcriptional modification of tenascin-C has been studied in cancer and during development where disease and tissue specific isoforms exhibit distinct adhesive, migratory and proliferative effects. However, little is known of how tenascin-C is expressed or alternatively spliced during inflammation. This study characterises inflammation and disease specific tenascin-C isoforms made by immune cells and fibroblasts, and investigates their functional relevance.

Biosynthesis and alternative splicing of tenascin-C was examined using standard curve qPCR, ELISA, Western blot and confocal immunocytochemistry in resting and activated primary human immune cells, dermal fibroblasts, and in synovial fibroblasts isolated from healthy controls and from osteoarthritis (OA) and rheumatoid arthritis (RA) patients. FnIII domains AD2-AD1, B-C-D and B-AD2-AD1-C-D were cloned, expressed and purified, and their impact on cell behaviour including adhesion, morphology and migration was assessed.

Basal tenascin-C expression was lower in myeloid and lymphoid cells than fibroblasts, and was induced in all following inflammatory stimulation. Tenascin-C expression was elevated in disease with RA and OA synovial fibroblasts containing higher levels than healthy controls. Alternative splicing following cell activation was cell-type specific: all FnIII except AD2 and AD1 were upregulated in dendritic cells and macrophages, in T-cells all FnIII were down-regulated and A1 was absent, and no change in splicing was observed in activated dermal fibroblasts. RA and OA fibroblasts predominantly expressed smaller isoforms, with FnIII-B and D specifically elevated in RA. Functional analysis revealed differences in the adhesion, morphology and migration of myeloid cells and dermal fibroblasts cultured on FnIII AD2-AD1, B-C-D, B-AD2-AD1-C-D and full length tenascin-C substrates.

For the first time, this study reveals differences in tenascin-C biosynthesis and alternative splicing by immune cells and fibroblasts following activation with inflammatory stimuli, and identifies cell-type specific responses to distinct FnIII that direct cell-matrix interactions.

P2538
Board Number: B690
Germline stem cell maintenance control by adipocyte collagen in adult Drosophila females.
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Stem cells reside in specialized niches that are a source of local signals and also receive a variety of systemic inputs. In adult female Drosophila, germline stem cells (GSCs) are physically attached to a somatic niche composed primarily of cap cells through E-cadherin. Local signals produced by cap cells, including bone morphogenetic protein (BMP) signals, are required for proper regulation of GSC function.
In adult females, a prominent collagen IV-containing extracellular matrix is maintained around the GSC niche; however, it remains unknown what the cellular source of collagen IV is in the adult GSC niche or how it affects GSC function. In the adult female fat body, collagen IV proteins are abundant and highly regulated by diet, leading us to ask whether collagen IV function in adipocytes is required for GSC function. Adipocyte-specific knockdown of collagen IV in adult females leads to increased GSC loss over time. Interestingly, we found that collagen IV produced in adult adipocytes is transported to and incorporated into the extracellular matrix in the GSC region during adulthood. Although BMP signaling from the niche is not perturbed, E-Cadherin levels are decreased at the GSC-niche interface when collagen IV is knocked down in adipocytes. We are currently testing the hypothesis that adipocyte-derived collagen IV regulates integrin/focal adhesion kinase signaling in the GSC region to regulate E-Cadherin levels and GSC maintenance. To our knowledge, this is the first example of an extracellular matrix component produced in adult adipocytes being transported to a stem cell niche in a distinct, fully established adult tissue. These findings are a major step in advancing our understanding of the wide range of mechanisms for how adipocytes control the function of other organs, and are widely relevant considering the devastating impact of the current global obesity epidemic on our health.

P2539

Board Number: B691

BMP4-induced differentiation sensitizes glioblastoma tumor initiating cells to mechanical inputs.

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Glioblastoma (GBM) is one of the most common and lethal primary brain cancers. Difficulty in treating this disease has been attributed to a rare but important subpopulation of cells that evade chemotherapeutic treatment, diffusely invade the surrounding tissue, and establish secondary tumors. These tumor-initiating cells (TICs) share characteristics with stem cells in that they self-renew and differentiate to produce the more specialized and heterogeneous cell populations that make up the bulk of the tumor. GBMs are typically stiffer than the surrounding brain tissue, and tumor cells follow stiffer structures such as vascular beds as they invade. Because of these observations, and because GBM is a disease of cell migration, we probed how these TICs respond to stiffness cues in their environment. In contrast to the majority of other cell types, TICs showed surprisingly little stiffness-dependent changes in cell shape and migration. By increasing cellular force generation via constitutive activation of RhoA, we were able to sensitize the TICs to mechanical cues. Furthermore, we found that TICs with heightened cellular force generation showed reduced invasion through three-dimensional materials, and when xenografted, produced small and circumscribed tumors, which translated into a 30% increase in survival time. Evidence from our lab and from others suggests that mechanosensitivity and differentiation state are correlated with TIC invasiveness and tumor generating potential. Others have demonstrated that bone morphogenetic protein 4 (BMP4) elicits a differentiation-like effect on GBM TICs and extends survival in a xenograft model. We therefore asked how the mechanosensitivity of these TICs changes as they are exposed to bone morphogenetic protein 4 (BMP4). We found that TICs treated with BMP4 showed increased mechanosensitivity of cell shape but not migration speed. To achieve a systems-level picture of the links between TIC differentiation state and mechanically-regulated signaling, we performed RNA sequencing. Interestingly, cells exposed to BMP4 on soft substrates showed a greater transcriptomic shift compared to those on stiff substrates, mirroring the observation that TICs exhibit a
“stiff” phenotype on soft gels and that sensitivity to soft gels is heightened upon BMP4 treatment. Of the genes differentially influenced by BMP4 treatment, pathways associated with cell-matrix interactions were particularly impacted. Immunoblotting and immunostaining confirmed differentiation-mediated changes in focal adhesion protein expression, including integrins and zyxin. Taken together, these findings suggest a link between differentiation, mechanosensitivity and invasion, and that mechanical signaling could be leveraged to control tumor progression.

P2540
Board Number: B692
Mechanical modulation of glycolysis through phosphofructokinase and its activators in a KRAS-dependent manner.
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Cells can actively sense the microenvironment by cytoskeletal remodeling and by actomyosin contractility. Both processes consume a significant amount of energy, which is supported by the cell’s metabolic activity. However, it is unclear if cells adjust their metabolism directly to the mechanical conditions of the environment. To begin to test such a link this study investigates metabolic changes as a function of extracellular matrix stiffness using untransformed human bronchial epithelial cells (HBECs) and non-small cell lung cancer cells (NSCLCs). We show that HBECs have more glucose metabolites and exhibit higher rates of glycolysis when placed on stiff substrate in comparison to soft substrate. Pharmacological inhibition of focal adhesion kinase and actomyosin contractility both disrupt glycolysis. Moreover, we show that expression of phosphofructokinase platelet (PFKP), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3 (PFKFB3), and AMP-activated protein kinase (AMPK) are down-regulated upon decreased focal adhesion or intracellular tension, which could be partially rescued by proteasome inhibition. However, both mutant Kras-transformed HBECs and NSCLCs with the KRAS mutations assume consistent rates of glycolysis independent of cell substrate stiffness. Interestingly, the expression of PFKP is maintained by the mechanical desensitization driven by the Kras mutations. Knockdown of PFKP in Kras-mutant NSCLCs hindered tumor growth in mice as expected. Thus, while untransformed lung epithelial cells appear to adjust their metabolic activity in response to the mechanical properties of the microenvironment, Kras-mutated, transformed lung cancer cells loose this responsiveness, which may support the conditions of tumorigenesis and metastasis.

P2541
Board Number: B693
A Cautionary Tail: Changes in Integrin Behavior with Labeling.
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All mesenchymal cell migration depends upon integrin heterodimers forming connections between the extracellular matrix (ECM) and the cytoskeleton to enable the cell to pull itself forward. Critical to this function is the ability of integrins to increase their affinity in response to ECM and redistribute into structural and signaling hubs called adhesions. Decades of conventional fluorescence microscopy (TIRF, FRAP, FRET, FLIP) have shown that genetically expressed fluorescently labeled integrins correctly respond to ligand and form adhesions, and this ability to respond to a ligand is independent of which subunit of the heterodimer is labeled. However, when we imaged the cells using live-cell single
molecule super-resolution, we discovered differences in the behavior of the integrin molecule that depend upon which subunit is labeled. When we expressed either αβ3 or αβ1 integrins in the null backgrounds of CHO-K1 and CHO-B2 cells, respectively, we found that labeling the beta subunit decreased the mobility of individual integrins, increased the size of the adhesions, and increased the extent of cell spreading. These results are indicative of elevated integrin affinity, and the increased size of adhesions and cell spreading were nearly identical to results obtained when cells expressing unlabeled integrin were experimentally forced into a higher affinity state by Mn^{2+} treatment. Moreover, direct measures of integrin affinity made with quantitative immunofluorescence of an antibody targeted against a site that is only accessible when the affinity is elevated, indicate that labeling the beta subunit significantly increases the affinity of the integrin heterodimer in comparison to labeling the alpha subunit. Labeling the longer cytoplasmic tail of the beta subunit likely spreads the two subunits apart to change integrin conformation and enhance the response of the cell to a ligand. Thus, our dense-field, live-cell measurements of single molecule behavior can detect changes in receptor conformation, bringing to light a cautionary tail regarding molecular labeling.

P2542
Board Number: B694
Directly measuring integrin conformational change on the cell surface using Interferometric Photoactivation Localization Microscopy (iPALM).
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Measuring protein conformational change has been the realm of soluble protein chemistry, NMR spectroscopy and X-ray crystallography. Here, we describe the use of super-resolution Interferometric Photoactivation and Localization Microscopy (iPALM) combined with a constrained photoactivatable fluorescent protein integrin fusion to measure the conformational change of the lymphocyte specific integrin LFA-1 on the cell surface. Our molecular scale measurements of the cell surface adhesion receptor agree with predicted distances derived from crystal structure models of bent and extended integrins. While measured LFA-1 conformation show that extension is ligand specific and inducible by small molecule allosteric modulators, adding additional support for the cytoskeletal force model of integrin activation.

P2543
Board Number: B695
A regulatory mechanism in the inside-out integrin signaling pathway.
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Integrins are cellular receptors that regulate cell adhesion, migration, and proliferation. Abnormal integrin activity has been linked to many life-threatening diseases. Integrins are activated via an inside-out pathway, which is induced by the Rap1, and mediated by Rap1-interacting adaptor molecule (RIAM) and talin. Kindlin further enhances integrin activity. We have previously elucidated the structural basis of the interactions among these proteins. However, more detailed physiological information is still lacking in this signaling pathway, and many in vivo studies have indicated that alternative mechanisms
may exist. The current focus of our efforts is to identify new regulatory mechanisms in the inside-out pathway that fine-tune integrin activation by structural and biochemical approaches. We will present data regarding the structural and mechanistic basis of these regulatory events.

Chaperones, Protein Folding, and Quality Control 1

P2544
Board Number: B697
Polyphosphate protects organisms against DNA-damaging agents.
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Organisms are continually exposed to stressors that specifically target and damage DNA, including ultra violet radiation or chemical mutagens. We now discovered that polyphosphate (polyP), a linear polymer consisting of up to 1000 inorganic phosphate (Pi) molecules linked by high-energy ATP-like bonds, protects bacteria against a variety of different DNA damaging agents. We found that deletion of ppk, the polyP-synthesizing enzyme in E. coli, sensitizes cells against DNA damaging agents ranging from UV light to cisplatin, mitomycin C and phleomycin. Cells lacking polyP present significantly higher mutagenesis rates in response to cisplatin treatment in comparison to cells deleted for the polyP-degrading enzyme (ppx). These results agree well with quantitative polyP measurements, which revealed that E. coli accumulates large amount of polyP in response to cisplatin treatment. Preliminary results suggest that polyP protects cells using a mechanism that is independent of its known function as oxidative stress protecting agent, and independent of modulating DNA-repair systems. Instead, genetic studies suggest that polyP might protect DNA by chromosome compaction through interaction with histone-like proteins. Altogether, these observations provide new insights on the global function of PolyP upon stress and might open new ways of co-treatment when using DNA-damaging drugs.

P2545
Board Number: B698
GIV/Girdin Mediates Cell Survival during Endoplasmic Reticulum Stress.
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Endoplasmic reticulum (ER) stress is a form of cellular stress that is experienced both under normal physiological conditions such as in professional secretory cells and disease states such as cancer, diabetes and neuro-degeneration. Upon facing ER stress, cells initially try to restore normal function by activating a conserved signaling pathway called the Unfolded Protein Response (UPR). However, if the stress is overwhelming and cells are not able to recover within a reasonable time frame, the UPR ultimately commits cells to apoptosis. How cells make this life-or-death decision remains an exciting yet poorly understood phenomenon. Here, we show that GIV (Go-Interacting Vesicle associated protein aka Girdin), a multimodular signaling protein, helps promote cell survival during ER stress via activation of the Akt pathway. HeLa cells treated with various ER stressors activate the Akt pathway and this activation is significantly diminished upon shRNA-mediated depletion of GIV. Furthermore, GIV-depleted cells show a significant decrease in cell survival during ER stress. We also found that GIV interacts with one of the key ER chaperones in an ER stress dependent manner. Taken together, these findings suggest that GIV may play an important role in helping cells survive ER stress. Our current and future studies are
focused on delineating the exact mechanism by which GIV enhances cyto-protective Akt signals during ER stress. This project is supported by the NIH grant # SC2GM121246 (DB) and in part by the NIGMS Award # R25GM071638 (RC and YR). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

P2546
Board Number: B699
Cytosolic proteostasis through importing of misfolded proteins into mitochondria.

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Loss of proteostasis underlies ageing and neurodegeneration characterized by the accumulation of protein aggregates and mitochondrial dysfunction. Although many neurodegenerative disease-associated proteins can be found in mitochondria, it remains unclear how mitochondrial dysfunction and protein aggregation could be related. In dividing yeast cells, protein aggregates that form under stress or during ageing are preferentially retained by the mother cell, in part through tethering to mitochondria, while the disagggregase Hsp104 helps to dissociate aggregates and thereby enables refolding or degradation of misfolded proteins. Here we show that, in yeast, cytosolic proteins prone to aggregation are imported into mitochondria for degradation. Protein aggregates that form under heat shock contain both cytosolic and mitochondrial proteins and interact with the mitochondrial import complex. Many aggregation-prone proteins enter the mitochondrial intermembrane space and matrix after heat shock, and some do so even without stress. Timely dissolution of cytosolic aggregates requires the mitochondrial import machinery and proteases. Blocking mitochondrial import but not proteasome activity causes a marked delay in the degradation of aggregated proteins. Defects in cytosolic Hsp70s leads to enhanced entry of misfolded proteins into mitochondria and elevated mitochondrial stress. We term this mitochondria-mediated proteostasis mechanism MAGIC (mitochondria as guardian in cytosol) and provide evidence that it may exist in human cells.

P2547
Board Number: B700
Hsp104 disaggregase: protein refolder or phase converter?

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In budding yeast, S. cerevisiae, many environmental stresses such as heat shock trigger aggregation of soluble proteins and RNA into massive particles, many of which are then recruited to stress granules (SGs). During the recovery period, a set of molecular chaperones called heat shock proteins resolubilizes the constituent proteins back to the cytosol. Although this aggregation has long been interpreted as resulting from stress-induced protein misfolding, recent results offer an alternative view. We recently demonstrated that poly(A)-binding protein (Pab1 in budding yeast), a classical marker for SGs, demixes into hydrogel droplets in vitro upon exposure to physiological stress conditions. Demixing involves phase separation and gelation, and is an adaptive response to stress. Here we show that the Hsp104 disaggregase system disperses Pab1 hydrogels with unparalleled efficiency relative to the model aggregate firefly luciferase. We isolate the precise proteins...
necessary for efficient dispersal. In an important methodological advance, we introduce a new fluorescence anisotropy assay for kinetic monitoring of the conversion of inert Pab1 hydrogels into soluble RNA-binding Pab1 monomers. Our assay reveals that hydrogel dispersal into functional monomers is markedly more rapid than disaggregation of a model misfolded protein. Our results raise important questions as to the identity of the native substrates of protein disaggregase systems, the mechanisms by which these systems recognize their substrates, and the molecular features which control the efficiency of molecular dispersal of phase-separated endogenous structures.

P2548

Board Number: B701

Ire1 RNase specificity separates transcriptional and post-transcriptional regulation of ER protein homeostasis.

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The endoplasmic reticulum (ER) is the major folding compartment for most secretory and plasma membrane proteins in the cell. A conserved signaling pathway, the unfolded protein response (UPR), senses and modulates the folding capacity of the ER. To maintain ER protein homeostasis under ER stress conditions, the ER membrane embedded sensor, Ire1, binds unfolded proteins through its ER-lumenal domain and initiates two distinct mRNA processing programs through its cytoplasmic kinase/RNase domains. First, in both metazoans and S. cerevisiae, Ire1 catalyzes the unconventional cytoplasmic mRNA splicing of XBP1 (metazoans) or HAC1 (S. cerevisiae)—thereby initiating a transcriptional response that increases the ER folding capacity. Second, in metazoans and S. pombe, Ire1 selectively degrades ER-localized mRNAs—thereby post-transcriptionally reducing the ER’s protein folding burden through regulated Ire1-dependent mRNA decay. Thus, Ire1 homologs in S. cerevisiae and S. pombe are specialized to only one of the two functional outputs, while Ire1 in metazoans can perform both. To understand how Ire1 can regulate protein homeostasis through distinct RNA processing programs mechanistically, we characterized Ire1 from S. cerevisiae and S. pombe with in vivo and in vitro experiments. Surprisingly, despite relatively low sequence conservation in the lumenal domains, these domains share conserved ER-stress sensing mechanism. Conversely, despite high sequence conservation, Ire1 cytoplasmic domains recognize distinct RNA sequence and structural features, which leads to functional divergence in RNA processing. Finally, by applying our new findings, we successfully reconstituted unconventional mRNA splicing in S. pombe cells. Therefore, we engineered S. pombe into a metazoan-like Ire1 system, where unconventional mRNA splicing and selective mRNA decay co-exist. Our results provide new insights into a mechanistic understanding of Ire1 function and its interplay with RNA substrates.
P2549
Board Number: B702
Clustering of IRE1α depends on sensing the type and level of ER stress, but not on its RNase activity.
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The sensors of the Unfolded Protein Response (UPR) react to Endoplasmic Reticulum (ER) stress by transient activation of their enzymatic activities, which then initiate various signaling cascades in order to restore the cellular homeostasis. However, if the stress exceeds a tolerable threshold, the UPR sensors initiate programmed cell death and therefore play a fundamental role in determining the cell fate. Following ER stress, the ER transmembrane sensor Inositol-Requirement Enzyme 1 α (IRE1α) undergoes several molecular changes to transmit the stress signal to the cytosol: dimerization, auto trans-phosphorylation and, finally, activation of its RNase activity. In addition, IRE1α exhibits stress-induced clustering in the same transient fashion as its endoribonuclease activities. Neither the function nor the regulation of IRE1α clustering is well-understood, so we characterized the molecular requirements for clustering. Our data show that clustering depends on the stress sensing mechanism of IRE1α, because it is prevented by mutations in the dimerization or kinase domains, and because ablation of the ER luminal chaperone BiP causes massive and irreversible clustering of IRE1α. Although IRE1α kinase activity is required for clustering, sustained phosphorylation through okadaic acid treatment does not extend the clustering response, suggesting that phosphorylation is necessary for initiation but not dispersal of clusters. Surprisingly, the endoribonuclease activity is not necessary for clustering, since both active and inactive forms of IRE1α cluster irreversibly. Furthermore, clustering is dispensable for IRE1α endoribonuclease activity because the flavonoid luteolin binds to and activates IRE1α RNase activity without inducing clustering. The clustering response differentiates between distinct sources and magnitude of ER stress. The calcium pump inhibitor thapsigargin induces significantly less clustering than the inhibitor of glycosylation tunicamycin or the disulfide bond reductant dithiothreitol, and it also inhibits the tunicamycin-induced clustering. This suggests that ER calcium homeostasis is required for cluster formation. We propose that IRE1α clustering is a cellular tool to measure the type and strength of ER stress, since its magnitude and timing correlate inversely with the cells’ ability to cope with the stress.

P2550
Board Number: B703
Nuclear-import receptors reverse aberrant phase transitions of RNA-binding proteins with prion-like domains.
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RNA-binding proteins (RBPs) with prion-like domains (PrLDs) phase transition to functional liquids, which can mature into aberrant hydrogels composed of pathological fibrils that underpin fatal neurodegenerative disorders. Several nuclear RBPs with PrLDs including TDP-43, FUS, hnRNPA1, and hnRNPA2 mislocalize to cytoplasmic inclusions in neurodegenerative disorders and mutations in their PrLDs can accelerate fibrillization and cause disease. Here, we establish that nuclear-import receptors specifically and potently chaperone and disaggregate wild-type and disease-linked RBPs bearing a NLS.
Karyopherin-β2 (Kapβ2) engages PY-NLSs to inhibit and reverse FUS, TAF15, EWSR1, hnRNPA1, and hnRNPA2 fibrillation, whereas importin-a plus KapB1 prevent and reverse TDP-43 fibrillation. Remarkably, Kapβ2 dissolves and liquidizes aberrant hydrogels formed by FUS and hnRNPA1 fibrils. Increased Kapβ2 expression prevents RBPs with PY-NLSs incorporating into stress granules, restores nuclear localization, and rescues degeneration caused by disease-linked FUS and hnRNPA2 in vivo. Thus, elevating nuclear import receptor expression could therapeutically restore RBP homeostasis and mitigate neurodegeneration.

P2551
Board Number: B704
Developing therapeutic protein disaggregases for Neurodegenerative Disease.
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The accumulation of abnormal protein aggregates in human brain is connected with neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease. The mammalian protein-disaggregase system comprised of Hsp110, Hsp70, and Hsp40 (Hdj1 and Hdj2) can protect cells from proteinopathies. However, this disaggregate system has limitations and activity declines with age. Therefore, as a therapeutic strategy for neurodegenerative disease we propose to introduce an exogenous, synthetic disaggregate machinery, based on Hsp104, that converts amyloid to soluble, functional protein. Hsp104 is a hexameric AAA+ ATPase that is critical for stress tolerance in yeast by facilitating the resolubilization of stress-damaged, aggregated proteins. The homologues of Hsp104 are highly conserved in bacteria, fungi, and plants, but absent from metazoa. The disaggregate activity of Hsp104 can be enhanced by the presence Hsp70 and Hsp40. Hsp104 can dissolve α-synuclein, β-amyloid, and tau aggregates, but impractically high Hsp104 concentrations are needed. We aim to rationally design potentiated Hsp104 variants that can reverse protein misfolding at low concentrations. By investigating the mechanism of Hsp104 disaggregation and its structural basis, we will engineer and evolve potentiated Hsp104 variants that eradicate α-synuclein, β-amyloid, or tau misfolding and toxicity. We have exploited new cryo-EM structures of Hsp104 to engineer key interfaces to yield several novel Hsp104 variants with tunable activity, which can reverse protein aggregation in the presence or absence of human Hsp70 and Hsp40. These novel Hsp104 variants effectively rescue the aggregation and toxicity of diverse human neurodegenerative disease proteins and could have therapeutic utility. Moreover, our engineered Hsp104 variants provide key mechanistic insights into disaggregate function.

P2552
Board Number: B705
Modification of the ribosome as part of the adaptive response to oxidative stress in yeast.
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Living organisms are constantly exposed to a variety of environmental and internal stressors that are detrimental to their cellular physiology and viability. One such condition, oxidative stress, is caused by abnormal amounts of Reactive Oxygen Species (ROS) that can lead to damage to proteins, nucleic acids, and lipids. Although the mechanisms to neutralize ROS have been widely studied, the understanding of ROS-mediated signaling for these mechanisms is rather incomplete and sparse. We have uncovered a previously undescribed phenomenon of yeast ribosomes to respond to elevated levels of ROS through a
site-specific endonucleolytic cleavage of the 25S rRNA in the c-loop of the expansion segment 7 (ES7c) regions. ES7c is a putative regulatory region located on the surface of the 60S ribosomal subunit. This cleavage was detected at the early stages of stress and is not a part of cell death. Additionally, we have seen that low level exposure to oxidative stressors can lead to a protective effect against this cleavage when cells are exposed to higher levels of the stressor. We conclude that ES7c cleavage represents an early and sensitive marker of increased ROS levels in yeast cells and propose that changes in ribosomes may signify an adaptive response to oxidative stress.

P2553
Board Number: B706
Engineering potentiated Hsp104 variants with enhanced substrate-specificity to counter neurodegeneration.
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Hsp104, a hexameric AAA+ protein disaggregase found in yeast, dissolves amorphous aggregates and amyloid using energy from ATP hydrolysis. With the Hsp70 chaperone system, Hsp104 reactivates misfolded protein substrates. Interestingly, an Hsp104 homolog is absent from metazoa, and as such humans lack ample machinery to eradicate protein misfolding in the context of neurodegenerative disease. Therefore, Hsp104 is an ideal therapeutic candidate for directly targeting underlying protein misfolding. We previously engineered potentiated Hsp104 variants that disassemble neurodegenerative disease-associated substrates α-synuclein (implicated in Parkinson’s disease) and FUS and TDP-43 (implicated in amyotrophic lateral sclerosis). Though robust disaggregases, these Hsp104 variants are not substrate-specific and likely unfold essential proteins, causing off-target effects. Here, by targeting conserved pore loop residues that contact substrate in the central channel of Hsp104, we engineered substrate-specific Hsp104 variants capable of specifically suppressing α-synuclein toxicity in yeast proteinopathy models. These α-synuclein-specific Hsp104 variants only minimally suppress toxicity of FUS or TDP-43. Interestingly, we isolated two sets of α-synuclein-specific Hsp104 variants that differ in their ability to restore proper localization to and suppress toxicity of α-synuclein in yeast, and have different biochemical features. As such, these substrate-specific Hsp104 variants may selectively suppress α-synuclein toxicity through different mechanisms. Excitingly, some α-synuclein-specific Hsp104 variants inhibit dopaminergic neuron loss in a C. elegans model of Parkinson’s disease. Our findings reveal key insights to tuning Hsp104 to therapeutically target specific misfolded proteins.

P2554
Board Number: B707
Yeast FIT2 homologs mediate the crosstalk between lipid droplet biogenesis, the unfolded protein response and cytoplasmic proteostasis.
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Lipid droplets (LDs) have long been regarded as inert cytoplasmic organelles with the primary function of housing excess intracellular lipids. More recently, LDs have been strongly implicated in conditions of lipid and protein dysregulation. Moreover, these conditions are major contributors to the
pathophysiology of metabolic diseases and concomitantly activate cellular stress response pathways, namely the unfolded protein response (UPR) and heat shock response (HSR). However, despite the increasing support for the involvement of LDs in other cellular processes, mechanistic insight into the fundamental process of LD biogenesis and its direct physiological relevance to the cell remains rudimentary. The fat storage inducing transmembrane (FIT) family of proteins comprises of evolutionarily conserved endoplasmic reticulum (ER)-resident proteins that have been reported to induce LD formation. Using Saccharomyces cerevisiae as a model, this study aims to dissect the role of LDs in cellular lipid and protein homeostasis through the yeast FIT homologs (ScFITs), SCS3 and YFT2. While LD biogenesis and basal UPR activation remain unaltered in ScFIT mutants, SCS3 was found to be essential in the absence of the sole yeast UPR sensor IRE1. Devoid of a functional UPR, Δscs3 mutants exhibited increased microsomal triacylglycerol levels, indicative of impaired ER-LD lipid partitioning and suggesting a UPR-dependent compensatory mechanism for LD biogenesis. Surprisingly, the absence of ScFIT results in the downregulation of the closely-related HSR pathway. In line with this observation, the turnover of cytoplasmic misfolded proteins is impaired in ΔScFIT cells, while a screen for interacting partners of Scs3 identifies components of the proteostatic machinery as putative targets. Taken together, these suggest that ScFITs may modulate proteostasis and stress response pathways with LD biogenesis at the interface between the two cellular processes.

P2555
Board Number: B708

Adaptive unfolded protein response facilitates melanogenesis.

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The endoplasmic reticulum (ER) is a network of membranous tubules within the cytoplasm of a eukaryotic cell that is involved in protein and lipid synthesis. The ER possesses oxidizing environment, chaperones, protein-disulfide isomerases and glycosylating enzymes that facilitate protein folding. Any perturbation that disrupts protein folding capacity in the ER activates a signaling cascade called the unfolded protein response (UPR). UPR functions to bring back ER protein folding homeostasis, dysregulation of which can eventually result in apoptosis. The paradoxical role of UPR has been examined in variety of cellular models and is particularly critical to secretory cell types such as plasma cells, pancreatic β cells etc. Melanocytes are unique pigment secreting skin cells. These cells synthesize the pigment melanin within organelles called melanosomes, which are subsequently transferred to keratinocytes. Melanosomes are Lysosome-related organelles & several key melanosomal proteins are heavily glycosylated and dependent on glycosylating enzymes in the ER-Golgi channel for their folding. Biosynthesis of melanin and melanosomes is activated during tanning resulting in increased secretory load on melanocytes. We set out to decipher the role of ER UPR during the melanogenesis process. We employed previously developed B16 pigmentation oscillator model, wherein cells can be triggered to produce melanin pigment in a cell autonomous way in 6-8 days. This model is particularly useful to study temporal regulation of pathways during the transition from ‘white’ to ‘black’ cells. While following the changes in the ER UPR markers during melanogenesis, we observed a concomitant activation of the UPR pathways; particularly the X-box binding protein-1 (Xbp1) splicing was upregulated. We could also identify other downstream UPR genes to be upregulated along with melanogenesis. Other than the canonical UPR pathways, our unbiased analysis of the pigmentation oscillator model revealed molecular signature for the regulation of Cyclic-AMP Response Element Binding Protein 3-like protein 2 (Creb3l2). CREBs are known to be crucial to the regulation of Microphthalmia-Induced Transcription Factor (MITF), a Master regulator of melanocyte biology. Activation of Creb3l2 is marked by protease-mediated release

Monday-434
of its N-terminus, which acts as a transcription factor. Both Creb3l2 expression and activation was regulated during early time points of pigmentation. Thapsigargin, an ER stress-inducing agent, also caused a clear activation of Creb3l2 in B16 cells. Pharmacological induction of ER stress increased melanogenesis, while rescue with chemical chaperones caused depigmentation. Overall, our study reveals the essential function of ER adaptation via the UPR for melanogenesis.

P2556
Board Number: B709
Exploring and enhancing the metazoan disaggregate system to combat protein aggregation.
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Proteins perform a myriad of essential and non-essential functions within the cell; however, proteins must be properly folded in order to function. During cellular stress, proteins may become misfolded and aggregate through aberrant intermolecular interactions. The 70 and 40 kDa heat shock proteins (Hsp70 and Hsp40) form one of the predominant molecular chaperone systems within the cell that unfolds and refolds misfolded proteins. However, aggregated proteins contain stable intermolecular interactions that are more difficult to break. Hsp110, a member of the Hsp70 super-family, collaborates with Hsp70 and Hsp40 to enable disassembly of protein aggregates and restoration of native protein function. Our lab has shown that the metazoan disaggregate system (MDS) comprising of Hsp70, Hsp40, and Hsp110 family members refolds disordered aggregates such as urea-denatured luciferase and heat-denatured GFP in vitro. The human genome contains 13 Hsp70 genes, over 50 Hsp40 genes, and 4 Hsp110 genes, giving rise to thousands of three-component combinations of the MDS. Select combinations more effectively disassemble α-synuclein amyloid than others, suggesting that different combinations of the MDS can have drastically different efficacy against the same substrate. This combinatorial phenomenon may be the key to understanding how a disaggregate system of under a hundred genes could chaperone a proteome of tens of thousands of proteins. We have purified a number of Hsp70, Hsp40, and Hsp110 proteins that are expressed in the cytosol of cells and systematically assessed the disaggregate activity of each combination against a variety of aggregated substrates. We are also screening a library of small molecules to identify enhancers and inhibitors of the MDS. Identified small-molecule enhancers are promising candidates for the treatment of neurodegenerative diseases. The results presented here further our understanding of the MDS and provide crucial therapeutic leads for a devastating class of diseases.

P2557
Board Number: B710
A guardian factor that protects folding polypeptides from promiscuous degradation.
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Newly synthesized proteins engage molecular chaperones that assist folding. Their progress is monitored by quality control systems that target folding errors for degradation. Paradoxically, chaperones that promote folding also direct unfolded polypeptides for degradation. Hence, a mechanism was previously hypothesized that prevents the degradation of actively folding polypeptides. In this study, using genetic and biochemical approaches, we show that a conserved endoplasmic
reticulum (ER) membrane protein complex, comprised of Slp1 and Emp65 proteins, performs this function in the ER lumen. The complex binds unfolded proteins and protects them from degradation during folding. In its absence, the degradation of misfolded ER proteins is aberrantly accelerated, and, more importantly, approximately 20-30% of newly synthesized normal proteins that could otherwise fold are degraded. Our further analysis revealed that Slp1 specifically associates with premature ER proteins to protect them from ER-associated degradation (ERAD). Slp1-Emp65 complex hosts a broad range of clients and protects both glycosylated and non-glycosylated proteins. However, the substrates of this complex are restricted to soluble proteins. Taken together, these studies demonstrate the vulnerability of newly translated, actively folding polypeptides and the discovery of a new proteostasis functional class we term “guardian” that protects them from degradation.

**P2558**

**Board Number: B711**

Defining conserved and divergent functions of Hsp104.
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Hsp104 is a hexameric AAA+ protein disaggregate from yeast that rapidly dissolves disordered aggregates, preamyloid oligomers, amyloids, and prions. These protein-remodeling activities facilitate resistance to and reversal of stress-triggered protein misfolding, and have allowed yeast to harness prions for adaptive purposes. Hsp104 homologues are found in all nonmetazoan eukaryotes and eubacteria, but the vast majority of these remain unexplored. Thus, Hsp104 sequence space, and the extent of protein-remodeling activities enabled by it, is unknown. Here, I present our efforts to functionally define diverse Hsp104 homologues from fungal, plant, algal, and protozoan lineages using a comparative biology approach. Hsp104 homologues perform various protein-remodeling activities performed by Hsp104 from *S. cerevisiae* (ScHsp104) via a conserved mechanism. However, we find that they are also able to antagonize unique and diverse proteotoxic misfolding events under conditions where ScHsp104 is inactive. I also present our work to understand the molecular mechanisms underpinning these activities. Our results establish that natural Hsp104 homologues can have therapeutic-disaggregate activity, and suggest that sequence variation among Hsp104 homologues may be a valuable resource for engineering therapeutic disaggregases to counter disease.

**P2559**

**Board Number: B712**

Recognition of transmembrane domains by HRD1 E3 ligase during the membrane protein quality control.
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A significant proportion of newly synthesized transmembrane proteins are misfolded and degraded by the endoplasmic associated quality degradation (ERAD) pathway. The mechanisms by which ERAD pathway selectively recognizes misfolded or non-native transmembrane domains (TMDs) of membrane proteins are unclear. To address this question, we identified a novel ERAD substrate, WRB, which has three TMDs and robustly degraded when it fails to assemble with its membrane subunit. We find that the conserved E3 ligase HRD1 recognizes a single lysine residue in the third TMD of WRB for ubiquitination and retrotranslocation into the cytosol. Replacing the lysine residue in the third TMD with
either a negative or hydrophilic residue can also be recognized by HRD1 for degradation. Unexpectedly, an efficient recognition of these aberrant TMDs by HRD1 is not occurring within the lipid bilayer, but rather requires the flipping of the TMD into the ER lumen. Unexpectedly, we find that the TMD flipping into the ER lumen is not only due to the low hydrophobicity but also due to its shorter cytoplasmic tail. Interestingly, HRD1 appears to recognize the overall hydrophobicity of the flipped TMD, but not specific charge or hydrophilic residues in the flipped TMD. Future studies are focusing on whether HRD1 directly recognizes the flipped TMD, which is still attached to the membrane through other two TMDs of WRB. We propose that flipping of TMDs may be a standard feature for discriminating between misfolded and folded multi-transmembrane proteins by E3 ligases in the ER.

P2560
Board Number: B713
Induction of Activator of G-Protein Signaling 3 Puncta: Role of Serine/Threonine Residues in the G-Protein Regulatory Domain and Lysosomal Inhibition.
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Activator of G-protein Signaling 3 (AGS3), a receptor independent activator of G-protein signaling, contains 7 tetratricopeptide repeats (TPR) and 4 G-protein regulatory motifs (GPR) connected by a linker region. Disruption of TPR organization by point and deletion mutations induce the formation of punctate structures facilitating entrance into the aggresomal pathway. To further define regulatory factors affecting AGS3’s subcellular “positioning”, we addressed the role of potential sites of protein phosphorylation in the GPR domain. AGS3 is a phosphoprotein with putative phosphorylation sites in the GPR domain. We generated a series of AGS3 constructs with mutations of potential serine/threonine (S/T) phosphorylation sites in the GPR domain and transiently expressed the cDNA constructs in COS-7 and HEK-293 cells. Mutation of all 24 S/T residues in the GPR domain to alanine (AGS3-GFP-PM1) resulted in the distribution of AGS3 to cytosolic punctate structures in marked contrast to the distribution of WT AGS3 (cell cortex and diffuse cytosolic). Proteasome inhibition shifted AGS3-GFP-PM1 puncta to a perinuclear aggresome. The subcellular distribution of AGS3-GFP-PM1 to the pre-aggresomal punctate structures was also observed with mutation of a single residue (T602A) located between GPR-III and GPR-IV. Coexpression of G[alpha]i3 antagonist gallein (10 uM) or with pertussis toxin (200 ng/ml) had no effect. Interestingly, the preaggresomal punctate distribution observed with AGS3-GFP-T602A was also observed with WT AGS3 upon cell treatment with the lysosome inhibitor (ammonium chloride - 25 mM, 24 hrs) and a protease inhibitor cocktail (Sigma; 1:100 dilution), but not with pharmacological manipulation of autophagy. These data are consistent with the hypothesis that the movement of AGS3 into or within the aggresomal pathway, and its potential functional role in this pathway, is a regulated process influenced by cell signaling mechanisms.
Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder caused by the selective loss of the upper and lower motor neurons. Only 10% of all cases are caused by a mutation in one of the two dozen different identified genes, the most common of which are C9orf72, SOD1, and TDP-43, together responsible for at least 60% of familial ALS. The remaining 90% of cases are likely caused by a combination of as yet unidentified genetic and environmental factors. Remarkably, despite the large degree of heterogeneity, all cases of ALS have protein aggregates in the brain and spinal cord that are immunopositive for SOD1, TDP-43, OPTN, and/or p62. Protein inclusions are normally prevented and cleared by heat shock proteins (Hsps), suggesting that ALS motor neurons have an impaired ability to induce the heat shock response (HSR). Accordingly, there is evidence of decreased induction of Hsps in ALS mouse models and in human post-mortem samples compared to controls. However, the role of Hsps in protein accumulation in human motor neurons has not been fully elucidated. Here we generated motor neuron cultures from human induced pluripotent stem cell (iPSC) lines carrying mutations in SOD1, TDP-43, or C9orf72. We show that despite a lack of overt motor neuron loss, there is an accumulation of insoluble aggregation prone proteins in iPSC-derived motor neuron cultures but that content and levels vary with genetic background. Additionally, protein aggregation corresponds to an incomplete induction of the HSR and minimal stress granule formation. We therefore conclude that ALS iPSC-derived motor neurons recapitulate key early pathological features of the disease and fail to endogenously upregulate the HSR in response to increased protein burden. As such, we believe that iPSCs represent a valuable model to further study the role of the HSR in ALS.

The unfolded protein response (UPR) is an intracellular signaling network that adjusts the abundance and protein folding capacity of the endoplasmic reticulum (ER) according to need. The most converged branch of the UPR is mediated by the ER-resident transmembrane kinase/endoribonuclease IRE1. It senses unfolded protein accumulation within the ER and transduces the signal via a non-conventional mRNA splicing mechanism. In response to direct binding of unfolded proteins in the ER lumen, IRE1 activates by oligomerization and accumulates in dynamic foci. IRE1 foci are not autophagosomes as they did not colocalize with the autophagosomal marker LC3. Fluorescence recovery after photobleaching (FRAP) experiments indicate that IRE1 molecules in the foci remain in equilibrium with IRE1 molecules in the surrounding ER network. We determined the structure of IRE1 foci in cells by whole cell correlative light – electron tomography. Our results show that IRE1 oligomers induce
membrane deformations, leading to the protrusion of narrow 30 nm ribosome-free tubes that remain connected to the ER and are twisted into glomeruli of complex topology. The tubes contain two parallel filaments in their lumen, likely representing oligomerized IRE1 ER-lumenal domains. Taken together, our findings define a previously unrecognized subdomain of the ER membrane and shed new light on the structure and organization of active mammalian IRE1 inside the cell.

P2563
Board Number: B716
Autoregulatory transcriptional control of prions by G-Quadruplex motifs in prion promoter. P. Pradhan, V. Perumal, B. Kundu; 1Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, New Delhi, India

Cellular prion protein (PrP\(^C\)) misfolds into an aggregated and infectious conformer, PrP\(^Sc\) (scrapie) that forms the hallmark of pernicious diseases commonly known as transmissible spongiform encephalopathies (TSEs). The association of prions with random quadruplex forming nucleic acid sequences has been recently identified but the exact physiological role of this interaction still remains obscure. Herein, we show that the promoter region of the human prion gene (PRNP) incorporates two G-rich sequences (Q1 and Q2) that could assume stable hybrid intra-molecular G-quadruplex structures (\(T_m\) >70 \(^\circ\)C). We found that both Q1 and Q2 specifically bind to native, \(\alpha\)-helical PrP\(^C\) with high affinity \((K_D \sim 70-100\ nM)\) but showed very weak or no association with \(\beta\) sheet rich-PrP oligomers (PrP\(^Sc\) like). To further probe the relevance of these interactions, a battery of kinetic and structural studies have been utilized that involved surface plasmon resonance (SPR), fluorescence, NMR, circular dichroism and molecular modeling. We demonstrated that the N-terminal unstructured tail of PrP (residues 23-88) is crucial for binding and unwinding of both the quadruplex sequences. Interestingly, cell-based luciferase assays showed that PrP\(^C\) auto-regulated its expression by binding to G-quadruplexes present in its own promoter sequence. Evidently, the autoregulatory function was lost on mutating one of the quadruplex sequences. Overall our data indicate the presence of feedback transcriptional regulation of PRNP gene by native PrP\(^C\) through the dynamic unwinding of quadruplex structures by native PrP\(^C\) that apparently gets affected by pathogenic PrP\(^Sc\) formation. The identification of feedback transcriptional regulation could be a crucial event in the pathogenic cycle of prions which may be targeted for developing newer therapeutic interventions.

Cell Death

P2564
Board Number: B717
Potential mechanisms of platelet-activating factor induced neutrophil NETosis. Y. Li, V.P. Werth, M. Liu; 1Department of Dermatology, Perelman School of Medicine at University of Pennsylvania, Philadelphia, PA, 2Department of Dermatology, Michael J. Crescenz V.A. Medical Center, Philadelphia, PA

Background/Purpose: Platelet-activating factor (PAF) is a proinflammatory lipid mediator, and plays a critical role in autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, psoriasis, and lupus, by affecting different immune cells. Increasing evidence indicates the importance of neutrophils in development of autoimmune diseases. Neutrophil NETosis is a newly described form of cell death, and NETotic neutrophils release neutrophil extracellular traps (NETs) that are involved in various human diseases. Although the PAF signaling cascades have been widely investigated in other human
pathophysiological conditions, the cellular and molecular mechanisms of PAF in the new type of cell death, NETosis, are still less understood. Our study explores the cellular and molecular mechanisms of PAF mediated neutrophil NETosis.

Methods: Primary healthy human neutrophils were isolated with Dextran and Histopaque 1077. The human promyelocytic leukemia (HL-60) cell line was differentiated with 1.2% DMSO for 5-6 days to develop HL-60-derived neutrophil-like cells. Protein kinase C-alpha (PKCα) and Rho-associated protein kinase (ROCK) inhibitors were used to study the potential involvement of PKCα and ROCK. Cells were fixed with 2% PFA and stained by Sytox Green after treatment with PAF in the presence/absence of PKC/ROCK inhibitors. NETosis was detected at 504/523 nm in the microplate readers, and NET formation was confirmed by Fluorescent Microscopy.

Results: In the current study, we found that PAF induced the formation of neutrophil NET and NETosis in both human primary and HL-60 neutrophils in a dose-dependent fashion. Our immunoblot results show that PAF treatment caused activation and phosphorylation of PKCα in a time-dependent manner during PAF-induced neutrophil NETosis. Moreover, several PKC inhibitors, including specific inhibitor peptide, could significantly attenuate PAF-induced neutrophil NETosis, suggesting that PKCα is involved in PAF-induced neutrophil NETosis. Interestingly, ROCK inhibitors could also attenuate PAF-induced neutrophil NETosis.

Conclusion: Our findings indicate that PKCα and ROCK activation play vital roles in PAF-induced neutrophil NETosis. These results suggested that ROCK or PKCα signaling pathways might be therapeutic targets for PAF-mediated autoimmune diseases via regulating NETosis.

P2565

Board Number: B718

Antiapoptotic proteins’ expression profile upon FGF1 and FGF2 translocation.

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Fibroblast growth factor 1 (FGF1) and 2 (FGF2), canonical members of the FGF family, act through specific tyrosine kinase receptors (FGFRs) and activate specific downstream signaling pathways. Unique feature of FGF1 and FGF2 among other fibroblast growth factors is their ability to translocate into cytosol and nucleus under stress conditions. Based on the function of novel FGF1 binding partners, we suggested that intracellular FGF1 plays a role in cell survival. This hypothesis is strongly supported by our observation that FGF1 and FGF2 are able to inhibit apoptosis induced by serum starvation or staurosporine treatment in the presence of specific FGFR inhibitor. To understand the mechanism of antia apoptotic activity of translocated FGF1 and FGF2, we studied the expression profile of 35 apoptosis-related proteins upon FGF1 treatment in serum starved U2OS cells stably transfected with inactive variant of FGFR1 K514R. We conducted protein level analysis using Proteome Profiler Human Apoptosis Array Kit (R&D Systems). We found that 16 h incubation with FGF1 led to upregulation of some antia apoptotic proteins including: clusterin, HSP60, HSP70, livin, survivin and XIAP, and downregulation of phospho-p53 and cleaved caspase-3. By the use of ApoLive-Glo Multiplex Assay (Promega) we confirmed that translocated FGF1 and FGF2 downregulate caspase-3 activity in serum starved U2OSR1K514R. Next, we conducted a more detailed analysis of selected proteins’ levels in serum starved or staurosporine treated U2OSR1K514R and NIH3T3 cells upon FGF1 and FGF2 translocation. Our data indicate that translocation of FGF1 and FGF2 evokes changes in expression profile of proteins mentioned above.

ACKNOWLEDGMENTS:
The work was supported by the National Science Centre, Poland (grant 2015/18/E/NZ3/00501).
P2566
Board Number: B719
The role of nuclear localization of translocated FGF1 and FGF2 for their antiapoptotic activity.
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Fibroblast growth factors 1 and 2 belong to a large family of fibroblast growth factors. FGF1 and FGF2 act through binding to four specific cell-surface tyrosine kinase receptors (FGFRs, FGFR1-4) followed by FGFRs' dimerization and activation. In contrast to other growth factors FGF1 and FGF2 possess the unusual property to cross cellular membranes and translocate into cytosol and nucleus in a receptor-dependent manner. Our data showed that translocated FGF1 and FGF2 inhibit apoptosis induced by serum starvation or staurosporine treatment in the presence of specific FGFR inhibitor. We also observed that the protective effect is more profound in the case of FGF2 than of FGF1. We assumed that these differences may result from diverse nuclear retention time. In order to study the relationship between the time of FGF nuclear localization and cell survival, we tested selected FGF1 mutants with different duration within the nucleus. FGF variants were expressed in bacterial system and purified using heparin-affinity chromatography. To verify proper folding of obtained mutants, we applied fluorescence and circular dichroism measurements. Subcellular localization of translocated FGFs in NIH3T3 cells was determined via fractionation. Antiapoptotic activity of translocated FGF1 variants was measured by flow cytometry annexin V assay and caspase-3/7 activity in serum starved NIH3T3 cells incubated with FGF variants in the presence of FGFR kinase inhibitor. The results were verified using U2OS cells stably transfected with FGFR1 and inactive variant of FGFR1-K514R. Our preliminary data suggest that subcellular localization of FGF1 and FGF2 may be important for their antiapoptotic activity.

ACKNOWLEDGMENTS:
The work was supported by the National Science Centre, Poland (grant 2015/18/E/NZ3/00501).

P2567
Board Number: B720
The cis-1 gene regulates physiological apoptosis in a CED-13 dependent manner within the germline of Caenorhabditis elegans.
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The mitochondrial localized, iron-sulfur binding, CISD proteins (mitoNEET/CISD1, NAF-1/CISD2, Miner2/CISD3) are involved with several human pathologies including diabetes, neurodegeneration, and Wolfram syndrome 2. Studies indicate that NAF-1/CISD2 binds to Bcl-2 and disruption of NAF-1 function leads to increased apoptosis, suggesting that NAF-1 has a role in apoptosis. We used C. elegans to examine the cisd genes role in apoptosis. The C. elegans genome contains three uncharacterized cisd genes that code for CISD-1 (homology to the vertebrate mitoNEET/CISD1 and NAF-1/CISD2), CISD-3.1 or CISD-3.2 (both have homology to vertebrate CISD3). Using CRISPR technology we determined that the cis-1p::GFP transcriptional reporter is expressed in the adult germline. In C. elegans, apoptosis occurs within the germline during the normal process of oogenesis. To test the hypothesis that the cisd genes have a role in physiological germline apoptosis we used DIC microscopy and programmed cell death markers (eg. CED-1::GFP, ACT-5::YFP, acridine orange staining) to quantify the number of cell corpses within the germline of the cis-1(tm4993) mutant and animals with RNA interference knock-down of the cisd-1, cisd-3.1 or cisd-3.2 genes. Disruption of cisd gene function results in a significant increase in the
number of cell corpses within the adult germline and additional germline defects (e.g. distal tip cell migration defects). To determine the function of cisd-1 in the context of the apoptotic pathway we examined the impact of altered core apoptotic machinery on the cisd-1(tm4993) mutant phenotype. In C. elegans the core apoptotic machinery involves the CED-9 (Bcl-2) protein which functions to prevent cells from dying by binding and regulating CED-4 (APAF); CED-9 and CED-4 interaction ultimately impacts CED-3 (Caspase) protein cleaving activity. We found that the cisd-1(tm4993);ced-3(RNAi) and cisd-1(tm4993);ced-9(n1950) animals have a reduced number of cell corpses in the germline relative to the cisd-1(tm4993) mutant. Note that the ced-9(n1950) is a gain-of-function mutant. In C. elegans, the egl-1 and ced-13 genes code for pro-apoptotic, BH3 (Bcl-2 homology region 3) containing, proteins that physically interact with the anti-apoptotic CED-9 protein. To test if cisd-1 functions upstream of ced-9, we examined the impact of egl-1 or ced-13 dysfunction on the cisd-1(tm4993) mutant phenotype. RNAi knockdown of ced-13, but not egl-1, significantly reduced the number of cell corpses observed in the cisd-1(tm4993) mutant. This work is significant because it demonstrates that the cisd genes function in the process of apoptosis and places the cisd-1 gene as a regulator of physiological germline programmed cell death acting through CED-13 function.

P2568
Board Number: B721
Two small GTPases function antagonistically in corpse removal of a developmental non-apoptotic dying cell.
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Programmed cell death and subsequent cell clearance are necessary in the development and maintenance of organisms and tissues. Defects in cell clearance can result in inappropriate inflammatory responses and autoimmune disorders. Much is known about apoptotic corpse removal; however, our understanding of mechanisms driving the clearance of cells dying by non-apoptotic means is rudimentary. Linker cell death in C. elegans is an excellent model of non-apoptotic cell death. The linker cell is a male-specific cell that is born in the second larval stage, leads the elongation of the developing gonad, and then dies in a programmed manner independently of known apoptosis, autophagy, or necrosis genes. Importantly, linker cell corpses are robustly engulfed in mutants lacking the capacity to engulf apoptotic bodies, suggesting utilization of a novel engulfment program. To elucidate the mechanism of linker cell corpse removal, we carried out a forward genetic screen to identify mutants that are defective in this process. F2 mutant males were propagated by artificial insemination, as surviving linker cells or corpses may block spermatid exit and promote male sterility. From this screen, we isolated two loss-of-function mutants in the RAB-35 guanine nucleotide exchange factor (GEF), rme-4, and a gain-of-function mutant in the small GTPase, arf-6. Using a variety of genetic and cell biological methods, we have uncovered the relevant GEFs, and GTPase activating proteins (GAPs) for RAB-35 and ARF-6 in this process, and have determined that these two proteins and their regulators have antagonistic functions; RAB-35 promotes corpse clearance, and ARF-6 inhibits it. Using a microfluidic imaging-device we developed, we imaged the dynamics of these proteins over a 20h period, capturing linker cell migration, engulfment, and degradation. We found that YFP-RAB-35 is localized to the extending pseudopod membrane, and remains at the membrane for the duration of phagosome maturation. While ARF-6-YFP is also localized to the extending pseudopods, it changes localization from the plasma membrane to intracellular vesicles after engulfment is complete. These results, together with our genetic and protein interaction studies, suggest that ARF-6 and RAB-35 localize to the plasma membrane during corpse recognition and engulfment, but RAB-35 then inactivates ARF-6, likely through
its GAP CNT-1, to allow phagolysosome maturation to proceed. Rab35 and Arf6 have been previously implicated together in the recognition and removal of foreign particles in mammals, but not in the degradation of programmed cell death corpses. *rab-35* and *arf-6* may work together to preferentially degrade corpses that die by non-apoptotic mechanisms.

P2569

**Board Number:** B722

**Modifiers of proteotoxicity associated with multisystem proteinopathy.**

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Multisystem proteinopathy (MSP) is a rare, genetic degenerative disorder that can affect bone, muscle, and the central nervous system. It is commonly referred to as inclusion body myopathy (IBM) associated with Paget’s disease of bone (PDB), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS), or IBM/PD/ALS, because clinically, it can present with single or multiple phenotypes encompassing all these disorders. Mutations in the prion-like domain of heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 and A2 are associated with MSP. These nuclear RNA-binding proteins normally function in RNA metabolism and processing, splicing, and nuclear-cytoplasmic shuttling. In disease, these proteins misbehave and form cytoplasmic aggregates which contribute to neuron death. However, the exact molecular mechanisms underpinning pathogenesis are not well understood. To better understand pathogenesis and possible ways to combat it, we used Saccharomyces cerevisiae to model disease. An unbiased gene deletion screen revealed forty modifiers of proteotoxicity. These modifiers implicate several classes of proteins in pathogenesis such as ribonucleoprotein-granule components, spliceosome proteins, and molecular chaperones. All suppressors of toxicity of hnRNP A1 also suppressed toxicity of hnRNP A2, suggesting mechanistic convergence. Interestingly, very few modifiers of hnRNP A1 and A2 overlapped with known modifiers of TDP-43 and FUS, RNA-binding proteins implicated in ALS, suggesting mechanistic divergence between these outwardly similar proteins and diseases. From these suppressors of toxicity, we generated a network of protein-protein interactions that advance our understanding of MSP. Importantly, these studies highlight pathways implicated in pathogenesis and suggest potential therapeutic targets.

P2570

**Board Number:** B723

**HMA induces changes in the nuclei and mitochondria of BHK21 cells in a caspase3-independent manner.**

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Apoptosis, also known as programmed cell death, is common control mechanism required for regulating the number and function of cells. Apoptosis is characterized by morphological changes in the plasma membrane, mitochondria, and nuclei of cells. Previous studies examined the maturation of erythroblasts, a process similar to apoptosis (Malik et al., 2013). This research examined apoptosis in Baby Hamster Kidney (BHK21) cells, an adherent cell line (Macpherson, 1961). To induce apoptosis, BHK21 cells were incubated with hexamethylene amiloride (HMA) (Altairac S, et al. 2003.). Control cells were incubated with dimethyl sulfoxide (DMSO). HMA-treated cells exhibited fewer number of cells per ml than stock cells. Cells were stained with MitoTracker (MT), fixed with formalin, permeabilized, and

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then stained again with an antibody directed against Caspase 3. Finally, cells were stained with 4,6'-diamidino2-phenylindole (DAPI). Additional controls consisted of cells stained with two of the three markers (Fluorescence Minus One [FMO] controls). All cells were analyzed by multispectral imaging flow cytometry and IDEAS software v6.2 (Amnis Corporation, Seattle, WA; Niswander et al., 2014). Compared to DMSO-treated cells, HMA-treated cells exhibited a higher mean fluorescence intensity (MFI) for DAPI and MT, indicating changes in the nuclei and mitochondria, respectively. There was no change in the MFI for Caspase 3 in HMA-treated cells, as compared to DMSO-treated cells, suggesting that Caspase 3 was not involved in HMA-induced changes in BHK21 cells. Brightfield images of HMA-treated cells revealed slightly more rounded cells, as compared to control cells. However, the majority of HMA-treated cells exhibited no significant plasma membrane changes. Taken together, these results suggest that the observed HMA-treated cells are not undergoing apoptosis in a caspase-dependent manner. Further studies will examine possible activation of other caspase enzymes in HMA-treated cells and inducement of apoptosis in BHK21 cells using staurosporine or etoposide, reagents used to cause apoptosis in other cell lines.

P2571
Board Number: B724
The importance of isoflavone metabolism to regulate lactating mammary epithelial cells.
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Isoflavones are a class of polyphenolic compounds present in legumes. After metabolic conversion by enteric bacteria, isoflavones and the metabolites are absorbed into the body. There are two primary isoflavone metabolic pathways. One is associated with formononetin, daidzein, equol and the other with biochanin A, genistein, and p-ethylphenol. These isoflavones show different influences in non-lactating mammary epithelial cells (MECs) and breast cancer cells. However, it remains unclear whether metabolic conversion of isoflavone is involved in milk production during lactation. In this study, we investigated that influence of isoflavones and their metabolite on behavior of lactating MECs by using a lactating MEC culture models. MECs isolated from the mammary glands of ICR mice (9-14 weeks) were cultured with prolactin and dexamethasone to induce milk production ability. Subsequently, lactating MECs were treated with each isoflavone. To evaluate the influence of isoflavones on milk production ability, we investigated the expression of β-casein and phosphorylated-signal transducer and activator of transcription 5 (pSTAT5) in MECs by western blot analysis. MECs treated with biochanin A or genistein at 25 µM for 2 days reduced β-casein production and pSTAT5, whereas biochanin A or genistein at 5 µM didn’t influence β-casein production and pSTAT5. However, MECs treated with biochanin A or genistein at 5 µM for 7 days reduced β-casein production and pSTAT5. To evaluate the influence of isoflavones on apoptosis, we investigated cellular viability by cell counting WST-8 kit and the expression of cleaved caspase-3 in MECs by western blot analysis. Treatment of biochanin A or genistein at 25 µM for 2 days decreased cellular viability. Treatment of biochanin A or genistein at 5 µM for 7 days also decreased cellular viability. Cleaved caspase-3 was detected in MECs treated with biochanin A or genistein at 25 µM for 2 days and at 5 µM for 7 days. However, p-ethylphenol didn’t influence β-casein production, pSTAT5, cellular viability and expression of cleaved caspase-3 any concentrations and treatment time. These results indicate that metabolic conversion of upstream isoflavones is detoxifying action to lactating MECs.
P2572

Board Number: B725
The intrinsic ability of the Bax family to activate caspase 8-mediated cell death.
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The majority of pro-apoptotic members of the Bax protein family induce cell death through targeting mitochondria. Interestingly, certain subgroup members within the Bax family, such as BaxΔ2 and BaxΔ2ω, induce cell death through the formation of cytosolic protein aggregates, and activation of caspase 8. However, the functional domain(s) that are responsible for triggering this noncanonical pathway were previously unknown. Recently, our data showed that C-terminal removal in BaxΔ2 and BaxΔ2ω did not inhibit the formation of cytosolic aggregates, but did abolish their ability to recruit caspase 8. In addition to the critical role the C-terminus plays in the cytotoxic action of these proteins, the data also indicated that the helical conformation of the C-terminus, rather than the primary sequence, was crucial for recruitment of caspase 8. Furthermore, we report that impairing the ability of the parental Baxα protein to target mitochondria via point-mutation, resulted in Baxα mimicking the aggregation behavior of BaxΔ2. Notably, the functional domains responsible for aggregation are shared by almost all Bax family members. Together, these results imply an intrinsic potential for aggregate-mediated caspase 8-dependent cell death in the Bax family.

P2573

Board Number: B726
Development of assays to detect apoptosis and necrosis in real time using a plate reader.
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We have developed a homogeneous assay for detecting apoptosis in real-time that is recorded using a standard plate-reader. The assay is based on binding of annexin V to phosphatidyl serine (PS) that is exposed on the outer leaflet of the cell membrane during apoptosis. We engineered two fusion proteins composed of annexin V linked to a small or large fragment of a luciferase. The annexin-luciferase fragment fusion proteins and a luciferase substrate are added as a reagent directly to cultured cells. The individual annexin V-luciferase fragment fusion proteins have very low background luminescence in the presence of non-apoptotic cells. When exposed to apoptotic cells, the annexin V-luciferase fragment fusion proteins bind to PS in close proximity on the cell surface, an active luciferase enzyme is reconstituted and a luminescent signal is generated. The reagent can be added to cells for extended periods of incubation enabling detection of the progress of apoptosis in the population of cells in real time. We have multiplexed the homogeneous luminescent assay with a vital fluorogenic DNA binding dye to simultaneously quantify the accumulation of apoptotic and necrotic cells from the same sample at progressing times. By recording data in a kinetic mode, we demonstrate the onset of annexin V binding precedes loss of membrane integrity and secondary necrosis in vitro. We further demonstrate detection of caspase-3/7 activity precedes annexin V binding by comparison to endpoint assays of caspase-3/7 activity as an orthogonal marker of apoptosis. We also demonstrate concordance of the real-time annexin V binding assay with traditional flow cytometric analysis of apoptosis and necrosis. The assay has been validated using a number of anchorage dependent and suspension cell lines and has been shown to correlate with activation of caspase-3/7 activity as an orthogonal marker of apoptosis.
Imaging the luminescent and fluorescent signals enables creation of time laps movies showing heterogeneity among individual cells in a population undergoing apoptosis. This new homogeneous apoptosis assay method represents a simplification and improvement over flow cytometry and endpoint assay methods by providing kinetic data from the same sample of live cells in real time using a multimode plate reader.

P2574
Board Number: B727
Co-receptors are dispensable for tethering receptor-mediated phagocytosis of apoptotic cells. J. Lee1,2, B. Park1,2, H. Moon1,2, D. Lee3, J. Cho4, D. Park1,2;
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During efferocytosis, phagocytic cells recognize dying cells by receptors binding to ligands specifically exposed on apoptotic cells. Multiple phagocytic receptors and some of their signaling pathways have been identified. However, the downstream pathways of tethering receptors that secure apoptotic cells remain elusive. It is generally assumed that tethering receptors induce signaling to mediate engulfment via interacting with co-receptors or other engulfment receptors located nearby. However, it is poorly understood whether co-receptors for tethering receptors exist during efferocytosis, and if they do, whether they are indispensable for this process. Here, we address this issue using glycophasphatidylinositol (GPI)-anchored annexin A5 (Anxa5-GPI), an artificial tethering receptor without a putative co-receptor. Phagocytes expressing Anxa5-GPI exhibited enhanced binding of apoptotic cells, resulting in promoted ingestion of apoptotic cells in a phosphatidylserine-dependent manner. Anxa5-GPI-induced phagocytosis of apoptotic cells relied on the known cytoskeletal engulfment machinery but partially depended on the Elmo-Dock-Rac module or the integrin pathway. In addition, Anxa5-GPI-mediated efferocytosis provoked anti-inflammatory responses. Taken together, our work suggests that co-receptors are dispensable for tethering receptor-induced efferocytosis and that tethering receptors mediate the engulfment of apoptotic cells through multiple engulfment signaling pathways.

P2575
Board Number: B728
Behavior of labile ferrous ions and reactive oxygen species during ferroptotic response of cells. M. Sato1, T. Hirayama2, T. Fujii1, H. Nagasawa2, I. Minoura1;
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Ferroptosis is a recently identified iron-dependent regulated cell death (Dixon and Stockwell 2014). Understanding mechanisms and regulations of this oxidative cell injury may bring us new therapeutic methods for cancer and neurodegenerative diseases. The injury may be caused by reactive oxygen species (ROSs) which are generated by Fenton reaction between labile ferrous ions (Fe2+) and peroxides. Therefore, Fenton reaction should be a key for ferroptotic responses of cells. However, other indirect pathways to generate ROSs should be also considered. To further understand the mechanisms how ROSs are generated during the ferroptotic responses, we tried to visualize labile Fe2+ and ROSs by using cell permeable, activatable fluorescent probes. As previously described, >20 μM of erastin caused
ferroptosis of HT-1080 cells. To visualize the responses for an enough time-period (~9 hours), we chose the erastin concentration of 30 μM and observed the behavior of labile Fe²⁺ and ROSs by fluorescence microscopy. To detect labile Fe²⁺, both commercially-available chemical probe FeRhoNox-1 (RhoNox-1, Hirayama, et al., 2013) and newly-synthesized RhoNox-4 which possesses a higher sensitivity with high specificity to labile Fe²⁺ were used. To detect ROSs, aminophenyl fluorescein (APF, Setsukinai et al., 2003) which fluoresces by either hydroxyradical, peroxinitrite, or hypochlorous acid, and OxiORANGE (Koide et al., 2007) which does by either hydroxylradical or hypochlorous acid were used. The fluorescence signals indicating labile Fe²⁺ reached the maximum at 3 hours after erastin stimulation. On the other hand, fluorescence signals of APF and OxiORANGE became maximal at 6 hours after the stimulation. The results were consistent with the simple model that hydroxyradical is generated by the reaction between cytoplasmic labile Fe²⁺ and peroxides and thus causes the ferroptotic cell death.

References

P2576
Board Number: B729
Peroxidasin (PXDN) Promotes Prostate Cancer Progression.
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Peroxidasin (PXDN), a human homolog of Drosophila PXDN, belongs to the family of heme peroxidases and has been found to promote oxidative stress in cardiovascular tissue. PXDN has been associated with melanoma, but its role in prostate cancer has not been previously elucidated. We hypothesized that PXDN promotes prostate cancer progression. Immunohistochemical staining showed that PXDN expression increased with prostate cancer progression as compared to normal tissue or cells. Stable knockdown of PXDN followed by proteomic analysis revealed that PXDN knockdown affected oxidative stress and gluconeogenesis pathways. PXDN knockdown also resulted in decreased cell viability and adhesion to collagen or fibronectin, along with an increase in reactive oxygen species (ROS) and apoptosis as shown by Annexin V assay. Finally, PXDN knockdown decreased colony formation on soft agar. Overall, we propose that PXDN plays a role in prostate cancer by modulating pathways involved in oxidative stress leading to decreased apoptosis possibly via scavenging ROS. Therefore, PXDN may be a biomarker associated with prostate cancer and a potential therapeutic target.
P2577
Board Number: B730
Assaying free radical scavenging abilities of herbal remedies and anti-apoptotic gene expression effects of *Ginkgo biloba* in an *in vitro* stroke model.
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Ischemic stroke is a leading cause of death and disability in the United States, and current treatment options are both limited and time-sensitive. In the brain, cellular apoptosis pathways are active for days following the initial ischemic insult, expanding the area of damage. Herbal remedies that possess antioxidative components may convey prophylactic protection against this secondary damage, and this study examined several commercially available herbals to assess their potential efficacy in blocking apoptotic injury. A particular focus of this study, *Ginkgo biloba* leaf extract, is an herbal remedy long used in traditional medicine, and previous work has demonstrated that it contains several components with antioxidant properties and that one particular purified extract (EGB761) has neuroprotective effects on cultured cells. This study used a spectrophotometric assay to directly test the free-radical scavenging ability of separate batches of an easily accessed, over-the-counter *Ginkgo* supplement; a dose-dependent effect was measured, and samples from different lots showed similar radical scavenging. This assay is currently in use to test additional herbal remedies for free-radical scavenging activity; while dietary supplements are not regulated as drugs by the FDA, these compounds may be clinically useful as neuroprotectants, and the assay provides a straightforward way to provide an initial screen of over-the-counter preparations. To further assess the *Ginkgo* supplement, primary brain and body *Gallus gallus* cell isolates served as a stroke model in which hydrogen peroxide was used to induce apoptosis, mirroring the cell death that occurs following the initial ischemic event. Prophylactic *Ginkgo* treatment of these cell isolates was assessed in cell viability assays using trypan blue exclusion at 0-24 hours post-treatment, and demonstrated that treated cells had improved survival compared to PBS-treated control cells. Gene expression differences were also noted in treated cells via two independent PCR arrays that indicated up-regulation of anti-apoptotic genes and down-regulation of pro-apoptotic genes such as caspases that are components of the Fas death-inducing signaling complex; Western blots will determine whether protein level changes are consistent with the observed gene expression changes. Together, these results suggest that a commercially available, crude *Ginkgo biloba* supplement may convey protection from cellular apoptosis following stroke, and initiates studies of other candidate herbals. Future work will include the direct inhibition or activation of the Fas death-inducing pathway to confirm its role in mediating the protective effects of *Ginkgo* extract, and cellular viability studies using the newly identified herbals.

P2578
Board Number: B731
Actin nucleation factors are important for caspase activation and cell death.
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Actin nucleation factors control many vital cellular functions, including morphogenesis, motility, and division, yet they remain relatively uncharacterized in relation to programmed cell death pathways. JMY is a versatile nucleation factor that can both polymerize actin directly and activate the Arp2/3 complex to assemble actin filament networks in the cytoplasm. Upon DNA damage, JMY is translocated into the
nucleus, where it cooperates with the transcriptional regulators p53 and p300 to promote apoptosis. To gain a better understanding of the relationship between actin nucleation factors and programmed cell death, we generated JMY knockout (KO) human fibroblasts and monitored their responses to DNA damage. Compared to JMY-expressing parental cells, JMY KO cell lines exhibited significantly lower levels of Annexin-V staining and cell death, indicating that the loss of JMY results in less apoptosis. Consistent with these observations, caspase cleavage was inefficient in cells lacking JMY. Interestingly, JMY inactivation had a broad effect on the transcriptome and caused a dramatic upregulation of RhoD, a small G-protein known to interact with the JMY homolog WHAMM. Similar to JMY-deficient fibroblasts, WHAMM KO cell lines displayed lower levels of apoptosis. In contrast, RhoD KO cells exhibited increases in caspase cleavage and cell death. These findings suggest that both JMY and WHAMM are capable of performing pro-apoptotic functions, but that their actions may be inhibited by RhoD.

Biophysical Approaches to Cell Biology

P2579
Board Number: B733
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Charcot-Marie-Tooth (CMT) disease is the most common hereditary neuromuscular disease characterized by progressive loss of muscle tissue followed by sensory loss and skeletal deformities. Approximately 1 in 2500 individuals is affected by CMT, a condition that is still incurable. Mutations in Hsp27, a small heat shock protein, have been shown to lead to CMT in humans. However, despite over a decade of research, there is no consensus as to how mutations in Hsp27 affect protein oligomerization and chaperone activity. My project aims to understand the disease mechanism by pairing cutting-edge Cryo-EM technology and negative stain EM with biochemical assays to resolve the long standing problem. We investigated the structure-function relationship of the wild type Hsp27 compared to the five disease-causing mutations: S135F, R127W, R136W, T151I, and P182L. The proteins were expressed in bacteria and purified to ~99% homogeneity by chromatography techniques. The chaperone activity of the purified proteins was assayed by measuring rescue of DTT-induced aggregation of alpha-lactalbumin in-vitro. Our results suggest that wt. Hsp27 was able to maintain chaperone activity, however, the mutants had compromised activity (<50% of wt.) and were not able to completely prevent protein aggregation. Could the reduced chaperone activity be due to the differences in oligomerization status of the mutant proteins? Negative-stained EM images coupled with data from size exclusion columns suggest that the wild type forms a 24-mer in a spherical ‘ball’ conformation. On the other hand, the mutant proteins formed fewer oligomers as the mutations appear to inhibit oligomerization. Furthermore, only the large oligomeric complexes had chaperone activity. Our data, for the first time, correlates the oligomerization status (complex size) of Hsp27 and the mutant proteins with chaperone activity with the aid of high resolution negative-stained EM images. Mutations in Hsp27 modulate the size of the complexes by regulating the oligomeric status and thereby reduce the chaperone activity of the proteins. Future experiments aim to obtain high resolution 3D- reconstructions of the complexes to drive the process of drug design to find a pharmacological cure for CMT.
We investigate the emergence of contractile behaviors in disordered non-muscle actomyosin networks, whose non-equilibrium dynamics remain largely unexplored when containing reversibly-bound passive cross-linkers and active myosin II motor filaments. Current understanding of how contractility emerges in disordered actomyosin networks of non-muscle cells is still largely based on the intuition derived from earlier works on muscle contractility. In addition, in disordered networks, passive cross-linkers have been hypothesized to percolate force chains in the network, hence, establishing large-scale connectivity between local contractile clusters. Our work, based both on analytical theory and detailed molecular simulations, shows that cross-linker binding dynamics plays a crucial role even at the level of elementary force generating elements in contractile actin networks. In particular, our results shed light on the non-equilibrium effects of transiently binding proteins in biological active matter, as observed in the non-muscle actin cytoskeleton, showing that highly efficient contractile force dipoles result from synergy of passive cross-linker and active motor dynamics. These findings begin to elucidate the tools available to this biological active matter to dramatically alter their micro-structural morphologies and generate active cellular forces.

The collagen fibrils are the main building block of connective tissues in mammals where they fulfill both structural and mechanical roles. The structure of a fibril is based on collagen molecules that self-assemble into micro-fibrils and sub-fibrils stabilized by hydrogen bonds and covalent crosslinks. The non-integer staggering of collagen molecules results in a characteristic D-band pattern along the fibril with a periodicity of 67nm. Besides this natural variation, localized damaged sites have been observed along the length of mechanically overloaded fibrils [1], which suggests the inherent existence of structural inhomogeneity along collagen fibrils. Recently, Dittmore et al. reached the same conclusion by tracking the diffusion kinetics of single fluorescently labeled MMP-1 molecules along collagen fibrils [2]. To explore this further we are using an atomic force microscopy (AFM)-based manipulation technique that allows us to perform tensile tests on a single fibril in bowstring geometry [3]. In this work, we are investigating the potential impact of structural inhomogeneity on the viscoelastic properties of single fibrils. 40 fibrils were extracted from two different bovine extensor tendons, around 50 microns long segments were isolated, stretched to a target strain of 5, 10, 15 or 20%, held at that strain for 150 seconds and then released. We observed two characteristic stress relaxation times as previously reported [4] and the absence of plastic strain, assessed optically, after release implying full axial recovery. However, AFM imaging of the released fibrils demonstrated a two to three-fold increase in the height fluctuations along the fibril length with a characteristic length scale larger than the D-band spacing, in the micrometer range. We propose that the inherent structural heterogeneity along the length of the fibril becomes a prominent feature after stress relaxation providing a new viscoelastic

P2582
Board Number: B736
Supergrowth: Effect of osmotic oscillations on the rate of cell growth and the regulation of the proteome.
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Cell growth is a complex process in which the expansion of cellular dimensions are globally coordinated with biosynthesis of cellular components. While much is known about how certain intracellular structures such as chromosomes are replicated, less is known about how the proteome itself is duplicated in the cell cycle. Most cell types, including the fission yeast S. pombe, exhibit exponential growth, in which the growth rate of individual cells scales with cell size, suggesting that the amount of some cellular component(s) dictates the rate of growth. As the majority of proteins are maintained at fixed concentrations during the cell cycle, protein synthesis rates may be coordinated with the rate of cellular expansion. The mechanisms responsible for exponential growth and the coordination of global protein synthesis with growth rate are not well understood. Here, we identify a condition in fission yeast in which the rate of protein synthesis is globally de-coupled from growth, causing cells to grow many times faster than normal (supergrowth). In studying the functions of turgor pressure, we subjected cells to osmotic oscillations and then released them back into normal media. Cells grew slowly under these oscillations, but upon release, grew 2-3-fold faster than control cells for a few generations; we term this unprecedented rapid growth “supergrowth.” Cells maintained normal cell morphology and size control, and these effects were independent of the osmotic response pathway. The concentrations of a large majority of representative proteins were found to increase 10-100% higher than normal during typical oscillations (depending on protein and condition), and then were depleted back to normal concentrations during the supergrowth period. Cell growth and protein synthesis were thus uncoupled in these cells, as during oscillations, proteins were synthesized at normal rates even though cell enlargement was stalled. We provide evidence that this abnormal accumulation of proteins may drive supergrowth, as blocking this accumulation with cycloheximide prevented subsequent supergrowth. Our current model is that growth rates are set by an effective amount of “growth materials,” which accumulate to abnormal levels during oscillations and thus provide extra fuel for growth. During oscillations, cells may lack a negative feedback mechanism for controlling protein homeostasis, as they may not be able to properly sense growth or size. These studies provide one of the first examples of decoupling protein synthesis from cell growth/size control, and begin to give insights into this coordination and the mechanisms that control growth rates and the global regulation of the proteome.

P2583
Board Number: B737
Temporal control of cellular phenotype.
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A hallmark of cellular stress response pathways is to protect cells from different environmental insults. While much is known about how cells are protected against acute stresses of different types, the
mechanisms underpinning how cells are protected against gradually changing stresses are poorly understood. Here we demonstrate that a linear stress gradient but not a step, pulse or a quadratic gradient of the same stressor and intensity causes a severe cell growth phenotype. We determined that this phenotype is caused by the failure of cells to activate the stress-activated protein kinase signaling pathway due to a threshold in the rate of stress application. This lack of response occurs despite cells physically detecting the relatively slow changes in stress signal. These findings have fundamental implications for understanding mechanisms of how temporally changing environments impact biological phenotype.

P2584
Board Number: B738
Soft matrix facilitates the activation of mesenchymal stromal cells into antifibrotic phenotypes by tumor necrosis factor-α.
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Mesenchymal stem or stromal cells (MSCs) have been shown to ameliorate scar formation in some animal models of fibrosis by secreting paracrine factors. However, mechanical rigidity of scars can activate MSCs into myofibroblasts, which promote fibrosis by collagen production. Culturing MSCs on soft substrates is known to prevent mechanical activation. However, it remains unknown whether soft substrates play an active role in priming MSCs into anti-fibrotic phenotypes when they are exposed to inflammatory signals emanating from injuries. We show that human bone marrow MSCs encapsulated in a normal parenchymal tissue-like, soft Arg-Gly-Asp (RGD)-alginate hydrogel produce more paracrine factors that have been implicated in ameliorating fibrosis than those in a scar-like, stiff hydrogel upon activation with tumor necrosis factor α (TNFα). The factors include metalloproteinase-1 (MMP-1) and TNF stimulated gene 6 (TSG-6), which have been previously shown to ameliorate fibrosis by degrading collagen and facilitating M2 macrophage polarization, respectively. A higher level of TNFα binding on the cell surface was observed with MSCs in the soft hydrogel compared to the stiff hydrogel, thereby suggesting that substrate stiffness may regulate TNFα receptor dynamics. Surprisingly, transient pharmacological inhibition of actin polymerization by latrunculin A, but not myosin-II activity by blebbistatin, increases the production of the TNFα-induced factors from MSCs in both soft and stiff matrices. Interestingly, inhibition of caveolin, but not clathrin, mimics the effect of inhibiting actin polymerization, and increases TNFα binding to the cell surface. The results thus suggest a novel pathway to regulate TNFα activation of MSCs by substrate stiffness, thereby modulating the induction of potential anti-fibrotic paracrine factors.

P2585
Board Number: B739
CRYPTOCHROMES: FROM LIGHT-DEPENDENT FLAVIN REDUCTION TO CIRCADIAN CLOCK REGULATION.
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The circadian clock regulates biological and behavioral adaptations in response to day-night cycles. Cryptochromes (CRYs) are members of a family of photolyases involved in DNA repair that are...
responsible for the oscillation of the circadian clock. The structure of the drosophila Cryptochrome (dCRY) has a complex, re-entrant topology made of two lobes, a photolayase homology (PH) domain and a FAD binding domain. FAD photoreduction causes the release of a C-terminal tail (CTT) from the core of the FAD binding domain. The CTT in the light-activated dCRY recruits other transcription factors important for the transition between night and day cycles. Here, we studied the underlying mechanisms responsible for the light-induced conformational changes of dCRY coupled to FAD reduction. We used optical tweezers to mechanically unfold dCRY in the presence and absence of FAD. We show that in the absence of FAD, dCRY remains unfolded most of the time, and occasionally only the PH domain transiently folds into a native-like structure. In contrast, when FAD is present the entire protein folds with high probability, indicating that the cofactor is essential for the protein to attain its native tertiary structure. The mechanical unfolding process of the protein reveals several intermediate states, reflecting the complex topology of the protein. Ongoing experiments include determining how FAD in the reduced and oxidized states affects the mechanism of folding and the stability of the protein.

P2586

Board Number: B740

Soft matrix enhances autocrine production of transforming growth factor-β1 to facilitate tumor necrosis factor-α activation of mesenchymal stromal cells.

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Mesenchymal stem or stromal cells (MSCs) have been tested in many clinical trials due to their ability to modulate the immune system during inflammation. Some disease conditions that involve immune cells, including fibrosis, show profound physical changes where tissues become rigid due to collagen deposition and crosslinking. How physical cues impact immunomodulatory functions of MSCs remains generally unclear. Transforming growth factor-β1 (TGF-β1) is known to play an important role in MSC differentiation into chondrocytes on soft substrates and into fibrotic phenotypes on stiff substrates. However, it remains unclear how TGF-β1 plays a role in regulating immunomodulation by MSCs. Here, we show that human bone marrow MSCs secrete a substantial level (~15 ng/ml per 100,000 cells per day) of endogenous TGF-β1 when they are cultured in a soft Arg-Gly-Asp (RGD)-alginate hydrogel, while MSCs in a stiff hydrogel produce ~2.5-fold less TGF-β1. MSCs in both soft and stiff hydrogels show a linear, constitutive kinetics of TGF-β1 secretion. The treatment of MSCs in the soft hydrogel with an inhibitor of TGF-β kinase, SB-431542, decreases the induction of chemokines, including CCL2 and interleukin-6 (IL-6), as well as vascular endothelial growth factor (VEGF) by exogenous recombinant tumor necrosis factor-α (TNF-α). In contrast, SB-431542 does not affect the TNF-α-mediated induction of paracrine factors from MSCs in the stiff hydrogel. Interestingly, inhibition of myosin-V normalizes the difference in endogenous production of TGF-β1 in MSCs in soft and stiff hydrogels. The results thus demonstrate a novel mechanism to regulate constitutive secretion of TGF-β1 in MSCs, which may play an important role in maintaining their immunomodulatory functions in an autocrine manner.
P2587
Board Number: B741
Integration of single molecule binding events in T cell activation.
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T cells are capable of responding to antigenic ligands presented on major histocompatibility complex (pMHC) molecules with single molecule precision. Discrimination between ligands ensures selective and robust immunological responses upon T cell receptor (TCR) triggering. It remains unclear how binding inputs are integrated into productive, activating signals during cell decision-making. pMHC:TCR complexes activate ZAP70 kinase, which phosphorylates linker for T cell activation (LAT) molecules that are crosslinked into extended assemblies. We developed an assay that maps the spatial and temporal features of every pMHC:TCR complex experienced by a primary T cell to signaling clusters and a binary readout of T cell activation. Antigen densities are precisely controlled to ensure physiological, single molecule densities. Activating cells experience an ensemble of discrete, stochastic binding events whose cumulative numbers and duration vary widely. By capturing activation of rare cells that respond to a low density of weak agonist pMHC, we demonstrate that activation may include spatially and temporally coupled binding inputs. We demonstrate that T cells can transform binding inputs to create events of longer duration. Thus, T cells cross a molecular threshold for activation whose mean dwell time duration requirement is an order of magnitude longer than the population, ensemble average dwell time. We also show that activating cells are marked by extensive LAT clustering, arguing that initial binding events and ZAP70 activation are the critical steps in distinguishing cellular outcomes. LAT clustering only occurs in response to receptor ligation, and, strikingly, LAT clusters outnumber binding events by several fold. Direct visualization of LAT assemblies shows that ZAP70 is localized in these clusters, without association of a pMHC:TCR complex. These findings are consistent with a model for dispersion of active ZAP70 from pMHC:TCR that phosphorylate LAT and amplify signaling through generation of multiple clusters. The biochemical and physical mechanisms of Zap dispersion are under investigation. A detailed, molecular understanding of T cell activation is expected to promote actionable insights into control of immunological responses such as those seen in the emergence of chimeric antigen receptor (CAR) T cells.

P2588
Board Number: B742
Inferring emergent evolutionary features from alignments of intrinsically disordered regions despite poor sequence conservation.
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Intrinsically disordered proteins and regions (IDPs) represent around 30% of eukaryotic proteomes and have been shown to play crucial role in a variety of complex processes, from cell signaling to cellular organization. Despite their importance for cellular function, IDPs show poor sequence conservation. This leads to an apparent paradox: why should such functionally important protein regions show so little conservation, assuming of course that sequence conservation is the only way to glean evolutionary importance. In this work, we use ideas borrowed from statistical physics with extant biophysical analysis to argue that conservation in IDPs exists in a space orthogonal to conventional sequence conservation. We suggest that sequence features that lead to specific emergent properties are conserved, even when sequences themselves are poorly conserved. We use our framework to evaluate the conservation of
disorder across a range of well-studied proteins, and showcase important implications for assessing how mutations in disordered regions might influence phenotype.

P2589
Board Number: B743
Mobility of DNA-binding species in the nucleus: the transient anomalous subdiffusion model.
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Single-particle tracking experiments have measured the distribution of dwell times of DNA-binding species diffusing in living cells, including CRISPR-Cas9, TetR, and LacI. The observed distribution, a truncated power law, implies transient anomalous subdiffusion, in which diffusion is anomalous at short times (mean-square displacement proportional to t^α, α < 1) and normal at long times (MSD proportional to t). Monte Carlo simulations are used to characterize the time-dependent diffusion coefficient D(t) in terms of the exponent α, the crossover time, and the limits D(0), D(∞), and to relate these quantities to the dwell time distribution. We examine how the underlying dwell time distribution is broadened by the statistics of escape to give the ideal observed distribution of dwell times. The actual observed distribution may be further modified by experimental uncertainties. We examine sampling in relation to the truncated power-law distribution, to see how long a Monte Carlo simulation is required and how many experimental observations are required to characterize the distribution. The simplest interpretation of the model is that the dwell times are actual binding times to DNA. One alternative interpretation is that the dwell times are the periods of 1D diffusion on DNA in the standard model combining 1D and 3D search. The model has several implications for cell biophysics. (1) The initial anomalous regime represents the search of the DNA-binding species for its target DNA sequence, so this regime is of fundamental interest. (2) Non-target DNA sites have a significant effect on search kinetics. False positives in bioinformatic searches are potentially rate-determining in vivo. For simple binding, the search would be speeded if false-positive sequences were eliminated from the genome. (3) Both binding and obstruction affect diffusion. The proper controls for obstruction are GFP as a calibration standard among laboratories and cell types, and the DNA-binding species with the binding site inactivated. Given the lack of a consensus on a model of chromatin, obstruction effects ought to be characterized experimentally as well as by modeling. (4) Overexpression of the DNA binding species reduces anomalous subdiffusion because the deepest binding sites are occupied and unavailable. (5) The model provides a coarse-grained phenomenological description of diffusion of a DNA-binding species, useful in larger-scale modeling of kinetics and FRAP. (Supported in part by NIH grant GM038133)

P2590
Board Number: B744
Oscillatory heat dissipation by cell cycle signaling during early vertebrate embryogenesis.
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During early embryogenesis, rapid cell proliferation poses an extra extraordinary challenge for metabolism to provide energy for DNA replication, chromosome segregation, and mitosis. Yet, we lack a global view of the energetics of embryogenesis. Here we have used isothermal calorimetry to measure the heat dissipation during the synchronous reductive cleavage divisions of zebrafish development. Embryos displayed cell cycle signaling dependent oscillatory heat dissipation atop a more strongly
Increasing component. Chemical perturbation and mathematical modeling supported the hypothesis that the phosphorylation and dephosphorylation events by cyclin-dependent kinase and its counteracting phosphatase generate the oscillatory heat dissipation. Thus, a signaling mechanism that coordinates cell growth and division contributes significantly to global energy metabolism during embryogenesis.

**P2591**

**Board Number: B745**

**Building the phase diagram of a cellular body in vivo.**

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Intrinsically-Disordered Proteins (IDPs) tend to drive molecules to phase separate, i.e. form liquid drops within a liquid mixture. Recently, phase separation was found to be important for the assembly of membrane-less organelles (cytoplasmic or nucleoplasmic granules). I have chosen a simple and highly tractable model organism to study phase separation in vivo. Bacteria do not have membrane bound vesicles so we can suppose that phase separation drives cytoplasmic granule assembly. It is predicted that E. coli’s genome has a relatively low proportion of IDP compared to mammals but transcriptional proteins are surprisingly enriched in IDP. Interestingly, RNA polymerase (RNAP) localization changes according to changes in metabolism such as growth in different media; it goes from being diffuse in poor growth media to punctate in rich growth media (known as transcriptional foci, TF). I hypothesize that prokaryotic TF assembly is regulated by phase separation in response to changes in growth conditions. Physical models predict that phase separation occurs at a discrete boundary between a mixed and demixed (i.e. phase-separated) solution. The following will directly test my hypothesis and identify relevant 1) environmental and 2) molecular parameters that drive TF assembly. I have developed a quantitative assay to measure the formation of TF in living cells through imaging of a rpoC::mCherry E. coli strain (RNAP). This assay will be use to establish a phase diagram in relation to different testable parameters. First, we are establishing whether TF assemble as a function of environmental conditions such as growth medium. A sharp transition in fluorescence distribution (from diffuse to punctate) in relation to growth rate would indicate the presence of a phase boundary. Predictions from phase diagrams suggest that at the phase boundary, TFs would disassemble. Therefore, this boundary could signal between cellular growth and TF assembly/disassembly. Second, I am performing a targeted screen on a list of E. coli transcriptional protein we have predicted to be disordered (DP). I am generating DP mutants (deletion) in the rpoC::mCherry strain in order to analyze TF assembly. Mutations in a protein required for TF assembly would be predicted to modify the phase diagram of TF assembly. In addition to my microscopy assays, I have purified hits to assess their phase separation activity in vitro. Thus, I am using high-resolution microscopy of a simplified bacteria system, a reverse genetic screen and biochemistry assay to determine how growth condition affects TF assembly.
P2592

Board Number: B746


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What sets the fundamental limits on speed of motility of a microscopic swimmer? Many freely swimming ciliates can exhibit an avoidance response in the form of a short, rapid backward swimming motion or a ‘jump’ which punctuates their normal swimming motion. This swimming behavior is exemplified in the common pond-water dwelling ciliate Halteria grandinella whose swimming motion is characterized by periods of forward swimming at a modest 10 body lengths/s alternating with extremely rapid backward ‘jumps’ reaching maximum speeds of more than 150 body lengths/s. ‘Jumps’ occur when the motile cilia transition to a high amplitude, high-frequency (~100Hz) beat-form with an associated reversal in the direction of thrust generation. Preliminary measurements and estimates show that these jumps are at the limits of metabolic rate of the cell. We also discovered that H. grandinella is capable of sensing hydomechanical signals in the ambient fluid and transitions to a jump when this signal exceeds a critical threshold value. Towards elucidating the energetics of this extreme swimming motion we use their sensitivity to hydomechanical signals to perturb the natural swimming behavior of the organisms by confining them to artificial micro-chambers of varying spatial periodicity (aka microfluidic pin-ball machine) which leads to a modified frequency of jumps. We combine such external perturbations with measurements of intracellular signaling molecules such as calcium to understand how jumps are triggered and how intracellular energy flows occur during a jump. Since we hypothesize that H. grandinella is operating at the limits of its metabolic rate, studying the underlying mechanism offers insights into the limiting factors governing energy storage and release. Concurrently their sensing apparatus allows an understanding of the physical limits of microscale hydrodynamical sensing.

P2593

Board Number: B747

mTORC1 controls rheology and phase separation by tuning ribosome concentration.

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Crowding within the cell is optimized to accelerate reactions, but not excessively impede diffusion. However, the mechanisms that regulate crowding are unknown. We developed genetically encoded multimeric (GEM) nanoparticles to study the physical properties of the cytoplasm. GEMs self-assemble into bright, stable fluorescent particles of defined size and shape. We used this system to discover signaling pathways that modulate crowding in yeast and mammalian cells. We found that the mTORC1 pathway tunes macromolecular crowding through regulation of ribosome concentration and thereby regulates the effective diffusion of macromolecules larger than 16 nm in diameter but has no effect on the diffusion of molecules at the 5 nm length-scale. Crowding tunes the degree of phase separation of model proteins in vitro and in vivo. Our results connect a central regulator of growth and metabolism to the biophysical properties of the cell.
P2594

**Board Number: B748**

**Impact of compressive stress on the C. albicans transdifferentiation.**

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Tensile and compressive stresses are the two main types of mechanical stresses experienced by cells. While tensile stresses arise from mechanical adhesion between cells, compressive stresses naturally emerge when cells grow and divide in a confined environment. Although tensile stresses have been extensively studied, and our understanding of their integration and downstream effect is good, much less is known about compressive stresses, despite their paramount importance in all living kingdoms, from microbes to metazoans. We have recently shown that Msb2, a mucin-like transmembrane protein, is a mechanosensor for S. cerevisiae under a compressive stress. Interestingly, this protein is one of the most upstream sensors controlling the transdifferentiation event of the yeast-to-hyphal transition in multiple organisms, including the human pathogen Candida albicans. Candida is the fourth most common bloodstream infection in the United States. Its pathogenicity is complex, but the yeast-to-hyphal transition has been shown to be important for the pathogen’s virulence. We show that the yeast-to-hyphal transdifferentiation event can be mediated by a compressive mechanical stress in C. albicans, and that a deletion of Msb2 causes a huge decrease of this transition. Interestingly, the efficiency of the transition depends on both the compressive stress applied to cells, as well as the pH of the growth media, in a complex and non-linear way, suggesting an optimal pH-compressive value at which the transition occurs. To the best of our knowledge, it is the first time that a compressive stress has been reported to impact transdifferentiation. We postulate that C. albicans virulence could then be influenced by the biophysical changes occurring when the pathogen infects a tissue, as the preferred infected organs present a wide range of pH and elasticity, the latter parameter impacting the mechanical stress. Thus, pathogenicity may not only be dependent on changes in the chemical environment. RNA-seq now allows us to determine key genes transducing compressive stress and it is appealing to use them as new therapeutic determinants to treat C. albicans infection. This study may well lead to new understandings of this important pathogen, but also place compressive stresses at the center of a new biomechanical differentiation paradigm.

P2595

**Board Number: B749**

**Cytoplasmic density dynamics in fission yeast studied by quantitative phase imaging.**

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The density of the cytoplasm may be a critical factor for many cellular reactions within the living cell. How cytoplasmic density is regulated and what happens to cellular processes when cytoplasmic density is altered are poorly understood. The rod-shaped fission yeast *S. pombe* is a powerful eukaryotic model for studying the cell cycle and growth because of its simple geometry and growth patterns. Recent evidence in the lab suggests that scaling of protein synthesis with cell growth is critical for maintaining cytoplasmic density and for determining the rate of cell elongation (B. Knapp et al, unpublished). We are developing the use of quantitative phase imaging (QPI) as a tool to estimate the density of individual yeast cells. Analysis of the intensity propagation of light along the z-direction based on brightfield image
stacks allows estimation of the refractive index of the living cell\(^1\), enabling quantification of the biomass of single cells\(^2\). We are currently validating this approach using a number of approaches. In preliminary findings, inhibition of the secretory pathway by brefeldin causes a inhibition of cell elongation accompanied by a steady increase in cytoplasmic density, consistent with previous findings with yeast secretory mutants\(^3\). Preliminary data also suggest that cytoplasmic density increases during mitosis/cytokinesis in the normal cell cycle. We are currently screening mutants for abnormalities in cytoplasmic density and hope to test the effects of altered density on cellular processes. The establishment of this simple quantitative microscopy-based assay will facilitate the study into the coordination of cytoplasmic density, cellular mechanics and cell growth.

References


P2596

Board Number: B750

A two-step mechanism to activate PARP-1 and stabilize it on a DNA break.

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PARP-1 cleaves NAD\(^+\) and transfers the resulting ADP-ribose moiety onto target proteins and onto subsequent polymers of ADP-ribose. An allosteric network connects PARP-1 multidomain detection of DNA damage to catalytic domain structural changes that reverse autoinhibition of catalytic activity; however, the mechanism of PARP-1 autoinhibition remains poorly defined. In this work, we utilized the NAD\(^+\) analog benzamide adenine dinucleotide (BAD), and a combination of Hydrogen-Deuterium Exchange Mass-Spectrometry (HXMS), x-ray crystallography, binding studies and catalytic activity assays, to reveal that PARP-1 autoinhibition results from a selective block on access to the NAD\(^+\) binding site.

One of these approaches, HXMS, is a solution-based method that provides structural and dynamic information that complements conventional structural approaches. HXMS revealed that BAD only gains access to the catalytic center of PARP-1 in the first step of the mechanism after it engages a DNA break causing the unfolding of the autoinhibitory HD domain. Surprisingly, BAD binding, in turn, leads to allosteric changes visible as changes in HX, culminating in HX protection back at its DNA binding surface that is \(~35\) Å away from the catalytic center of PARP-1. Indeed, we found that in the second step of the mechanism the binding affinity of PARP-1 toward damaged DNA site is increased after BAD binds, providing the first evidence of a reverse allosteric mechanism. Overall, this work provides new insights into PARP-1 domain assembly on DNA damage and how a two-step PARP-1 allosteric activation network ultimately influences persistence on DNA damage.
P2597

Board Number: B751

Morphological Changes of Epithelia Cells Induced by Viscous Conditions.
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Cells are highly sensitive to their microenvironments. While many chemical effects on the microenvironment are known, most biophysical effects are yet to be studied. One example is the effect of viscosity on cells. Understanding the effects of viscosity is pertinent to cancer research as increased viscosity is a prominent mechanical effect observed in the tumor microenvironment. In this study, we used media containing a 2% thickening agent to examine the effects of viscosity on a variety of cell lines, including EPH4, 67NR, 4T1, NIH-3T3, and MDA-MB231. Using reflectance imaging, we observed that cells exhibit a significant morphological change when viscous conditions are introduced by replacing regular medium with medium containing a thickening agent. Parameters such as the percentage of filopodia positive cells, number of filopodia per cell, the length of the filopodia, the basal area of the cell, and the length of the cell were quantified before and after the viscosity change. We found the under viscous conditions all cell lines became 100% filopodia positive. All cells exhibited an increase in the average number of filopodia per cell. The greatest increase in the average number of filopodia occurred in 4T1 and MDA-MB231, which were 8.28 fold and 13.66 fold respectively. The average length of filopodia per cell also increased in all cell lines. EPH4 had the largest increase in average filopodia length, which was 2.78 fold. The average cell area increased in all cell lines except MDA-MB231 which decreased by 30%. The average aspect ratio increased significantly in EPH4, 67NR and MDA-MB231. Despite the changes in the basal area and the length of the cells, the volume of the cells remained constant. Furthermore, the cell motility of NIH-3T3 cells was compared between control and viscous conditions and no significant differences were observed. These results suggest a potentially distinct mechanism for cells navigating at high viscosity as under viscous conditions all cells exhibited significantly denser and longer filopodia. It is especially interesting that metastatic breast cancer cell lines, MDA-MB231 and 4T1, had the greatest increase in filopodia per cell, indicating that this may aid in the ability to navigate the tumor microenvironment. Further investigation could lead to the mechanistic insight for how cancer cells adapt to the tumor microenvironment with higher success, as well as potentially novel treatment strategies for breast cancer.

P2598

Board Number: B752

Molecular mechanisms of perilipins targeting to lipid droplets.
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Lipid droplets (LDs) are organelles regulating cellular energy metabolism and homeostasis. While most organelles are bilayer-bounded, LDs have an oil core covered by a single phospholipid monolayer containing proteins, forming thereby intracellular oil-in-water emulsion droplets. Thus, because of their oil/water interface, LDs cannot bear proteins with transmembrane domains. Instead, most LD proteins use hairpins or amphipathic helices for targeting. How proteins specifically localize to LDs is among the main questions of LD biology. One of the first class of identified LD proteins is the Perilipin family (PLIN 1 to 5); they are the most abundant LD proteins, and regulate the accessibility of enzymes to LDs. PLINs do not all perform the same functions, as they are expressed at different levels in different tissues. Despite
their early identification and critical function in metabolism, how PLINs specifically target LDs is still not fully understood. PLINs bind with multiple domains containing amphipathic helix motifs. However, while they share similar structural binding motifs, they target different LDs within a same cell, which suggests their selectivity for LD surface. Such unique surface selectivity could be the driving force for the occurrence of distinct LD pools, with distinct fates, marked by PLINs. We capitalized on our knowledge of emulsion and membrane biophysics to address the molecular mechanisms of PLIN proteins binding to LDs. We deciphered in cells and in vitro how the binding of PLINs to LDs is regulated.

P2599
Board Number: B753
PEA-15 phosphorylation homeostasis and allosteric regulation of cell proliferation and apoptosis.
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PEA-15 (phosphoprotein enriched in astrocytes, 15 kD) is a small, non-catalytic, death-effector domain (DED) containing protein, that is widely expressed in different tissues and highly conserved among mammals. Although the overall expression level of PEA-15 is mostly constant in most tissues, its phosphorylation states of the two serine residues on the C-terminal tail vary significantly depending on cell and tissue types and/or cellular environment and conditions. The phosphorylation states control the interactions of PEA-15 with other protein targets in various pathways, including Fas-associated death domain (FADD) and procaspase-8 (apoptosis), extracellular signal-regulated kinase (ERK) 1 and 2 (cell cycle entry), and phospholipase D (PLD) 1 and 2 (diabetes). We have previously reported a surprising conformational change of PEA-15 DED upon interaction with ERK2 using nuclear magnetic resonance (NMR) dynamics and residual dipolar coupling (RDC) data. We recently demonstrated again that the DED conformation is also modulated by the phosphorylation states of the C-terminal serine residues. Upon phosphorylation, mimicked by serine to aspartic acid mutations (PEA-15DD), the DED conformation is very distinct from the wild type protein, and its binding specificity switches from ERK1/2 to FADD. Additionally, the PEA-15DD structure is not significantly different from the FADD complex. Based on our most recent results, we propose that the DED conformation is allosterically controlled by the phosphorylation states of the C-terminal tail, which in turn determines the binding specificity of the protein. We further propose that the balance between phosphorylated and unphosphorylated PEA-15 is strictly regulated in different cell types and tissues to control the cellular outcomes, which we termed phosphorylation homeostasis, and any disruption of the delicate balance could lead to various diseases, such as cancers and neurodegenerative diseases.

P2600
Board Number: B754
Understanding the Role and Regulation of Cell Wall Hydrolases in Bacillus subtilis.
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The main goals of this project are to the determine the roles that different cell wall hydrolases play in bacterial growth, and to understand how they are regulated. Most bacteria are encased in a rigid meshwork called the cell wall. In order to grow, cells must break bonds in this meshwork. These breakages are mediated by critical enzymes called cell wall hydrolases, whose activity must be carefully regulated in space and time. If too many bonds are broken, or if bonds are broken in the wrong place,
the cells will lyse. If too few are broken, the cell cannot grow. We currently know little about the specific roles that cell wall hydrolases play during cell growth or how their activity is regulated. I have developed a microscopy-based assay to track the flow of cell wall material through the wall with high spatial and temporal resolution using fluorescent probes that are covalently incorporated into the cell wall. This assay allows me to measure 1) the rate of incorporation of new material, 2) the rate of cell growth, and 3) the rate of removal of old material. With this assay, I have shown that two hydrolases, LytC and LytD, are involved only in the removal of old material and do not contribute to cell growth, while two other hydrolases, CwIO and LytE, contribute only to the expansion of the meshwork required for growth. Additional work will extend these observations to understand the role and regulatory mechanisms of other highly expressed hydrolases.

P2601

Board Number: B755

Fluorescence spectroscopy reveals two types of binding sites for endogenous ligands in CISD proteins.

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The dimeric proteins CISD1 and CISD2 belong to a unique family of iron-sulfur cluster containing proteins and are implicated in a variety of disorders including type-2 diabetes, Wolfram syndrome 2, and neurodegeneration. Localized at the mitochondrion, endoplasmic reticulum, and mitochondria-associated ER membranes, CISD proteins are directly or indirectly involved in cellular energy homeostasis, but the specific mechanism(s) of regulation remain ill defined. Our lack of a mechanistic understanding how CISD proteins integrate into cellular bioenergetics is in part due to the fact that interacting proteins and ligands are still mostly uncharacterized. Recent studies have suggested that both proteins, CISD1 and CISD2, are involved in redox sensing via binding to NADPH, but a more comprehensive study of interaction with other cellular metabolites is missing. We demonstrate that the soluble portion of both proteins have one tyrosine and tryptophan residue per monomer which undergo fluorescent emission quenching upon ligand binding. This feature allows for the development of a simple fluorescence-based assay to characterize binding of physiologically relevant organic ligands to CISD1 and CISD2. Excitation at l = 280 nm results in fluorescent emission of both tyrosine and tryptophan, while excation at l = 295 nm generates only tryptophan emission. By monitoring the fluorescent emission of CISD1 and CISD2 excited at l = 280 nm or l = 295 nm, we could demonstrate that reduced nicotinamide adenine dinucleotide (NADH) binds with significant affinity to two different binding sites, while the oxidized form and adenine nucleotides such as ATP and ADP only interact with one type of site. The physiological concentration of NAD+ ranges between 0.3 - 0.4 mM, only approximately 2 – 3-fold lower than the apparent Kd = 1.1 mM and 0.7 mM, respectively). Furthermore, binding affinities of CISD1 to ATP and ADP are within the physiological range of the phosphorylated nucleotides (Kd = 3.4 ± 0.2 mM and 1.5 ± 0.1 mM). Our approach offers a versatile system to characterize physiological relevant binding partners and to rapidly screen for potential therapeutics that interact with CISD proteins. Importantly, we demonstrate that CISD1 and CISD2 could be governing or responding to the cellular redox state by binding with physiologically significant affinities to a variety of compounds that are directly or indirectly related to the cellular energy state (Supported by NSF CHE-160944).
P2602

Board Number: B756

Crystal structure of thioredoxin reductase from Cryptococcus neoformans in complex with FAD a potential target for antifungal drug.

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In resource-limited regions, invasive fungal infections constitute a major public health problem, especially in immunocompromised patients. Cryptococcosis is an important opportunistic fungal infection caused by Cryptococcus neoformans, an encapsulated fungus that causes an initial asymptomatic pulmonary infection and can spread systemically especially in patients with AIDS. Cryptococcosis affects about one million people each year and kills about 625,000. Despite the tireless search to the development of new antifungal strategies, there are few drugs to treat systemic and superficial fungal infections available. Relatively the few drugs are toxic, present undesirable secondary effects and poor absorption and efficacy. Using comparative genomics strategy it was identified the potential of new drugs targets for human fungal pathogens. One of those is the thioredoxin reductase, a essential gene in C. neoformans. In this study, we present the heterologous expression, purification, enzymatic characterization and crystallographic structure of oxidized TrxR at 2.25 Å, the first human pathogenic fungi Cryptococcus neoformans structure. The expressed product was purified by affinity chromatography on nickel column. It was possible to identify a band of 39 kDa. The enzymatic activity of the recombinant protein was confirmed by assays of enzyme kinetics. CnTrxR was concentrated to 10 mg/mL, then it was incubated with FAD and finally concentrated to 35 mg/mL in 10 mM Tris-HCl, pH 8.5 and 300 mM NaCl. The purified recombinant protein CnTrxR was crystallized at 20°C. The CnTrxR crystals diffract to 2.25 Å resolution primitive orthorhombic space group P21 21 21, considering the asymmetric unit, two CnTrxR molecules were expected in the asymmetric unit. X-ray diffraction data were collected using the Brazilian synchrotron source and the structure was solved by molecular replacement and refined to a crystallographic R factor of 0.16 and a free R factor of 0.22. The overall structure of C. neoformans TrxR is very similar to that the S. cerevisiae TrxR1 as the research model. The root-mean square deviation of the 300 α atoms of the two structures superimposed was found to be 1.82 Å. Sequence alignment shows that CnTrxR shares a sequence identity of 71%, 64%, 48% and 24%, with TrxRs of S. cerevisiae, A. thaliana, E. coli and H. Sapiens (high-molecular-mass TrxR) respectively. In addition, small molecules that interact with a TrxR model were selected from the virtual screening library, among the best hits, 2 of them showed antifungal activity in vitro against C. neoformans. In summary, our findings described here represent advancement and may contribute to the development of new drugs with broad-spectrum activity for the treatment of fungal infections.
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P2603
Board Number: B758
Sheath Cell Invasion and Trans-differentiation Repair Mechanical Damage Caused by Loss of Caveolae in the Zebrafish Notochord.
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The notochord, a conserved axial structure required for embryonic axis elongation and spine development, consists of giant vacuolated cells surrounded by an epithelial sheath. During morphogenesis, vacuolated cells maintain their structural integrity despite being under constant mechanical stress. We hypothesized that the high density of caveolae present in vacuolated cells could buffer mechanical tension. Caveolae are 50- to 80-nm membrane invaginations lined by cage-like polygonal structures formed by caveolin 1 (Cav1) or Cav3 and one of the cavin proteins. Recent in vitro work has shown that plasma membrane caveolae constitute a membrane reservoir that can buffer mechanical stresses such as stretching or osmotic swelling. Moreover, mechanical integrity of vascular and muscle cells is partly dependent on caveolae. However, the in vivo mechano-protective roles of caveolae have only begun to be explored. Using zebrafish mutants for cav1, cav3, and cavin1b, we show that caveolae are essential for notochord integrity. Upon loss of caveola function, vacuolated cells collapse at discrete positions under the mechanical strain of locomotion. Then, sheath cells invade the inner notochord and differentiate into vacuolated cells, thereby restoring notochord function and allowing normal spine development. Our data further indicate that nucleotides released by dying vacuolated cells promote sheath cell vacuolization and trans-differentiation. This work reveals a novel structural role for caveolae in vertebrates and provides unique insights into the mechanisms that safeguard notochord and spine development.

P2604
Board Number: B759
Wnt5b regulates basal constriction during neuroepithelial tissue folding.
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Epithelial cell shape changes are regulated by intracellular and extracellular signals that tightly control cytoskeletal dynamics, which together are needed for proper development of tissue and organ structure. We have utilized the zebrafish neuroepithelium as a model to identify and understand the mechanisms that control neuroepithelial cell shape. Using the highly conserved structure known as the midbrain-hindbrain boundary (MHB), we identified a novel cell shape change required to fold the neuroepithelium, basal constriction. While we have previously shown that basal constriction requires laminin, and is likely to depend on non-muscle myosin II function, the signaling cues that are required, and how they regulate the cytoskeleton to mediate basal constriction are not well understood. Here, we have identified an upstream regulator of basal constriction, Wnt5b. Wnt5b is an extracellular signaling molecule with known roles in planar cell polarity and cell migration and it is expressed specifically at the
MHB during morphogenesis. Using live confocal imaging and a digital sectioning morphometric technique that we developed, we have uncovered a role for Wnt5b in the regulation of basal constriction. During MHB morphogenesis in wild-type embryos, we observed that cells simultaneously become deeper in the dorsal-ventral direction and narrower in the anterior-posterior direction at the basal end of the cell. This polarization of cell shape basally is lost with knockdown of wnt5b. 2D-Gel based proteomic studies and Western blot analysis revealed changes in alpha-tubulin levels following wnt5b knockdown. Therefore, we investigated the role of the microtubule cytoskeleton in mediating Wnt5b-directed basal constriction. Live confocal imaging of microtubule dynamics using a plus-end binding protein, EB3-GFP, and cell shape analysis after drug-induced manipulation of microtubule polymerization, suggests that Wnt5b signaling may be required for modulating microtubule stability during basal constriction. Next, we investigated a potential role for JNK, a known regulator of microtubule dynamics and downstream mediator of Wnt5b signals, in this process. We observed a decrease in phosphorylated JNK with knockdown of wnt5b, and hypothesize that Wnt5b mediates microtubule cytoskeletal activity through JNK signaling. Together, we have identified Wnt5b as an upstream mediator of basal constriction that is required to fold the neuroepithelium. Our results further suggest that Wnt5b modulates basal constriction through regulation of the microtubule cytoskeleton and JNK activity during embryonic brain morphogenesis.

P2605
Board Number: B760
Apical cell-cell adhesions reconcile symmetry and asymmetry in zebrafish neurulation.
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The symmetric tissues and body plans of animals are paradoxically constructed with asymmetric cells. To understand how the yin-yang duality of symmetry and asymmetry are reconciled, we asked whether or not apical polarity proteins orchestrate mirror-symmetric neural tube development in zebrafish by hierarchically modulating apical cell-cell adhesions. We found that apical polarity proteins localize by a pioneer-intermediate-terminal order. Pioneer proteins establish the mirror symmetry of the neural rod by initiating two distinct types of apical adhesions: The parallel apical adhesions (PAAs) cohere cells of parallel orientation, and the novel opposing apical adhesions (OAs) cohere cells of opposing orientation. Subsequently, intermediate proteins selectively enhance the PAAs when the OAs dissolve by endocytosis. Finally, terminal proteins inflate the neural tube by generating osmotic pressure. Our findings suggest a general mechanism to construct mirror symmetric tissues: Tissue symmetry can be automatically established by simply aligning cellular asymmetry opposingly via adhesions.

P2606
Board Number: B761
Rho signaling is fine-tuned by multiple feedback mechanisms during epithelial morphogenesis.
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While hexagonal packing represents a low energy arrangement of cells in an epithelium, such a simple geometry is rarely useful in vivo. Instead, anisotropic forces are deployed within and across cells to generate the complex geometries necessary for physiological function. Rho GTPase signaling is a conserved mechanism for generating forces through activation of contractile actomyosin. How this pathway is tuned to produce different morphogenetic outputs is poorly understood. To investigate this,
we study an event in the *Drosophila* embryonic epithelium (termed “alignment”) that generates rectilinear columns of cells where the cell-cell interfaces separating the columns align. We found that this cellular rearrangement is driven by contractility of supracellular actomyosin cables that elevate the local tension along aligning interfaces. Our data suggest that Rho signaling planar polarized to aligning interfaces promotes the formation of the actomyosin cables. Rho-GTP and two of its effectors, Rho Kinase (ROK) and Diaphanous (Dia), are enriched along aligning cell-cell contacts. Inhibition of any of these three factors caused loss of alignment. Depletion of ROK or Dia led to distinct defects in actin and Myosin-II (Myo-II) planar polarity. Expression of constitutively active forms of ROK or Dia produced ectopic furrows in the epithelium, suggesting that precise regulation of this pathway is necessary to achieve the aligned geometry. There is emerging evidence that feedback mechanisms can modulate Rho-GTPase signaling for morphogenesis. Therefore, we tested whether feedback between components in the pathway could play a role in controlling signaling outcomes during alignment. We found a mutual dependence for F-actin and Myo-II: cytochalasin D treatment resulted in a loss of Myo-II planar polarity and Myo-II knockdown led to depletion of cortical actin. Surprisingly, each of these conditions had different effects on Rho signaling. Consistent with findings in starfish and *Xenopus*, the disruption of F-actin led to upregulation of Rho-GTP and Dia, suggesting that negative feedback allows for fine tuning of Rho signaling during alignment. However, Myo-II knockdown had no impact on Rho activation or polarity. This contrasts with findings in the early embryo where actomyosin contractility positively feeds back to the Rho pathway. Yet, we did find that Dia is depleted from aligning interfaces with ROK inhibition, suggesting an interaction between these two effectors that is independent of contractility. This work suggests that multiple feedback mechanisms factor into maintaining polarized Rho signaling and driving coordinated cell shape changes.

P2607

**Board Number: B762**

Direct quantification of swelling pressures that drive chick neural tube morphogenesis.

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Fluid pressure has been shown to be an important contributor to the morphogenesis of several organs including lungs, vasculature, and kidney. A number of studies have also shown qualitatively that altering the pressure in the embryonic neural tube dysregulates morphogenesis. Increasing pressure increases the size and alters the shape of the neural tube whereas decreasing pressure leads to neural tube collapse and tissue death. However, few attempts have been made to quantify the pressures in the neural tube during development. By quantifying how neural tube pressures change over gestation, physical models can be created to understand how these mechanical forces direct morphogenesis and may identify downstream mechanotransduction pathways relevant in dysregulated neural tube development. In these studies we performed measurements of chicken neural tubes at a range of gestational ages and directly assessed the effect of beta-D-xyllose on neural tube pressure. *Gallus gallus* fertilized chicken eggs were incubated at 37° C under periodic rotation and staged chicken embryos were then extracted and cultured *ex ovo* for 24 hours on an agar/albumin hydrogel prior to pressure measurements at HH 18 and HH 22 gestational stages. To measure pressure, a pulled glass microneedle was wetted and connected to a differential pressure transducer. Continuous pressure measurements were captured by computer and data was recorded and analyzed with custom LabVIEW and MATLAB programs. To measure pressure, the embryo hindbrain was cannulated with the microneedle using a micromanipulator. Our pressure measurement method was extensively calibrated and validated with a measurement resolution of 5 Pa. Mean baseline pressures found in the neural tube at HH 18 gestational stage were approximately 20 Pa. Physical and pharmacological methods were used
to perturb neural tube pressures and alter morphogenesis. Pressures were recorded as neural tubes were physically “popped” by creating a hole with a glass microneedle. Pressure decreased and the wound healed with the neural tube reinflating. To increase pressure in the neural tube, embryos were treated with either beta-D-xyloside to dysregulate chondroitin sulfate synthesis or vehicle control at the start of ex ovo culture. Treatment with beta-D-xyloside increased neural tube pressure and led to corresponding increases in neural tube volumes. The methods we have developed to measure pressure in the developing chick neural tube provide quantitative data of luminal fluid forces to be used in the development of computational models of morphogenesis over gestation. Further, our techniques can be adapted to quantify the magnitude and role of fluid pressures in several other organs during embryonic development.

P2608
Board Number: B763
Interphase localization of Abnormal Spindle to the nucleus is important for proper brain size.
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Autosomal recessive primary microcephaly (MCPH) is a neurodevelopmental disorder characterized by reduced brain size and life span. While the clinical aspects of the disorder are well characterized, the molecular mechanism remains poorly understood. Previous models favored cell division defects induced by mitotic spindle errors as the cause of the disorder, leading to reduced neuron/glia numbers and a smaller brain. The most commonly mutated gene in human MCPH patients, *Abnormal Spindle-Like, Microcephaly Associated (ASPM)* is known to be important for proper centrosome and mitotic spindle function during mitosis. However, our recent analysis of the Drosophila melanogaster ortholog, *Abnormal Spindle (Asp)*, showed that mitotic spindle & cell division defects are not the primary cause of MCPH in Asp mutant animals, suggesting the current model needs to be revised. We now provide evidence that Asp contributes to proper brain size through a novel role in the interphase nucleus. Using a combination of transgenic rescue assays and high resolution microcomputed tomography (micro-CT) of intact animals, we have identified the minimal fragment of Asp’s N-terminus (AspNMF, 597 aa) required for proper brain size and morphology. Mutation of a highly conserved asparagine residue located within the ASH domain of the AspNMF fragment abolishes the rescue phenotype. Subcellular localization of AspNMF within the developing larval & adult brain revealed an unexpected localization to the interphase nucleus of distinct neural stem cell populations and mature neurons. Intriguingly, RNA-Seq analysis of Asp mutant brains revealed a significant downregulation of actin-related genes, including *myosin heavy chain (Mhc)* and the troponin complex member *wupA*, whose expression could be restored to wildtype levels in the AspNMF background. In accordance with Asp’s role as a nuclear protein, we identify and characterize a novel interaction between AspNMF & umbra (HP6), a heterochromatin binding protein located exclusively in the interphase nucleus. Together, our data highlights the first interphase role for Asp and suggests that other MCPH genes may contribute to the disorder through non-canonical pathways that funnel through the nucleus.
P2609
Board Number: B764
iPSC Derived Cerebral Organoids Reveal Early Developmental Malformations In Schizophrenia.
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Improper migration and premature maturation of neuronal precursor cells into neurons have been suggested to alter brain structure and destabilize function in schizophrenia patients. Cerebral organoids are 3D organ structures that mimic human brain development during the first trimester and allow insight into early developmental processes and cortical organization. We generated cerebral organoids using Induced Pluripotent Stem Cells (iPSCs), which are derived from adult human fibroblasts. They were reprogrammed into a pluripotent state using specified transcription factors. Organoids are used to study brain structure and protein expression and can give insight into how neurological disorders that appear later in life manifest in fetal development, as proper neuronal migration, maturation, and organization during development plays a critical role in brain structure and function. Two week and 35 day old cerebral organoids derived from patients with Schizophrenia were analyzed for proliferative marker protein Ki-67, neuronal transcription factor TBR1 and regulatory protein Fibroblast Growth Factor Receptor 1 (FGFR1). Organoids embedded in gelatin were sliced into 30μm sections and stained and by fluorescent immunohistochemistry for each protein of interest. Schizophrenia derived organoids showed a marked increase in cells expressing Ki-67 as well as a disorganization of proliferative rosettes when compared to control samples. A computational minimum spanning tree analysis studying inter-cellular distance distances between neighboring cells demonstrated a significant increase in distance spread within proliferative neural progenitor. Moreover, the percentage counts of nuclear TBR1 and FGFR1 in the outer cortical plate were significantly lower in Schizophrenia organoids, indicating premature advancement of neuronal development when compared to control organoids. Supported by NYSTEM (C026415, C026714), National Science Foundation (CBET-1555720, CBET-1706050) and Patrick P. Lee Foundation.

P2610
Board Number: B765
Smc5/6 complex maintains genome stability in the stem and progenitor cells of developing mouse cerebral cortex.
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The Structural Maintenance of Chromosomes (SMC) 5/6 complex plays a central role in maintaining genome integrity (Gallego-Paez et al., 2014, Gómez et al., 2013, Wu and Yu, 2012). Previous reports showed that mutations in the Smc5/6 complex cause severe developmental defects, including microcephaly and primordial dwarfism in humans (Payne et al., 2014). However, it is not known exactly how the loss of Smc5/6 disrupts the molecular pathways in the developing brain. We have developed a novel mutant mouse model harboring a conditional knock-out (cKO) allele of Smc5. These mice exhibit microcephaly during embryonic development through adulthood, thus mimicking the defect seen in humans. We confirm our previous observation that cKO of Smc5 results in the destabilization of the entire Smc5/6 complex (Pryzhkova and Jordan, 2016). We compared brain morphology and neural stem cell status in Smc5 cKO and control mice during fetal development, neonatal development and adulthood. We found that Smc5 cKO mice had severely underdeveloped cerebral cortex and showed
reduced numbers of neural stem cells and progenitors within the ventricular zone (VZ) and lower cortical layers (VI-IV), respectively. In our current work, we are closely assessing development of the fetal brain to determine the direct consequences of destabilizing the Smc5/6 complex. We are also assessing the genetic causes of the apoptotic response that is responsible for the development of microcephaly. Ultimately, our research will contribute to understanding of the molecular functions of the Smc5/6 complex during brain development in humans.

P2611
Board Number: B766
Glial cell remodeling and peripheral nerve re-organization during metamorphosis in Drosophila.
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The larval nervous system of the fruit-fly Drosophila melanogaster is reorganized during the 4-day transition period of metamorphosis, which is crucial for establishing adult specific behaviors. A prominent anatomical restructuring is the fusion of five pairs of posterior abdominal nerves to form a terminal nerve trunk (TNT). Segmental nerves defasciculate from the TNT at regular intervals to innervate the body wall. Glial cells that ensheath individual nerves are likely to play a significant role in nerve fusion. Our recent studies using confocal and EM approaches have shown that layer specific remodeling occurs during the 4-day period of pupal development. Our current work focuses on the most external glial layer, the perineurial glia (PG), which undergoes four-fold increase in number during the first day of metamorphosis (25% of development). Coincident with this increase in PG numbers is the breakdown of the adjacent extracellular matrix-the neural lamella (NL). It is possible that disintegration of NL during day 1 of metamorphosis triggers signaling mechanisms to bring about PG proliferation. Our experiments will test the hypothesis that FGF signaling plays a role in PG proliferation. Preliminary experiments using the Gal80ts target system to temporally control transgene expression, have identified that disruption of the FGF receptor-heartless in PG cells leads to eclosion defects in animals. Additionally, a significant number of animals that emerge have defective TNT patterns. Our research on glial remodeling in Drosophila will lay the groundwork for future studies on the role of glia-glia and glia-neuron communication during TNT formation, and could lead to the establishment of a model system to study gliopathies.

P2612
Board Number: B767
Tissue macrophages modulate alveolar epithelial and myofibroblast differentiation during fetal lung sacculcation.
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Tissue macrophages are fundamental effectors in the modulation of several organs development, mainly in processes as branching morphogenesis and vasculature formation. Although these cells are present throughout lung developmental stages, their contribution to its formation is unknown. Accordingly, we
hypothesize that fetal tissue macrophages play critical roles in lung developmental events, such branching morphogenesis, differentiation and maturation. Using a macrophage-deficient mouse model (colony stimulating factor-1 receptor (Csf1r) knockout (KO) mice), we performed morphological analysis at different lung developmental stages, which evidenced a disruption of distal lung morphology in KO mice, with a significantly increase in lung non-epithelial-like volume and decrease in air space compartments at saccular stage. Since, lung saccular stage is largely characterized by differentiation of several cell lineages (e.g. alveolar epithelial, myofibroblasts) with the continuous vascular network formation, we evaluated distal epithelium, myofibroblasts/smooth muscle and vasculature differentiation. Transcripts expression levels of distal epithelial markers evidenced a clear impairment in macrophage-deficient mice in this compartment. Deregulation in relative transcripts expression of myofibroblasts/smooth muscle cell related molecules was also observed in the lung of these animals. Alveolar markers type I and II protein expression investigation showed a significant reduction in alveolar differentiation in KO mice. On the other hand, a substantial increase in mesenchymal cell marker and myofibroblast marker expression was observed, suggesting a disruption in this cellular lineage differentiation. Vascular mediators analysis showed an imbalance of transcript expression levels in the KO mice. However, protein expression evaluation of two specific vascular markers showed no evident impairments in vasculature formation in KO mice at this stage. Our data demonstrate the influence of fetal tissue macrophages in the modulation of lung sacculation and alveologenesis, namely shaping distal epithelial and myofibroblast lineage differentiation. These results strongly suggest that fetal tissue macrophages act as paracrine suppliers indispensable to the formation of a functional pulmonary system.

Supported by: NORTE-01-0145-FEDER-000013; POCI-01-0145-FEDER-007038; FCT Exploratory Research Project IF/00959/2014

P2613
Board Number: B768
A Novel Role for Paxillin during Mammary Gland Morphogenesis.
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Abstract:
Paxillin is a scaffold protein that localizes to focal adhesions and has previously been implicated in multiple cellular processes including integrin-mediated signaling, polarized trafficking, directional cell migration and maintaining Golgi integrity. Our study aims to understand the role of paxillin in vivo, during mammary gland development. Global paxillin knockout in mice causes embryonic lethality so we generated a conditional paxillin knockout mouse that drives cre expression under the Mouse Mammary Tumor Virus (MMTV) promoter to specifically ablate paxillin in the mammary epithelial cells. To assess the role of paxillin during mouse mammary gland development, we performed whole mount staining and immunohistochemistry (IHC), as well as ex-vivo organoid 3D culture. The whole mounts of 6-week-old mammary glands revealed that paxillin^fl/fl cre glands have significantly less branching and dilated ducts. Similarly, reduced branching was observed in the paxillin^fl/fl cre organoids. Furthermore, epithelial cell shape in both paxillin^fl/fl cre mammary glands and organoids were more elongated as compared to controls. IHC analysis of the paxillin^fl/fl cre ducts revealed that the Golgi apparatus is mislocalized and apical polarity proteins also have a dispersed distribution, indicative of disrupted apical-basal polarity in paxillin^fl/fl cre glands. Interestingly, our preliminary data also showed both mislocalization and reduction of acetylated-tubulin in the paxillin^fl/fl cre glands. Taken together these data suggest paxillin is required for mammary gland morphogenesis by regulating epithelial cell apical-basal polarity formation potentially via microtubule acetylation-associated trafficking.
P2614
Board Number: B769
Smooth muscle differentiation guides domain branching in the embryonic mouse lung.
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During branching morphogenesis, a simple cluster or tube of cells proliferates and branches to generate an arborized network that facilitates the flow and exchange of gases or fluids. Branched organs are found throughout the animal kingdom, and while their function varies, their morphogenesis is driven by similar molecular programs. To achieve a specific final morphology, physical cues are required to guide branching of the epithelium into the surrounding mesenchyme. During murine lung branching morphogenesis, the airway epithelium develops concomitantly with a layer of smooth muscle, which is derived from the mesenchyme and wraps around the airways. Specific spatial patterns of smooth muscle differentiation are required for terminal bifurcation. Here, we examined the role of smooth muscle differentiation in shaping emerging domain branches (branches that bud laterally off the side of an existing branch) during early lung development. We found that the position and morphology of domain branches are highly stereotyped: branches begin as wide buds that thin at their base as they elongate. At the same time, there is an increase in the amount of smooth muscle wrapped around the parent branch at the base of the new domain branch. Perturbing the pattern of smooth muscle differentiation results in abnormal branch positioning and morphology. Loss of smooth muscle results in ectopic branching events and slows branch thinning, and enhanced smooth muscle differentiation suppresses branch formation and elongation. Our work uncovers a role for smooth muscle differentiation in sculpting emerging domain branches, and sheds light on the physical mechanisms of branching morphogenesis of the mouse lung.

P2615
Board Number: B770
Extracellular matrix remodeling and activation of focal adhesion kinase direct airway epithelial branching morphogenesis.
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Cells integrate biochemical and biomechanical cues from the microenvironment to execute developmental programs during organogenesis, including airway branching morphogenesis in the lung. To understand how cells in the developing lung sense and respond to the local microenvironment, we carried out RNA-sequencing analysis of critical stages of branching of the embryonic chicken lung, which revealed changes in expression of genes associated integrin signaling and extracellular matrix (ECM) remodeling. Therefore, we sought to define the relationship between ECM synthesis, remodeling, and airway branching morphogenesis. We found that prior to branching, the basement membrane (BM) is a uniform sheath wrapping the airway epithelium with no observable BM turnover prior to branch initiation. However, the BM is significantly reduced at the tip of elongating airway branches after of branch induction. Cultures of embryonic lung explants reveal that BM thinning at branch tips is mediated by matrix metalloproteinases (MMPs). Inhibiting MMPs suppressed branch extension, but did not prevent the initiation of new branches. As epithelial branches extend, cells in the surrounding

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mesenchyme become highly elongated in shape and activate focal adhesion kinase (FAK). This local activation of FAK is associated with the synthesis of tenascin-C (TNC) at branch tips. Moreover, disrupting FAK activity blocks initiation of new branches. Based on these data, we propose a model by which the airway epithelium initiates branches in a FAK-dependent manner. While matrix remodeling is not required for branch initiation, BM depletion at branch tips is required for branch extension. As branches extend, neighboring mesenchymal cells are deformed, which promotes a change in local ECM composition via FAK activation, ultimately shaping the growing branch.

P2616  
Board Number: B771  
A microfluidic ex vivo culture model of neonatal mouse lungs to investigate alveolar development.  
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Preterm infants are born with an underdeveloped lung that is unable to meet their tissues’ oxygen demand. Standard care of these infants requires enrollment into neonatal intensive care units and the use of mechanical ventilation (MV) which is known to expose the developing lung to altered mechanical forces. Even with recent therapies, MV can result in alveolar injury and long-term pulmonary issues. The most common complication associated with MV is bronchopulmonary dysplasia (BPD). MV induced BPD is characterized by the presence of fewer, enlarged alveoli indicating a failure of the alveoli to undergo septation. A common co-morbidity occurring in one-third of BPD diagnoses is pulmonary hypertension (PH), which has a three-year mortality rate of 75%. Unfortunately, new therapies for BPD and PH are hindered, as current models are unable to study mechanical forces in an intact organ; therefore, the genetic pathways linking altered mechanical forces to cellular behaviors remain largely unknown in the developing lung. We have created a novel, microfluidic, ex vivo organ culture platform that enables long-term culture of neonatal mouse lung explants. In this device, the trachea of the explant is intubated and the organ vasculature cannulated with custom fabricated microneedles. Each compartment is connected to separate pump systems allowing for independent control of cyclic airway ventilation and vascular perfusion during culture. The culture model was validated by quantification of explant ventilation and serial perfusion of dye throughout the vasculature. Histological analysis of lungs cultured in our device over 48 hours show increased alveolar septation compared to bath cultured samples. Additionally, the septation of lungs culture in our ex vivo device more closely resemble that of age-matched, in vivo, sibling controls. Similarly, EdU staining demonstrates uniform cellular proliferation throughout a lung lobe in device cultured explants compared to an absence of proliferation in the central one-half lobe volume in explants cultured using traditional methods. This microfluidic culture platform enables future studies to directly quantify the dynamics of alveolar morphogenesis. Additionally, combining this model with pharmacological interventions and established genetic methods can elucidate the role of mechanotransduction pathways in BPD and PH and allow for the development of adjuvant therapies for infants undergoing mechanical ventilation.
P2617
Board Number: B772
Epithelial deformation during lung development as a driver of localized growth signaling.
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During development, the lung is unique in its ability to form a stereotyped branched architecture. It is well accepted that during the pseudoglandular stage of development, when the main conducting airways are being formed, reciprocal signaling between the epithelium and mesenchyme at the distal tips is a major contributor to the normal branching architecture. However, it is not well understood how the signaling events are initiated at specific locations within the developing epithelial tree. Our work and the work of others have documented airway smooth muscle contractions that push luminal fluid to the distal airway tips causing significant cyclic stretch at these sites. Importantly, these distal tips are also regions that have been identified as active sites for several key reciprocal molecular signaling pathways responsible for regulation of airway branching morphogenesis. Whereas it has long been believed that cyclic stretch of the distal epithelium may be responsible for regulation of molecular signaling events in the developing lung, no one has directly demonstrated this is true, nor which signaling molecules can be induced by this stretch. To investigate these questions, we have developed a technique to manually dissociate embryonic mouse lung explants (E12-14) into its three main functional tissue compartments: epithelium, mesenchyme, and mesothelium. Using qPCR, we demonstrate that the tissue compartments were properly separated, and we developed in vitro culture methods to stably maintain isolated cells. To specifically investigate the role of stretch, we adapted a custom microfluidic flex cell that allows dynamically controlled stretch of a thin polydimethylsiloxane (PDMS) membrane between 0 and 15% strain. Isolated embryonic primary cells were seeded onto this PDMS membrane and allowed to form a confluent layer. Cells were stretched statically and dynamically for 12-24 hours and collected for downstream processing by IF and qPCR to determine the change in growth signaling molecules. Our results indicate that the key signaling molecules, HIF1a and VEGF, are increased when epithelial cells are exposed to physiological levels of stretch. These molecules have long been investigated for their role in branching morphogenesis, but have not been directly connected to stretch in the tissue. We can use this device and tissue separation technique to directly stretch isolated tissues in an effort to uncouple the reciprocal signaling pathways between tissue layers found in the embryonic lung and assess the tissue specific molecular signaling changes due to stretch.

P2618
Board Number: B773
Precision abscission for cell surface integrity and plant fitness.
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Organ separation, or abscission, in plants is critical for discarding leaves, flowers, and to conserve resources, and as a form of defense. Little is known about the mechanism guiding the spatiotemporal precision of abscission, nor how protection of the newly formed surface is maintained. Here, we identify two neighboring cell types in Arabidopsis that coordinate their activities to ensure precise organ abscission. One cell type produces a honeycomb structure of lignin, which acts as a mechanical brace to localize cell wall breakdown and spatially restrict abscising cells. The second cell type forms a layer of new epidermis with a protective cutin coat, defects in which lead to an imperfect surface barrier.
susceptible to infection. This transdifferentiation event demonstrates de novo specification of epidermal cell identity, which was thought to be restricted to embryogenesis.

**P2619**

**Board Number: B774**

Alterations in indices of internal anatomical structure of Brachypodium distachyon and Kazakhstani varieties of soft wheat under the action of Puccinia recondita pathogen.

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According to the Food and Agriculture Organization of the United Nations Kazakhstan is one of the world leading producers of high-quality wheat grain. Puccinia (P.) recondita pathogen can lead up to 40-60% in yield loss. The objective of study is the assessment of influence of P. recondita infection on the quantitative indices of internal anatomical structure of leaves, stalks and roots of a wild cereal model plant Brachypodium distachyon (standard line Bd21) and soft wheat varieties. Kazakhstanskaya 19 (K19), Kazakhstanskaya early (KE) with varying resistance to a brown rust (15 and 60%) and Bd21 were used as study materials. For anatomic studies above and underground vegetative parts of soft wheat and Bd21 collected on experimental and twice infected fields were used. Anatomic specimens (over 70) were prepared using a microtome with freezing unit; thickness of anatomic cuts was 10-15 microns (µ). Measurement of morphometric indexes was taken by eyepiece micrometer (ocular 10x22); microphotographs (lens 4x0.10) of anatomic cuts were produced on a trinocular microscope with the digital video camera. Statistically significant decrease by 13% in diameter of conductive bundles of leaf blades, increase by 24% in diameter of xyalary fibers are observed in Bd21 under the pathogen action, while the primary cortex is nearly reduced. Infected wheat plants show similar changes towards increase in the sizes of all tissues of leaf blade and dissimilar for stem (reduction in K19 and prevailation at KE, susceptible to brown rust). Increase in thickness of primary cortex (537.99 µ) and diameter of xyalary fibers (69.86 µ) are observed in internal structure of roots and the flag leaves of K19 causing increase in diameter of the central cylinder (770.41 µ). KE plants, on the contrary, display reduction of the sizes of primary cortex (269.34 µ) and the central cylinder (345.35 µ). Formation of anatomical structure of plants – growth process, sympathetic on the action of external conditions, especially during the early period of plant development, plays an important role in the course of plant adaptation to biotic factors.

**P2620**

**Board Number: B775**

Epithelial Cell Reintegration: Stray Cells Find Their Way Home.

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Mitotic epithelial cells exhibit changes in cell position as well as cell shape. Although interkinetic nuclear migration is primarily studied in vertebrate pseudostratified neuroepithelium, apical-directed mitotic movement is also observed in cuboidal and columnar tissues. In some conditions, one of the two mitotic products will not inherit a connection to the basement membrane. That cell must therefore rebuild neighbour cell contacts de novo as it reintegrates into the layer. In Drosophila, reintegration is driven by the lateral cell adhesion molecule Neuroglian. L1-Cam, the vertebrate homolog of Neuroglian, is also expressed in vertebrate epithelial tissues in which reintegration behaviours have been observed. We are
currently investigating whether the reintegration mechanism is conserved, and whether it is impacted by the cell cycle.

P2621
Board Number: B776
MafA transcription factor as a marker of differentiated β-cells of islets of Langerhans in rats. M. Kaligin1, A. Plushkina2, M. Titova1, A. Titova1, A. Gumerova1, A. Kiyasov1;
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One of the markers of progenitor cells (PC) of β-cells is stem cell factor receptor- C-kit (CD117). MafA transcription factor is also indicated as the marker of PC of β-cells. It is assumed that it activates the expression of insulin gene in the PC of β-cells. According to other data, MafA is a marker of differentiated β-cells, because it is required exclusively for regulation of insulin secretion by adult β-cells. The aim of the work was to study the role of MafA during experimental diabetes in rats by analyzing the expression of MafA, C-kit, glucagon and insulin. The study was made on 33 rats, which were injected intraperitoneally with alloxan at dose of 180 mg / kg. Paraffin sections of pancreas stained immunohistochemically with antibodies against MafA, C-kit, glucagon and insulin. Statistical analysis was made with the help of Statistika program. In normal rats pancreas we observed the maximum amount of MafA+ and insulin+ cells in islets. After alloxan injection, the number of both cells populations decreased on early stages (1, 2, 3 days) of experiment. At the late stages (14, 21, 28 days) the number of MafA+ cells continued to decrease, while the amount of insulin + cells began to increase, but did not reach the norm. This increase in the number of insulin+ cells may be associated with the onset of regeneration of insulin-producing cells from progenitor cells after 14 days of diabetes. The continued decrease of MafA+ cells during the increase of insulin+ cells casts doubt on the involvement of MafA in activating the expression of insulin gene in the progenitor β-cell. In this case we would have to observe an increase in the number of MafA+ cells, earlier than the number of insulin + cells increased. There were no C-kit+ cells in normal pancreas. During experimental diabetes their number began to grow within 1 day, the amount of C-kit+ cells decreased at the 2 day, then increased again at7 day. At 14 day they almost disappeared and reappeared at the end of the experiment (21 day). It is interesting that the dynamics of changes in the population of glucagon + cells on the first days (1-5) completely corresponds to that for C-kit+ cells. Then the number of glucagon+ cells decreases (7 days), followed by an increase (14 days) and a gradual decrease in the last 24 hours. Fluctuations in the number of populations of insulin+, glucagon+ and C-kit+ cells we associate with the process of differentiation of C-kit+ cells into insulin-producing cells through the stage of glucagon+ cells. We can make the conclusion that MafA is not a marker of progenitor β-cells, because from 1 day of diabetes dur

P2622
Board Number: B777
Cxcl12a induces Snail1b expression to initiate collective migration and sequential FGF-dependent neuromast formation in the zebrafish Lateral Line primordium. U.M. NEELATHI1, D. Dalle Nogare1, A.B. Chitnis1;
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The zebrafish posterior Lateral Line (pLL) primordium migrates under the skin along the horizontal myoseptum following a path defined by the chemokine Cxcl12a, periodically depositing neuromasts, to pioneer formation of the lateral line system. We show that snail1b, previously known for its role in promoting Epithelial Mesenchymal Transition and cell migration, is expressed in leading cells of the
primordium and in underlying cells of the horizontal myoseptum. Expression in leading cells is promoted by Cxcl12a and inhibited in trailing primordium cells by FGF signaling. Knockdown of snail1b delays initiation of collective migration of the primordium. This delay is associated with aberrant expansion of epidermal cell adhesion molecule (epcam) into the leading zone and a simultaneous loss of cadherin2 expression in leading cells. Co-injection of morpholinos with a relatively low dose of snail1b mRNA prevents the initial delay in migration and restores normal expression of epcam and cadherin2. Cell transplantation experiments show that function of snail1b in the leading cells of the primordium, not underlying horizontal myoseptum cells, is critical for initiating collective migration. The delay in initiating collective migration of the primordium in snail1b morphants is accompanied by a delay in sequential formation of trailing FGF signaling centers and associated proto-neuromasts that normally form within the migrating primordium. Intriguingly, the delay in sequential formation of proto-neuromasts and associated FGF signaling centers is not specifically related to knockdown of snail1b function but also associated with other manipulations that delay migration of the primordium without affecting snail1b expression. These observations reveal an unexpected link between migratory behavior and sequential formation of proto-neuromasts in the primordium

P2623

Board Number: B778

A Strain Map of Zebrafish Gastrulation Describes the Mechanics of Convergence and Extension and Emergence of Left-Right Chirality during Epiboly.

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During the development of the zebrafish embryo, the major embryonic axis and tissue patterns are organized by different morphogen gradients, such as Nodal (Xu et al. 2014) and BMP (Hardt et al. 2007), and biomechanical coupling through cell-cell and cell-matrix adhesions (Tada and Heisenberg 2012). How collective migration and local mechanical stress are coordinated globally over the entire embryo remains unknown. We have adapted the high spatial-temporal resolution of light sheet microscope system (Keller et al. 2008; Bhattacharya et al. 2012) for in toto imaging and a 2D tissue tectonics algorithm (Blanchard et al. 2009) to generate a 3D strain map of zebrafish gastrulation. The resulting strain maps reveal the time-course and location of areas undergoing deformation in 3 perpendicular directions during well-known morphogenetic movements, including convergence and extension, and somite formation. In addition, we detect rotational or curl strain, a new type of coordinated motion, as early as mid-epiboly. Rotational strain arises concurrently with axial and equatorial strain and contributes unique mechanical signatures of convergence and extension during gastrulation. Curl strain suggests that left-right asymmetry has been established and is mechanically coupled across the entire developing embryo.
P2624
Board Number: B779
Histone methyltransferase G9a is essential for osteoblastic differentiation and skull bone formation during development.
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The expression of cell lineage specific genes during cell differentiation and tissue development is subjected to epigenetic as well as genetic regulations. Posttranslational modifications of histone tails are critical epigenetic marks. Among them, methylation at histone 3 lysine 9 (H3K9) is a crucial modification that indeed affects gene expression and cell differentiation. The methylation of H3K9 is catalyzed by several H3K9 methyltransferases, such as G9a. Previously, we showed specific localization of G9a in the epiphyseal cartilages of long bones (Ideno et al. 2013). Therefore, we hypothesized G9a plays a role in bone formation. In this study, we used G9a-floxed mice to elucidate the function of G9a in bone formation, since G9a-null mice showed early embryonic lethality. We employed Sox9-Cre mice which expresses Cre in the cranial neural crest-derived tissues in vivo. At 3weeks after birth, G9a-fl/fl:Sox9-Cre mice showed reduced body size. The micro-CT analysis revealed mineralization defects and significant reduction of size in calvarial bone in G9a-fl/fl:Sox9-Cre mice. The skeletal preparation of G9a-fl/fl:Sox9-Cre mice at embryonic days (E)16.5, E18.5 and postnatal day1 also had the ossification defects of calvarial bones. The decreased expression of Osteocalcin (OC) was observed by in situ hybridization. However, the expression of Runx2, a transcription factor essential for osteoblastic differentiation, was not affected, suggesting G9a is required for Runx2 activities. We also generated G9a-knockout osteoblasts (G9a-KO OB) using Cre-recombinase expressing adenovirus in vitro. Proliferation assay showed the inhibition of proliferation in G9a-KO OB. OC promoter-Luc assays showed that G9a enhanced transcriptional activity of Runx2. G9a-KO inhibited accumulation of Runx2 on endogenous binding sites within the promoters of osteogenic marker genes in chromatin immunoprecipitation assay using a Runx2 antibody. These results suggest that the H3K9 methyltransferase G9a regulates osteoblastic differentiation and skull bone formation.

P2625
Board Number: B780
Effect of Sugar Cane Extract (SCE) Supplementation on Corticosterone Secretion in induced by ACTH injection Male Rats.
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Sugarcane extract(SCE) is the residue after removing glucose, fructose and sucrose from molasses. It is known that sugarcane extracts have various beneficial physiological function, such as anti-oxidative and anti-stress effects. Corticosterone is a main glucocorticoid produced in rodent, involved in regulating energy metabolism, immune reaction, and stress responses. This study investigated the effect of SCE on corticosterone production in male rats. Different time period (0, 30, 60, 120, 180minutes) of ACTH agonist injection was used to stimulate the production of corticosterone in the in vivo and in vitro
experiments. Three weeks male Wistar-Imamichi rats were fed with SCE-free diet or 2.16% SCE diet for 7 weeks. Blood samples were drawn for corticosterone, testosterone, LH and FSH measurement; mRNA of adrenal glands were analyzed for steroidogenic enzymes (P450scC, 3β-HSD, P450c21, P450c11) and ACTH receptor MC2-R gene expression. Plasma concentrations of corticosterone were significantly higher after 30 minutes of ACTH agonist injection in SCE group than control group (P<0.05). The basal mRNA level of the steroidogenic enzymes and ACTH receptor MC2-R showed higher expression in SCE group than control group. Results suggested that SCE supplementation increased adrenal response capacity of corticosterone secretion by enhancing the expression level of steroidogenic enzymes in male rats.

P2626
Board Number: B781
Systematically modulating cell-cell adhesion reveals cellular mechanisms of epithelial remodeling in Drosophila.
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During development, simple epithelia reorganize and remodel into functional tissues with complex form and structure. Adhesion at cell-cell contacts and contractile tension generated by actomyosin contractility are thought to be key parameters that control tissue mechanics and remodeling during epithelial morphogenesis. How the balance between tension and adhesion determines the structure, mechanics, and remodeling of epithelial tissues, however, remains unclear. In the Drosophila embryo, oriented cell rearrangements in the embryonic epithelium rapidly elongate the body axis from head to tail. To gain insight into how the balance between adhesion and contractility influence tissue structure, mechanics, and remodeling, we are systematically modulating levels of E-Cadherin expression using multiple approaches, including transgenic overexpression. We are using live confocal imaging and quantitative image analysis to study the effects of modulating E-Cadherin expression on cell rearrangements, cell shapes, and tissue elongation. In embryos expressing increased levels of E-Cadherin, we find that cell rearrangements within the tissue are decreased compared to in wild-type control embryos. However, macroscopic tissue elongation is not significantly reduced in the embryos with decreased cell rearrangement. This appears to be due in part to differences both in the effectiveness of cell rearrangement and in cell shape changes, which can also contribute to macroscopic tissue elongation, as E-Cadherin expression is increased. These systematic, quantitative experimental studies reveal how cell-cell adhesion influences the cell behaviors that contribute to tissue remodeling during epithelial morphogenesis, and will be essential for building and testing theoretical models of epithelial mechanics.

P2627
Board Number: B782
Microtubule Dynamics in Developing Mammary Epithelium.
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Branching morphogenesis closely coordinates cell proliferation and collective cell migration to expand tubular epithelial structures. In the mammary gland, early branching relies on cell proliferation to establish a stratified Terminal End Bud (TEB). Once this unit forms, coordinated migration of TEB cells acutely drives elongation and branching. Continued large scale expansion of the ductal network requires
complementary migration and proliferation. The involvement of microtubules in both mitosis and migration implies their critical role in directing this developmental process.

To investigate the dynamic contributions of microtubules to branching morphogenesis, we use three-dimensional culture of mammary organoids--epithelial cell clusters embedded within extracellular matrix. This culture system recapitulates the events of branching morphogenesis in a controlled and observable environment. When microtubule targeting drugs are added to murine mammary organoids at culture day 0 when proliferation is acutely required, they blocked branching (Nocodazole IC50 = 40nM, Paclitaxel IC50 = 5nM). However, nocodazole treatment at the branching IC50 does not affect the overall growth of the organoid suggesting that microtubule disruption alters organ morphology without affecting cell proliferation. We continue to investigate these results using several imaging modalities. Immunofluorescence demonstrates the relative alignment of the microtubule cytoskeleton, nuclei, golgi, and microtubule organizing center as well as the distribution of microtubule posttranslational modifications in three-dimensional epithelium. In addition, vital dyes and molecular biosensors in three-dimensional confocal time-lapse microscopy reveal microtubule dynamics in cell mitosis and migration within TEBs. These results offer novel insights into multicellular microtubule organization and dynamics in developing epithelial tissue.

**Tissue Mechanics**

P2628

**Board Number: B783**

**Emergence of tissue mechanics from cellular processes: shaping a fly wing.**

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Nowadays, biologists are able to image biological tissues with up to many thousand cells in vivo where the behavior of each individual cell can be followed in detail. However, how precisely large-scale tissue deformation and stresses emerge from cellular behavior remains elusive. Here, we study this question in the developing Drosophila wing. To this end, we first establish a geometrical framework that exactly decomposes tissue deformation into contributions by different kinds of cellular processes. These processes comprise cell shape changes, cell neighbor exchanges, cell divisions, and cell extrusions. As the key idea, we introduce a tiling of the cellular network into triangles. Our approach also reveals that tissue deformation can additionally be created by correlated cellular motion. Based on quantifications using these concepts, we developed a novel continuum mechanical model for the Drosophila wing. In particular, our model includes active anisotropic stresses and a delay in the response of cell rearrangements to material stresses. Taken together, our work helps to elucidate how tissue mechanical properties and thus organ shape emerge from the interplay of large numbers of cells.
P2629
Board Number: B784
Role of dynamics on the formation of zebrafish organ of asymmetry.
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In an embryo, epithelial cells actively change their shapes to generate emergent macroscopic patterns. How these programmed cell shape changes are regulated via a combination of mechanical forces and biochemical signaling pathways remains an open question in biology. The left-right organizer in the zebrafish embryo, Kupffer’s vesicle (KV), provides a simple system not only to investigate asymmetric organ formation but also to understand the origin of left-right asymmetries of vertebrates. During KV morphogenesis, monociliated epithelial cells in the KV undergo asymmetric cell shape changes along the anterior-posterior (AP) axis. These cell shape changes position more cilia in the anterior region of the KV, which is crucial for generating left-right asymmetries in the embryo. The developing KV and neighboring tissues, including the extending notochord and migrating mesodermal cells, are highly dynamic. While previous studies of the KV have successfully contributed to our understanding of how epithelial cells are regulated by differential interfacial tensions that are generated via localization and activation of cytoskeletal and ECM molecules, the role that dynamic cell motion plays in asymmetric cell shape changes has not been investigated. In the present work, using a self-propelled Voronoi model which unify tissue mechanics and cell motility, we explore the motion of the KV through the neighboring cells. Our model shows that in the presence of posteriorly-directed KV motion, with no other asymmetries, the KV becomes asymmetric along the AP axis with more ciliated cells packed into the anterior region. Our results suggest that these dynamical processes could help regulate KV cell shape changes and consequently play a role in establishing left-right asymmetries in the embryo.

P2630
Board Number: B785
Coordinated cell area fluctuations drive junction extension in epithelial cell quadruplets.
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Epithelial cell rearrangement underlies the complex choreography of large-scale morphogenetic tissue movements during development and regeneration. The minimal functional unit consists of a cell quadruplet with two old and two new neighbors. The stereotypic neighbor exchanges are driven by autonomous contractile activities in the old neighbors but presumably also depend on the surrounding cells. We analyzed the role of coordination between the four cells for neighbor exchange. We found that area fluctuations between old and new neighbors are antagonistically coupled especially during neighbor exchanges. xit and E-Cadherin RNAi mutant embryos have lost this coordination and are impaired in cell intercalation. Experimental interference to coordinated behaviour by optically induced cell contraction in new but not old neighbors prevented neighbor exchange. Our analysis reveals the essential function of coordinated cell behavior within the quadruplets and suggest a potential mechanotransduction mechanism by E-cadherin for concerted cell activities driving visible changes in morphology.
P2631
Board Number: B786
Ultra-fast contractions and emergent pattern dynamics: Primitive epithelium in Trichoplax adherence as a “living active solid”.
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Epithelium contractions are often associated with embryogenesis. Conventionally, these cellular contractions are slow and precisely controlled in space and time, patterning the shape and form of a developing embryo. In this work we report the discovery of ultra-fast epithelial contractions (50% cell area in 2 seconds, at least an order of magnitude faster) in a “simple” primitive marine invertebrate, Trichoplax adherence lacking neurons or muscles. Using a theoretical framework, we demonstrate that this speed can be explained by existing biophysical models of acto-myosin contractility, in a regime of low contractile load. We show that load reduction is possible due to the unique epithelial architecture of “T-shaped" cells and their dynamic stiffness variability. Furthermore, live in-toto imaging of the whole animal in vivo reveals emerging patterns including propagating radial and axial waves. Our work establishes a framework of studying primitive epithelium in early animals as “active solids”. We hypothesize that these contractile patterns are part of an ‘active cohesion’ mechanism, providing robustness. We further study the non-linear response of this ‘material’ to external stress using rheometry and simulation.

P2632
Board Number: B787
Identifying the molecular and mechanical requirements for coordinated tissue invagination.
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Epithelial folding mediated by apical constriction provides a fundamental mechanism that converts flat epithelial sheets into multilayered tissues. It remains elusive how forces generated near the apical surface mechanically drive tissue folding. We address this question by studying Drosophila ventral furrow (VF) formation, a well characterized model for epithelial folding. During VF formation, the prospective mesoderm cells constrict apically and subsequently invaginate into the interior of the embryo. Previous studies have demonstrated that apical constriction and invagination occur at temporally distinct phases, suggesting that apical constriction by itself cannot fully account for invagination. To elucidate the additional requirements for tissue invagination, we performed an RNAi-based candidate screen to identify genes that specifically regulate the invagination phase of VF formation. To measure the dynamics of tissue invagination, we developed a quantitative live-imaging approach to measure velocities of tissue movement on the surface of the embryo induced by apical constriction and invagination. Using this assay, we identified a unique class of mutants that exhibit relatively normal rate of apical constriction but greatly reduced rate of invagination. Interestingly, in the mutant embryos, the lateral, non-constricting cells flanking the constricting domain become hyper-stretched during apical constriction, as if they lose their rigidity and can no longer resist deformations imposed by the constricting cells. Consistent with this view, we found that the spatial distribution of cortical actin is misregulated in the mutant flanking cells. Further analysis has revealed an anti-correlation between the rate of invagination and the extent of stretching of the flanking cells. Together, our findings demonstrate that apical constriction alone is not sufficient to account for invagination;
instead, this process may depend on the mechanical integrity of the flanking, non-constricting cells. Further studies will be focused on understanding how cell rigidity is regulated and how such regulation may contribute to coordinated tissue invagination.

P2633
Board Number: B788
A collective solid-fluid transition in confluent 3D tissues.
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Understanding how the mechanical properties of tissues emerge from cellular behavior is vital to elucidate mechanisms that guide development, cancer growth, and wound healing. A very basic question is whether a tissue is fluid-like, where cells change neighbors and migrate, or solid-like, where neighbor relationships are maintained. For confluent monolayers, it has recently been shown that a simple model correctly predicts a fluid-solid transition governed by cell shape; though the mechanism for solidification has remained unclear. Also, we’d like to understand whether a similar transition is predicted in fully 3D tissues. Therefore, we develop and study a 3D self-propelled Voronoi (SPV) model that takes into account cell shape, cortical elasticity, cell volume constraints, and motile forces generated by individual cells. In the limit that motile forces are small, we find a fluid-solid transition that is controlled by a cellular parameter describing the preferred cell shape. In particular, we show that solidity can be predicted from a purely geometrical observable, which characterizes the actual cell shape, and can thus be extracted from imaging data. Moreover, we demonstrate that the onset of solidity in this model is a collective cellular effect, and that the mechanism creating solidity is fundamentally different from other disordered materials. When active motile forces are significant they help fluidize the tissue, although the observed cell shapes still robustly predict solidity. Thus, our model suggests that image data can be sufficient to quantify whether a 3D tissue is solid or fluid.

P2634
Board Number: B789
Epithelial cells spatiotemporally coordinate molecular activities and mechanical forces to drive radial intercalation during ductal elongation.
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During pubertal development, the mammary epithelium undergoes extensive elongation and bifurcation to form a ductal network. Elongation is led by a stratified, low-polarity structure known as the terminal end bud, which transitions to a polarized duct at the end of puberty. This process is regulated, in part, by receptor tyrosine kinases (RTKs). We sought to determine how cells coordinate the molecular activities in single cells with mechanical forces during elongation. Using organotypic culture and real-time confocal imaging, we first used fluorescent molecular biosensors to test whether the molecular logic of cell migration is conserved in cells within a tissue by visualizing the downstream signaling events of the RTK pathway. We observed individual cells asymmetrically enriching Ras activity, PIP3, and F-actin in protrusions, consistent with a migratory molecular polarity. In the protrusions, both Ras activity and PIP3 enriched concurrently at the site of F-
actin enrichment. Inhibiting actin dynamics prevented protrusion formation and morphogenesis, but did not prevent PIP3 enrichment.

For a duct to elongate, the basal surface must expand, which solely internal migration cannot accomplish. We therefore sought to examine the dynamics of migration as a cell approached the basal surface. We observed ductal elongation driven by radial intercalation in which migratory internal cells insert into the most superficial cell layer. We observed enrichments of Ras activity, PIP3, and F-actin in protrusions during intercalation. These dynamics are consistent with a transition from migratory front-rear polarity back to a stationary apico-basal polarity.

Next, we used the Cellular Force Inference Toolkit in 3D to analyze force balance equations at each cellular junction within an organoid. We found that migration through a tissue requires specific ratios of protrusion tension and posterior interfacial tension gradients. For intercalation, the duct requires high basal tension and a time-varying, increasing posterior interfacial tension gradient as protrusions are initiated. Finally, using finite element modeling, we generated in silico organoids with cells undergoing migration and radial intercalation to elongate a duct, solely based on mechanical alterations.

We conclude that ductal elongation results from an epithelial motility program that uses directed enrichments of Ras activity, PIP3, and F-actin in protrusions to drive migration and radial intercalation. These molecular dynamics create a spatial gradient of tensions across cells and time-dependent changes within the tissues that together accomplish morphogenesis. We now seek to understand how cancer cells re-access these developmental epithelial motility programs during metastasis.

P2635

Board Number: B790

Exploring the cell mechanics behind the T1 transition.

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Embryonic tissues elongate through cell-cell junction rearrangements within stereotypical T1 topological transitions. T1 transitions are characterized by dorsal-ventral junction contraction into a four-cell vertex, from which a new junction is synthesized in the anterior-posterior axis. Junction deformation in response to contractile forces is determined by junctions’ viscoelastic properties. And yet, a detailed understanding of how cells modulate such material properties during T1 transitions is lacking. We hypothesize that the molecular mechanisms mediating actin cytoskeletal reorganization differentially affect force transmission at cell-cell junctions, effectively modulating mechanical behaviors to drive vertex formation. To test this hypothesis, we use the TULIP optogenetic system to recruit RhoA-specific GEF Larg to cell-cell junctions within 2D confluent monolayers for local activation of actomyosin contractility. Live imaging shows rapid light-mediated recruitment of Larg followed by junction contraction. Surprisingly, constant junctional Rho activation was not sufficient for vertex formation. Rather, contraction stalled and reached a steady state length. Junction relaxation following activation was either elastic or contractile, while multiple activation periods showed only contractile behavior. The mechanical behaviors described here point to the regulation of actin dynamics and the coupling of cell-cell and cell-ECM interactions as an exciting mechanism for effective cell shape change. These data will directly test our current models of cell mechanics in a model tissue, allowing us to construct dynamic 3D models of multicellular force transmission that underlie T1 transitions.
P2636
Board Number: B791
A role for desmosomal cadherins in creating complex tissues.
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The evolution of multicellularity was facilitated by the emergence of cell-cell adhesion molecules in the cadherin family, which couple cell-cell adhesion sites to the actin cytoskeleton to regulate tissue integrity, polarity, and morphogenesis. As metazoans evolved, tissue morphologies with increasingly specialized functions appeared in concert with desmosomal (DSM) cadherins. One of the best examples is the epidermis, in which multiple DSM cadherins give rise to specific types of intercellular connections and cytoskeletal attachments throughout the cell layers. We hypothesized that the functional overlay of these patterned DSM cadherins onto classic cadherins facilitated new mechanisms to increase tissue structural and functional complexity during evolution.

We addressed this question for the DSM cadherin desmoglein 1 (Dsg1), which is first expressed as keratinocytes initiate a program of epidermal differentiation and exit the basal layer, and is later concentrated in the superficial layers. Using a genetically manipulable epidermal reconstitution model, we show that Dsg1 promotes delamination of basal cells, inducing stratification. We identified two Dsg1-associated modules required for Dsg1 function: a dynein motor complex and an Arp2/3/cortactin actin remodeling complex. Using genetic, pharmacologic, FRET, and laser ablation approaches, we show that dynein-dependent positioning of Dsg1/cortactin/Arp2/3 drives actin remodeling to reduce cortical tension and promote delamination of basal cells. Importantly, ectopic expression of Dsg1, normally absent in simple epithelia, is sufficient to induce stratification and formation of a second adherent cell layer in simple epithelial MDCK cells. Based on its role in regulating cortical tension during stratification, we tested whether Dsg1 coupling to the actomyosin system also contributes to suprabasal functions.

Molecular indicators of tension and microablation experiments revealed the existence of a mechanical gradient in stratified control epidermis, with highest tension in the superficial layers. Loss of Dsg1 shifts the mechanical gradient towards the basal layers of the epidermis. Dsg1 silencing altered indicators of the force-sensitive adherens junction component vinculin and tight junction (TJ) component ZO1, the latter which contributes to formation of the superficially restricted TJ barrier. Moreover, our data suggest that Dsg1 regulates TJs through the ErbB family member ErbB2, which in human skin is localized to cell-cell contacts in the TJ-forming layer. Together, our data suggest a model in which Dsg1 regulates the formation and function of the epidermis by controlling the distribution of mechanical inputs to drive morphogenesis and the development of the life-essential epidermal barrier.

P2637
Board Number: B792
Apical myosin activation induces Rab11 puncta accumulation near the apical cortex.
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During tissue morphogenesis, cell shape changes driven by mechanical forces usually involve changes in cell surface area, which often require active regulation of intracellular membrane traffic. It is not fully understood how membrane traffic is modulated by mechanical stimuli. To address this question, we investigated the behavior of Rabs, the master regulators of membrane traffic, during apical constriction-mediated Drosophila mesoderm invagination. We found that Rab11, previously known to regulate
exocytosis and membrane recycling, undergoes dynamic reorganization during this process. Prior to apical constriction, Rab11 is enriched at the perinuclear region. As the cells apically constrict and elongate apical-basally, Rab11 becomes enriched near the apical membrane as small, dynamic puncta. The puncta formation is not dependent on dynamin-mediated endocytosis, but requires intact microtubules. Genetic or pharmacological inhibition of myosin, the major force generating protein underlying apical constriction, abolishes puncta accumulation. Conversely, enhanced myosin activation results in more puncta. The accumulation of apical Rab11 puncta also occurs in the endodermal cells as they undergo apical constriction during posterior midgut invagination, suggesting that the process is not mesoderm-specific. In wild-type embryos, Rab11 puncta are broadly dispersed near the constricting apical surface, with a substantial fraction of them adjacent to the cell-cell boundaries. However, in mutant embryos where apical myosin is disconnected from the adherens junctions, Rab11 puncta become clustered at the center of the apical domain where myosin coalesces. In contrast to wild-type Rab11, constitutively active Rab11 locked in the GTP-bound form is enriched along the cell-cell boundaries, and this enrichment becomes more prominent as the cells undergo apical constriction. We speculate that wild-type Rab11 is also recruited to the cell-cell boundaries in its GTP-bound form, but only transiently. Indeed, we were able to detect Rab11 along the cell-cell boundaries in fixed embryos.

In summary, our work demonstrates that apical myosin activation triggers accumulation of Rab11 puncta at the vicinity where myosin is enriched. We hypothesize that the accumulation of Rab11 puncta facilitates vesicle delivery to the cell-cell boundaries to support cell membrane expansion, allowing the cells to elongate as they constrict apically.

P2638
Board Number: B793
Differential Regulation of Actin Dynamics during Collective Cell Migration.
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Collective cell migration is a specialized form of coordinated cellular migration, where cells maintain cell-cell contacts, group polarization and coordinated behavior. Collective cell migration is important in numerous processes during development, including neural tube closure, blood vessel branching and neural crest cell migration, as well as during adulthood in wound healing. As cells migrate, they must extend protrusions to interact with the extracellular environment, sense chemotactic cues, act as points of attachments, and signaling centers. The regulators of protrusive behavior have been widely studied in populations of cells that migrate individually; however, how protrusive behavior is coordinated throughout collectives is not well understood. Specifically, it has been difficult to define protrusive behavior of cells inside the collective, as cells are tightly packed due to cell-cell contacts. We are using advantages of the zebrafish model system, including amenity to live imaging and genetic approaches, to define protrusive behavior in collectively migrating cells. Towards this goal, we sparsely labeled filamentous actin in a small numbers of cells in the collective. Surprisingly, we discovered an abundance of brush-like, actin-based protrusions in multiple cells across the migrating cluster. Live imaging revealed that these structures were highly dynamic, oriented towards the direction of migration, and prevalent in the leading third of the collective. To identify factors that modulate this protrusive behavior, we have examined expression of genes that are regulated by the canonical Wnt signaling. We and others have previously showed that the canonical Wnt signaling is required for collective cell migration. We have found that an unusually high number of Wnt target genes regulates actin dynamics; these genes are expressed in distinct regions of the collective and their expression pattern is perturbed under Wnt-
deficient conditions. We are currently testing functional significance of these factors using CRISPR-Cas9 loss-of-function approach. We will present analysis of these mutant strains and how they regulate cellular movement and protrusive activity during collective cell migration.

P2639
Board Number: B794
A Rho GAP with a curved membrane-binding domain regulates morphogenesis via CDC-42.
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Rho GTPases are important in regulating cytoskeletal dynamics. Members of this protein family had been implicated in cell migration, polarization, nutrient intake, and epithelial morphogenesis. In C. elegans, loss of different Rho GTPases causes the embryo to die during different stages of development. While the processes regulated by Rho GTPases are subject to intense research, the regulators upstream of these Rho GTPases are still poorly characterized. For example, C. elegans has 23 Rho GAPs but the roles of most of these are still not understood. In this study, we found genetic evidence that the Rho GAP Y34B4A.8, which on its own has a mild embryonic morphogenesis phenotype, regulates Cdc-42 Rho GTPase, but not CED-10/Rac1. Besides the Rho GAP motif, Y34B4A.8 also has a BAR motif, which binds to curved membranes. A vertebrate homolog, SH3BP1, has been implicated in cell migration and adhesion. We used CRISPR to generate a full deletion of Y34B4A.8, and found a similar phenotype as depletion via RNAi. We also used CRISPR to tag Y34B4A.8 and found a complex localization pattern. While most cells of the embryo and adult express low levels of this GAP, it is strongly enriched in a few tissues that may give us a clue as to how it regulates development. For example, some of the strongest signal is at the apical pharynx and intestine, during the beginning stage of gut polarization. Y34B4A.8 appears to localize basal to ERM-1, but apical to DLG-1/AJM-1 complex (DAC) in the lumen, yet we have not detected an effect in apical lumen polarization. Y34B4A.8 has slight enrichment at the front of migrating epidermal cells, similar to CDC-42, ARP-2 and epidermal F-actin. This suggests Y34B4A.8 may regulate processes mediated by cytoskeletal dynamics. Further experiments are underway to identify the processes affected by CDC-42 in Y34B4A.8-dependent manner.

P2640
Board Number: B795
Dynamic imaging of actin cytoskeleton in cardiac progenitors reveal the importance of intercellular tension during organogenesis.
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The embryonic heart tube arises from the coherent migration and fusion of bilaterally symmetric populations of cells in the anterior lateral plate mesoderm (LPM). These bilateral progenitors undergo a myriad of structural alterations as they organize into the asymmetric linear heart tube. Traditionally, the specification and migration of cells and tissues during the formation of functional structures has been singularly considered from a biochemical signaling perspective. However, recent studies have established that mechanical forces play significant roles in the processes of tissue morphogenesis and organogenesis. Here, we explore the crucial contributions of biomechanical signals and intercellular forces to the morphogenesis of the LPM during embryonic heart tube formation in zebrafish. Using the
dynamics of the actin cytoskeleton as a readout of intercellular forces, we have acquired cellular resolution, three-dimensional, time-lapses of the actin fluorescent reporter, LifeAct-GFP in the developing LPM. Our 4-dimensional \((x,y,z, t)\) data has revealed a number of dynamic cellular interactions which is suggestive of differential force exertion from the cells as they undergo morphogenesis. These actin dynamics are asymmetrically distributed and are dependent on both biochemical signaling and biomechanical interactions. The asymmetries can be uncoupled genetically and are required for leftward placement of the heart tube. Taken together, these data suggest that multiple cellular traction forces provide key positional cues in the LPM as it undergoes migration and morphogenesis to form a leftward positioned linear heart tube.

P2641

**Board Number: B796**

**Tissue architecture of the *C. elegans* syncytial germline is maintained by actomyosin contractility of an extra-cellular inner tube.**

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Syncytial architecture is an evolutionary-conserved feature of the germline of many species and it plays a crucial role in their fertility. In *Caenorhabditis elegans*, multiple germ cell nuclei line the periphery of the gonad arm and remain connected to a common cytoplasm through stable openings (“bridges”) to a cell-free shaft called rachis. Actomyosin has been shown to be enriched at these bridges, and recent studies implicated actomyosin regulators in the stabilization of germ cell rachis bridges. However, the function of contractility in the maintenance of germline architecture remains poorly understood. Here, we combine genetic manipulations, laser ablations and 3D imaging to investigate the mechanobiology of the *C. elegans* syncytial germline. Importantly, 3D imaging revealed that the actomyosin network is not only enriched at bridges but is also present between bridges, outside the germ cells, forming an extra-cellular actomyosin-rich tube, akin to an inner corset. Laser ablation experiments demonstrated that the rachis tube has high contractility, which is dependent on myosin activity. We identified several key actomyosin regulators that control rachis tube contraction, and go on to show that there is a balance of mechanical forces between rachis tube contractility and germ cell membrane extension. Increasing contractility, by depleting the myosin phosphatase *mel-11*, leads to longer germ cell membranes and a reduced rachis core. Conversely, reducing contractility, by depleting the formin *cyk-1* or non-muscle myosin II (*nmy-2*), leads to shrinkage of germ cell membranes and a vastly increased rachis tube diameter. A mutant lacking the actin cross linker plastin (*pist-1*) displays non-homogenous distribution of myosin II along the rachis tube and clearly demonstrates the correlation between myosin levels and tube diameter. Taken together, our findings identify a novel multi-cellular actomyosin structure within the syncytial germline, map the balance of forces in the germline, and establish the critical role of rachis tube contractility in the preservation of a functional germline structure.
P2642
Board Number: B797
Cell-nonautonomously tunable actomyosin flows orient distinct cell division axes.
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Extrinsic controls of cell division orientation—whereby one cell instructs another to divide in a spatially organized manner—is a key mechanism for multicellular assembly. Indeed, cell divisions are oriented in diverse angles during stem cell division, organogenesis and embryogenesis, and anomalies in this process are associated with important human diseases including microcephaly, deafness, and cancers. Thus far, dynein-dependent pulling forces acting on astral microtubules are the only known extrinsically tunable force generation mechanism that drives oriented cell division. However, fruit flies or mice without centrosomes and astral microtubules still have relatively normal body plans; thus there might be unidentified additional force generation mechanisms underlying the division axis diversity observed in vivo. By focusing on oriented divisions that are independent of microtubule/dynein pathways, we have found that the non-muscle myosin II motor is an extrinsically tunable force generator that orients cell division axes through cortical actomyosin flows. Using a novel experimental approach to reconstitute multicellular environments with adhesive beads and isolated individual Caenorhabditis elegans cells, we identified three extracellular cues that generate different actomyosin flows. First, a single contact site locally inhibited myosin regulatory light chain (RLC) phosphorylation and myosin activity in a mechanosensitive manner, to generate asymmetric equatorial myosin flow specifically in the region proximal to the contact site. The actomyosin flow generates forces to trigger cell surface movements, thereby orienting AB cell division in parallel to the contact site at 2-cell stage. Second, size asymmetry of two contact sites polarized myosin activity and flow, thereby regulating left-right oriented ABA cell division at 6-cell stage. Third, an evolutionary conserved Wnt signaling also polarized myosin activity and flow, but in a manner overriding mechanosensitive effects, to specify the anterior-posterior oriented EMS cell division at the 7-cell stage. Notably, the contact-induced mechanosensitive myosin pathway also oriented cell division in mouse 2-cell embryos. To summarize, our results indicate that actomyosin flows constitute a new extrinsically tunable force generation mechanism that orients distinct cell division axes in response to different cues. Furthermore, we have documented an evolutionary conserved physical basis underlying the 4-cell stage architecture formation in C. elegans and mouse embryos. Tunable actomyosin flows acting in concert with microtubule/dynein pathways may specify diverse division axes during development to establish multicellular architectures. Preprint: Sugioka and Bowerman, bioRxiv 164186 doi: https://doi.org/1

Stem Cells and Pluripotency

P2643
Board Number: B798
Cell Type-Specific Response to Spindle Misorientation and Effects on Tissue Growth.
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Coordination of cell polarity and spindle orientation with cell growth and proliferation ensures mitotic fidelity and thus proper animal development. Mitotic errors have been associated with aberrant tissue growth in both epithelial cells and stem cells. Mutations in cell cycle-promoting genes in neural stem cells cause a mild increase in the Drosophila central nervous system (CNS). Conversely, identical
mutations in Drosophila imaginal wing discs (IWD), terminally differentiated cells, lead to massive tissue overgrowth. The mechanisms underlying the differential responses of these cells to errors in cell division, however, are unknown. Here we seek to build a stem cell model and a differentiated cell model to elucidate the varied tissue-specific responses. We found that mutated growth-promoting genes cause substantial overgrowth in epithelial cells, while no significant change in the CNS was observed when expressed in neural stem cells. Additionally, loss of Mud, a gene essential for proper mitotic spindle orientation, results in apoptosis-mediated inhibition of IWD growth in response to mutated growth-promoting genes. Our results further highlight the differential response of epithelial cells and stem cells to mutated growth-promoting genes. Taken together, these results point to an overgrowth-inhibitory mechanism in epithelial cells stemming from errors in spindle orientation caused by these mutations. Further analysis will provide a better understanding of the cell signaling pathways that govern tissue level responses to defective cell division, which will be important to improving our understanding of the underlying molecular bases for numerous human diseases.

P2644
Board Number: B799
Phosphatidylinositol transfer proteins control Apical Golgi Distribution, Polarity and division of Neural Stem Cells During Neurogenesis.
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Phosphatidylinositol (PtdIns) transfer proteins (PITPs) stimulate PtdIns-4-P synthesis and signaling in eukaryotic cells, but to what biological outcomes such signaling circuits are coupled remains unclear. Herein, we show that two highly related StART-like PITPs, PITPNA and PITPNB, act in a redundant fashion to support development of the embryonic mammalian neocortex. PITPNA/PITPNB cooperate in maintaining radial polarity of neural stem cells (NSCs) via a PtdIns-4-P and GOLPH3-dependent mechanism that promotes asymmetric distribution of the Golgi system into the apical processes of NSCs. We exploit an in utero electroporation approach to investigate the role of PITP-dependent inositol lipid signaling in the embryonic neural stem cell (NSC) pool. We found that the combination of a PITPNA null mouse line and PITPNB silencing evokes a dramatic depletion of NSC pools in embryonic brain. And, in an experiment using Emx1-Cre driver, which is forebrain specific driver, eviction of both PITPNA and PITPNB in forebrain leads to a mouse that is born but has an amazing microcephaly that is due to virtual loss of the forebrain. Neither PITPNA nor PITPNB eviction alone has any such effect. This experiment result is consistent with in utero electroporation data. Thus, we demonstrate that PITPNA and PITPNB redundantly control NSC homeostasis in mouse brain development. Moreover, while a plasmid which is isogenic to the wild-type PITPNA or PITPNB rescue eviction of both PITPs induced NSC depletion, PITPNA mutant clones which defeat phosphatidylinositol or phosphatidylcholine binding failed to rescue the NSC depletion in utero electroporation experiment. And saccharomyces cerevisiae SEC14 gene which stimulate PtdIns-4-P synthesis failed to rescue NSC depletion. We observed carefully daughter cell fate during NSC division and confirmed PITPs deficiency defeats NSC division program to accelerate differentiating cell divisions. We propose a mechanism where PITPNA/PITPNB drive PtdIns-4-P-dependent recruitment of GOLPH3 to Golgi membranes so as to promote asymmetric MYO18A- and F-actin directed loading of the Golgi network to apical processes of NSCs. And both PITPNA and PITPNB redundantly regulates daughter cell fate during NSC division.
P2645  
**Board Number: B800**  
Regulation of cell division by the intrinsic and extrinsic activities of small ovaries is required for germline development.

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A balance between stem cell self-renewal versus differentiation is required for tissue homeostasis, the disruption of which is associated with tumorigenic or degenerate phenotypes. Both cell autonomous and extrinsic signals cooperate to maintain stem cell homeostasis. The *Drosophila* ovary is a valuable model to study stem cell division and differentiation. The ovary comprises multiple ovarioles, assembly lines of progressively maturing egg chambers, with a germarium residing at the anterior of each ovariole. Within the germarium, somatically derived niche cells physically contact and support the 2-3 germline stem cells required for oogenesis. Using clonal knockdown approaches, we show small ovaries (sov), a predicted Zn-finger protein, is differentially required both within the soma and germline to permit germline development. Sov is an essential niche factor required to support germline differentiation. Loss of sov within the niche results in ovarian tumors, tissue degeneration, and failure to produce functional oocytes. In contrast, germline depletion of sov permits oogenesis, yet results in inviable embryos. Our data suggest ovarian tumorigenesis and embryonic lethality are products of centrosome dysfunction, as well as disrupted spindle orientation and morphology. Further, gene expression profiling shows Sov functions to repress mobile genetic elements, or transposons. Excessive transposable element activity disrupts germline genome stability resulting in cell death. Mutant and expression analyses are consistent with a role for Sov in the piRNA/PIWI small RNA defense pathway. Through examination of sov, we aim to clarify the longstanding relationship between the piRNA pathway, cell division control, and centrosomes to determine how small RNAs contribute to the regulation of stem cell proliferation and differentiation.

P2646  
**Board Number: B801**  
Activity of the Arp2/3 complex is necessary for embryonic stem cell differentiation.

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Despite abundant knowledge of the developmental cues and transcription factors controlling stem cell differentiation, how actin cytoskeleton dynamics drive the substantial morphological changes that occur with early differentiation remains poorly understood. New actin cytoskeleton filaments are predominantly generated by two distinct molecular machines, the Arp2/3 complex and formins, but neither has been reported as a regulator of stem cell differentiation. We found that substantial changes in actin filament architectures occur with differentiation of naïve mouse embryonic stem cells (mESCs) to primed epiblast cells (epiSCs), which require activity of the Arp2/3 complex but not of formins. Real-time imaging over 72 h of the differentiation process showed that naïve mESCs have a static circular colony morphology, large nuclear-to-cytoplasmic volume ratio, compact polygonal cell shape with a contiguous cortical ring of actin filaments and lack of plasma membrane protrusions. We found substantial changes in these properties with differentiation to epiSCs, which have a dynamic colony morphology, decreased nuclear-to-cytoplasmic volume ratio, elongated cell shapes and a dense ribbed

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actin filament network in abundant highly dynamic plasma membrane protrusions. All of these changes are blocked by the Arp2/3 complex inhibitor CK666 but not the formin inhibitor SMIFH2. Additionally, CK666 but not SMIFH2 attenuates the transition from naïve to primed epiSCs as indicated by decreased expression of epiSC microRNAs, differentiation markers Fgf5 and Brachyury, and nuclear translocation of MRTF. We used a dual reporter mESC cell line expressing developmentally regulated miRNA clusters tagged with distinct fluorophores for fluorescence-activated cell sorting of differentiation stages. Neither CK666 or SMIFH2 affects the percentage of self-renewing mESCs. However, after 72h of differentiation in the presence of CK666 but not with SMIFH2 there is a significantly higher percentage of naïve cells compared with controls. We used qRT-PCR to show that CK666 attenuates changes in the expression of differentiation markers after 72h in differentiation medium but not naïve pluripotency markers. To our knowledge, these data are the first to show that Arp2/3 complex activity is necessary for the differentiation of naïve stem cells. Our findings open new directions for determining how the Arp2/3 complex and actin filament remodeling contribute to stem cell differentiation, with insights relevant to embryonic development, induced pluripotency and regenerative medicine.

P2647
Board Number: B802
The Expression of Sarcomeric Proteins During Myogenesis in C2C12 Myogenic Stem Cells.
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Myogenesis is a tightly regulated process resulting in the sequential change in gene expression leading to the expression of muscle cell specific proteins. During the process of myogenesis the basic units of contraction, sarcomeres, are assembled into to the extremely precise structures that provides for the slide filament mechanism of muscle contraction. This study examined the timing of expression of a number of muscle specific proteins that are relevant to myogenesis/sarcomerogenesis over the course of myotube development, using C2C12 mouse myogenic stems cell in culture. Using quantitative PCR, titin expression and specifically expression of the cardiac specific isoform, N2-B, was studied. The expression of several myosin isoforms were also examined. In addition to these sarcomeric components we also looked at the expression of myogenic transcription factors and the cell cycle regulator of the G2/M phase transition (cdk1). At the onset of myogenesis (confluency and lowered growth factors) the expression of cdk1 as well as a transitory increase in the myogenic transcription factor MyoD. In this study we have taken two approach to assessing gene expression in myogenesis and sarcomere genesis, normalize to the expression of GAPDH (expression relative to the cell) or actin (expression relative to the sarcomere assembly). Gene expression after re-exposure of myotubes to high serum was examined.

P2648
Board Number: B803
Glutamine sustains Cripto expression and function, enabling MyosinII/RAB11A-dependent endosomal/exosomal trafficking in stem cells to promote tissue regeneration.
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Stem cells are necessary for proper development, tissue homeostasis and regeneration while dysregulation of their activity leads to diseases such as diabetes and cancer. We hypothesized that the
stem cell marker, Cripto (or TDGF1), is a regulator of tissue regeneration. Using the zebrafish model of caudal fin wound healing, we show that expression of the zebrafish Cripto homolog, one-eyed pinhead (or oep), is increased in blastemal stem cell tissue at 96 hours post amputation. We further demonstrate that Cripto is necessary and sufficient for stem cell-mediated regeneration in this in vivo model. We identified non-muscle Myosin IIIs (MYH9/10) as novel Cripto-binding proteins using proteomics and confirmed these interactions by co-IP/Western blot and immunofluorescence (IF) in mesenchymal stem cells. Notably, dual pharmacological inhibition of Cripto and Myosin IIIs did not reduce caudal fin regeneration beyond that of either inhibitor alone, suggesting that these proteins function in the same pathway during this process. Consistent with these findings, we further report that Myosin II inhibition reduces cell surface localization and production of soluble (GPI anchor-cleaved) Cripto. Our group and others have previously demonstrated that soluble Cripto promotes tissue-specific stem cell functions via cell-surface binding to GRP78 (glucose-regulated protein 78). Since GRP78 primarily functions as an ER chaperone that responds to cellular stresses, we tested whether nutrient deprivation affects Cripto expression, sub-cellular localization or binding to Myosin IIIs in mesenchymal stem cells. Interestingly, we found that glutamine is necessary, but not sufficient, to sustain normal Cripto expression and its Myosin II-associated functions. Using the BioGrid database, we discovered Rab11A as a co-binder of GRP78 and Myosin IIIs. Given the endosomal/exosomal trafficking functions of Rab11A, we hypothesized that Cripto may in turn help traffic GRP78 or other cell surface proteins in a Myosin II-dependent manner. Inhibition of Myosin IIIs in mesenchymal stem cells transfected with wild-type, constitutively active or dominant negative GFP-tagged Rab11A revealed that Myosin IIIs act upstream of and in concert with Rab11A to regulate Cripto localization to Myosin II-rich membrane protrusions. Based upon these data, we propose a new model of glutamine-dependent Cripto function whereby Myosin II/Rab11A activities promote membrane localization of Cripto to facilitate a unique endosomal/exosomal transport processes and autocrine/paracrine signaling during stem cell-mediated tissue regeneration.

P2649
Board Number: B804
Quantification of Nuclear Morphology Changes During Induced Pluripotent Stem Cell Differentiation.
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Understanding stem cell differentiation offers insight into organismal development with potential medical applications. Although cellular differentiation is known to involve a variety of activities, such as interactions with growth factors and the use of various transcription factors, questions remain about the underlying mechanisms. While nuclear size and shape have been noted to change during differentiation, it is unknown if and how these nuclear morphology changes contribute to the process. As a starting point, we used fluorescence microscopy and two- and three-dimensional quantification techniques to measure nuclear morphology and the nuclear-to-cytoplasmic ratio in human iPSC cells and stem cells differentiated into the three germ layers. Using these methods, we find that iPSC cells and the cells of these germ layers show statistically significant differences in their nuclear morphology in terms of overall area, shape factor, perimeter, and length to breadth ratio. More specifically, we observed that, compared to iPSC cells, ectoderm nuclei tend to be larger as evidenced by a 17.9% increased nuclear area and a 9.5% increase in nuclear perimeter whereas mesoderm nuclei tend to be smaller and more irregularly shaped as is shown by their 13.0% smaller nuclear area and 15.8% decrease in shape factor. We also observed that endoderm cells exhibit a longer, thinner morphology compared to iPSC cells, having a 22.5% larger nuclear length to breadth ratio and 12.3% smaller shape factor.

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Having carefully quantified changes in nuclear size and shape during cellular differentiation, our future work will focus on understanding what controls these nuclear morphology changes and whether these changes contribute to differentiation. Toward the latter goal, we will use previously identified nuclear scaling factors to determine if manipulating nuclear size affects the ability of iPS cells to differentiate into cells of the three germ layers. Factors of particular interest are the nuclear lamins that are subject to a variety of post-translational modifications. Our group previously demonstrated that PKC-mediated phosphorylation of human lamin A at S268 influences nuclear size. We are currently using genome editing in human iPS cells to EGFP tag lamin A and introduce phosphorylation site mutations at S268. With these tools, we will quantify nuclear size and lamina dynamics, assess how PKC activity impacts these parameters, and investigate the differentiation potential of mutant cells with altered nuclear size.

P2650

Board Number: B805

Nuclear transport protein, Transportin Serine-Arginine rich, is required for germline stem cell proliferation and self-renewal in Drosophila.

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Steroid hormones are critical for stem cell self-renewal and progenitor cell proliferation in diverse tissue systems. The mechanism by which they regulate tissue-specific stem cells, however, remains unclear. The Drosophila ovary harbors a population of tissue-specific stem cells known as germline stem cells (GSCs), which are a well-studied model for tissue-resident stem cell function. GSCs self-renew to maintain an undifferentiated cell population and give rise to differentiated germ cells. These actions are, in part, regulated by the steroid hormone ecdysone. Ecdysone binds to its receptor, ecdysone receptor (EcR), which transcriptionally regulates expression of many target genes. EcR is necessary for GSC self-renewal and proliferation, but the exact mechanism by which it regulates these processes is largely unknown. Multiple studies have identified a nuclear transport protein encoded by Transportin Serine-Arginine Rich (Trn-SR), as responsive to ecdysone signaling. Trn-SR is a highly conserved transporter that has been implicated in the targeting of proteins containing nuclear localization signals for transport into the nucleus. In vitro studies have identified some Trn-SR interacting proteins, but its cell biological role is not well understood. In this study, we show that like EcR, Trn-SR is necessary for GSC self-renewal. Trn-SR mutant GSCs also exhibit decreased proliferation rates and increased cell size as compared to wildtype GSCs. Further, Trn-SR mutant GSCs phenocopy loss of CycE and CDK2, which are also necessary for GSC self-renewal and proliferation. Indeed preliminary data indicate a strong genetic interaction between Trn-SR and CycE, suggesting a model wherein Trn-SR modulates key cell cycle controls to promote GSC self-renewal. Future studies will also address whether Trn-SR’s function is restricted to stem cells, or if its biological functions are imperative for differentiated cell populations as well. Together, these data will provide insight into the intrinsic cues that regulate cell function, as well as the actions remote cues take to control stem cell self-renewal and proliferation.
P2651

Board Number: B806

Placenta derived mesenchymal stem cells increases invasion ability of trophoblast via alteration of mitochondrial function.
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Trophoblast plays an important role in early embryo fertilization and placental development. Especially, trophoblast functions such as invasion, proliferation and differentiation was important factor to successful implantation in the early development. Mitochondria are highly dynamic organelles which continually fuse and divide in response to diverse stimuli. Alterative mitochondrial function has been reported to be a key for the multi-functions of trophoblast. We previously reported that placenta derived mesenchymal stem cells (PD-MSCs) have several potentials for differentiation, immunomodulation, and affects the invasion ability of trophoblast. However, invasion mechanisms of trophoblast via mitochondrial functions are still unclear. Therefore, the objectives of this study are to analyze the functions of trophoblast with co-cultured PD-MSCs on proliferation, death and invasion ability, mitochondrial mechanism and compare their correlation between trophoblast functions and mitochondrial changes according to PD-MSCs co-cultivation. Finally, we investigated their ability of trophoblast by mitochondrial dynamics. Several markers related to proliferation, death and mitochondrial function include ATP production, ROS levels, membrane potential, mass and metabolism in static condition of trophoblast with PD-MSCs co-cultivation were analyzed by quantitative RT-PCR (qRT-PCR), western blot, immunofluorescence and Elisa. Mitochondrial DNA (mt DNA) was analyzed by qRT-PCR. In addition, invasion ability of trophoblast with PD-MSCs co-cultivation was analyzed by invasion assay. PTEN-induced putative kinase 1 (PINK1) and PARKIN, which are markers for mitochondrial autophagy (mitophagy), were analyzed in trophoblast by qRT-PCR and IF. Although, there are no difference for the survival and death of trophoblast, but PD-MSCs induce to change static condition of trophoblast. The mt DNA copy number was significantly increased in trophoblast with PD-MSCs co-cultivation (p<0.05). Also, PD-MSCs co-cultivation was significantly increased the invasion ability of trophoblast by increasing MMP-2/-9 activities and upregulated Rho family gene expression (p<0.05). Interestingly, mitophagy of trophoblast was increased in invaded trophoblast with PD-MSCs co-cultivation. Taken together, PD-MSCs can regulate trophoblast invasion by dynamic mitochondrial functions via autophagic mechanism. Theses results support the fundamental mechanisms of mitochondria function contribute to trophoblast invasion and suggest new therapeutic strategy in infertility.

P2652

Board Number: B807

BCP1 is a positive regulator of Notch signaling pathway in the regulation of mammalian neural stem cells.
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In the developing central nervous system, it has been widely known that Notch signaling preserves progenitor pools, inhibits neurogenesis, and drives astrocyte differentiation. BCP1 is known as an ion transporter protein containing membrane spanning regions. In this study, we assessed the role of BCP1 in the mammalian brain development. We first found that Notch intracellular domain increased
transcription of BCP1. Interestingly, BCP1 also synergistically increased transactivational ability of Notch. We used a retroviral vector system to investigate the effects of BCP1 expression on the properties of neural stem cells in vivo as well as in vitro. BCP1 induced efficient neurosphere formation, a high level of neural stem cell proliferation, and localization of neural progenitor population in the VZ and SVZ of developing mouse brain. Taken together, our data indicate that BCP1 is a novel Notch target gene and also a regulator in the canonical Notch signaling pathway.

P2653

Board Number: B808

A Hoxc dependent skin zip code controls regional adult stem cell regeneration.

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Stem cell regeneration ability is different in distinct body regions. Here, using an unbiased multi-step screening approach, we identified a single gene cluster, Hoxc, with expression associated with the regional activation of hair follicle stem cells (HFSC). Hoxc genes are only highly expressed in regions where HFSCs exhibit long-term regenerative ability. Lentivirus mediated in vivo over-expression in epithelial cells excludes the intrinsic ability of Hoxc gene in promoting HFSC activation. A chromosome 15 inversion results in ectopic expression of multiple Hoxc genes in dermis and results in regional activation of otherwise dormant HFSCs. Using CRISPER/Cas9 mediated gene knockouts and lentivirus mediated gene over expression in functional studies, we conclude niche expressed Hoxc genes function redundantly to determine the regenerative capacity of HFSCs. The region-specific expression pattern of Hoxc genes is controlled by antagonistic switch between PcG-dependent repression and enhancer activation epigenetic modifications. Circularized Chromosome Conformation Capture (4C) results reveal the chromosome 15 inversion leads to ectopic interaction of Hoxc cluster with an active regulatory chromatin domain and increased Hoxc genes expression. Dermis expressed Hoxc genes switch on the epithelial HFSCs regeneration through activating Wnt signaling. Together, these data delineate an altered epigenetic landscape in dermis from different skin regions that controls the differential expression pattern of Hoxc genes, which encode the positional identity of adult HFSCs and regulate their regenerative ability.

P2654

Board Number: B809

Tousled-like Kinase 1-Regulated Differentiation in Murine Embryonic Stem Cells.

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Embryonic stem cells (ESCs), derived from inner cell mass of blastocyst, are defined by their abilities of pluripotency and self-renewal. Core transcription factors, Oct4, Nanog and Sox2, play a crucial role in maintaining stemness in ESCs and these factors should be properly inhibited as cells differentiate. A serine/threonine kinase Tlk1 is evolutionarily conserved and plays an essential role in cell cycle progression, DNA damage response and chromatin assembly. In C.elegans and Drosophila, TLK is required for development but the role of Tlk1 during early lineage commitment in murine ESCs (mESCs) remains undefined. In this study, we knocked down Tlk1 using a specific short hairpin RNA and overexpressed Tlk1 using Tet-On inducible expression system in E14 mESCs to determine its function.
Tk1 downregulation resulted in delayed repression of core pluripotency factors and formed irregular shape of embryoid body during differentiation. Conversely, elevated expression of Tk1 causes downregulation of core pluripotency factors and correlated with decreased self-renewal activity with low AP activity and flattened morphology even in the presence of LIF. Moreover, Tk1 overexpression impairs cell growth with accumulation of G2/M phase population. Thus, our gain and loss-of-function studies in E14 mESCs revealed a novel function of Tk1 in modulating ESC stemness.

P2655
Board Number: B810
High Throughput Automated Patch Clamping and Cytotoxicity Investigations for Cardiac Safety: CiPA Compound Effects in Stem Cell-Derived Cardiomyocytes.
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Drug induced arrhythmia is one of the most common causes of drug development failure. Human induced pluripotent stem cell-derived cardiomyocytes (iPSCMs) show great promise for cardiovascular research and predictive in-vitro cardiac safety screening. In the light of the new Comprehensive in Vitro Proarrhythmia Assay (CiPA), a FDA directed initiative to improve guidelines and standardize assays and protocols, the use of hiPSC-CMs may become critical in determining the proarrhythmic risk of potential drug candidates. Human stem cell-derived cardiomyocytes (hiPSC-CMs) have recently proven to recapitulate key features of human cardiac electrophysiology in vitro. Patch clamp assays, the gold standard of ion channel research, are distinguished by high complexity. Conventional patch clamp is technically demanding and is unsuitable for high-throughput screening (HTS) experiments. Chip-based approaches allow parallel patch clamp recordings without compromising data quality or technical sophistication. We present high-throughput voltage and current clamp recordings of CiPA reference compounds. Since drug efficacies may vary with temperature, we present recordings at room and at physiological temperatures. In addition to patch-clamping experiments, we present hybrid impedance (cell contractility) with MEA-like extracellular field potential (EFP) recordings. Pharmacological effects of a number of CiPA reference compounds including those designated high risk (e.g. Dofetilide), intermediate risk (e.g. Cisapride) or low risk (e.g. Verapamil) were evaluated and will be presented. Experiments were complemented with optical stimulation of monolayers of hiPSC-CMs expressing the light-gated cation channel Channelrhodopsin2. This approach allows frequency-dependent drug screening and detection of potential side effects on Na+, Ca2+ and repolarizing K+ channels. Furthermore, investigations of potential breaks in excitation-contraction coupling can accompany the ion channel screening with the final aim to enable a reliable automatized cardiac toxicity screening platform.
P2656
Board Number: B811
Toxic effect of titanium dioxide nanoparticles (TiO2) on Wharton Jelly mesenchymal stem cells.
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Titanium dioxide is one of the most used mineral pigments in the world in the last decades, its applications stand out in the field of paints, cosmetics, sun blockers, plastics, paper, food, etc. It has been reported that TiO2 at different nanometric scales is considered toxic, depending on the concentration and type of cell being exposed. TiO2 nanoparticles (TiO2-NPs) have the ability to cross biological barriers. In vivo models, it cross the blood-brain barrier and also it could to cross the fetoplacental barrier. On the other hand, human mesenchymal stem cells (hMSCs) are referred to as highly relevant cells because they have an immunomodulatory profile, preventing their rejection as well as the ability to regenerate various tissues. In most organs and tissues, hMSCs are forming niches where they can remain quiescent, self-renewing, or differentiated to the needs of the tissue that houses them. The effect of TiO2 in the capability for differentiation of hMSC remain unknown. In this work it was investigated if the TiO2-NPs exert toxic effects on Wharton’s jelly hMSC. We evaluated the morphology by cell staining and electronic microscopy, cell viability was measure by alamar blue and possible changes in cell differentiation was evaluated by specific staining oil red or alizarine-S red (adipogenic or osteogenic differentiation respectively). The results showed that cell viability of hMSC significantly decreased with the TiO2 treatment in a concentration and time dependent way. When treated cells were observed by microscopy, it was observed that hMSC had morphological changes, and more specific, that distribution of the TiO2-NPs inside the cell had a characteristic pattern of perinuclear TiO2-NPs accumulated, forming a characteristic halo. Finally, treatment of hMSC with TiO2-NPs interfered the osteogenic and adipogenic differentiation when hMSC were induced with the specific culture media. This results showed that TiO2-NPs has a significant impact on the viability and capability in cell differentiation of the hMSC. The use of nanotechnology in different products that contains TiO2 have to be regulated so that is does not affect the public health.

P2657
Board Number: B812
Is a microtubule-dependent mechano-transduction involved in early human Hematopoietic Stem Cells differentiation?
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Hematopoietic Stem Cells (HSCs), through their ability to differentiate into hematopoietic lineage cells and self-renew, are essential to maintain hematopoiesis throughout life and have major potentials for treatment of haematological diseases. Yet, how HSCs integrate cues from their environment to control self-renewal and differentiation is still poorly understood. It has been recently shown that actin cytoskeleton of adherent stem cells is central to this process. Actin can transmit external forces and modulate nucleus architecture, chromatin organization and gene expression controlling cell fate. HSCs are poorly adherent cells and it is not known whether similar or alternative mechano-transduction
mechanisms play a role in their differentiation.
To investigate this question, we have quantitatively analysed cell architecture during the early
differentiation of human HSCs into committed progenitors. We show that HSCs differentiation is marked
by nucleus reshaping. Initially ovoid, the nucleus exhibits large invaginations in progenitors. This
reshaping associates with chromatin reorganization, as shown by the spatial redistribution of
heterochromatin marker H3K9mE3. It also correlates with changes in gene expression program. In
parallel, HSC differentiation associates with a dramatic microtubule network reorganization, while actin
cytoskeleton remains unchanged. Microtubules, initially radiating in the cytoplasm, form bundles that
tightly clamp and deform the nucleus of committed progenitors. Using living imaging approaches, we
could show that the shape of HSC nucleus is fluctuating and that these fluctuations are microtubule-
dependent. Finally, pharmacologically perturbing microtubule dynamics during long tem in vitro HSC
differentiation significantly reduced nucleus deformations that normally take place during this process.
Taken together, our observations indicate that microtubules exert mechanical constraints on the
nucleus of HSCs and can deform it, and suggest that HSC early differentiation involves an original
microtubule-dependent mechano-transduction mechanism.

P2658
Board Number: B813
Molecular and cellular phenotyping of mouse embryonic stem cells from diverse inbred
laboratory mouse strains reveals strain-specific differences in cellular traits.
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The naturally occurring genetic variation that distinguishes the genomes of inbred laboratory mouse
strains provides a powerful platform to elucidate the role of genetic variation in mammalian
development and disease. To determine if systems genetics approaches can be applied to cellular traits
that can be assayed in vitro, we created embryonic stem cell (mESCs) lines from a diverse panel of
inbred laboratory mouse strains, including the founders of the Collaborative Cross and Diversity Outbred
mapping populations. These include a strain background in which the naïve, ground pluripotent mESC
state is known to be unstable (NOD/ShiLtJ). Here we report our initial efforts to characterize the
phenome and transcriptome of these cell lines under pluripotent culture conditions (LIF, 2i) and for a
subset of lines, after removal of LIF and 2i. We found strain-specific differences in basic cellular and
molecular traits including gene expression, morphology, growth rate, self-renewal capacity, homology
directed repair of CRISPR-cas9 induced double strand breaks, and potentially early differentiation. These
data demonstrate that strain-specific molecular and cellular phenotypes are distinguishable in mESCs
from different inbred mouse strains and provide an initial set of resources for exploring in vitro systems
genetics approaches to cell biology, pluripotency and early lineage commitment. These data also
provide technical insight into the optimization of genome engineering across a variety of inbred strain
backgrounds.
P2659
Board Number: B814
Silencing Spermine Synthase in Mesenchymal Stem Cells mimics Snyder-Robinson Syndrome by showing impaired osteogenesis.
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Snyder-Robinson Syndrome (SRS) is a rare disease where patients harbor mutations in the spermine synthase (SMS) gene, resulting in multiple defects, including inadequate bone development. This defect has been suggested to be due to impaired osteogenic differentiation of bone marrow-derived mesenchymal stem cells (MSCs). In order to elucidate how defective SMS leads to impaired osteogenesis, we generated a lentivirus to deliver shRNA to silence SMS expression. Effective silencing of SMS expression was confirmed by RT-PCR. Interestingly, we found that silencing SMS strongly inhibits cell proliferation. To determine how silencing SMS affects osteogenesis, MSCs were cultured in osteogenic media and assessed for commitment, maturation and mineralization. Commitment is marked by an increase of Runx2 at day 1 of differentiation. However, silencing SMS had no significant effect on Runx2 expression. In contrast, while studying maturation at day 14, we observed a significant inhibition of bone sialoprotein (BSP) expression in MSCs transduced with the shRNA against SMS, as compared to controls. BSP is a major component of the bone extracellular matrix. Consequently, mineralization in MSCs with shRNA targeting SMS, showed lower calcium precipitation, as assessed at day 28 using Alazarin Red S staining. To elucidate the underlying mechanisms, mass spectrometry of primary metabolites was performed in MSC transduced with either control or SMS shRNA. RNAseq studies to determine altered gene expression are currently in progress. In anticipation to improve bone formation in SRS patients, our study aims to more clearly understand the role of SMS, especially in the context of inefficient osteogenic differentiation of MSCs.

P2660
Board Number: B815
Isolation and Expansion of Human Skeletal Stem Cells.
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Bone remodeling and repair requires the activity of osteoblasts, which orchestrate bone formation. During remodeling or after fracture, osteoblasts need to be replenished, because osteoblasts mature into post-mitotic osteocytes, which are embedded in bone salts. The existence of a resident stem cell in the bone marrow responsible for bone homeostasis and repair has been largely acknowledged, but to date these cells have not been isolated from human bone marrow, due to the lack of surface markers to isolate them. Based on recent work in mice, we have now found that the combination of markers CD45-CD51+ CD146+ CD200+ strongly enriches for cells capable of initiating colony forming units-fibroblast (CFU-F), a hallmark property of SSC. Here we show the results from isolating SSC from 7 different donors (varying from 24 to 52 years old). We determined the frequency of SSC, their ability to form CFU-F, and compared them to the frequency of total CFU-F, obtained by plating unsorted bone marrow mononuclear cells. Since SSC are present in very limited numbers in bone marrow, we also plated SSC and expanded them as CFU-F. In culture, SSC acquire all properties of regular Mesenchymal Stem Cells (MSC), including morphology, immune phenotype and differentiation potential into osteoblasts and adipocytes. However, we also found that sorted SSC after 14 days in culture expressed consistently lower levels of Gremlin1 and Mx1 as compared to the unsorted cells. These results suggest that even
after cell expansion, SSC retain differences from MSC. Human SSC are likely to lead to novel diagnostic and therapeutic tools in orthopedic medicine.

P2661
Board Number: B816
Successful enrichment of cardiac progenitors following differentiation of EGFP-expressing transgenic mouse ES cells.
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Cardiovascular diseases are among the leading causes of deaths worldwide. In recent years, stem cell-based approaches to treat, for example, ischemic heart disease are being explored. Among the various cell types studied, cardiac progenitors (CPs) are the most promising ones. This is because CPs are multipotent, and they are capable of differentiating into cardiac cell types such as cardiomyocytes, endothelial cells and vascular smooth muscle cells. However, the major limitation has been the lack of optimized experimental system to enrich and expand CPs that are compatible for cell transplantation. In this study, we adopted a two-pronged approach to enrich and expand CPs, differentiated from our in-house-derived and well-established transgenic mouse embryonic stem cell line i.e., GS-2 ESCs (Singh et al, A. J. S. C.s, Vol 1(2) : pp 163-73 ; 2012). We developed experimental systems for enrichment of ESC-derived CPs by exploiting Sca-1 cell surface marker. We exploited this strategy because Sca-1 is identified as one of the key molecular markers associated with CPs’ plasma membrane. First, we assessed the use of extended cultures of ESC-derived embryoid bodies (EBs) in order to achieve better differentiation of GS-2 ESCs to CPs. We performed suspension cultures of EBs from the standard day 3 (D3) to D5 or D7 prior to plating EBs onto 0.1% gelatin coated-dishes. By flow-cytometry, we assessed the percentage of Sca-1⁺ cells obtained from EB outgrowths. Second, we enriched the CPs, derived from EB outgrowths (D7) by adopting the MACS approach exploiting Sca-1⁺ cell selection, following the depletion of CD31⁺ cells from the total cultured cells. The enriched Sca-1⁻/CD31⁻ cells were then assessed for their expandability and differentiation potential. Molecular phenotyping was performed for the differentiation associated markers by RT-PCR, immunofluorescence and FACS analyses. Our results show that the extended suspension cultures of EBs (D7 & D9) produced about a 2-fold increase in the percentage of Sca-1⁺ cells vis-à-vis standard attachment cultures. Moreover, MACS-based enrichment strategy showed a 2-4 fold enrichment of CPs (81.5 % ± 3.5; n = 4). Interestingly, the enriched CPs exhibited spontaneous differentiation to functional cardiomyocytes, evidenced by their rhythmic beating behaviour and expression of cardiac contractile protein markers such as αMHC and MLC-2v. Overall, we successfully developed an experimental strategy for efficient enrichment and expansion of CPs which could be exploited for engraftment studies in ischemic heart diseases and they have immense clinical significance in the context of regenerative medicine (Support: Dept. of Biotechnology, GoI, New Delhi).
P2662
Board Number: B817
Genome-wide CRISPR-Cas9 Screens Reveal Genes Promoting Entry into a G0-like State in Human Neural Progenitors.
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Mammalian tissue homeostasis is maintained by balancing cell division with cell quiescence during stem and progenitor cell lineage commitment. Recent data further suggests that even continuously dividing cells enter a transient G0-like state each cell cycle and that the duration of G0 determines the proliferation rate for any given cell type. Here, we performed genome-wide CRISPR-Cas9 screens in human neural progenitors to identify genes rate-limiting for their in vitro expansion. The screen revealed ~90 candidate growth-limiting genes, which were highly enriched in single nucleotide variant analysis across 7330 cancers, representing 29 distinct cancer types. Characterization of top scoring overlapping screen hits, including, for example, CREBBP, NF2, PTPN14, TAOK1, and TP53, revealed that knock out (KO) of the target genes invariably caused neural progenitors to grow faster by blocking entry into a G0-like state, regardless of cell density. Hallmarks of G0, including variable length G0/G1 transit times, low CDK2 activity, and high p27 levels were lost in KO cells, causing dramatically shortened G1-phases and cell cycles. Single-cell- and population-based gene expression profiling of KO cells revealed the impact of these genes on G1 subpopulations and also on development potential of the cells in differentiation assays. Taken together, our results demonstrate that a portion of the top pan-cancer mutated genes may act to limit cell growth by allowing entry into a G0-like state, possibly by permitting overall CDK2 activity to drop as cells exit mitosis.

P2663
Board Number: B818
Analysis of marker gene transcripts and 35 cytokines in adipose tissue mesenchymal stem cells spanning 15 consecutive passages.
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Mesenchymal stem cells (MSC) are multipotent stem cells, typically isolated from bone marrow, adipose tissue, and umbilical cord. But they have also been isolated from other fetal and adult tissues. MSC can differentiate into many different cell types, among them adipocytes, chondrocytes, and osteoblasts. The therapeutic potential of MSC has been assessed for a number of conditions, including conditions characterized by inflammation. MSC have been found to promote tissue regeneration and modulate immune responses involving inflammation. Thus, the cytokine secretion profile of MSC is important in these cells’ ability to modulate inflammation. However, with increasing in vitro passages, MSC progressively lose their potency. To ascertain the changes in their cytokine secretion profile during in vitro propagation, we grew adipose tissue MSC for 17 successive passages, and for each passage quantified 35 secreted cytokines by the Luminex microbeads multiplex assays. The results show that the cytokine concentrations in the culture media increased, with various cytokines peaking around passages 8-11. Then the levels decreased, in some cases precipitously. The variations in cytokine levels from passage to passage ranged from relatively small to dramatic. For example, eotaxin concentration at passage 10 was nearly 30-fold greater than that at passage 6, and it was below detection limit at
passage 17. The hepatocyte growth factor (HGF) and interleukin 1 receptor antagonist (IL-1RA) exhibited a different profile; both had early passage peak levels and then gradual drop. EGF, MIG, IL-3, and IL-15 could not be detected at any passage.

We also assessed the expression profile of the main MSC marker genes and several negative marker genes. The expression of the positive markers CD29, CD44, and CD73 expressions did not have appreciable changes across the span of passages, CD 90 expression generally increased with passage number, and CD166 had significantly higher levels at passages 4 and 5, but much lower at other passages. The most remarkable of the negative markers was CD34; its level at passage 15 was nearly 100-fold greater than its lowest levels at some earlier passages.

### Host-Pathogen/Host-Commensal Interactions 1

**P2664**

**Board Number: B820**

**Cell surface vimentin is involved in matrix stiffness dependent infection of endothelial cells by Listeria monocytogenes.**

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Vascular endothelial cells line the inner lumen of vessels forming a protective barrier against spread of bacterial infection. Endothelial cells display remarkable phenotypic heterogeneity in part due to the different mechanical stimuli transmitted from their extracellular environment. Although endothelial cell response to mechanical signals has been studied extensively, how bacterial uptake occurs in endothelial cells residing on varying stiffness environments was hitherto largely unknown. We exploit Listeria monocytogenes (Lm), a facultative intracellular bacterial pathogen, to determine the biophysical relationships between endothelial cells, the stiffness of their matrix and bacterial infection. We find that adhesion of Lm on human microvascular endothelial cells (HMEC-1) increases with increasing matrix stiffness in a focal adhesion kinase (FAK) dependent manner. We find that HMEC-1 residing on stiffer as compared to softer environments show higher FAK activity and that FAK activity is critical for uptake of Lm by HMEC-1. Through isolation of the surface receptors of HMEC-1 treated with a FAK inhibitor and mass spectrometry analysis, we find that the amount of cell surface vimentin is reduced when FAK activity is inhibited, which leads to reduced Lm uptake by HMEC-1. Our results identify a novel pathway wherein increased extracellular matrix stiffness sensed by endothelial cells leads to enhanced FAK activity that augments the amount of vimentin exposed at the surface of endothelial cells that in turn increases uptake of Lm.
Identification of polyubiquitinated species at sites of Listeria monocytogenes cell-to-cell spreading.
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The cell-to-cell dissemination of the pathogenic bacteria Listeria monocytogenes (L. monocytogenes) relies on their ability to hijack the host cell actin polymerization machinery. The spreading process of the bacteria begins inside the host cell where they simultaneously hijack and activate, to their surface, the Arp2/3 complex. Consequently, the bacteria cloak themselves in a branched filamentous actin (F-actin) cloud. Asymmetrical polymerization of this actin cloud causes the formation of long and slender actin-rich structures called comet tails that propel the bacteria throughout the host cell. Propulsion of these bacteria towards the host cell plasma membrane ultimately causes them to protrude out from it into neighboring cells via filopodia-like structures called listeriopods. Importantly, the molecular mechanisms governing listeriopod engulfment by neighboring cells remains largely unexplored. Since bacterial pathogens often exploit post-translational modification of proteins during their disease process, we investigated whether L. monocytogenes would also co-opt these modifications during their cell-to-cell spread. To test this, we infected human cervical epithelial (HeLa) cells with wild type L. monocytogenes and then stained fixed cells with an antibody which recognizes solely polyubiquitinated proteins. Interestingly, we saw no evidence of an enrichment of polyubiquitinated species at actin clouds surrounding stationary bacteria or at comet tails of motile bacteria. We also saw similar results when observing fully extended listeriopods that had yet to reach any nearby cells. Most importantly, when we examined listeriopods that had clearly invaginated into neighboring cells, we saw that the membrane region surrounding the protrusions was highly enriched with polyubiquitinated proteins. In summary, we provide evidence for a potential role of cellular post-translational modifications, specifically the polyubiquitination of proteins, during the dissemination process of the pathogenic bacteria L. monocytogenes.

Spatial organization of the human tongue dorsum microbiome at the micron scale.
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Humans have co-evolved with the microbes comprising our microbiome but the significance of this symbiotic relationship for human health is only beginning to be appreciated. The individual microbiomes of the body may be considered the physiological equivalents of multi-cellular organs. As with other body organs, an understanding of their anatomy is essential for understanding their physiology. In previous work using multiplexed, spectral imaging and fluorescence in situ hybridization, we discovered a distinctive, multi-genus consortium in human supragingival plaque consisting of a radially arranged nine-taxon structure (Mark Welch et al 2016. PNAS 113 E791-E800). The distinctive structure permitted us to generate specific hypotheses for how the consortium develops, functions and is maintained. We now apply the multiplexed, spectral imaging approach to the microbiome of the human tongue. We designed oligonucleotide probes for taxa both abundant and prevalent on the human tongue dorsum as determined by oligotyping re-analysis of data from the Human Microbiome Project. Multiplexed imaging
revealed a highly structured spatial organization ranging in linear dimension from fifty to hundreds of microns. However, the organization differed from supragingival plaque in both structural detail and taxonomic composition. Featured on the tongue were consortia whose members were primarily cocci and bacilli drawn from the genera Streptococcus, Rothia, Neisseria, Actinomyces and Veillonella. Fifteen additional genera were detected at lower abundance and prevalence. The consortia radiated outward from a core of epithelial cells, with individual taxa clustering in domains suggestive of clonal expansion. The range expansion/contraction of these domains permits inferences regarding the selective advantage of the taxa during consortium development. Variations in taxon abundance from sample to sample and person to person were used to calculate correlations in relation to cell-cell and cell-host interactions. Finally, species-level analysis revealed distinctive tropisms in the oral microbiome. For example, the Rothia genus member on the tongue was determined to be almost exclusively R. mucilaginosa. Species level identification in combination with genomic information revealed that several of the tongue taxa possess nitrate reductase, a metabolic capacity not encoded by the human genome. This suggests that the tongue microbiome plays an important symbiotic role in nitric oxide homeostasis which is of significance for systemic control of vasodilation and blood pressure. Our work illustrates how high resolution analysis of micron-scale organization can provide insights into physiological functions and microbiome-host interactions. Supported by NIDCR Grant DE 022586.

P2667  
Board Number: B823  
Proteins in soy resistant to the digestive protease enzymes interact with intestinal epithelial cells and modulate metabolite absorption and microbiome profile in gut.  
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Proteins are important nutrient for our body. Most of the food proteins are digested after they are consumed and processed along the digestive tract. Some food proteins, however, are resistant to the digestive proteolytic enzymes. For example, glycinin, a major protein in soy, is resistant to the proteolytic enzymes. Regarding this fact we hypothesized that the proteolytic enzyme resistant food proteins (PRFP) can interact with the intestinal tract, metabolites and/or with gut microbes. The interaction can change the physiological function of the intestinal epithelial cells, changes the profiles of the gut microbiome, and the absorption of metabolites in the digestive tract. The interaction of the FITC-labeled PRFPs with intestinal cells showed specific bound to the intestinal epithelial cell line, CaCo2, but not to the hepatoma cell line, HepG2. Interacting proteins on the membrane of CaCo2 cell and murine intestinal mucosa to PRFP were isolated by nearest-neighbor photo-affinity cross-linking and affinity purification, and identified by LC/MSMS. The list of interactor proteins includes membrane bound proteins involved in cholesterol uptake. The fact that PRFPs interfere the interaction of bacteria to intestinal cells can modulate the growth of the bacteria and change the profiles of the microbes in the gut.
P2668
Board Number: B824
Chlamydia uses ARF GTPases as a switch to control microtubule stabilization and actin polymerization.
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Both actin and microtubules are major cytoskeletal elements in eukaryotic cells that contribute to many cellular functions, including vesicle and organelle movement. During infection with the intracellular bacterium Chlamydia trachomatis, both of these cytoskeletal elements are drastically reorganized. For example, once inside the cell, Chlamydia develops into a parasitic compartment called an inclusion, which surrounds itself with actin and microtubule cages. Here, we address how one particular Chlamydial protein expressed on the surface of the inclusion, called CT813/InaC, controls both actin polymerization and the stabilization of microtubules around the inclusion in order to promote Chlamydia replication. To accomplish this task, CT813 binds the small GTPases ARF1 and ARF4, and uses ARF as a switch to coordinate both actin and microtubules. Finally, we establish that both actin polymerization and microtubule stabilization are necessary for Chlamydia 1) to build a physical scaffold around its inclusion, which re-enforces the inclusion membrane, and 2) to co-opt host organelles that are required for inclusion growth, respectively. Altogether, these results show for the first time that a single protein coordinates two major cytoskeletal elements using ARF GTPases as a switch.

P2669
Board Number: B825
A Chlamydia trachomatis protein mediates the remodeling of epithelial cytoskeleton and cell-cell junctions.
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Chlamydia trachomatis is an obligate intracellular bacterial pathogen and a leading cause of sexually transmitted infections. Chlamydia delivers proteins to target epithelial cells to promote invasion and the formation of its replication vacuole, termed an “inclusion.” Infection of epithelial cells leads to the activation of multiple tyrosine kinases. We identified a bacterial protein TepP (translocated early phosphoprotein) that is translocated to the host epithelial cell and tyrosine-phosphorylated by Src kinases during early infection. TepP acts as a scaffold to recruit Crk adaptor proteins, which regulate the organization of the actin cytoskeleton, and the PI3-kinase complex to modify the early inclusion membrane and innate immune signaling. Significantly, Chlamydia lacking TepP (∆tepP) show decreased levels of tyrosine phosphorylation of multiple unknown proteins. Here, we performed a global phosphoproteomic analysis of host and bacterial proteins to identify proteins that are tyrosine phosphorylated in A2EN endocervical epithelia infected with wild type or TepP-deficient Chlamydia. We identified that Eps8, a filamentous actin-binding protein that localizes to epithelial tight junctions and apical microvilli, as a prominent TepP-dependent target of tyrosine phosphorylation. High-resolution microscopy shows that Eps8 is recruited to punctate F-actin structures associated with early inclusions in a TepP-dependent manner. Eps8 recruitment is dependent on its C-terminal actin-binding domain and coincides with altered F-actin organization, epithelial cell morphology and cell-cell junction integrity. Interestingly, we observed early inclusions in close association with epithelial cell-cell junctions, and the recruitment of additional tight junction proteins (e.g. ZO1). Consistent with the targeting of cell-cell
junctions, infection with TepP-expressing Chlamydia induces transient epithelial cell scattering. To validate these observations in primary cells, we developed a novel endometrial organoid infection model and examined the early infection process in a polarized, three-dimensional setting. In mock-infected organoids, Eps8 localizes to the apical cell surface and tight junctions, but re-locallizes to early inclusions in infected organoids, which show disrupted epithelial morphology and possibly integrity. Taken together, these studies demonstrate that TepP secretion during early infection regulates cytoskeletal organization by subverting Eps8 activity.

P2670
Board Number: B826
*Neisseria gonorrhoeae* modifies its infectivity based on the properties of human cervical epithelial cells.
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Gonorrhea, caused by *Neisseria gonorrhoeae* (GC), is one of the most common sexually transmitted infections. It has recently re-emerged as a public health crisis due to increases in multidrug resistant strains. However, the pathogenesis of this bacterium is not well understood due to a lack of infection models that mimic the human disease and the phase variation of multiple molecules on this bacterium, including opacity-associated protein (Opa). GC infection in the female reproductive tract starts from the cervix. The mucosal surface of the human cervix consists of three types of epithelial cells: non-polarized multilayer squamous at the ectocervix, polarized monolayer columnar at the endocervix, and transforming epithelial cells in between. This study examined the cellular mechanisms by which GC overcome the mucosal epithelial barrier to establish infection in the human cervix. We developed an ex vivo infection model using human cervical tissues and bacterial strains expressing no Opa or Opa proteins that cannot undergo phase variation. Using three-dimensional immunofluorescence microscopy, we found that GC that express phase variable Opa selectively penetrate into the subepithelium of the transformational zone and the endocervix but not that of the ectocervix. However, GC colonize the mucosal surface at all three regions, but the level of colonization at the transformation zone is higher than those at the ectocervix and endocervix. These findings are consistent with previous clinical observations using patients’ biopsies. The expression of phase invariant Opa that binds to the host adhesion molecules CEACAMs enhances GC colonization at the ectocervix and the transformation zone but inhibits GC penetration into the endocervical epithelium. In contrast, Opa expression does not affect GC infectivity in the transformational zone. Surprisingly, epithelial cells in the transformation zone do not express CEACAMs on their surface. GC break the cervical epithelial barrier by disrupting their adherens junction, which leads to GC penetration and epithelial cell shedding. Opa expression restores the epithelial adherens junction in a CEACAM-dependent manner, thereby inhibiting GC penetration and epithelial cell shedding. Our results collectively suggest that GC modify their infectivity based on the availability of the host cell receptors CEACAMs on cervical epithelial cells and the expression of Opa isoforms on GC. Our cervical tissue explant model is a useful tool for studying pathogenesis and developing treatments of sexually transmitted pathogens. This work has been supported by NIH grants.
**P2671**

**Board Number: B827**

**Toxoplasma gondii dysregulation of endothelial cell barrier function.**

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*Toxoplasma gondii* is an obligate intracellular parasite that infects an estimated one-third of the global population causing significant morbidity and mortality. Humans are typically infected by consuming contaminated food or water, or through vertical transmission, at which point the parasite crosses formidable biological barriers to infect tissues of the central nervous system (CNS), such as the brain and the eye. Brain endothelial cells can provide a replicative niche for *T. gondii* and facilitate entry into the host CNS, where the parasite establishes a chronic infection. However, our current understanding of the molecular interactions occurring at the interface between endothelial cells and *T. gondii* remains limited. In the current study, we demonstrate that *T. gondii* induced marked reduction in filamentous actin (F-actin) stress fibers and the reorganization of the intermediate filaments and microtubules in primary human endothelial cells during infection in both static and shear stress conditions. In addition, parasite infection disrupted endothelial cell planar polarity and the localization of the Golgi apparatus under physiologic shear stress, reinforcing an effect of infection on the actin cytoskeleton. The destabilization of the cytoskeleton in infected endothelial cells was associated with a reduction in the localization of the adherens junction protein VE-cadherin and β-catenin to the cell periphery and a functional dysregulation of endothelial barrier integrity, as determined by TEER assays and increased permeability to low molecular weight polymers. Collectively, these findings reveal that *T. gondii* dysregulates endothelial barrier function and remodels the host cytoskeleton during infection. This study contributes to our understanding of *T. gondii* pathogenesis and more broadly, the regulation of endothelial barrier permeability, the dysregulation of which is a hallmark of many diseases.

**P2672**

**Board Number: B828**

**Studying the effect of the obligate endocellular bacteria Wolbachia on its host.**

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Wolbachia pipientis is an intracellular obligate α- proteobacteria present in 65-70 percent of insects. Depending on the host, Wolbachia can be a reproductive parasite or a mutualistic endosymbiont. Permanence within the host includes strategies such as feminization, parthenogenesis, cytoplasmic incompatibility and complementation of metabolic needs. Some hosts, such as filaria are unable to function properly when the bacterium is killed. In these cases different host mitochondrion-related proteins are overexpressed, suggesting that there is a close Wolbachia-mitochondrion interaction. In contrast, there are reports that Wolbachia is highly sensitive to ROS. The metabolism of isolated Wolbachia has not been studied probably due to the difficulty in obtaining large quantities of the bacterium, and also because as a true obligate endosymbiont, Wolbachia may survive outside a host cell for about a week, remaining capable of reinfesting new cells, but unable to divide. In cells infected with Wolbachia, mitochondrial oxidative phosphorylation activities are extended, suggesting that Wolbachia are determinant for longer mitochondrial survival. This is reflected in long-lasting high concentrations of cytochromes b, c, and a, in higher rates of oxygen consumption and in higher respiratory coupling during aging.

Monday-507
P2673

Board Number: B829

Shedding light on redox-sensitive MarR protein regulating EmrB and RND efflux pumps in B. thailandensis.

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Many species of the genus Burkholderia are opportunistic pathogens and are difficult to treat due to a high level of resistance to a wide range of antibiotics. Bacteria have intrinsic multidrug efflux pumps to withstand lethal doses of antimicrobials. Different efflux pump genes as well as virulence genes are regulated by multiple antibiotic resistance regulators (MarR). Since production of reactive oxygen species (ROS) is the first line of host defense and many transcription factors respond to such ROS, we set out to determine if BtMarR encoded by BTHI0021 contributes to oxidative stress responses. Our data suggest that in transposon mutant of marR, expression of rnd and emrB increases approximately 2.1 ± 0.1 and 25.2 ± 0.7 fold respectively indicating that BtMarR is a transcription repressor. Both rnd and emrB is upregulated in presence of H2O2 (2.6±0.2 and 3.5 ± 0.4 fold respectively). Conversely, CuCl2 treatment caused repression of both genes (0.47 ± 0.1 and 0.39 ± 0.01 fold respectively). SDS-PAGE analysis revealed that BtMarR forms different oligomeric species upon oxidation. By electrophoretic mobility shift assays we have determined that BtMarR binds to the intergenic operator DNA of its own gene and a divergently oriented gene, hypo (genomic locus BTHI0020) encoding a conserved hypothetical protein. marR deletion leads to the upregulation of this gene. Moreover in mutant strain, expression of rnd was upregulated by 4.6 ± 0.2, 3.5 ± 0.1 and 6.5 ± 0.1 fold after addition of 2mM H2O2, CuCl2 and ZnCl2 respectively while no change was seen in case of emrB. This data suggests that along with BtMarR, another transcription factor is involved in regulation of rnd. Differential expression of hypo under oxidative stress in wild type and mutant strain suggests that in addition to marR, hypo encoding a hypothetical protein of unknown function may play a significant regulatory role to control adjacent oriented rnd. Collectively, the above data suggest that BtMarR is the repressor of both rnd and emrB under reducing conditions and regulation of rnd might involve another transcription factor along with BtMarR. Further studies will uncover the impending regulation of RND and EmrB efflux pumps by targeting stress responsive transcription factor BtMarR.

P2674

Board Number: B830

Thermodynamic protein instability is a pathogen-associated molecular pattern targeted by human defensins.

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Human defensins are innate immune defense peptides with a remarkably broad repertoire of activities. They disintegrate bacterial membranes and modulate immune response, inflammation, and angiogenesis. Moreover, remaining relatively benign towards human proteins, defensins inactivate bacterial toxins and intercept various viruses at different stages of their life cycles. We hypothesized that defensins recognize and target a common and essential property shared by many bacterial toxins and viral proteins – the intrinsically low thermodynamic stability. We showed that defensins inactivate susceptible proteins by taking advantage of their low thermodynamic stability and acting as natural
“anti-chaperones”, i.e. destabilizing the targets’ native conformations. To understand the detailed mechanisms of pathogen proteins inactivation by defensins, we have proposed a testable experimental model, where the initial transient interactions of defensins with proteins is mediated by electrostatic interactions; whether or not the electrostatic interactions advance to the unfolding stage will depend on the thermodynamic stability of a targeted protein. Due to the overall low specificity of these interactions, defensins integrated into disordered regions of thermodynamically unstable proteins would stabilize their partially unfolded states with high aggregation propensity. Stable proteins will remain either unaffected or stabilized by defensins. To test this model in the present study, we employed site-directed mutagenesis, chemical modifications, and other biochemical and biophysical approaches. Our data indicate that the initial electrostatic interactions can indeed account for different susceptibility of homologous toxins to defensins. Both C. difficile Toxins A and B (TcdA and TcdB) are thermodynamically unstable, but only TcdB with higher density of negative patches on its surface is effectively neutralized by defensins. Reduction of a net positive charge via acetylation of surface lysines on resistant TcdA increases its susceptibility to defensins. Next, a covalent stabilization of a toxin known to be both thermodynamically marginally stable and vulnerable to defensins (Actin Crosslinking Domain (ACD) of V. cholerae MARTX toxin) reduces its precipitation by defensins. In agreement with the proposed model, we show that the affinity of defensins to toxins is drastically increased near the melting temperature. Moreover, in complex mixtures, lower stability predisposes proteins for preferable precipitation by defensins. Understanding the defensins’ protective mechanisms is essential for rationally utilizing and boosting natural immune potentials of the human body and is expected to lead to a new generation of rationally designed synthetic therapeutic antimicrobials.

P2675
Board Number: B831
BPX-01 Topical Minocycline Gel Reduces P. acnes-induced Inflammatory Lesions in Mice.
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A novel, hydrophilic topical minocycline gel (BPX-01) has been developed for the treatment of inflammatory acne vulgaris. Minocycline, an antibiotic, is known to be effective against bacterium implicated in the pathogenesis of inflammatory acne, such as Propionibacterium acnes (P.acnes). Currently, minocycline is administered orally for this disease, but presents undesirable systemic side effects. Existing antibiotics used for the treatment of acne lesions (Clindamycin, Erythromycin, etc.) have already presented antibiotic resistance in patients.

The advance of a topical minocycline gel allows for direct delivery of the drug to the skin, significantly reducing or evading systemic exposure, thus potentially decreasing the incidence of antibiotic resistance. The topical minocycline also allows for targeted drug delivery into the affected areas (pilosebaceous unit) where P. acnes typically reside, potentially reducing the excess accumulation of bacteria and the inflammation caused. The efficacy of minocycline’s impact on P.acnes and lesion count was further evaluated in a Phase 2a and Phase 2b clinical trial, respectively. Furthermore, minocycline was detected in the plasma of only one subject out of the 259 tested in these studies. This indicates the potential for a decreased systemic antibiotic resistance risk with a topical minocycline gel.

In the present study, we investigated anti-inflammatory properties of minocycline in skin inflammation and hyper-proliferation induced by P. acnes using immortalized human keratinocytes (Ker-CT) and sebocytes. The anti-bacterial and anti-inflammatory effects of topically applied minocycline in vivo was further evaluated in an animal model. Live P. acnes were injected dermally into SKH1 mice resulting in inflammatory acne-like lesions. Physiological parameters including behavior, erythema and acne-like lesion features were observed. Histological examination of the lesions indicated characteristics.
commonly observed in clinical inflammatory lesions such as dermal inflammation, hyperkeratosis and inflammatory cell infiltrates, supporting the acne-like inflammatory aspects of the model. The daily application of BPX-01 reduced erythema and lesion size indicating a clearance of the P. acnes and inflammation in the skin. Other topical products, such as the antibiotic Clindamycin, were included. A high-resolution cross-sectional imaging method was also applied to provide a precise and less subjective approach to measure lesions. In conclusion, these data support the potential activity of minocycline for the treatment of acne vulgaris which may be attributed to its direct antimicrobial effect, and its ability to modulate the secretion of proinflammatory cytokines associated with acne pathology.

P2676
Board Number: B832
Innate immune variants of the surfactant protein in airway function after Klebsiella pneumoniae infection.
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Surfactant Protein-A (SP-A) is a major surfactant protein component and plays a role in surfactant related function and innate immunity. The important contribution of SP-A in innate immunity becomes evident with increased susceptibility of SP-A knockout (SP-A-KO) mice to a variety of infections. Human SP-A consists of two functional genes, SP-A1 and SP-A2, and each is identified with a number of genetic variants. SP-A1 and SP-A2 variants are shown to differentially enhance bacterial phagocytosis, with SP-A2 being more effective than SP-A1. Here, we investigated the role of SP-A variants on airway function after Klebsiella pneumoniae (Kp) infection. We measured the lung function of transgenic mice each carrying different SP-A1 and SP-A2 variants using the forced oscillation technique (FOT). Lung mechanical parameters including total respiratory system resistance (Rrs), compliance (Crs), elastance (Ers), central airway resistance (Rn), tissue damping (H), and tissue elastance (G) were obtained by analyzing pressure and volume signals acquired in response to predefined, small amplitude, oscillatory airflow waveforms applied at the subject’s airway opening. Lung function was assessed after infection (baseline), and following inhaled methacholine dose response (0-50 mg/mL). We found that: 1) in the absence of methacholine (baseline) no significant differences were observed between SP-A1 and SP-A2 variants and/or KO. 2) In response to methacholine, i) SP-A2 (1A3) gene variant in males exhibited increased total and central airway resistance (Rrs and Rn) versus all other variants; ii) In females, SP-A2 (1A3) and SP-A1 (6A2) variants had similar increases in total and central airway resistance (Rrs and Rn) versus all other variants; iii) Allele specific differences were observed, a) with SP-A2 (1A3) exhibiting significantly higher total and central airway resistance (Rrs and Rn) versus SP-A2 (1A0), in both males and females, and b) SP-A1 (6A2) had significantly increased total and central airway (Rrs and Rn) lung function versus 6A4 in both males and females. We conclude, in response to infection and methacholine, SP-A variants differentially affect lung function and exhibit sex specific differences, consistent with previously reported findings of functional differences of SP-A variants. Thus, the changes in respiratory function mechanics provide insight into the role and importance of genetics of innate immune molecules, such as SP-A, in mechanical consequences in lung function after infection. This work is supported by the CHILD fund.
**P2677**

**Board Number: B833**

Plasma membrane remodeling by *Neisseria meningitidis* is driven by a wetting process along type IV pili fibers.

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*Neisseria meningitidis* is a bacterial pathogen that colonizes human blood vessels and causes septic shock and meningitis. Adhesion of meningococci to host endothelial cells via type IV pili is accompanied by a potent remodeling of the host cell plasma membrane, forming filopodia-like protrusions that intercalate between aggregated bacteria. While the mechanism behind plasma membrane remodeling remains unknown, the plasma membrane protrusions have been shown to increase microcolony resistance to shear stress. Here we first show that plasma membrane remodeling takes place during bacterial colonization of human blood vessels *in vivo*. In *vitro*, we find that remodeling occurs at the level of the single adhering bacterium as discrete short-lived protrusions, which then stabilize in between aggregated bacteria in the microcolony. Cortical actin polymerization is not necessary for protrusion formation but stabilizes them. Several observations point to a scaffolding mechanism exerted by type IV pili. First, plasma membrane protrusions disappear upon pilus retraction. Second, electron microscopy shows that membrane protrusions grow alongside type IV pili fibers. Third, perturbing the architecture of the type IV pili meshwork hinders plasma membrane remodeling. Accordingly, we show that wetting onto adhesive nanofibers can drive tubulation of phospholipid vesicles, and that the plasma membrane of human endothelial cells deforms locally in response to adhesive nanotopographical cues. Therefore, we propose that the human endothelial cell plasma membrane deforms onto type IV pili fibers through a linear wetting mechanism which reflects a general property of biological membranes in contact with adhesive nanofibrillar structures.

**P2678**

**Board Number: B834**

Cell-specific defense cascade in *Verticillium*-infected grafted tomato.

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Although the hypersensitive reaction in foliar plant diseases has been described extensively, little is clear regarding plant defence strategies in vascular wilt diseases. *Verticillium* is a vascular fungus that affects more than two hundred economically important plants worldwide with billions of dollars lost. In *Verticillium* wilt, the tomato Ve-resistance locus is thought to encode a receptor (NLR), which binds a pathogen effector and sets off an immune cascade that curtails pathogen colonization. Breeding strategies have inactivated the Ve-R locus to improve yield and fruit quality. In turn, susceptible stems often are grafted on resistant roots to provide both an enhanced and resistant crop. To better
understand the contribution of genes in the grafted plants we have undertaken genomic and proteomic studies of defense/stress gene expression in grafted plants consisting of both susceptible (Ve1-) and resistant (Ve1+) tissue. Analyses indicate that in the susceptible plant a very heroic but unsuccessful systemic response occurs involving many known plant defense/stress genes, while surprisingly more modest changes occur in the resistant tomato. Since a number of the highly elevated proteins are known to participate in a plant hypersensitive response as well as natural senescence, the results suggest that some or all of the disease symptoms, including ultimate plant death, actually are the result of this exaggerated defense response. Analyses in grafted plants indicate that induction of the defense cascade is cell-specific, and not transduced throughout the plant. Instead, effectively curtailed fungal colonization in the root is sufficient to permit the enhanced crop production by susceptible tissue.

**P2679**

**Board Number: B835**

High Content Screening Implicates the PI3K-Akt Pathway in FAST Protein-Mediated Cell-to-Cell Fusion.

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Cellular fusion is central to many physiologic and pathophysiologic processes but remains a poorly understood phenomenon. The reovirus fusion-associated small transmembrane (FAST) proteins, the smallest proteins known to trigger cell-to-cell fusion, are useful tools in the dissection of this critical cellular process. High-content phenotypic siRNA and small molecule screens of FAST protein-mediated cellular fusion were developed to identify endogenous proteins and pathways involved in syncytium formation. HT1080 cells expressing the baboon reovirus p15 FAST protein were treated with a library of 258 small molecule kinase inhibitors (at 0.025, 0.25 and 2.5μM) and an automated fluorescence microscopy assay was developed to quantify syncytogenesis. In this fashion, phosphoinositide 3-kinase (PI3K) p110α subunit inhibitor Pik-75 and AKT kinase inhibitor A-443654 were identified as agents that dramatically inhibited syncytogenesis. A similar kinome screen based on RNAi also implicated the PI3K-AKT pathway in cellular fusion. The necessity and isoform specificity of the p110alpha/AKT signaling pathway to FAST protein-mediated cell fusion was validated through genetic intervention using shRNA (targeting p110 and p110 isoforms) and a transgenic knockout cell line DLD-1 (AKT1-/-/AKT2-/-), and via pharmacologic inhibition using PI3K isoform specific inhibitors TGX-221 (p110 inhibitor) and A525424(p110 inhibitor). Interestingly, Pik-75 and A-443654 also inhibited syncytogenesis mediated by the measles virus F+H fusion protein complex. The lack of structural and evolutionary relatedness between the FAST proteins and the measles virus F+H fusion protein complex suggests p110alpha/AKT involvement in a common cell-cell fusion mechanism. Immunoblot analyses indicate FAST protein expression leads to AKT activation through the PI3K p110α isoform, and fluorescence videomicroscopy revealed colocalization of AKT1-PH-GFP (a biosensor of phosphatidylinositol 3,4,5-phosphate) and the reptilian reovirus p14 FAST protein at areas of cell-to-cell contact, and this colocalization increased immediately prior to the formation of a cell-cell fusion synapse. Treatment with Pik-75 disrupted p14/AKT-PH-GFP colocalization and altered actin dynamics at areas of cell-to-cell contact resulting in a failure to form a cell fusion synapse. These studies identify the PI3K-AKT pathway as critical player involved in cell-cell fusion.
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P2680
Board Number: B837

**c-Abl kinase in Niemann-Pick type A disease: its implication in the pathogenic mechanisms leading to neurodegeneration.**

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**Introduction:** Niemann-Pick type A (NPA) disease is a lysosomal neurodegenerative disorder characterized by the deficiency in acid sphingomyelinase (ASM), accumulation of sphingomyelin and alterations in autophagy. Previously, we described that the c-Abl/p73 pathway is activated and mediates neurodegeneration in other lysosomal diseases. In addition, it has been recently described that c-Abl has a role in autophagy regulation. Our aim was to evaluate the participation of c-Abl in the autophagic alterations and neuronal pathology in NPA disease.

**Materials and Methods:** We used the ASM deficient mouse and desipramine treated-SHSY5Y neurons as *in vivo* and *in vitro* NPA models, respectively. We evaluated Purkinje cell loss, inflammation, markers of the autophagy flux and the c-Abl/p73 pathway at different ages in the NPA cerebellum. In treated SHSY5Y neurons we evaluated viability and c-Abl levels. The same parameters were evaluated in both NPA models using the c-Abl inhibitor Imatinib.

**Results:** We found progressive neurodegeneration, inflammation, alterations in the levels of autophagic markers and activation of the c-Abl/p73 pathway at the cerebellum of the NPA mice. Accordingly, the cellular neuronal NPA model presented activation of this signaling pathway. Treatment with Imatinib reduced neuronal death and caspase 3 active levels in the cellular neuronal NPA model and preserved Purkinje neurons and reduced inflammation in the NPA cerebellum.

**Conclusion:** c-Abl is activated and relevant in NPA neurodegeneration, supporting the potential use of Imatinib for clinical treatment of NPA patients.

P2681
Board Number: B838

**Analysis and modulation of cathepsin B and D in liver damage in *in-vitro* and *in-vivo* models of Niemann-Pick type C disease.**

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**Introduction:** Niemann-Pick type C (NPC) disease is a genetic storage disorder, characterized by cholesterol accumulation in lysosomes. NPC patients present progressive neurodegeneration and liver damage. We are interested in understanding the mechanisms involved in liver damage in NPC disease. Interestingly, increases in cathepsin B and D, two proteases involved in liver fibrosis and apoptosis, have been reported on different NPC models. **Aim:** to study the contribution of cathepsins B and D on the liver damage in NPC.

**Material and Methods:** We used NPC1 deficient mice (Npc1/−) at different ages. *In-vitro* models; i) Pharmacological: Hepa1-6, LX-2 or primary cultures of hepatic stellate cells (HSC) from WT mice treated with U18666A (U18) and ii) Genetic: HSC primary cultures of WT and NPC mice. We modulated cathepsin B or D, using the CA074 and pepstatin A inhibitors, respectively, or shRNAs, and analyzed the pro-fibrotic phenotype.
**Results:** We found increases on cathepsins levels in several NPC models, and a correlation with the pro-fibrotic phenotype in the in-vitro NPC models. Inhibition of cathepsin B activity reduced the pro-fibrotic phenotype in hepatic in-vitro NPC models, and inhibition of cathepsin D activity or levels reduced the pro-fibrotic phenotype in hepatic in-vitro and in-vivo NPC models.

**Discussion:** Increased levels of cathepsins B and D were found in several hepatic NPC models analyzed. Modulation of these cathepsins decreased the hepatic pro-fibrotic phenotype. Cathepsins B and D are potential therapeutic targets for NPC liver disease.


**P2682**

**Board Number: B839**

**REVEALING NOVEL FUNCTIONS OF GLUTAMATE CARBOXYPEPTIDASE II, A DIAGNOSTIC AND THERAPEUTIC TARGET IN NEUROPATHOLOGIES AND PROSTATE CANCER.**

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Glutamate carboxypeptidase II (GCPII) is becoming to be recognized as an important diagnostic and therapeutic target. Overexpression of GCPII, also known as prostate-specific membrane antigen (PSMA), has been implicated in the prostate cancer while inhibition of GCPII activity has been shown to improve neuropathologies such as neurodegenerative brain damage, glutamate neurotoxicity and peripheral nerve neurodegeneration in animal models. GCPII is involved in several physiological processes as a transmembrane glycoprotein with large extracellular part possessing carboxypeptidase activity. Despite extensive research, the function of GCPII is understood only in the brain and small intestine but not in other GCPII-expressing tissues. Several different attempts to generate GCPII knock-out (KO) mice were reported with rather controversial results, ranging from embryonic lethality to generation of viable GCPII KO mice with no obvious phenotype. In order to dissect this controversy, we attempted to generate GCPII KO mice by targeting the active site of the enzyme using TALEN technology. We established reliable genotyping method based on nested PCR and show that no GCPII protein is expressed in our KO mice as determined by Western blot and carboxypeptidase activity analysis. Our GCPII KO mice are viable, breed normally and do not show any obvious phenotype. This reliable mouse model could be further used for revealing yet unknown physiological functions of GCPII in the kidney, prostate or elsewhere.

**P2683**

**Board Number: B840**

**PYROXD1; a novel cause of congenital myopathy highlights oxidative distress as a core mechanistic pathway in muscle and neurodegenerative disorders.**

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Monday-514
Our laboratory recently identified PYROXD1 as a novel genetic cause of congenital muscle disease (myopathy) that is characterized by generalized weakness, difficulty feeding and swallowing in the neonatal period, respiratory problems and distinctive nasal speech. Histologically, PYROXD1 myopathy is distinctive, with patient muscle biopsies bearing all of the pathological features that usually sub-classify the myopathies; internalized nuclei, zones devoid of mitochondrial activity, myofibrillar disorganization and protein inclusions. The two eldest patients (now aged 24 and 28) have emerging evidence for nerve and cardiac involvement.

PYROXD1 is an uncharacterized pyridine nucleotide-disulphide oxidoreductase (PNDR). PNDRs are a family of powerful reductases that reduce redox-reactive thiols in their substrates; often other crucial enzymes and signalling proteins that become oxidized as a result of their functional activity. Recent studies have identified PYROXD1 as one of ~1800 genes essential for cell survival, and our studies confirm targeted knock out of PYROXD1 results in embryonic lethality in mice. Therefore, PYROXD1 has a non-redundant reductase activity that no other reductase can compensate for. PYROXD1 myopathy highlights the power of rare, genetic disorders to impart new insight to basic biology. PYROXD1 either lends its reductive powers to a novel redox pathway, or alternately, PYROXD1 represents a new upstream regulator of a known redox pathway vital for cell viability. Both possibilities are of fundamental significance.

Our studies are now targeted on defining the oxidative pathway and vital substrates reduced by PYROXD1, using proximity-dependent biotin identification (BioID2) and comparative thiol proteomics. BioID2 utilizes a PYROXD1-biotinylase fusion protein, whereby a brief biotin pulse results in biotinylation of protein interactors. Using mass spectrometry, the PYROXD1 biotinylated interactome is then cross referenced with proteins showing differential thiol oxidation between wild-type and PYROXD1 knockout tissues.

PYROXD1 myopathy provides evidence that development of several pathological endpoints in the myopathies are linked to redox dysfunction, and gradual accumulation of oxidative stress in long-lived muscle fibers. Interestingly, we show that protein aggregates in PYROXD1 patient muscle also stain positively for markers of neurodegenerative aggregates. We are now studying mechanistic parallels in aggregate pathobiology between myopathies and neurodegenerative disorders using histological staining and biochemical assays with specific intent to evaluate clinical merit of existing anti-oxidant therapies for myopathy patients.

P2684
Board Number: B841
Inhibition of histone acetyltransferases specifically targeting H4K5 acetylation rescue myogenic differentiation of emerin-null myogenic progenitors.
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Emery Dreifuss Muscular Dystrophy (EDMD) is a disease characterized by skeletal muscle wasting, contractures of the major tendons, and cardiac conduction defects. The symptoms generally begin during adolescence and slowly worsen over time. As a result, it is predicted that the disease results from a failure of muscle fibers to properly regenerate after injury. The inability to properly regenerate skeletal muscle is predicted to result from impaired muscle stem cell differentiation. Mutations in the gene encoding emerin cause EDMD. In previous experiments, myogenic progenitors derived from emerin-null mice confirmed their impaired differentiation by observing cell cycle exit, myosin heavy chain expression, and myotube formation. The emerin-null myogenic progenitors showed decreased ability to exit cell cycle, decreased expression of myosin heavy chain, and decreased myotube formation. It was
previously shown that emerin interacts with and increases the catalytic activity of HDAC3. Treatments with theophylline, a HDAC3 activator, rescued myotube formation in differentiating emerin-null myogenic progenitors. Furthermore, addition of RGFP966, a HDAC3 inhibitor, decreased myotube formation. This suggests that HDAC3 is important for the later stages of myogenic differentiation. To confirm the deacetylation function of HDAC3 by theophylline treatment was responsible for rescue of myogenic differentiation in emerin-null progenitors, we tested inhibitors of H4K5 histone acetyltransferases (HATs). Nu9056, L002, and windorph were added to differentiating wildtype and emerin-null myogenic progenitors and differentiation was assessed using multiple assays. Treatments with the HAT inhibitors showed an increased formation of myotubes in emerin-null myogenic progenitors. Importantly, HAT inhibition rescued emerin-null myogenic progenitor differentiation. Thus, we conclude emerin regulation of HDAC3 activity is important for myogenic differentiation.

P2685

Board Number: B842
Myosin replacement in myofibrils is induced by Hsp90 activity.
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Myosin is a fundamental force generating protein in skeletal muscles. In myofibrils, a single thick filament consists of approximately 300 myosin molecules, which are constantly substituted in myofibrils. Our previous study showed that myosin replacement rate is significantly reduced by an inhibition of myosin synthesis. However, it remains largely unknown which factor(s) regulates myosin substitution in myofibrils. In this study, we investigated whether myosin replacement rate was induced by the activity of heat shock protein 90 (Hsp90) which functions as myosin chaperone to correctly fold myosin subfragment-1. Fluorescence recovery after photobleaching experiments showed that the replacement rate of green fluorescence protein fused myosin heavy chain was significantly increased in myotubes overexpressing Hsp90. However, an inhibition of Hsp90 activity in the presence of geldanamycin reduced myosin replacement rate. These results indicate that Hsp90 activity is required for up-regulation of myosin substitution rate in myofibrils. To ask how Hsp90 promoted myosin replacement rate, unincorporated myosin content in the myocytoplasm was semi-quantified. The cytosolic myosin content was increased in myotubes overexpressing Hsp90 but not in geldanamycin treated myotubes. Furthermore, the gene expression level of myosin heavy chain was up-regulated by Hsp90 overexpression but down-regulated by inhibition of Hsp90 activity. Taken together, our results demonstrated that myosin substitution is facilitated by Hsp90 activity through elevated levels of the cytosolic myosin content and the myosin gene expression.

P2686

Board Number: B843
The lipid kinase PIKfyve in cardiac fibroblasts activation: a potential target to control cardiac fibrosis.
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Fibrotic remodeling of cardiac tissue is a key determinant in the progression of heart failure, a leading cause of death worldwide. Cardiac fibrosis is characterized by the activation of cardiac fibroblasts and
Posters Monday 5-17

P2687
Board Number: B844

Mutational and biochemical analysis of the dual kinase region of UNC-89 (Obscurin).

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In C. elegans, unc-89 loss of function mutants display reduced locomotion, disorganized myofibrils and lack of M-lines. The largest UNC-89 isoforms at approximately 900,000 Da consist of 53 Ig domains, 2 Fn3 domains, a triplet of SH3, DH and PH domains near their N-termini, and two protein kinase domains (PK1 and PK2) at their C-termini. Nematode UNC-89 is the founding member of the UNC-89/obscurin family of proteins. UNC-89 is localized to M-lines. Homology modeling suggests that PK1 is catalytically inactive, whereas PK2 is catalytically active. Although we have found that both PK1 and PK2 interact with SCPL-1, a novel CTD-type protein phosphatase, and that PK1 interacts with LIM-9 (FHL2), the actual functions of these protein kinase domains is unknown. To begin to explore the in vivo functions of UNC-89 kinase domains, we have used CRISPR/Cas9 gene editing to create nematodes having an in-frame deletion of PK1 (unc-89(sf23)), or a putative catalytically inactive PK2 (unc-89(sf22)). To make the catalytically inactive PK2, we changed a conserved lysine (K) that is involved in ATP coordination to alanine (A). We have shown that PK2 has catalytic activity in vitro, and we are in the process of verifying that this KtoA mutation eliminates kinase activity. unc-89(sf22) and unc-89(sf23) worms have normal sarcomere structure as assessed by immunostaining using antibodies to various sarcomeric proteins. Nevertheless, both these mutants display reduced whole body locomotion (swimming in liquid or crawling on agar). To help explain how these mutants move more slowly despite having normal myofibrils, we hypothesized that mitochondria might be defective in organization or function. We used two methods (Mitotracker staining, and muscle-specific expression of mitochondrial-localized RFP) to assess the organization of mitochondria. Each method shows that, as compared to wild type, unc-89(sf22) and unc-89(sf23) have mitochondrial networks that are broken or fragmented. The mitochondrial protein content (assessed by quantitative western blotting using antibodies to ATP synthase, ATP5A), however, is unaffected. As mitochondrial fragmentation usually indicates mitochondrial dysfunction, we are in the process of measuring ATP levels in these mutants.
P2688
Board Number: B845
Nuclear receptor interaction protein (NRIP) maintains Z-disc width through α-actinin-2 bound with CapZ.
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Previously, we demonstrated a gene, nuclear receptor interaction protein (NRIP, also named as DCAF6 or IQWD1), which could interact with nuclear receptors such as androgen and glucocorticoid receptors. NRIP acts as a transcriptional cofactor to activate nuclear receptor-driven gene expression. NRIP is a Ca2+-dependent calmodulin binding protein to stimulate calcineurin phosphatase and CaMKII kinase for muscle contraction; hence global NRIP knockout mice (NRIP gKO) revealed muscle dystrophy (JCS, 2015). Here we further examined heart functions of gKO that revealed cardiac hypertrophy. Specifically, the muscle-restricted NRIP knockout mice (cKO) displayed the enlarged outlook of heart at 22 wk. The echocardiography assay showed the diminished fractional shortening [FS] and EF [the ration of end-systolic diameter to end-diastolic diameter [ESD/EDD] in cKO in comparison with WT; indicating loss of NRIP may cause cardiac hypertrophy. The sarcomere structure by using electronic microscopy revealed the reduction of I-band width, extension length of Z-disc and shortened sarcomere length in heart at age 10 wk. The calcium transient of primary cardiomyocytes from adult mice revealed that the deficiency of NRIP decreased the amplitude of calcium transient and shortened sarcomere contraction that was consistent with shorten length sarcomere by EM. To verify the regulatory mechanism of NRIP, we found that α-actinin-2 (ACTN2) (a biomarker of muscular Z-disc complex), was one of NRIP-interacting proteins from the yeast two-hybrid system using NRIP as a prey. In vitro and in vivo assays confirmed NRIP directly bound with ACTN2. Loss of NRIP, the ACTN2 bounded less CapZ than wild type; but CapZ expression was comparable between WT and cKO; indicating that NRIP might stabilize Z-complex formation at least through CapZ to anchorage actin in sarcomere structure. On the other hand, we are in progress to generate AAV encoding NRIP and will see the therapeutic effect on the model of NRIP cKO-inducing cardiomyopathy model. In conclusion, NRIP is a Z-disc protein and has function for maintenance of sarcomere integrity structure those involved in calcium transient and muscle contraction.

P2689
Board Number: B846
Roles of Interleukin-1 in the regulation of myoblast fusion and actin dynamics.
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Myogenesis is a highly organized process involving cellular fusion of myogenic progenitors into multinuclear myotubes, a process requiring dynamic actin remodeling in response to appropriate regulation of intracellular signals including Rho-family small GTPases (Rac1, Cdc42, etc). Recently, we found that interleukin-1 (IL-1) α/β -double knockout (IL-1KO) mice exhibited impaired muscle regeneration after
cardiotoxin-induced muscle injury, characterized by delayed infiltrations of immune cells and delayed appearance of satellite cells as compared with those of wild-type (WT) mice. While sophisticated interactions between inflammatory microenvironments and satellite cells are apparently prerequisite in vivo, the potential direct involvement of IL-1s in the regulation of satellite cell functions has yet to be clarified. Herein, we took advantage of IL-1KO satellite cells obtained from the IL-1KO mice to directly investigate the regulatory role of IL-1 in the fundamental functions of satellite cells in vitro. We found that IL-1KO satellite cells displayed obvious impairments in growth and myogenic fusion in vitro as compared to WT satellite cells and that, importantly, these defects were both dose-dependently normalized by exogenous IL-1β. Despite the delayed myogenic fusion process in IL-1KO cells, they highly expressed key myogenic transcription factors such as myogenin and MyoD, indicating the transcriptional myogenic program to be appropriately activated in the absence of endogenous IL-1. On the other hand, biochemical analysis of intracellular signaling intermediates revealed the phosphorylation status of group I PAK proteins (e.g. PAK2/Thr402), direct effectors of Cdc42 and Rac1 involving myogenic regulation, to be persistently higher in IL-1KO than WT cells during the course of myogenesis, while cellular contents of N-WASP were slightly diminished. Furthermore, intravital imaging analysis of actin dynamics using LifeAct-Venus revealed that IL-1KO cells exhibited stable formation of micro-spikes with a quiescent state of actin remodeling activity under basal conditions, which differed strikingly from WT cells displaying active ruffling formation. As expected, administration of IL-1β markedly restored actin dynamics in IL-1KO cells as evidenced by vigorous remodeling of actin filaments underneath the plasma membrane, promoting membrane ruffling, which lasts at least for 16 hrs. Taken together, these data demonstrate that fundamental satellite cell properties are intrinsically regulated by endogenous IL-1α/β and that lack of IL-1 related signals results in derangements of their actin regulatory systems, most likely accounting for the compromised myogenic fusion process.

P2690
Board Number: B847
Glucose-dependent insulinotrophic polypeptide stimulates the differentiation of mammalian skeletal muscle cells.
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Glucose-dependent insulinotrophic polypeptide (GIP, also known as gastric inhibitory polypeptide), is an incretin, and is synthesized in K-cells of the duodenum and the proximal portion of small intestine. Oral ingestion-associated elevation of blood glucose level stimulates the secretion of GIP in to the circulation. Thereafter blood GIP binds GIP receptor (GIPR) expressed in pancreatic β-cells, induces insulin secretion from them, and facilitates uptake and utilization of glucose in targeting tissues, such as skeletal muscles. Secretion of glucagon from a-cells of the pancreas is also stimulated by GIP. It is reported that the inhibition of GIP as well as the silencing of GIPR impairs glucose homeostasis in mice. In addition, GIP directly stimulates glucose uptake in rat skeletal muscles via GIPR that is also expressed in skeletal muscle cells. Recently, we have demonstrated that the administration of GIP increases the protein content and myotube diameter of C2C12 cells. Therefore, GIP-GIPR intracellular signal(s) may play a role in myogenic differentiation. The purpose of this study was to investigate a physiological role of GIP-GIPR intracellular signal(s) in the myogenic differentiation using mouse myoblasts-derived C2C12 cells. Expression of GIPR was observed in not only myotubes but also undifferentiated myoblasts of C2C12
cells. The expression level of GIPR in C2C12 myoblasts cells was lower than that in C2C12 myotubes. In this study, GIPR of C2C12 myoblasts was knock down using small interfering RNA (siRNA) immediately before the initiation of differentiation. Knock down of GIPR induced the down-regulation of both MyoD and Pax7 in C2C12 myoblasts. Furthermore, GIPR-knock down-associated depression of both the increase in muscle protein and myotube formation of C2C12 cells were observed during differentiation. Evidences from this study strongly suggest that GIP-GIPR intracellular signal(s) might stimulate myogenic differentiation via inhibiting of proliferation of myoblasts. This study was supported, in part, by Grants-in-Aid for challenging Exploratory Research (16K13022), and Grants-in-Aid for Scientific Research (C, 17K01762) from the Japan Society for the Promotion of Science, the Science Research Promotion Fund from Promotion and Mutual Aid Corporation for Private Schools of Japan, the research grants from Graduate School of Health Sciences, Toyohashi SOZO University, and All Japan Coffee Association.

P2691
Board Number: B848
Effects of acute hypoxia on zebrafish cardiac tissue.
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Human cardiomyocytes are only capable of an inadequate proliferative and regenerative response to damaged cardiac tissue. In contrast, it is known that cardiomyocytes in damaged Danio rerio (zebrafish) hearts are capable of complete regeneration in response to pathological injury and stress. We have repurposed a simple anaerobic incubation tank to induce pathological hypoxia in zebrafish hearts. After exposing fish to a severely oxygen-depleted environment for 2 hours, we found that the dissolved oxygen (DO) in fish water is reduced by an average of 81%, when compared to water exposed to normal atmospheric conditions—presumably sufficient to cause detectable cardiomyocyte damage. Following re-exposure to normal oxygen levels, we dissect hearts to study specific effects on cardiac tissue. We have used fluorescent labels to detect nitroreductase activity and reactive oxygen species (ROS) production—both indicators of hypoxic damage. Repeatedly, Epicardial, pericardial, and myocardial tissues appear to exhibit detectable ROS production, while only myocardial tissue appears to demonstrate nitroreductase activity. When cardiomyocytes are damaged by prolonged hypoxia exposure, they are typically programmed for apoptosis, and should exhibit an increase in the level of mono- and oligonucleosomes in the cytoplasm prior to actual cell death and dissolution of the cell membrane. Here, we demonstrate our efforts to assess the level of cardiomyocyte damage inflicted using our hypoxia model, by testing for evidence of apoptosis programming with an ELISA assay to detect cytoplasmic histone-associated DNA fragments. If we can achieve detectable cardiomyocyte damage using our hypoxia model, we will conduct future investigations of the cardiac regeneration and cardiomyocyte proliferation process in zebrafish. These findings could be translatable into discovery of molecular targets for therapies to stimulate human cardiomyocyte regeneration and repair.
Nearly 30 years ago George Brooks proposed the existence of a lactate shuttle in skeletal muscle between fast twitch fibers and slow fiber. According to his model, what was previously considered a mere byproduct of glycolysis, had acquired a role in sustaining energy metabolism during exercise. The transporters responsible for shuttling lactate were discovered only a decade later. The monocarboxylate transporters (MCTs) are proton-coupled transporters expressed in the plasma membrane of different tissues. The preferential localization of MCT4 in the sarcolemma of fast, glycolytic fibers and of MCT1 in slow, oxidative fibers makes them important components of the lactate shuttle in skeletal muscle. Based on their distribution, affinity and capacity to transport lactate, MCT1 primarily regulates the transport of lactate into the cells, while MCT4 predominantly exports lactate outside the cells. However, their directionality can change according to local lactate ions gradient. MCT1 and MCT4 require an accessory protein, Basigin (Bsg), for maturation and translocation to the sarcolemma. It has been shown that in Bsg−/− mice the MCTs do not localize at the plasmamembrane. Given the importance of lactate for energy metabolism and muscle physiology, we hypothesized that interfering with the shuttle will impair muscle function and cause exercise intolerance. To test this hypothesis, we used a global SLC16A3 (MCT4) knock-out mouse model and an inducible skeletal muscle specific Bsg knock out model (iMS Bsg−/−) created by crossing Bsg+/− animals with the a-skeletal actin-MerCreMer mice. MCT4−/− and iMSBsg−/− 4 months old mice performed poorly on a treadmill assay, suggesting exercise intolerance. When tested for voluntary activity with running wheels, MCT4−/− animals showed a significant decrease in activity compared to controls. Surprisingly, iMS Bsg−/− mice activity was similar to control littermates. This suggest that in the MCT4−/− an additional behavioral component may play a role since MCT4 is expressed also in astrocytes. To study the effects of lack of MCT4 on muscle mechanical properties we assayed ex vivo contractile properties of isolated extensor digitorum longus (EDL) muscles. MCT4−/− muscles generated less force than controls. Analysis of compound muscle action potential (CMAP) showed a decreased in CMAP amplitude with normal latency and duration in MCT4−/−, suggestive of axonal degeneration. Further experiments are necessary to evaluate the functionality of the neuromuscular junctions of the animals. Our studies on the MCT4−/− mouse demonstrate for the first time the importance of the lactate shuttle in supporting motor function and energy metabolism during endurance exercise. Funding source: Grant EY012042 to NJP

Systemic iron homeostasis is maintained by regulation of iron absorption in the duodenum, recycling from erythrocytes, and mobilization from the liver and is controlled by the hepatic hormone, hepcidin.
Hepcidin expression is induced via the bone morphogenetic protein (BMP) signaling pathway that preferentially uses two type-I (ALK2 and ALK3) and two type-II (ActRIIA and Bmpr2) BMP receptors. Hemojuvelin (HJV), HFE, and transferrin receptor-2 (TfR2) facilitate this process presumably by forming a plasma membrane complex with BMP receptors. Matriptase-2 (MT2) is a protease and key suppressor of hepatic hepcidin expression and uses MT2 as substrate. Previous studies have therefore suggested that MT2 exerts its inhibitory effect by inactivating HJV. Here, we report that MT2 suppresses hepcidin expression independently of HJV. In Hjv/- mice, increased expression of exogenous MT2 in the liver significantly reduced hepcidin expression similarly as observed in WT mice. Exogenous MT2 could fully correct abnormally high hepcidin expression and iron deficiency in Mt2/- mice. In contrast to MT2, increased HJV expression caused no significant changes in wild-type mice, suggesting that Hjv is not a limiting factor for hepcidin expression. Further studies revealed that MT2 cleaves ALK2, ALK3, ACTRIIA, BMPR2, HFE, and to a lesser extent HJV and TfR2. MT2-mediated Tfr2 cleavage was also observed in HepG2 cells endogenously expressing MT2 and TfR2. Moreover, iron-loaded transferrin blocked MT2-mediated Tfr2 cleavage, providing further insights into the mechanism of Tfr2’s regulation by transferrin. Together, these observations indicate that MT2 suppresses hepcidin expression by cleaving multiple components of the hepcidin-induction pathway.

P2694
Board Number: B851
Predictors of inflammatory obesity and the protective role of catecholaminergic receptors expression in immune cells for its development.
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Central obesity (CO) is a systemic inflammatory condition linked to immunometabolic disease clusters promoting premature death. Health risk assessment associated with excess adiposity requires identification of predictors of human subclinical inflammatory obesity (SIO) and understanding the immune-metabolic regulation. Immune cells, neurons and adipocytes share the language of catecholamines (CA), potent modulators of neuro-endocrine-immune/inflammatory networks. We aimed to identify SIO and hypothesized CA immunomodulation in obesity. A low-cardiovascular risk population was studied concerning anthropometric, metabolic, endocrine and inflammatory parameters. Waist circumference (WC) measure defined CO after the International Diabetes Federation criteria. With a flow cytometry analysis, we studied monocyte subsets, staining for CD14, CD16, CD36 and CD11b. Expression of catecholaminergic receptors and tyrosine hydroxylase (TH) was assessed by real-time PCR in peripheral mononuclear cells (PBMC). A ratio (R) between adrenoceptors (AR), dopaminergic receptors (DR) and TH mRNA of CO and no-CO<0.5 was considered under and >2.0 over expression. CD16+ monocytes in CO showed a more inflammatory pattern without expansion, as compared to no-CO subjects. WC was sensitive to lipidemia and, in CO group, lipidemia was associated with a more inflammatory phenotype of CD16+ monocytes. In CO, adrenaline was correlated with CD16+ monocyte expansion with a lower inflammatory pattern. Leptin, VLDL-C plasma levels, and CD14 expression of CD16+ monocytes were found to be CO predictors (R² =0.337; Z (3) = 6.258; p=0.002).

Monday-522
\( \beta_2 \text{AR transcripts were under expressed in CO relatively to no-CO group (R=0.08; p=0.009). Logistic regression analysis (LRA) demonstrated a lower association for CO development if } \beta_2 \text{AR mRNA} \geq 2 \times 10^{-6} \text{[OR 0.177 (95%CI) (0.040-0.796)]}. \beta_2 \text{AR expression inversely correlated with plasma triacylglycerol, VLDL-C and leptin and positively with HDL-C. PBMC from CO subjects showed lower expression of DRD2, DRD4 and DRD5 as well as lower levels of TH mRNA in comparison to the counterpart. DRD2 and DRD5 expression strongly correlates with lower weight, BMI and WC, lower plasma levels of leptin and with a lower inflammatory pattern. In CO model, LRA showed a protective role for CO development for DRD2 mRNA \geq 0.0000000455 \text{[OR 0.018 (95%CI) (0.002-0.195)]. In conclusion, we identified predictors that explain SIO in more than 33%. Our work suggests that in SIO, dyslipidemia and leptin induce monocytes maturation rather than an independent arise of CD16+ monocytes. The expression of catecholaminergic receptors in PBMC has a different pattern in SIO and associate with a better metabolic profile and with a lower inflammatory state, suggesting CA immunomodulation in obesity.}

P2695

**Board Number: B852**

**Expression and regulation of the novel adipocyte enriched gene NRNP1.**

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Obesity and Type II diabetes are co-morbidities that are increasing at an alarming rate in the United States. A thorough understanding of the function of white adipose tissue (WAT) is critical to attaining new insights into the development of these diseases. One approach to increase our understanding of adipose tissue function in health and disease is the identification and functional assessment of novel genes that are induced as preadipocytes convert to mature adipocytes. To identify new genes with potential importance in adipocyte function, we carried out transcriptional profiling studies using the 3T3-L1 *in vitro* model of white adipogenesis. Transcript for a novel gene, which we have named NRNP1 (Nutritionally Regulated Nuclear Protein 1), was found to be expressed at >20-fold higher in 3T3-L1 adipocytes vs. 3T3-L1 preadipocytes. NRNP1 was also similarly induced during preadipocyte to adipocyte conversion in an *in vitro* model of brown adipogenesis. Assessment of a panel of mouse tissues indicated expression in WAT depots and brown adipose tissue (BAT). Importantly, NRNP1 was enriched in the adipocyte cell fraction vs. the stromal vascular cell fraction of mouse WAT, indicating the adipocytes of WAT are the cell type expressing NRNP1. *In vivo* studies revealed an increase in NRNP1 transcript expression in subcutaneous WAT (12-fold) and BAT (3-fold) of mice that were fasted and then refed vs. NRNP1 transcript levels in fasted mice. We also noted increased expression of NRNP1 transcript in fatty liver of *ob/ob* mice vs. C57BL/6J mice. Transfection of an NRNP1-eGFP fusion protein revealed nuclear localization. Together, these data indicate that expression of the transcript for the novel nuclear protein NRNP1 closely parallels adipogenic conversion and states of lipid accumulation in *vivo*. Given the nuclear localization of NRNP1, we hypothesize that NRNP1 may have a regulatory role in these processes.
P2696
Board Number: B853
Blocking Ca\(^{2+}\)-channel \(\beta_3\) subunit reverses diabetes.
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Voltage-gated Ca\(^{2+}\) channels (\(\text{Ca}_n\)) has an important function in change of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) level which lead to insulin exocytosis in the pancreatic islet. The \(\beta_3\) subunit of Ca\(_n\) (Ca\(_n\)\(\beta_3\)) is regulatory subunit of Ca\(^{2+}\) channel, Ca\(_n\)\(\beta_3\) is known to modify Ca\(^{2+}\) channel currents. In the previous report showed that islets from Ca\(_n\)\(\beta_3\)-deficient (Ca\(_n\)\(\beta_3^{-/-}\)) mice exhibited increased [Ca\(^{2+}\)], oscillation frequency, better insulin secretion, and more glucose tolerant compared to wild-type mice. However, there is lack of information about function of Ca\(_n\)\(\beta_3\) in Ca\(^{2+}\) dynamics and insulin secretion in diabetes. So we focused on function of Ca\(_n\)\(\beta_3\) in the diabetes model. Here, we observed that islets from ob/ob mice model and high fat diet feed mice showed decreased [Ca\(^{2+}\)], dynamics amplitude and oscillation, also impaired insulin secretion in glucose stimulation. Also overexpression of Ca\(_n\)\(\beta_3\) in islets alters Ca\(^{2+}\) dynamics and impairs insulin secretion. We implemented the reduced Ca\(_n\)\(\beta_3\) in ob/ob mouse’s islet using antisense oligonucleotide and then discovered the recovery of [Ca\(^{2+}\)], dynamics, insulin secretion. In the development of diabetes, to define the Ca\(_n\)\(\beta_3\) function, inducing diabetes to the Ca\(_n\)\(\beta_3\) knocking out mice. Feeding a high fat diet to the Ca\(_n\)\(\beta_3^{-/-}\) mice show more tolerance to glucose stimulated [Ca\(^{2+}\)], dynamics and insulin secretion compared to normal mice fed on high fat diet. In vivo study, we transplanted Ca\(_n\)\(\beta_3^{-/-}\) mice islet to the diabetic mice, we found improved glucose tolerance. So we could suggest that Cav\(\beta_3\) is crucial for regulation of islet function in diabetic condition. This study will discover the potential value of the VDCC Ca\(_n\)\(\beta_3\) as a target of diabetes treatment.

P2697
Board Number: B854
A role in olfaction for DLK in resistance to diet induced obesity.
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The physiological role of Dual Leucine Zipper Bearing Kinase (DLK)--a MAP 3-kinase that interacts with JNK--remains incompletely known. To investigate DLK function, we generated DLK hypomorphic mice that circumvent early neonatal death observed in DLK null mice. DLK hypomorphs (\(Dlk^{neo/neo}\)) were resistant to diet induced obesity. Our results showed that the difference in body weight gain observed on a hypercaloric diet in \(Dlk^{neo/neo}\) vs. control mice was due predominantly to a dramatic difference in adipose accumulation and not due to a difference in the rate of lean mass growth. While DLK hypomorphs had smaller body surface area than wild type, experiments done in a thermoneutral environment confirmed resistance to diet induced obesity (DIO). DLK hypomorphs exhibited increased energy expenditure that resulted from increased resting metabolic rate and increased spontaneous motor activity. They consumed less food than WT controls, (although \(Dlk^{neo/neo}\) mice consumed food at a rate similar to control when food consumption was normalized to either body mass or lean mass) and did not demonstrate intestinal fat malabsorption. In considering mechanisms by which DLK deletion results in resistance to DIO, we were intrigued by observations by Riera et al. (Cell Metab, July 2017) that loss of olfactory neurons in mice protects them against DIO by altering energy homeostasis. We found that DLK null and DLK hypomorphic mice lacked brain anterior commissures and DLK hypomorphs
had reduced olfactory bulb volume compared to wild type control. DLK null neonates had reduced milk bubbles yet attached normally when shown the mother’s nipple. Given these observations, we examined the hypothesis that DLK deleted mice exhibit an olfactory functional defect. In food finding assays, mature Dlk<sup>neo/neo</sup> mice demonstrated impaired olfactory function relative to wild type control. These observations were confirmed in adult conditional DLK null mice (Dlk<sup>lox/lox</sup> x Ubiquitin-Cre<sup>SRT2</sup>), where DLK was deleted following tamoxifen injection. We speculate that at least in part, DLK is necessary for normal olfaction dependent regulation of energy homeostasis.

**P2698**

**Board Number: B855**

**High Throughput Idiosyncratic Drug-Induced Hepatotoxicity and Investigations of Chronic Proliferation of Cells with a Non-Invasive Approach.**

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Hepatic toxicity has accounted for 15 of the 47 drugs withdrawn from the market in the last two decades<sup>1</sup>. More specifically, Drug Induced Liver Injury (DILI) is the major cause of acute liver failure in the USA and Europe and is one of the main reasons for regulatory actions<sup>2</sup>. DILI is classified as intrinsic (or dose-dependent) or as idiosyncratic. A prominent example of idiosyncrasy is acetaminophen (paracetamol), with a variable time of onset and not directly dependent on dose. We present a non-invasive DILI assay approach based on impedance measurements in monocyte-derived hepatocyte-like (MH) cells from MetaHeps®. Although improvements have been made to cellular and animal models to predict intrinsic (dose-dependent) DILI, it is almost impossible to predict idiosyncratic DILI. MH cells have been developed as a tool to investigate long-term hepatotoxicity, metabolism and drug interactions. Furthermore, patient-derived MH cells could provide a tool for diagnosis or exclusion of idiosyncratic DILI, and provide the causative agent in polymedicated patients. MH cells were used on a 96-well screening system that monitors changes in impedance (or cell monolayer resistance). Once the monolayer is exposed to a cytotoxic agent, the impedance changes and measures of toxicity can be quantified long-term. Cells are monitored under physiological conditions for temperature, humidity and CO2 incubation. We investigated the hepatotoxic effects of paracetamol on MH cells when exposed for 24 and 48 hours. In agreement with other standard toxicity assays, such as lactate dehydrogenase release assay, low doses of paracetamol caused transient toxicity and ‘adaptation’ was observed. At higher doses, hepatotoxic effects of paracetamol could be reversed upon washout after 24 hours, but continued exposure caused increased hepatotoxicity. In addition to hepatotoxicity, another validation of the principle is shown for chronic proliferation of cancer cells. Traditional cell proliferation assays involve labeling cells of interest with compounds that become reduced in the environment of metabolically active cells, or by incubating cells with radioactive labels. In this study, murine mammary carcinoma cells (H8N8 and H8N8 T3.2) were used and changes in impedance, and therefore confluency, were used as a measure of toxicity. Intrinsic (dose-dependent) effects of the standard clinical treatment regimen cyclophosphamide, doxorubicin and 5-fluouracil could be identified consistent with other methods of live cell analysis systems. Therefore, the utilized 96-well impedance system in combination with murine mammary carcinoma cells provides a novel tool for investigating therapy resistance of cancer cells in vitro. Ref: 1.Stevens,JL et.al.,Drug Disc Tod, 2.Benesic, A.,et.al 2015. Dig.Dis.
P2699

Board Number: B856
Mutation of dgat2 uncouples lipolysis and lipoprotein synthesis in the zebrafish embryonic digestive organ resulting in excess ectopic lipid droplets.
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During zebrafish development, maternally-deposited yolk is the source of nutrients for embryogenesis prior to digestive system maturation. The yolk syncytial layer (YSL) is the cytoplasmic layer surrounding the yolk mass that metabolizes, re-packages and exports yolk nutrients to the developing embryo. Export of lipid from the YSL is via triglyceride-rich lipoproteins. We show that blocking lipoprotein production, by inhibiting the activity of microsomal triglyceride transfer protein (MTP), results in aberrant accumulation of lipid droplets in the YSL. The dense packing of lipid droplets refracts light such that the yolk takes on a dark appearance. Paradoxically, we have also found that zebrafish embryos with a nonsense mutation in exon 2 of diacylglycerol acyltransferase 2 (dgat2), an ER resident enzyme that synthesizes triacylglycerol from diacylglycerol and fatty acyl-CoA, also exhibit dark yolks indicative of excess lipid. mRNA in situ hybridization indicates dgat2 is expressed in the YSL, as well as in the intestine and liver at later developmental stages. Unlike DGAT2-null mice, which are lipopenic and die soon after birth, zebrafish dgat2 mutants are viable and fertile. Similar to inhibition of MTP, dgat2 mutant embryos have visible accumulation of lipid droplets in the YSL. These droplets vary in size, stain positively with oil-red-O and some can even persist after yolk depletion. Biochemical analyses reveal that the YSL still has remaining dgat activity and lipid droplets are rich in triglycerides and cholesterol esters. Residual dgat activity cannot be explained by the expression of other known dgat isoenzymes (e.g. dgat1a and dgat1b are not expressed in the YSL). Dgat2 mutant embryos synthesize fewer and smaller beta-lipoproteins, and transmission electron microscopy indicates the ER in the YSL of dgat2 mutants is abnormally swollen and electron dense, suggesting possible accumulation of lipid within the leaflets of the ER membrane. Experiments are currently underway to identify the source of the remaining dgat activity and determine why YSL triglyceride export is attenuated. These data suggest that blocking triglyceride export uncouples the rates of yolk degradation and lipid export, forcing the YSL to pack the accumulating lipid species into lipid droplets, likely to prevent lipotoxicity. Future work will be aimed at understanding whether the embryo, YSL and the yolk degradation machinery communicate to ensure the correct provision of nutrients to the body to promote proper embryonic development.

Hematopoietic System

P2700

Board Number: B857
Targeting lipoprotein(a)-induced endothelial cell metabolic changes to reduce inflammation and leukocyte migration.
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Lipoprotein(a) [Lp(a)] is plasma lipoprotein composed of apolipoprotein(a) [apo(a)] covalently bound to apolipoprotein B-100 (apoB) of LDL. Lp(a) is an independent, genetically determined risk factor which is causally related to myocardial infarction, stroke and peripheral artery disease. Despite the fact that one-
fifth of the general population has elevated Lp(a) levels, the lack of established treatment may be attributed to little understanding of the pathophysiology of Lp(a) mediating cardiovascular disease. Recent research substantiated that activation of immune cells lead to metabolic changes due to altered glycolysis-associated gene expression. Although data exists showing Lp(a)-induced inflammation in endothelial cells (EC), the exact molecular pathways remain to be elucidated. Recent in vivo studies from our group showed that patients with elevated levels of Lp(a) had increased metabolic activity in plaque regions, as shown by 18F-FDG PET/CT imaging. This suggests that Lp(a), besides activating the inflammatory phenotype of immune cells, may also be crucial for increasing metabolic activity of the endothelium. Based on these data, we hypothesize that Lp(a) mediates endothelial activation via a marked metabolic shift, facilitating a pro-atherogenic environment. Here, we set out to investigate that endothelial cell inflammation can be reduced by altering EC metabolism. Lp(a) (100mg/dl)-induced endothelial gene expression of key-adhesion molecules E-selectin, ICAM-1 and VCAM-1, the chemokine MCP-1 and the cytokines IL-6 and IL-8 were all significantly upregulated when compared to low-level (10mg/dl) Lp(a)-stimulated EC. Additionally, Lp(a) exposure revealed a marked increase in the production of cytokines IL-6 and IL-8. Functional transendothelial migration (TEM) assays substantiated these findings and showed that Lp(a) stimulation led to increased leukocyte migration. Additionally, we showed that all these effects were attributed to the oxidized phospholipids that were bound to recombinant apo(a). These Lp(a)-induced changes coincided with increased metabolic activity, as shown by the increase in gene expression of key-glycolytic enzymes PFKFB3, HK2 and PFKM. Interestingly, partial glycolytic inhibition using 3PO, an inhibitor of PFKFB3, a concomitant decrease in inflammation was found, suggesting that glycolysis is driving lipoprotein-induced endothelial inflammation. To conclude, these data suggest that Lp(a)-induced inflammation and endothelial metabolic changes are closely intertwined. Inhibition or interference of glycolytic metabolic pathways would be of great clinical interest in order to reduce atherosclerosis.

P2701
Board Number: B858
Plasma sdLDL(small dense LDL) induced to modification of cellular cholesterol transport in foam cells.
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[Objective] Plasma Small dense LDL (sdLDL) was often found in type IIB Hyper cholesterolemia and Hyper triglyceridemia patients, produced by TG rich VLDL. Some correlation between IHD (ischemic Heart Disease), convalescence of IHD, and sdLDL already reported. To elucidate a possibility of activation of ABC transporters and removing FC from foam cells, several PPAR agonists, ARB and vascular statins can be examined as activator to ABCA1 transcription and translocation. [Method] sdLDL was prepared from human plasma by KBr density gradient separation. Foam cells were prepared by J774 cells incubating with sdLDL for 24-72hr, and cultured up to 3-6 days in the presence of LPDS (Lipoprotein deficient serum) +/- HDL and PPAR agonists, ARB or statin. FC rich lipid droplets were detected by Filipin staining. [Results & Discussion] Plasma sdLDL strongly induced lipid droplets in foam cell, as well as acetylated LDL(AcLDL), especially, so many FC-rich lipid droplets were shown in filipin stained cells. HDL treatments activated reverse cholesterol transport in AcLDL-induced foam cells, whereas, FC-rich lipid droplets still remained in sdLDL-induced one. Maybe, HDL defects the FC-removing activity in sdLDL-induced one and its metabolites may reflect on cellular FC transport including ABC transporters transcription and/or translocation. Some ABC transporter activator were tested: PPAR a agonist (Fenofibrate, Ciprofibrate) had a potent activity of inducing ABCA1 and its translocation, was also
known to avoid foam cell death, FC rich lipid droplet formation in AcLDL-induced cells. However, these fibrates molecules did not enough to reduce cellular FC in sdLDL-induced cells. Vascular statin molecules (Simvastatin, Atorvastatin) were well known as a potent activator of ABCA1 translocation, also reduced FC-rich lipid droplet formation, and increase ABCA1 expression level in AcLDL-induced foam cells. However, reverse cholesterol transport activity was reduced in sdLDL-induced one. ARB molecules (Angiotensin II AT1 receptors blockers; Losartan, Irbesartan, Telmisartan) had a potent activity as well as PPAR α, γ agonists. sdLDL-induced foam cell resists to reduce FC from the cells, filipin stained FC-rich lipid droplets still remained. These signify relationship in cellular cholesterol transport in sdLDL-induced foam cells, show the possibility cellular cholesterol transport regulates by the property of lipoproteins and its lipid profiles (apoB modification, PC oxidation, etc) and also accumulation process( scavenger receptors). Among the FC-rich lipid droplet formation, regulation of reverse cholesterol transport and rupture of foam cells in lesion may reflect to development of atherosclerosis correlated in IHD patients. Contact me by; mmori@cis.ac.jp

P2702
Board Number: B859

Oxygen Concentration-Dependent Regulation of Erythropoiesis in Human Erythroblasts.

It is estimated that approximately 2.3 million human erythroblasts extrude their nucleus every second in the hypoxic environment (5% O2 and its concentration) of the bone marrow. Enucleation requires multiple signal transduction pathways and local assembly of a contractile actomyosin ring. We have previously shown that human erythroblasts produce ATP by anaerobic glycolysis based on the following observations: i) the Ser300 residue in pyruvate dehydrogenase (PDH) is phosphorylated by PDH kinase 1 and 4 isozymes; ii) inhibition of lactate dehydrogenase by stiripentol reduces ATP level in the cells and decreases enucleation ratio. However, at which extent hypoxia-inducible factor (HIF1)-α regulates ATP production in human erythroblasts remains unknown. In the present study, we assessed the role of HIF1-α on ATP production in human colony-forming unit-erythroblasts (CFU-Es) and mature erythroblasts generated from purified CD34+ cells treated with the HIF1-α-specific inhibitor, KC7F2 (N,N'-(Dithiodi-2,1-ethanediyl) bis [2,5-dichlorobenzene-sulfon-amide]). CFU-Es cultured in a 5% O2 environment were capable of proliferating and extruding their nucleus as well as at 21% O2. In contrast, in a 1% O2 (hypoxic) environment, proliferation of CD34+ cells was impaired, the cell number being reduced by about 90% compared with a 5% O2 environment after 7 days in culture. Additionally, in a 1% O2 environment, cell morphology after the Day 7 CFU-E (terminal differentiation) stage was dramatically changed and the number of macropaghe-like cells was increased. These results supported that human erythroblasts require at least 5% O2 concentration for proper proliferation and terminal differentiation. HIF1-α expression was confirmed at the mRNA and protein levels during terminal differentiation (Day7 ~ Day13). KC7F2 blocked the proliferation of CFU-Es and enucleation rate in a dose-dependent manner (0 ~ 100 μM). The ATP concentration in CFU-Es was also significantly reduced by the inhibitor treatment. However, KC7F2 did not affect cell morphology nor expression of the erythroblast surface markers glycophorin A (GPA) and CD71. We conclude that human erythroblasts require 5% O2 concentration at least for proliferation despite their ability to produce ATP through anaerobic glycolysis. Our results also suggest that ATP production may be modulated by HIF1-α during terminal differentiation of human erythroblasts.
This work was supported by JSPS KAKENHI Grants 15K09448 (WN), 15K19540 (KU), 17K16177 (YG) and 15K09516 (HW) and by a private donation from Dr. Ken Satoh, Satoh Naika Clinic, Sakata 998-0013, Japan.

P2703
Board Number: B860
Pharmacological activation of LPA receptors regulates murine erythro-megakaryocytic differentiation in myeloid lineage.
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Myeloid cell differentiation is one of the most crucial physiological processes among hematopoiesis to form blood components and replenish blood system. Our previous studies demonstrated that lysophosphatidic acid (LPA) regulates myeloid cell differentiation through activating multiple G protein coupled receptors. However, the underlie mechanism in vivo remains unclear. In this study, we aimed to validate the effects of LPA and its corresponding receptors, LPA₂ and LPA₃ on erythropoiesis/megakaryopoiesis in vivo. By profiling LPA receptors in murine myeloid cells, LPA₂ and LPA₃ were found to express at different stages of progenitors. Mice injected with LPA₂ agonist, GRI977143, showed severe perturbation in both erythroid and megakaryocytic lineages. Further, GRI treatment suppressed populations of common myeloid progenitor (CMP) and megakaryocyte erythrocyte progenitor (MEP), suggesting that LPA₂ inhibited myeloid differentiation at early stage of myelopoiesis. In contrast, treatment with LPA₃ agonist, 1-oleyl-2-O-methyl-rac-glycerophosphoethionate (OMPT), skewed toward erythroid lineage with increased erythroid populations and RBC numbers at the expense of megakaryocyte populations. Finally, OMPT injection successfully restored PHZ-induced acute haemolytic anaemia. Taken together, this study demonstrated for the first time that LPA₂ and LPA₃ acted at different stages to regulate hematopoiesis in vivo, and provide a potential therapeutic strategy for future treatment of anemia.

P2704
Board Number: B861
Changes in the Proliferation and Gene Expression of HUVECs in Response to Treatment with Plant Secondary Metabolites.
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Medicinal plants have historically been a valuable source of new drugs and Southern California possesses a rich collection of native plants which have been used as medicines by native people groups for thousands of years. Angiogenesis is the biological process of new blood-vessel growth from endothelial cells. It is an essential part of the wound-healing process, and increased angiogenesis has also been implicated in the growth of some types of cancerous tumors. In this study, extracts of the Southern Californian native plants Red shanks (Adenostoma sparsifolium) and the alkaloid extract of Jimson weed (Datura wrightii) were tested for their angiogenic effects in human umbilical vein endothelial cells (HUVECs) using a cell proliferation assay, nitric oxide production assay, and qRT-PCR. Treatment of HUVECs with extracts from Red shanks and Jimson weed increased proliferation at concentrations of 10 ng/µL and 1 ng/µL, but no changes in nitric oxide production were measured for any treatment. Changes in expression of the genes vegfr2 and mmp9 were measured by qRT-PCR and
suggested that proliferation may be induced by these plant extracts through factors that bypass normal nitric oxide-mediated proliferative signals.

P2705
Board Number: B862
Characterization of the mechanically-induced shape change of erythrocytes into polyhedrocytes.
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Polyhedral structures have been observed in nature since the time of Aristotle; however, most research has focused on Platonic and Archimedean solids rather than irregular polyhedra. Moreover, these tightly packed cells were recently observed in contracted blood clots, where the highly deformable erythrocytes were compressed into the core of the clot and formed a tessellated network. This resulted in a shape change from the normal biconcave cell to a polyhedral shape; consequentially the terminology polyhedrocytes was coined. Since hemostatic and thrombotic disorders are leading causes of death and disability worldwide, exploring the nature of the shape change from biconcave erythrocytes to polyhedrocytes has the potential to inform therapeutic targets. Here, we use histology, transmission electron microscopy, scanning electron microscopy and confocal microscopy to visualize and quantify the mechanical deformation of erythrocytes. We determined that this shape change is linked to the presence of contractile forces that are generated by the platelets and highly influenced by the composition of the clot. The presence of more platelets or more platelet activity, as induced by a higher thrombin concentration, resulted in a direct correlation with the percentage of polyhedrocytes present. Likewise, the inhibition of contractile proteins or the ability of platelets to propagate contractile force resulted in softer clots with fewer polyhedrocytes present on the inside of the clot. Through the use of confocal microscopy, we were able to reconstruct the three-dimensional structure of erythrocytes from contracted blood clots and compare bioconcave cells, polyhedrocytes and intermediate forms. Interestingly, as the erythrocytes undergo deformation, there was no change in the surface area, volume, or sphericity of the cells. The deformed erythrocytes and polyhedrocytes became less oblate and more prolate than the biconcave cells. Collectively, these studies reveal that polyhedrocytes are convex, irregular polyhedra. The polyhedrocytes had a total number of faces that ranged from 10 to 16 with a median of 13 faces. The faces were made up of polygons with 3 to 6 sides, with the majority of the faces being quadrilaterals. Taken together, these results point to the importance of studying the deformation of erythrocytes as a result of clot contraction into a tightly packed network, since these structures could provide a key function of replicating the barrier function of the endothelial layer post injury and/or influence the outcome of thrombotic conditions.

P2706
Board Number: B863
Immunosuppressive drug causes Hepatic iron overload.
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The introduction of calcineurin inhibitor tacrolimus greatly reduced the rate of allograft rejection. Along with having, immunosuppressive properties it can also cause adverse side effects. Recipient's iron status is an important determinant of clinical outcome in transplantation medicine. The current experimental
setup was designed to analyze the tacrolimus induced hepatic iron overload in wistar rats. Aqueous suspension of tacrolimus powder (3 mg/ml) was orally given to four experimental groups of wistar rats. While control group was provided with normal drinking water and dissections were done after 6, 12, 24 and 48 h of tacrolimus suspension administration. Real-time PCR, ELISA and Prussian blue iron staining were performed to evaluate hepatic iron contents. Real-time PCR analysis showed an initial upregulation in the expression of hepcidin at 6h (1.95±0.2 fold) and 12h (2.1±0.05 fold) time points. Ferritin-L expression found to be significantly upregulated at 6h (1.82±0.14 fold) and 12h (1.69±0.15 fold) time points in response to tacrolimus dose and after 12h start to decline towards baseline level. Ferritin-H expression was also found to be significantly upregulated at 12h (2.08±0.29 fold) and 24h (2.08±0.20 fold) time points when analyzed by one way ANOVA. Transferrin gene expression was found significantly upregulated at 12h (1.92±0.19 fold) time point. Early upregulation at 6h (1.81±0.19 fold) was also observed but this difference remained statistically non significant. An early statistically significant upregulation of transferrin receptor 1 expression was observed at 6h (5.30±0.9 fold) and 12h (4.89±0.56 fold) time points as compared to control. Serum Hepcidin concentration was measured via quantitative sandwich ELISA, showed no significant alterations in counter to toxic effect of tacrolimus (p=0.8968). Hepatic iron staining showed blue stained hemosiderin granules within the hepatocytes, sinusoidal spaces and portal areas at 12 and 24h time points. These clearly noticeable bluish granules were directed consideration towards the storage of iron in the liver. A mild level of iron deposition was also observed at 48h time point as compared to control. These findings indicate that tacrolimus use resulted in hepatotoxicity and causes iron overload in hepatic cells of wistar rats.

P2707
Board Number: B864
Apoptosis Signal-Regulating Kinase 1 (ASK1) is a novel regulator of heparin-induced thrombocytopenia and thrombosis in mice.
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Heparin-induced thrombocytopenia and thrombosis (HITT) is a life-threatening (20-30% mortality risk) disease in which IgG antibodies against the heparin-PF4 complex bind and activate platelets via FcγRIIA. Our laboratory has identified that the Apoptosis Signal-Regulating Kinase (ASK1), a MAP3K, is present in both human and murine platelets and potentiates many platelet functions. Given that ASK1 regulates platelet function, and that platelets are known to play a major role in the pathogenesis of HITT, we hypothesized that ASK1 is a novel regulator of HITT.
To establish if ASK1 is activated downstream of FcγRIIA, we first stimulated washed human platelets with anti-CD9 (700ng/mL). Anti-CD9 induces the activation of FcγRIIA. We found that anti-CD9 induced a robust activation of ASK1, as measured by phosphorylation of ASK1 Thr845 (a marker of ASK1’s kinase activity). ASK1 exclusively activates p38 in platelets; therefore we also measured phosphorylation of p38 a marker of ASK1’s signaling activity. We found that anti-CD9 also induced a robust phosphorylation of p38.
To determine the role ASK1 plays in platelet-FcγRIIA signaling and HITT, we crossed Ask1−/− mice to FcγRIIA+/+ (hFcR) mice. We found that genetic ablation of Ask1 did not have any effect on anti-CD9-induced activation of PLCγ2 or Syk in hFcR/Ask1−/− (KO) platelets compared to hFcR/Ask1+/+ (WT).
However, loss of Ask1 did result in the complete absence of p38 activation in KO platelets following activation of FcγRIIA by anti-CD9 (500ng/mL). Further, we observed that anti-CD9-induced integrin αIIbβ3 activation, α-granule secretion, and platelet aggregation were all significantly attenuated in KO platelets.

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(P<0.01). These in vitro results strongly suggested that ASK1 plays a prominent role in FcγRIIA-mediated platelet activation.

To further investigate the role of ASK1 in HITT pathogenesis, WT and KO mice were subjected to an in vivo model of HITT by injecting anti-CD9 IgG. Platelet counts were measured in samples of whole blood collected at various time points post-injection, and compared to resting platelet counts taken before HITT was initiated. We found that KO mice were significantly (P<0.01) protected from thrombocytopenia compared to WT mice when injected with 250μg/kg anti-CD9. However, when injected with 500μg/kg anti-CD9, both WT and KO mice displayed the same level of thrombocytopenia. Despite not protecting from thrombocytopenia, when observed for signs of shock, we found that KO mice were significantly (P<0.01) protected from anti-CD9 induced shock compared to WT mice regardless of the dose.

Taken together these in vitro and in vivo data strongly suggest that ASK1 regulates FcγRIIA-mediated platelet activation, and that ASK1 plays a key role in the pathogenesis of HITT.

P2708
Board Number: B865
Vitronectin regulates the fibrinolytic system and inflammation during the repair of cerebral cortex in stab-wounded mice.
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Vitronectin (VN), one of the serum proteins, is known to be involved in the regulation of blood coagulation, fibrinolysis, and cell migration. It has been proposed that the regulation of fibrinolysis by VN promotes the blood brain barrier (BBB) recovery from brain injuries such as traumatic injury and subarachnoid hemorrhage. However, the effects of VN on fibrinolysis in the injured brain remain unclear. Here, we examined the effects of VN on the fibrinolytic system in the stab-wounded cerebral cortex of VN-knockout (KO) mice. First, hemorrhage and recovery from BBB breakdown in the wounded regions were assessed by serum immunoglobulin G (IgG) extravasation. The level of IgG extravasation increased 3–7 days after the stab wound (D3–7) in the cortex of VN-KO mice, compared with that in wild type (WT) mice, indicating that VN deficiency inhibited the recovery from BBB breakdown. VN deficiency decreased fibrin fiber deposition at D1–3, suggesting that VN deficiency tilts the balance between fibrinogenesis and fibrinolysis towards fibrinolysis. Next, the effects of VN deficiency on the fibrinolytic factors were analyzed in the stab-wounded cortex. VN deficiency impaired the activity of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of the fibrinolytic system, at D3–5. Moreover, VN deficiency up-regulated the mRNA and protein expression levels of tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA). Furthermore, we examined whether VN regulates the activity of astrocyte, which plays an important role in the recovery of BBB. To address this problem, we analyzed the expression level of GFAP in stab-wounded VN-KO mice. The expression level of GFAP mRNA was increased in VN-KO mice at D5, compared with that of WT mice. Then, immunostaining of GFAP around the lesion showed that activity of GFAP was increased in VN-KO mice at D3 - D7. It was suggested that VN regulates the expression level of astrocyte in the wounded cerebral cortex. Moreover, we analyzed the effect of VN loss on the mRNA expression level of pro-inflammatory cytokines, IL-1beta, TNFalpha, and IL-6 in the lesion at D0-7. These cytokine expression profiles in VN-KO mice were increased from these of WT mice, suggesting that VN deficiency affects the inflammation
after the stab wound. These results demonstrate that VN contributes to the regulation of the fibrinolytic system and the inflammation, and recovery from BBB breakdown in the wounded brain.

P2709
Board Number: B866
Neuropeptide Y induces hematopoietic stem/progenitor cell mobilization by regulating matrix metalloproteinase-9 activity through Y1 receptor in osteoblasts.
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Hematopoietic stem/progenitor cell (HSPC) mobilization is an essential homeostatic process regulated by the interaction of cellular and molecular components in bone marrow niches. It has been shown by others that neurotransmitters released from the sympathetic nervous system regulate HSPC egress from bone marrow to peripheral blood. In this study we investigate the functional role of neuropeptide Y (NPY) on this process. NPY deficient mice had significantly impaired HSPC mobilization due to increased expression of HSPC maintenance factors by reduction of matrix metalloproteinase-9 (MMP-9) activity in bone marrow. Pharmacological or endogenous elevation of NPY led to decrease of HSPC maintenance factors expression by activating MMP-9 in osteoblasts, resulting in HSPC mobilization. Mice in which the Y1 receptor was deleted in osteoblasts did not exhibit HSPC mobilization by NPY. Furthermore, NPY treatment in ovariectomized mice caused reduction of bone loss due to HSPC mobilization. These results suggest a new role of NPY on HSPC mobilization, as well as the potential therapeutic application of this neuropeptide for stem cell-based therapy.

P2710
Board Number: B867
Partial Refractoriness of Platelets in Thrombotic Conditions.
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Platelets, the smallest blood cells, play a crucial role in formation of thrombi associated with hemodynamic disorders and dysfunction of tissues and organs. In addition to the pro-coagulant membrane surface of activated platelets and their ability to adhere and aggregate, platelets can cause a volume shrinkage (contraction or retraction) of blood clots, driven by the contractile forces generated by activated platelets attached to fibrin fibers. Any changes in platelet count and functionality can lead to an increase or decrease of the risk of thrombosis and affect its course and outcome. The aim of this study was to assess the functional state of platelets in patients with venous thromboembolism (VTE) and arterial thrombosis, such as acute ischemic stroke (AIS). Platelets were isolated from the blood plasma by gel filtration. Using scanning electron microscopy, morphological signs of activation of platelets isolated from 4 patients with VTE, 6 patients with stroke and 7 healthy donors were examined. At least 10 randomly selected fields were analyzed for each platelet preparation and a total of 4211 individual cells from VTE patients, 892 cells from AIS patients, and 791 cells from healthy donors were examined. The functional state of quiescent and activated platelets was evaluated using flow cytometry by the expression of P-selectin (probed by fluorophore-labeled antibodies to CD62p) and the active αIIbβ3 integrin (probed using fluorophore-labeled fibrinogen) before and after addition of the thrombin receptor-activating peptide (TRAP). The fractions of platelets with morphological signs of activation
(formation of filopodia, loss of the discoid shape) were much greater in patients with VTE (75%) and AIS (34%) compared to healthy donors (9%, p <0.0001). Flow cytometry revealed that platelets both from the AIS and VTE patients displayed a reduced response to stimulation by TRAP. The fractions of TRAP-activated platelets expressing P-selectin in AIS (71±7%) and VTE (30±7%) were significantly smaller compared to normal platelets (84±4% P<0.0001). The fractions of stimulated cells expressing the fibrinogen-binding active αIIbβ3 integrin (43±7% in AIS and 41±8% in VTE) were also significantly smaller compared to TRAP-activated normal cells (60±7% p <0.01). The results show that platelets in thrombotic conditions, such as VTE and AIS, undergo chronic continuous activation, which can lead to their exhaustion and secondary dysfunction manifesting as the reduced responsiveness to a chemical stimulus, i.e., partial refractoriness and a decrease of the overall activation potential. Research supported by the Program for Competitive Growth at Kazan Federal University.

Therapies: Design and Mechanisms for Normal and Diseased Organs 1

P2711
Board Number: B868
A non-saponin fraction from Korean red ginseng prevents TNF-α-induced muscle atrophy via regulation of Akt/mTOR signaling in C2C12 myotubes.
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Korean red ginseng is a representative tonic herbal medicine that help to strengthen body muscles and bones making them stronger. In this study, we investigated the effects of a non-saponin fraction from Korean red ginseng (RGNSF) on muscle atrophy and the underlying mechanisms involved. Tumor necrosis factor alpha (TNF-α)-induced myotube atrophy was accompanied by increased muscle specific ubiquitin E3 ligase markers, such as, Atrogin-1 and myostatin, and decreased MyoD in C2C12 myotubes. Co-treatment of RGNSF with TNF-α was significantly inhibited TNF-α-induced myotube atrophy in C2C12 myotubes. Consistent with these results, RGNSF effectively prevented atrogines atrogin-1 and muscle RING-finger protein 1 upregulations by TNF-α, and increased the expression level of myoblast differentiation markers (myoD and myogenin). Furthermore, RGNSF also resulted in the upregulation of the phosphorylation of Akt and mammalian target of rapamycin (mTOR). These findings suggest that RGNSF prevents TNF-α-induced muscle atrophy via regulation of Akt/mTOR signaling pathway, suggesting that RGNSF is invaluable for the development of therapeutic medicines to prevent disuse muscle atrophy and its accompanying muscle weakness.

P2712
Board Number: B869
Transmembrane BAX Inhibitor Motif-6 (TMBIM6) protects against Cisplatin-induced testicular toxicity.
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Study question: What is the molecular mechanism by which cisplatin causes reproductive toxicity?
Summary answer: TMBIM6 protects against cisplatin-induced testicular toxicity through heme oxygenase-1 (HO-1)–associated ROS inhibition. Moreover, TMBIM6 maintains the levels of steroidogenic enzymes through control of ER redox balance and its associated ER stress.

What is known already: Testosterone production is highly suppressed as a main complication of cisplatin (cis-diaminedichloroplatinum) anticancer therapy.

Study design, size, and duration: After exposure to cisplatin for 3 days, testis and serum were collected from Tmbim6 knock-out (KO) and wild type (WT) mice. In the Tmbim6-lentivirus-mediated testicular expression-rescued KO mice, phenotypes were analyzed. Tmbim6-expressing TM3 mouse Leydig cells were also used in this study.

Participants/materials, setting, and methods: In this study, 8- to 10-week-old Tmbim6 KO and WT male mice received cisplatin (30 mg/kg, single IP). On the 3rd day, blood and testis tissue samples were collected. Testosterone level and testicular weight and structure were compared between the groups. Quantitative PCR, immunoblot, and assays for ROS, HO-1 activity, and PDI carbonylation were performed.

Main results and the role of chance: p-Akt, Nrf2, and its downstream heme oxygenase-1 (HO-1) and the levels of testosterone synthesis-associated enzymes, including steroidogenic acute regulatory protein (StAR), a rate limiting enzyme for testosterone production, were significantly expressed and maintained in the presence of Tmbim6. Excessive post-translational oxidation of protein disulfide isomerase (PDI), altered folding capacitance and ROS accumulation, and ER stress were also regulated in the presence of Tmbim6. Higher levels of ER stress and protein hypercarbonylation were consistently observed in KO testes compared with WT testes. In the Tmbim6 KO mice, lentivirus-mediated testicular expression of Tmbim6 rescued the above phenotypes. Furthermore, the regulatory role of Tmbim6 against testis toxicity was consistently shown in Tmbim6-overexpressing TM3 Leydig cells, testosterone producing cells. In this study, TMBIM6 is suggested to have a regulatory role against cisplatin-induced testis toxicity by inducing HO-1 and enhancing ER folding capacitance.

Wider implications of the findings: The findings implicate cisplatin as having a role in organ toxicity, including that of the kidney. Furthermore, ER stress and redox imbalance should be considered as other anticancer therapy-associated toxicity mechanisms.

P2713
Board Number: B870
Immune Enhancement of Fucoidan in Raw264.7 cells.
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For a long time, some types of brown algae have been a regular part of the diet in many Asian countries, especially Japan and Korea. Fucoidan is a complex sulfated polysaccharide which is found in the cell walls of several edible brown algae. The compositions and structure of fucoidan vary among different brown seaweed species. But generally the compound consists primarily of L-fucose and sulfate, along with small quantities of D-galactose, D-mannose, D-xylene, and uronic acid. Many previous reports have shown that fucoidan exerts anti-bacterial, anti-viral, anti-coagulant, anticancer, antioxidant, anti-inflammatory, and immunomodulatory effects. We experimented with fucoidan samples separated into 80-90 kDa and 200 kDa (or more). We treated fucoidan in Raw264.7 at different concentrations and measured nitric oxide production using Griess Reagent. As a result, nitric oxide production increased at treatment of 200 kDa (or more) fucoidan, meaning that Raw264.7 cells were differentiated into M1 type macrophages. Based on this, mRNA and cytokines such as IL-1β, IL-6, IL-10 and TNF-α were measured in

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supernatants of Raw 264.7. We also confirmed COX2 and NF-kB protein expression. Taken together, fucoidan could be used a potential nutrition therapeutic agent for immune deficiency patients.

P2714
Board Number: B871
In vivo cellular reprogramming to restore respiratory function after SCI.
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The injured adult mammalian spinal cord is incapable of significant repair. This limitation is due in part to two major neuropathological consequences of spinal cord injury (SCI): i) the formation of growth-inhibitory glial scars, of which activated astrocytes are a key component, and ii) the destruction of intraspinal neuronal connectivity, contributed to by the loss of the interneurons in the spinal circuitry. Previous cell-based strategies have traditionally been focused on transplantation of various neural stem cells into the injury site to replace lost neurons, improve the inhibitory environment and modulate inflammation; however, there are significant hurdles to their applications. For example, obtaining sufficient amounts of purified cells for transplantation may be difficult; the procedure often requires the use of immuno-suppression, which has detrimental effects on the host; and successful implementation of such a strategy needs to address the challenges of cell survival and appropriate cell differentiation without formation of tumors. Here we applied an in vitro and in vivo direct conversion strategy to reprogram activated astrocytes to potential functional interneurons by introducing into the injured site a single lentiviral vector encoding Ascl1, which serves as a major neurogenic regulator CNS interneurons. Our in vitro data revealed that Ascl1 is a potent reprogramming factor that was able to convert more than 40% of the activated astrocytes into functional neurons in cultures. These cells not only were stained positive for pan-neuronal markers, but also were mature enough to bear typical neuronal electrophysiological properties. Successful in vivo conversions in adult rats were also achieved using the same strategy, yet with a much more diminished efficiency (On average, 4 converted neurons from each animal were identified) 6 weeks after the viral injection followed by the contusive cervical SCI. As we expected, functional examinations such as plethysmography and terminal diaphragm electromyography showed no significant improvement. However, a substantial reduction of the glial scars was identified in Ascl1 treated animals. Based on the data obtained so far, we posit that to achieve more efficient in vivo conversions, a longer duration may be required following the viral injection. Despite the lack of functional recovery from our current treatment strategy for SCI, our pilot study serves as a proof-of-concept for its potential translational applications. Meanwhile, we are adjusting our viral content in order to achieve a more efficient in vivo conversion for SCI therapies. (This work was funded by a grant from the Craig H. Neilsen Foundation to LQ).

P2715
Board Number: B872
The study of new quaternary ammonium compounds specific activity to gram positive and gram negative bacteria in vitro and in vivo.
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Prevention and successful treatment of infectious diseases are the critical aspects of modern healthcare. Quaternary ammonium compounds (QAC) are currently one of the promising classes of antiseptics and
disinfectants. However, its common use for medical application is limited due to bacterial resistance to these compounds. Development of new antimicrobial agents by functionalization of QACs with different substituents can solve this problem. In this work, we studied new potential antiseptics based on quaternary ammonium pyridoxine derivatives. As part of this study, in vitro and in vivo bactericidal activity of the obtained compound against gram-positive (Staphylococcus aureus ATCC209p) and gram-negative (Escherichia coli CDCF-50) bacteria in comparison with benzalkonium chloride, miramistin and chlorhexidine digluconate was evaluated. The mechanisms of membrane-damaging action of these compounds and their influence on intracellular processes of bacterial cells were also studied. Bactericidal efficacy of quaternary ammonium pyridoxine derivatives against several museum strains of microorganisms was evaluated by serial dilution method, quantitative suspension test method, metal surface disinfection test, and in vivo rat skin test. For all compounds a relatively high bactericidal activity against gram-positive and gram-negative bacteria has been observed. The ability of the obtained compounds to change the electric potential of the bacterial membrane was studied using membrane-potential-sensitive cyanine dye DIOC2(3). Outer membrane permeability activity of gram-negative bacteria was determined using a fluorescent assay with N-phenyl-1-naphthylamine dye. It was demonstrated that the treatment by the studied compounds led to rapid and dramatic damage in structure of the bacterial membrane. In particular, a concentration-dependent response in permeabilizing the outer membrane was observed. It was also established that the synthesized compounds can cause/initiate strong depolarization of bacterial membrane. The obtained results suggest that the developed compounds are promising candidates for development of promising antiseptics and disinfectants.

P2716
Board Number: B873
Ametryn causes alterations of the prostatic components percentage of adult Wistar rats.
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Recently, investigations of the association of various diseases to environmental and/or occupational exposures to pesticides have increased substantially. Ametryn is one of the most widely used herbicides in the world, probably because of the excellent performance of its selective effect on weeds. However, knowledge of its effect on reproductive parameters is still scarce. Several natural compounds are traditionally used in popular medicine. Many have shown antitumor and antioxidant properties. Brazilian green propolis is a natural compound widely used by local populations and has been shown to act as an effective anti-free radical agent, DNA protector and immune system stimulant. Thus, the present study evaluated, through morphometric, stereological and biochemical analyses, the effect of ametryn herbicide on the prostate of adult Wistar rats and the possible protective effect of green propolis on the prostate in these rats treated with ametryn. 36 animals were used, distributed equally in 3 groups: C (control), A (ametryn), P+A (propolis + ametryn). The C group received distilled water (0.5 ml), whereas A group received ametryn (15 mg/kg/day) and P+A group received propolis and ametryn (6 and 15 mg/kg/day, respectively), all by gavage. The animals received commercial rat chow and water ad libitum and after 56 days, the animals were euthanized, the ventral prostate was collected, weighed and processed for histological analysis. Subsequently, it was photographed with a camera attached to a light microscope (Olympus BX-40). Morphometric and stereological analyses were performed with IMAGE PRO PLUS 6.0 software. Blood was collected in a vacuum tube (Vacuette) with a separator gel and centrifuged for serum separation, the samples were refrigerated (2 to 8°C) until analysis. Total serum PSA and testosterone levels were measured by the ELISA technique. All statistical analyses were
performed with PRISM 5 STATISTIC Program. No significant differences of PSA hormone or ventral prostrate weight were found for the groups evaluated. These results are consistent with the measurement of testosterone levels, which were also not significantly altered. Stereological evaluation showed a significant reduction in volumetric ratio (%) of Muscular Stroma found by comparing the A and P+A groups with the C group. Thus, it can be affirmed that the ametryn treatment used in this experiment, significantly affects rat’s prostatic tissue, therefore could cause reproductive abnormalities in males. Green propolis has no protective effect on the damage caused by ametryn.

P2717

Board Number: B874

Polyglutamine length dependent structural properties and phase behavior of huntingtin exon 1.

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Expansion of the polyglutamine (polyQ) stretch within the first exon of the huntingtin protein (Httex1) above 36-37 glutamines leads to Huntington’s disease (HD). The existence of a pathogenic polyQ-length threshold has led to the hypothesis that monomeric Httex1 undergoes a sharp conformational change when the polyQ length crosses 36-37 residues. Poor solubility of Httex1 has prevented the testing of this hypothesis using high-resolution structural studies. We overcome this limitation by performing single molecule Förster resonance energy transfer measurements on site-specifically labeled Httex1 proteins of five different polyQ lengths. By integrating the results of these measurements with atomistic simulations, we find that irrespective of polyQ length, monomeric Httex1 adopts tadpole-like architectures. Our results argue against a sharp, polyQ-length-dependent conformational change within monomeric Httex1 — a result that is further supported by hydrogen-deuterium exchange mass spectrometry experiments. Instead, we propose that the continuous increase in surface area of the polyQ domain with increasing polyQ length modulates the drive to form homotypic and heterotypic interactions. Particularly, we find that increasing the polyQ length decreases the concentration threshold at which Httex1 aggregation is observed. Together these results suggest structure-based drug design is a challenging route to pursue for HD therapeutics. Alternatively, specifically targeting distinct aggregation phases has the possibility to homogenize the underlying aggregation distribution and shift the distribution away from toxic species. Profilin, an abundant cellular protein, has been shown to reduce Httex1 aggregation and toxicity in cells. We find that profilin reduces Httex1 aggregation by preferentially binding and thus stabilizing one of Httex1’s soluble aggregation phases. This result suggests that the use of ligands to shift phase boundaries might be a promising route to designing therapeutics for treating diseases associated with protein aggregation.
P2718

Board Number: B875

Human adipose-derived multipotent mesenchymal stromal cells as a new target for a cell-mediated drug delivery.

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Rationale: Lately, a number of preclinical and clinical studies have demonstrated the efficiency of mesenchymal stromal cells to serve as an excellent base for a cell-mediated drug delivery system. Cell-based targeted drug delivery has received much attention as a delivery systems to facilitate the incorporation and transfer of active substances to specific organs and tissues with high efficiency. Human adipose-derived multipotent mesenchymal stromal cells (AMMSCs) represent an ideal target cell because of their ease of isolation and phagocytic function.

Methods: AMMSCs were isolated from the adipose of healthy donors with signed informed consent according to the guidelines approved by the Ethics Committee, Immanuel Kant Baltic Federal University (Kaliningrad, Russian Federation). Isolated AMMSCs in culture exhibited positive staining for osteoblast, chondroblast and adipocyte markers, confirming their origin. Capsules, with average size of 5 microns were made using alternated adsorption of polyelectrolytes (calcium carbonate) to generate microparticles labeled by FITC-BSA. The isolated AMMSCs were incubated with FITC-labeled capsules at various ratios for 16 hrs, then cells were either analyzed for surface markers by flow cytometry or were cultured for up to 190 hrs. Cultured cells were enumerated and analyzed for viability, motility and phagocytosis using Cell-IQ v2 MLF integrated platform for a continuous real-time phase-contrast imaging. AMMSCs incubated with FITC-labeled antibodies to CD90 were used as control.

Results: AMMSCs incubated with FITC-capsules at ratio 1:5, 1:20 and 1:45 did not change their phenotype profile, however at ratio 1:90 cells shifted their phenotype towards macrophages (increased CD45, CD34, CD14, CD20 and decreased CD90, CD105, CD73). FITC-capsules were taken up by AMMSCs cells at all time points up to 30 hrs. Cell motility (pxl/h) was capsules-loading dependent. Cells with ratio 1:45 capsules per cell exhibited 94, 90, 88 % and cells with ratio 1:90 exhibit 89, 87 and 84 % of motility from control for 2, 6 and 10 hours incubation post phagocytosis, respectively.

Conclusions: Here we identified that human AMMSCs can be efficiently labeled with microencapsulated FITC-BSA with up to 90 capsules per cell. Further, capsules loaded cells can survive up to 6 days in vitro, maintaining their motility up to 30 hrs post phagocytosis. Thus, the strategy of using AMMSCs cells as a drug delivery vehicle across the tissue-blood or tumor-blood barrier to facilitate treatment of stroke, cancer or inflammatory diseases may open a new prospective therapeutic approach.

P2719

Board Number: B876

Ultrastructural studies of phagocytosis of synthetic microcapsules by human polymorphonuclear leukocytes.

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Rationale: Neutrophils or polymorphonuclear leukocytes (PMNLs) are the most abundant leukocytes in human blood and are potential carriers of diagnostic or therapeutic agents to areas of acute inflammation. A neutrophil-based drug delivery system, therefore, has great potential for the treatment of several types of brain disorders that involve neutrophil infiltration, such as multiple sclerosis, Alzheimer’s disease, stroke and traumatic brain injury.

Methods: Human PMNLs were isolated from the blood of healthy donors with signed informed consent according to the guidelines approved by the Ethical Committee of Kazan State Medical Academy (Kazan, Russian Federation). Capsules with an average size of 2 microns were fabricated using alternating adsorption layers of polyelectrolytes onto calcium carbonate micro-particles used as a sacrificial template. Capsule shells were labelled either with FITC-BSA or with gold nanoparticles incorporated in between polyelectrolyte layers. The isolated PMNLs were enumerated and then incubated with labeled capsules at various cell/capsule ratios ranging from 1:1 to 1:10 for 20 minutes at 37°C. Immediately after incubation, the cells were washed with cold phosphate-buffered saline and collected by centrifugation. PMNL incorporation of capsules was analyzed by flow cytometry and transmission electron microscopy.

Results: Flow cytometry analysis of PMNLs demonstrated successful intracellular capsule incorporation at all ratios, with maximum yield at a cell/capsules ratio of 1:6. Transmission electron microscopy demonstrated that 1-5 capsules were observed per cell and appeared as variable oval (deformable) structures ~0.5-2.5 µm in size with an electron-dense contour. Some capsules were located inside phagolysosomes, while others were in the cytosol. At a cell/capsule ratio of 1:5 or less various signs of cell damage were observed, such as reduction of microvilli, rounding of the cell contour, condensation of heterochromatin in the nucleus, and partial disintegration of the plasma membrane.

Conclusions: Here, we have demonstrated that capsules can be successfully incorporated into human PMNLs. Further, we have established the appropriate conditions to maximize the internalization without damaging cell integrity. These steps establish the possibility of using patient-derived neutrophils as a vehicle for microencapsulated drug delivery. This may be a promising strategy for targeted therapies in human diseases in which PMNLs play a role, such as stroke and traumatic brain injury.

Funded by Russian State project «Science», № 4.5752.2017/8.9 and by the Program for Competitive Growth at Kazan Federal University.
P2720

Board Number: B877

Inhibitory effects of oleuropein on interleukin-4-induced asthmatic inflammation and emphysema in a smoke/OVA mouse model.
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Chronic obstructive pulmonary diseases such as chronic bronchitis and emphysema are progressive lung diseases characterized by irreversible airflow obstruction and chronic inflammation of the airways. The current study investigated that oleuropein, a phenylethanoid found in olive leaves, inhibited eosinophilic airway inflammation and emphysema in interleukin (IL)-4-exposed airway epithelial cells and in cigarette smoke/OVA-exposed BALB/c mice. Nontoxic oleuropein at 1-20 μM attenuated the induction of eosinophil chemotactic protein eotaxin-1 and its receptor CCR3 in 50 ng/ml IL-4-stimulated epithelial cells. The IL-4 production and CCR3 induction increased in smoke/OVA-induced emphysema. Oral supplementation of 10-20 mg/kg oleuropein reduced the number of eosinophils and neutrophils elevated in bronchoalveolar lavage fluid of cigarette smoke/OVA-exposed mice. In addition, oleuropein suppressed the induction of CD11b(+), F4/80(+) and mucin SAC with inhibition of small airway destruction in cigarette smoke/OVA-exposed mouse airways and lungs. These results demonstrated that oleuropein inhibited eosinophilia and inexorable emphysema associated with asthmatic inflammation prompted by cigarette smoke and OVA irritants. Therefore, oleuropein may be a potential agent targeting against inflammation-associated obstructive pulmonary disease.

P2721

Board Number: B878

Developing Novel Inhibitors of Gamma-Glutamyl Transpeptidase.
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Inhibitors of gamma-glutamyl transpeptidase (GGT1, a.k.a. gamma-glutamyl transferase) are being sought due to their therapeutic potential in the treatment of asthma, reperfusion injury, cardiovascular disease and cancer. GGT1 is a cell surface enzyme that cleaves extracellular gamma-glutamyl compounds including oxidized and reduced glutathione and glutathione S-conjugates. Among the many substrates of GGT1 is leukotriene C₄, which GGT1 metabolizes to leukotriene D₄, a potent mediator of inflammation. GGT1 on the surface of the renal proximal tubules functions in the reclamation of cysteine from glutathione as the blood is filtered through the kidney, and metabolizes glutathione S-conjugates as part of the mercapturic acid pathway. The inhibitors of GGT1 that have been evaluated in the clinic are glutamate analogs and are too toxic for clinical use. To pursue a structure-based drug-design strategy in the development of less toxic inhibitors, we determined the first crystal structure of human GGT1 (hGGT1). We have co-crystallized human GGT with a series of compounds that bind within the active site and identified residues within the active site that are critical for inhibitor binding. We are developing new classes of GGT inhibitors that are not glutamate analogs. These structural data provide new insights for continued development of a potent, specific, non-toxic inhibitor of GGT that can be used clinically. Supported by an Institutional Development Award (IDeA) from the National Institutes of Health under grant P20GM103640.
P2722
Board Number: B879
Differentiation of human induced pluripotent stem cells (hiPSCs) with a 57 kb ctns deletion.
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Cystinosis is an autosomal recessive disease caused by mutations in the ctns gene, which encodes for cystinosin, a membrane transport protein responsible for the extrusion of cystine from lysosomes. In cystinosis, the cystine levels increase in all cells in the body due to defective cystinosin function. However, infantile cystinosis is characterized in particular by defective renal proximal tubule (RPT) reabsorptive function. Infants with this disorder to exhibit increased urination, thirst, dehydration, and ultimately a limited lifespan. To develop new therapies, we developed hiPSCs from cystinotic fibroblasts with a 57 kb deletion in ctns (as well as normal counterparts), having transduced the fibroblasts with 3 vectors (pCXLE-hUL encoding for L-myc and Lin28; pCXLE-hSK encoding for Sox2 and Klf4, and CXL encoding for Oct3/4). The resulting normal and cystinotic hiPSCs exhibited a morphology typical of hiPSCs, and an infinite lifespan. However, the cystinotic hiPSCs exhibit some differences, including an altered morphology, and a decreased growth rate (presumably due to oxidative stress, as indicated by their differing ascorbate requirements). Indicative of their pluripotent state, both mutant and wildtype hiPSCs not only express pluripotent markers (Oct3, Sox2, SSEA4 and Tra-1-60), but also exhibit the capacity to form embryoid bodies with 3 germ layers (as indicated by the expression of α-fetoprotein (endoderm), β3-tubulin (ectoderm), and α-smooth muscle actin (mesoderm)). Both the normal hiPSCs as well as hiPSCs with the 57 kb deletion have been differentiated into RPTs, by treatment first with CHIR99021 (to form mesoderm), then with FGF2 and retinoic acid (to form inner mesoderm), and finally with insulin and transferrin alone (to form proximal tubules) (Lam et al., JASN 25:1). RPTs derived from normal hiPSCs possess the polarized morphology and brush border typical of this nephron segment, as well as transport markers including the Sodium/Phosphate cotransport system (NPT2a), and the associated NHE3 Regulatory Factor (NHERF). While cystinotic hiPSCs have been observed to form tubule by fluorescence microscopy, the relative level of expression of transport markers including NPT2a still needs to be determined. Long-term goals include correction of the genetic defect in the ctns gene so as to achieve normal renal function in vivo.

P2723
Board Number: B880
Use of submicron vaterite particles serves as an effective delivery vehicle to the respiratory portion of the lung.
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Microencapsulation has proven to be a useful technique for the construction of drug delivery vehicles for use in vascular medicine. However, the possibility of using these techniques within the lung as an inhalation delivery mechanism has not been previously considered. A critical element of particle delivery to the lung is the degree of penetration that can be achieved with respect to the airway tree. In
this study we examined the effectiveness of labelled particles of 5, 1.3, and 0.7 μm diameter in reaching the respiratory portion of the lung. Microparticles, coated with Cy7 labelled Albumin as a model compound, were delivered to mouse lungs via tracheostomy with subsequent imaging performed 24, 48, & 72 hours after delivery by in vivo fluorescence. Subsequently the distribution of the particles 45 mins after administration was examined by serial section and S.E.M. The delivery of fluorophore to the blood was assessed using Cy7 labelled 0.7 μm particles. Pulmonary fluorescence was observable at 24, 48, and 72 hrs post administration of 0.7 μm particles, with secondary fluorescence in the kidney and liver. The predominance of fluorescence was observed in the gut following use of 5 μm particles. Biodistribution was confirmed by IVIS imaging of excised organs post 72 hrs. 45 mins post administration particles of all three sizes were visible in the lung, with the deepest penetration observed with 0.7 μm particles. S.E.M. images show 0.7 μm particles reaching the alveolar space and associating with surfactant in aggregates as large as 7.2 μm. 5 or 1.3 μm particles were only found in the upper airways. Cy7 labelled 0.7μm particles efficiently delivered fluorescent material to the blood with a peak 3 hrs after particle administration. The kinetics of delivery will be shown. These studies establish that by using 0.7 μm microencapsulation particles we can efficiently access the respiratory portion of the lung, which represents a potentially efficient delivery mechanism for both the lung and the vasculature. This work was partially supported by the Russian State project «Science», № 4.5752.2017/8.9 and by the Program for Competitive Growth at Kazan Federal University.

P2724

Board Number: B881

Overproduction of biologically active human bone morphogenetic protein-4 in Chinese hamster ovary cells.

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Bone morphogenetic protein-4 (BMP-4), originally identified as one of the growth factors inducing new bone formation, has been demonstrated to have therapeutic potential for various diseases including cancer. In spite of increasing demands of recombinant human BMP-4 (rhBMP-4) for therapeutic purposes, efficient production procedures of biologically active BMP-4 have not yet been developed. In the current study, a recombinant Chinese hamster ovary (rCHO) cell line overexpressing rhBMP-4 as well as a pilot scale manufacturing process using 7.5-l bioreactor (5 l working volume) were established. We selected the optimized combination of a chemically defined (CD) medium and a nutrient supplement solution for high expression of rhBMP-4 in bioreactor cultures. The production rate was 32 mg/l from the 11-day fed-batch cultures of the established rhBMP-4-expressing rCHO cells. The mature rhBMP-4 was purified to homogeneity from the culture supernatant using a two-step chromatographic procedure, resulting in a recovery rate of approximately 55% and a protein purity greater than 95%. The mature purified rhBMP-4 has been proved to be functionally active with an effective dose concentration of EC50 of 2.93 ng/ml.
Tuesday, December 5
New Technologies in Single Molecule and Super-Resolution

P2725
Board Number: B1
A DNA origami platform for quantitative super-resolution microscopy.
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Single molecule localization based super-resolution microscopy offers a unique opportunity for quantifying protein copy numbers at the nanoscale level [1,2]. While fluorescent proteins have been extensively characterized for quantitative imaging using calibration standards, similar calibration tools for small organic fluorophores used in conjunction with immunofluorescence-based super-resolution techniques are missing. The development of a suitable calibration method represents the best way to address the challenges of molecular counting using super-resolution [3,4]. Here we demonstrate that DNA origami in combination with GFP antibodies is a versatile platform for quantifying protein copy number in immunofluorescence-based super-resolution microscopy. We show that this calibration method, besides quantifying the average protein copy number in a cell, allows determining the abundance of different oligomeric states. Furthermore, we apply this calibration method to quantify nucleoporins (NUP107) [5] and molecular motors (dynein intermediate chain) [6] in vivo. Overall, we provide a versatile strategy [7] for quantifying a large number of proteins of interest using various labeling approaches.

P2726
Board Number: B2
A New Methodology for Quantitative Measurements of Single Cell Mechanics.
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Using modified atomic force microscopy (AFM), a new method is developed for measurements of single cell mechanics as well as applying designed mechanical perturbation. Attaching a hard microsphere to an AFM probe and compressing the cell at a designated location, this method enables force-deformation profile to be measured for living cells under incubation conditions. The optical microscopy guides the location of an AFM probe and then monitors the deformation of cell shape, while micro-sphere modified AFM probes compress the cell while acquiring force-deformation profiles. In conjunction with viable mechanics models, such as Hertzian model, one could extract quantitative information such as Young’s modulus of the cellular membrane, cytoskeleton, as well as the bending constant of the membrane at single cell level. Combined multimodal imaging and analysis provide important readouts of cellular biostatus, including migration, activation, and malignancy. Combined readouts lead to a much higher degree of accuracy and completion of cellular status, and provide new insights into cellular signaling processes. This presentation will also provide several specific examples form primary cells and cell lines to demonstrate the enabling aspects and advantages of this new approach.

P2727
Board Number: B3
An algorithm to obtain blinking-corrected super-resolution images.
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In super-resolution microscopy techniques PALM and STORM, the fluorophore can stochastically switch between an activated state and dark states, leaving gaps in the temporal emission of the single molecule before photobleaching. These gaps create multiple localizations belonging to the same molecule, a phenomenon known as blinking. Blinking distorts the true image leading to the appearance of fake nanoclusters, which are often incorrectly interpreted as real biological structures. Here we present a model-free method to eliminate blinking without the need of additional experiments or arbitrary thresholds. The approach relies on the observation that the true pairwise distance distribution can be obtained using the distances of localizations separated by a time longer than the survival time of the fluorescent molecule. The approximate probability for a molecule being a blink is then determined from the deviation of the distance distributions at varying frame differences. Using this information and performing a phase space search to minimize the differences between the pairwise distance distributions for all frame differences, results in an accurate reconstruction of the true image. To test this new methodology, we used simulated PALM data with different underlying true structures and compared our methodology with traditional methods to eliminate blinking. We then applied the algorithm on real experimental data, which show identical trends as simulation, justifying the new approach.
P2728

Board Number: B4

Distinguishing Biological and Non-Biological Networks in Single Molecule Localization Super Resolution Microscopy.

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Single molecule localization microscopy (SMLM) is based on the repeated activation (blinking) of small numbers of discrete fluorophores whose precise localization is determined using a Gaussian fit of the point-spread function (PSF). However, repeated blinks derived from the same molecule form non-biological nano-clusters or networks (NBNs). Distinguishing NBNs from biological networks (BNs) that reflect the distribution of component molecules of the biological structure is a major challenge for SMLM.

Importantly, in contrast to BNs, NBNs are related to the physical properties of the fluorophores and the labeling process and therefore similar and homogeneous. Further, while the number of molecules or proteins in a BN is constant, additional blinks acquired during the imaging process are centered and spread around a molecule to form a NBN with a particular spatial scale due to microscope drift and localization error. As more blinks are collected, the scale plateaus and we perform multi-scale, dynamic network analysis to estimate this scale. Spatial and iterative merging of blinks within that scale effectively eliminates repeated blinks, thereby differentiating between BNs and NBNs.

We validated our approach, first, on synthetic data. We position a set of in-silico molecules on a 3D grid and generate repeated blinks with known Gaussian distribution parameters around each synthetic molecule. The distance between the molecules is modified to mimic the different levels of overlap between neighboring molecules in real biological settings. Using our network analysis method, we retrieved the NBN scale of the repeated blinks even with moderate levels of overlap between blinks of different molecules. Applying the NBN scale in the iterative merging module accurately estimated the location of synthetic molecules on the grid. We then validated our approach using a real dataset of caveolin-1 antibody labeling of HeLa cells imaged using a home-built dSTORM microscope with real time drift-correction (Tafteh et al., Opt Exp 24:22959, 2016). After scale-detection and merging, the estimated number of caveolin-1 molecules per caveolae was on average 142, very similar to the reported 145 (Pelkmanns et al., Nature, 436:78, 2005). Multi-threshold modularity analysis of Cav1 domains in HeLa cells described the molecular structure and architecture of non-caveolar scaffolds that group together to form the caveolae coat.

Finding NBNs and their features is important for many applications in SMLM. We foresee our method being useful for molecular counting, providing non-biased bio-signatures for subcellular structures, defining imaging parameters (e.g. stopping criteria based on the saturation of the collected blinks), etc. Supported by grants from the CIHR, NSERC and CFI/BCKDF.
P2729
Board Number: B5
A Cryosectioning Technique for the Observation of Intracellular Structures and Immunocytochemistry of Tissues in Atomic Force Microscopy.
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The use of cryosectioning enabled the morphological analysis and immunocytochemistry of cells in tissues in atomic force microscopy (AFM). The cantilever could access all parts in cryosection of a tissue sample after the embedding medium (sucrose) has been replaced with phosphate-buffered saline, and this approach has enabled the production of a type of high-resolution image. The images resembled those obtained from freeze-etching replica electron microscopy (EM) rather than from thin-section EM. In retinal tissue, the AFM images showed disks stacked and enveloped by the cell membrane in rod photoreceptor outer segments at EM resolution. In addition, three-dimensional architecture of synaptic ribbons, and the surface of the post-synaptic membrane facing the active site were revealed, which were not apparent using thin-section EM. AFM could depict the molecular binding of anti-opsin antibodies conjugated to a secondary fluorescent antibody bound to the disk membrane. The specific localization of the anti-opsin binding sites was verified through correlation with immunofluorescence signals in AFM combined with confocal fluorescence microscope. To prove reproducibility in other tissues besides retina, cryosectioning-AFM was also applied to elucidate molecular organization of sarcomere in a rabbit psoas muscle. In particular, Z-band M line and cross bridges between myosin heads and actin filaments were observed more clearly than in thin section EM.

P2730
Board Number: B6
The Stoichiometry of AMPA Receptors Measured By Single Molecule Imaging.
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AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, which are ionotropic glutamate receptors, conduct fast excitatory synaptic transmission at the postsynaptic membrane of neurons. It is known that AMPA receptors are tetramers and are assembled from four subunits GluA1-4 either as homomers or heteromers. The assembly and the stoichiometry of AMPA receptors are fundamental to fast neuronal signal transduction. However, existing studies of AMPA receptor stoichiometry either suffered from the ensemble measurement or disrupted the functionality of the receptors. To directly measure the stoichiometry of AMPA receptors, we employed a single molecule imaging technique to count the number of different subunits labeled with different fluorescent proteins. With this method, we are able to measure the stoichiometry of heteromeric AMPA receptors at the single molecule level. We found that in GluA1/A2 heteromeric receptors, a 2:2 stoichiometry is preferred. Unlike NMDA receptors which have an obligatory heteromeric assembly, our results showed the existence of 3:1 and 1:3 stoichiometries for AMPA receptors. By measuring chimeras with swapped domains, we find that the transmembrane domain is decisive for the 2:2 preference. Our data suggests possible assembly mechanisms and helps us to understand synaptic integration in the central nervous system.
P2731
Board Number: B7
Visualization of transcriptional dynamics at single-cell resolution with a genetically-encoded fluorogenic RNA.
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Advancement of single-cell analysis techniques enabled the monitoring of the transcriptional activity at single-cell resolution. However fluorescent or luminescent protein has been mostly used for the indirect monitoring of the gene transcription living cells so far, because practical probes for direct monitoring of RNA transcription have been missing. Combinations of fluorescent protein-tagged RNA-binding proteins and RNA stem-loops, such as MS2 or PP7 stem-loop, have been developed for the visualization of RNA in living cells, but there is a drawback that their total fluorescence reflects the amount of the expressed protein, not of the target RNA. Fluorogenic RNA would be ideal for measuring the transcriptional activity, because its fluorescence signal directly reflects the amount of the target RNA. Spinach, Broccoli and their derivatives have been developed as fluorogenic RNAs that emits green fluorescence upon binding to a small molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). However, their application was limited, mainly because their fluorescence signal was too weak to detect mRNA in living mammalian cells. Here we report a new fluorogenic RNA probe Romanesco, an improved version of Broccoli. The improved brightness of Romanesco enabled us to measure transcription activity in living mammalian cells in culture. Reporter constructs that contains a coding sequence of iRFP followed by Romanesco at the 3′ end successfully reported the rise and fall of green fluorescent signal from Romanesco reflecting the promoter activity, while the iRFP signal was delayed. The linearity of Romanesco signal was verified by qPCR analysis, but the iRFP signal intensity was not proportional to the Romanesco signal, which would demonstrate the cell-to-cell variance in the translational activity. Transcriptional activity of endogenous genes was also visualized by knocking-in Romanesco at the 3′-UTR of the target genes. Furthermore, the subcellular localization of the fluorescent signal visualized the RNA translocation from nucleoplasm to cytoplasm as well as the accumulation of newly synthesized RNA molecules around the nuclear speckles. Thus, Romanesco would serve as a versatile visualization probe for the transcription, dynamics and localization of RNA in living cells.

P2732
Board Number: B8
Single-molecule Fluorescent Amplification of RNA using ClampFISH Probes.
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RNA signatures can be used to define cell types, cell states, and disease phenotypes. However, RNA expression within a given cell population is heterogeneous and these differences can be important for cellular function. The ability to separate cells based on their RNA signatures would provide sufficient material to perform biochemical assays that will help to tease out the functional differences between cells. However, the current state of RNA imaging technology (i.e. single-molecule FISH) provides signal that is too weak for current separation methods such as flow cytometry. We present a new method for the fluorescent detection of RNA that combines the specificity of oligonucleotides and bioorthogonal click chemistry in order to achieve highly specific and high-gain signal amplification. Using this method, we have achieved >400-fold signal amplification of individual transcripts while maintaining minimal off-
target binding. The signal intensity is so strong that it can detect and separate cells based on RNA signal using flow cytometry and low-magnification microscopy, enabling the analysis and separation of large numbers of cells. This method circumvents enzyme-based amplification schemes that suffer from poor cell penetration and exceeds the reported amplification of nucleic acid based amplification methods. Perhaps most importantly, the chemical ligation step enables stringent wash conditions to reduce background. Our results demonstrate the power of RNA amplification in situ and open up the single-cell and RNA fields for mechanistic studies using high-throughput, analytical methods.

P2733
Board Number: B9
Optical imaging and labelling of individual biomolecules in dense clusters.
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Recent advances in fluorescence super-resolution microscopy have allowed sub-cellular features and synthetic nanostructures down to ~15 nm in size to be imaged. However, direct optical observation of individual molecular targets (~5 nm) in a densely packed biomolecular cluster remains a challenge. Here, we show that such discrete molecular imaging is possible using DNA-PAINT (points accumulation for imaging in nanoscale topography) - a super-resolution fluorescence microscopy technique that exploits programmable transient oligonucleotide hybridisation - on synthetic DNA nanostructures. We examined the effects of high photon count, high blinking statistics, and appropriate blinking duty cycle on imaging quality, and developed a software-based drift correction method that achieves <1 nm residual drift (r.m.s.) over hours. This allowed us to image a densely packed triangular lattice pattern with ~5 nm point-to-point distance, and analyse DNA origami structural offset with angstrom-level precision (2 Å) from single-molecule studies. By combining the approach with multiplexed Exchange-PAINT imaging, we further demonstrated an optical nano-display with 5x5 nm pixel size and three distinct colours, and with <1 nm cross-channel registration accuracy. Combined with photo-activated crosslinker, we further demonstrated selective labelling on single molecule targets with custom patterns and sub-diffraction (~30 nm) resolution. These methods open up possibilities for direct and quantitative optical observation and perturbation of individual biomolecular features in crowded environments.

P2734
Board Number: B10
Developing a single-molecule imaging technology for early cancer detection in blood samples.
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Over 40% of people will develop cancer during their lifetimes, and nearly 600,000 die of cancer each year in the United States. It is estimated that over 90% of cancer cases can be managed effectively if found early on. However, early cancer detection, especially for solid tumors, remains an elusive goal. In our recent studies using mouse tumor models, we discovered that the solid tumor releases a trace amount of its cytoplasmic and nuclear contents into the peripheral circulation, possibly through
apoptosis or unconventional secretion processes. Thus, the tumor projects a “molecular shadow” of itself into the bloodstream. Because the nucleocytoplasmic proteins of cancer cells—including oncoproteins, signaling complexes, tumor suppressor proteins and transcription factors—are in general the ultimate mediators of oncogenesis and progression, they in principle provide the most definitive identification of the tumor. Therefore, we are developing an ultra sensitive, single molecule imaging-based technology, which we term as SMAC (for single molecule analysis and counting), to detect the molecular shadow of tumor cells in the peripheral circulation. We have demonstrated that cytoplasmic GFP expressed in tumor cells is released to bloodstream in mouse preclinical models. We also successfully applied SMAC assay to detection of multiple biomarkers in ovarian cancer samples. We believe that the concept of probing the molecular shadow of tumor cells in blood samples represents a paradigm shift in the field of cancer detection and diagnosis, and opens the door to the prospect of true tumor-specific (in contrast to tumor-associated) biomarkers.

P2735
Board Number: B11
Obtaining 3D Super-resolution Information from 2D Single-molecule Localizations through a 2D-to-3D Transformation Algorithm.
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Currently it is still challenging to obtain three-dimensional (3D) super-resolution information in studies of structures in fixed specimens as well as transport kinetics in live cells with a high spatial resolution (< 10 nm). Without using real-time 3D particle tracking or making extensive optical microscopy modifications, here we introduce an approach to achieve 3D super-resolution information with resolution ~ 1 nm by converting 2D single molecule localizations to 3D spatial density maps through a 2D-to-3D density transformation algorithm. The method has been successfully applied to obtain structural and functional information for 25-300 nm bio-channels that have rotational symmetries. In this article, we will provide a comprehensive analysis of this new method by using experimental data and computational simulations.

P2736
Board Number: B12
Absolute Quantification of Transient Membrane Protein Interactions in Single Living Cells Using Co-Immunomobilization.
S. Park1, D. Kim1, D. Kim2, M. Jeong3, J. Noh1, Y. Kwon1, K. Zhou1, N. Lee6, S. Ryu1;
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Quantitatively understanding protein-protein interactions in living cells holds a key to unravel how diverse cellular processes are properly generated in space and time. Equilibrium dissociation constants (K<> of protein-protein interactions must be determined in living cells to objectively compare and rank the strengths among the interactions. However, this remains challenging because of the transient nature of protein-protein interactions, which has hindered their quantitative analysis using conventional methods. Single-particle tracking (SPT) allows to capture rapid molecular events and provide diffusional information, yet biochemical interpretations of trajectory data are complicated because multiple
molecular processes are convoluted in single trajectories. Here, we develop a direct method enabling the absolute quantification of transient interactions between membrane proteins by utilizing the synergy between SPT and antibody-induced protein immobilization. Interaction moments of a interaction partner with specifically immobilized a protein of interest was visualized as co-immunoimmobilization (Co-II) using single-particle tracking photoactivated localization microscopy (sptPALM) and counted to calculate the in vivo $K_D$ value of protein-protein interactions in the membrane of single living cells. Using Co-II, we revealed that epidermal growth factor receptor (EGFR) and beta-2 adrenergic receptor (β2-AR) homodimerization are dominantly regulated by the intramolecular conformation and membrane microenvironment, respectively.

P2737
Board Number: B13
High Depth, High Precision, Three-Dimensional Super-Resolution Imaging and Particle Tracking with the Double Helix SPINDLE™ Module.
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Double Helix Light Engineering™ modifies the response of a microscope such that, instead of an Airy Disc, the image of each point source is in the form of two well separated lobes. The two lobes rotate around their midpoint as the emitter is moved along the axial dimension. Thus, the center of the pair of lobes determines the lateral position of the emitter and the angle between the lobes determines the axial position. We present an implementation of this technique in the form of an add-on module, the Double Helix SPINDLE™, that can be attached between most scientific microscopes and a high sensitivity camera. The SPINDLE™ in conjunction with the Double Helix TRAX™ software extends the imaging capabilities of most scientific microscopes to nanometer scale 3D localization for imaging and tracking. In conjunction with single molecule localization microscopy (SMLM) methods, such as Stochastic Optical Reconstruction Microscopy (STORM) or Photoactivated Localization Microscopy (PALM), the SPINDLE™ enables high precision 3D reconstructions. Double Helix Light Engineering™ also extends the depth of field over which emitters can be localized, enabling collection of more data with high precision axial information. Similarly, in single particle tracking applications, the Double Helix SPINDLE™ facilitates collection of tracks over an extended depth with high precision 3D information. Here, we show super-resolution reconstructions of sub-cellular structures using the SPINDLE™ module and TRAX™ software. With this method, we were able to achieve average precision values for the localizations below 20 nm laterally and below 25 nm axially. Reconstructed images represent more than two microns of depth of field with no axial stitching. Additionally, we show 3D super-resolution reconstructions of stress granule cores, which were first visualized using Double Helix Light Engineering™. Finally we show results from 3D particle tracking of beads traversing in an inverse opal structure. We demonstrate improved tracking precision and accuracy of diffusion measurements using the Double Helix. In both these applications, Double Helix Light Engineering™ enables researchers to collect more information with high axial and lateral precision with up to three times the depth of conventional methods.
New Technologies in Cell Biology: Fluorescence

**P2738**

**Board Number: B14**

Imaging Minimally-Engineered mRNA and long non-coding RNA transcripts at the single-molecule level using phosphorothioate-optimized 2'-O-methyl RNA molecular beacons.

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Recently, we developed a new molecular beacon (MB) architecture composed of 2'-O-methyl RNA (2Me) and a fully phosphorothioate (PS)-modified loop domain with a phosphodiester stem (2Me/PSLOOP MB) and showed that this new MB exhibits a marginal false-positive signal in living cells. In addition, 2Me/PSLOOP MB could accurately image the dynamics and localization of single mRNA molecules harboring 32 tandem repeats of an MB target sequence without affecting gene expression or cell viability. In this study, we evaluated the sensitivity of MBs for detecting minimally engineered RNAs in cells, and found that the MBs can detect single mRNA transcripts containing as few as 8 tandem target repeats without compromising accuracy. In both the nucleus and the cytoplasm, mRNAs harboring 8 repeats moved faster than those with 32 repeats, suggesting that intracellular activities are less impeded in RNA molecules with smaller engineered insertions. The MB/8-repeat system was then further shown to enable, for the first time, live-cell visualization of long non-coding RNAs (lncRNAs) and could do so without altering their expected physiological activities. We envision the proposed MB-based technology for live-cell single RNA imaging can be useful for discovering new RNA functions and activities.

**P2739**

**Board Number: B15**

Genetically Encoded Tools to Control and Reveal Cellular Dynamics in IPSC Disease Modelling and Drug Screening.

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Induced pluripotent stem cells (iPSC) models hold great promise for disease modeling and drug discovery. Genetically encoded light emitting reporters offer the prospect of multichannel resolution of events within subcellular compartments. Optogenetic technology allows us to control neurons depolarisation and heart coupling with the aid of photo-controllable proteins, channelrhodopsin2 (ChR2), which is a cation channel activated by blue light irradiation. Combining genetically encoded optogenetic control and spectrally compatible calcium indicator tools into a single adenoviral vector allows the analogous capability for cell control with simultaneous cellular phenotyping without the need for contact. This combination can be applied to human stem cell derived cardiomyocytes as single cells enabling contactless small molecule evaluation in isolated stem cell derived cardiomyocytes for inhibitors of sodium, potassium and calcium channels suggesting it may be useful for early toxicity studies. We also reveal cellular disease phenotype in dish by using iPSC patient specific model. A family
carrying the point mutation in L-type calcium channel Cav1.2 display long-QT syndrome or hypertrophic cardiomyopathy will be discussed briefly.

P2740
Board Number: B16
Immunoprecipitation high performance liquid chromatography (IP-HPLC) analysis in the postoperative exudate of bisphosphonate related osteonecrosis of mandible.
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Recurrent bacterial infections in cases of bisphosphonate-related osteonecrosis of jaw (BRONJ) frequently occur. Therefore, BRONJ are usually treated by radical saucerization followed by intensive antibiotic medications without bisphosphonate therapy. The postoperative exudate (POE) from BRONJ lesions may directly indicate the inflammatory status of osteomyelitis in patients, but so far, the POE has rarely been examined for its expression of various cytokines and wound healing proteins. A total of 27 cases of BRONJ, which involved the mandible, were selected and their individual POE collected 6 h, 1 day, and 2 days after surgical intervention was analyzed by immunoprecipitation high performance liquid chromatography (IP-HPLC). The different protein expressions in the BRONJ POE were compared with findings from ten cases of chronic mandibular osteomyelitis (CMO) exudate as the control group. For the protein expressions for inflammation, osteogenesis, and angiogenesis, in the 6 h POE sample, the BRONJ exudate exhibited more expression of IL-10, IL-28, OPG, and osteocalcin, but less expression of TNFa and LL-37 than the control. In the 1 day POE sample, the BRONJ exudate showed more expression of TNFa, IL-6, 8, 12, 28, a1-antitrypsin, VEGFA, and VEGF-C, but less expression of CD68, lysozyme, bFGF, RANKL, bFGF, and ALP than the control. In the 2 day POE sample, the BRONJ exudate consistently showed more expression of LL-37, b-defensin-1, and VEGF-A than the control. The present BRONJ POE revealed the rapid progress of bony wound healing through increased molecular signaling for inflammation, angiogenesis, and osteogenesis compared to the control. Therefore, it was suggested that the POE obtained from the postoperative bony lesions should be collected and analyzed by the IP-HPLC method to predict the prognosis of seriously complicated inflammatory bony diseases such as BRONJ. *This study was supported by a Grant of the Korean Health Technology R&D Project, Ministry of Health and Welfare, and Republic of Korea (HI15C0689).

P2741
Board Number: B17
Engineering the MS2 System to follow the Life Cycle of Single mRNAs.
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The MS2-MCP system allows imaging multiple steps of the mRNA life cycle with high temporal and spatial resolution. However, for short-lived mRNAs, the tight binding of the MS2 coat protein (MCP) to the MS2 binding sites (MBS) protects the RNA from being efficiently degraded, confounding the study of mRNA regulation. Here, we describe a reporter system (MBSV6) with reduced affinity for the MCP, allowing mRNA degradation while preserving single molecule detection determined by smFISH or live
imaging. Highly-regulated mRNAs endogenously tagged with MBSV6 in S. cerevisiae degrade normally and were imaged throughout their complete life cycle. The MBSV6 reporter revealed that coordinated recruitment of mRNAs at specialized structures such as P-bodies during stress did not occur. MBSV6 provided single molecule detection of the molecular chaperone HSP70 mRNA in live mammalian cells. Supported by NIH GM57071 to RHS.

P2742

Board Number: B18

Microfluidic Imaging Windows: A New Method for Imaging and Controlling the Tumor Microenvironment.

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Intravital Windows are powerful tools capable of elucidating the complex tumor microenvironment. Advancements in protocols for intravital windows have recently led to rapid development in the ability to observe structures and mechanisms of metastasis in vivo. The utility of intravital window technology has been amplified by modifying the window itself to feature microfluidic functionality. Microfluidic Intravital Windows (MFIWs) have been engineered to contain pre-loaded drug reservoirs and microchannels capable of controlling the tumor microenvironment through factors delivered locally into the tumor microenvironment. Two modes of devices have been developed: the Passive MFIW (P-MFIW) and the Active MFIW (A-MFIW). P-MFIWs were fabricated using soft-lithography techniques and utilize hydrogel-facilitated diffusion as the mechanism for drug delivery. Elongated channels control the timeframe of the experiment and allow drug-release from multiple reservoirs both in parallel and in series. The P-MFIW has been validated for imaging, drug-release and collection. Imaging was conducted through windows installed in mice under a multiphoton microscope. Drug release was characterized using for diffusion studies and gradient formation studies using fluorescent hepatocyte growth factor (fHGF). Release dynamics were characterized in vitro as well as through solid tumors harvested from chorioallantoic membranes (CAMs). fHGF was loaded in 4% alginate hydrogels for hydrogel-mediated release. A microneedle protocol has been optimized to allow controlled depth-of drug release from the P-MFIWs, as well as for uptake of metastatic cells. The Active MFIW utilizes light energy to control the spatial and temporal release of factors. Azobenzene liquid crystal polymer (LCP) films have been generated in LCP photopolymerization cells made using soft-lithography techniques. These LCP films demonstrate stress localization and macroscopic bending when exposed to 488 nm light. The degree and extent of bending is determined by light intensity and polarization. Normally Open (NO) microfluidic valves have been fabricated with azobenzene functionality. Valves have been validated to demonstrate discrete “ON” and “OFF” states. Induction of GFP expression in human epidermoid carcinoma (T-HEp3) tumors via the release of doxycycline from the A-MFIW at controlled timepoints will be reported.
P2743
Board Number: B19
Fluorescent cell-labelling strategies live-cell analysis.
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In the pursuit of more relevant and translational cell-based assays, researchers are increasingly turning to real-time live-cell analysis for their studies. Live-cell analysis provides cell biologists with greater insight and productivity by enabling quantification of cell function over hours, days and weeks via time-lapse image analysis, all automatically and within the controlled environment of the incubator. Many cell functions, such as proliferation, migration and neurite outgrowth, can be quantified in simple monocultures without fluorescent cell labels by analyzing features of phase-contrast images. This ‘label-free’ approach is desirable since it avoids complications associated with cell labels. However, there are limitations to what can be achieved without cell labels. For example, it is difficult to extract true cell count metrics from phase-contrast images in dense cultures, and in co-culture models cell labels are absolutely required to identify different cell types to observe their interactions. Accordingly, a range of different cell-labelling strategies for live-cell analysis have been developed. Protein-based fluorophores have been used for several decades to label cells and are ideally suited to real-time live-cell analysis. Fluorescent proteins (FPs) provide long-term and stable-cell labelling, are generally non-toxic and not depleted by repeated fluorescent excitation. By using specific targeting sequences in the expression constructs, proteins can be directed to label different cell types or organelles. Via this approach, we have developed a range of different vectors and delivery methods including nuclear-targeted FPs, cytoplasmic FPs and neuronal-specific FPs. These are packaged for delivery with either lentiviral or baculoviral (BacMam) expression constructs. Alternatively, in certain scenarios, it may be difficult or undesirable to transduce cells with fluorescent proteins. In these cases, cells can be labelled with cytoplasmic or nuclear specific fluorescent dyes. When used correctly, these chemical probes are non-perturbing to cells and produce uniform and homogeneous labelling. These dyes have very different properties and use protocols to each other, and to the protein labels, and are best aligned with different applications. In this poster, we will describe the use of these fluorescent protein and dye-based labelling strategies and how they can be used most effectively in live-cell analysis applications including cell proliferation, cytotoxicity, apoptosis, immune cell killing, and neurite outgrowth in both primary rodent and human iPSC-derived neurons.

P2744
Board Number: B20
An enclosed system for long-term brain slice culture: viral-mediated transgene expression and precisely localized repetitive cell imaging.
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Cultured rodent brain slices are useful for studying the cell biology and behavior of neurons and glia in an environment that maintains many of their normal interactions. Expression of fluorescently tagged proteins or reporters by infection with replication deficient virus in wild-type hippocampal slices allows for high-resolution imaging by fluorescence microscopy. Although several methods have been developed for imaging brain slices, combining slice culture with repetitive high-resolution imaging of
identical cells in live slices over long time periods has posed problems. This is especially true when viral vectors are used to express exogenous proteins since viruses are best used in an enclosed system to protect users and prevent cross contamination. Here we report simple modifications made to the roller tube brain slice culture method that allows for repetitive high-resolution imaging of slices over many weeks/months. Furthermore, culturing slices on photo etched coverslips provides the fiducial marks required for precise localization and imaging of the identical field of cells over time before and after different treatments. Applications are shown for the use of this method combined with specific neuronal staining and expression to observe long-term maintenance of hippocampal architecture, viral-mediated neuronal expression of fluorescent proteins, and, in response to slice treatment with oligomers of amyloid-β peptide, the development of coflin pathology observed in brains of human and mouse models of Alzheimer disease.

P2745
Board Number: B21
Flagella standards for quantitative fluorescence microscopy.
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Despite available quantitative analytic programs, it remains challenging to estimate molecule numbers based on fluorescent intensity. To address this challenge, we converted a ruler-like microtubule-based structural scaffold in flagella into fluorescent intensity standards, akin to protein or DNA markers for electrophoresis. Specifically, we engineered transgenic biflagellate green algae, Chlamydomonas, expressing fluorescent proteins tagged to a cytoskeletal protein of a defined periodicity throughout the length of flagella. The fluorescent intensity had at least a 10 fold linear range. Carrier effects were not evident for four fluorescent proteins and two carrier proteins tested. Quantitative applications were demonstrated by comparing fluorescent flagella with fluorescent objects in Chlamydomonas and Saccharomyces cerevisiae. Methanol fixation, which diminished chloroplast fluorescence, improved the signal/background ratio. These observations demonstrate the feasibility of broad applications of biologically inert fluorescent flagella as microscopy intensity standards.

P2746
Board Number: B22
Properties of Near-Infrared Fluorescent Proteins Engineered From Bacterial Photoreceptors in Mammalian Cells.
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Non-invasive in vivo imaging demands for near-infrared (NIR) fluorescent probes. Numerous NIR fluorescent proteins (FPs) were recently engineered from bacterial photoreceptors but lack of their systematic comparison makes researcher’s choice rather difficult. We evaluated side-by-side several modern NIR FPs, such as blue-shifted smURFP and miRFP670, and red-shifted mIFP and miRFP703. We found that among all NIR FPs, miRFP670 had the highest fluorescence intensity in various mammalian cells. For instance, in common HeLa cells miRFP703, mIFP and smURFP were 2-, 9- and 53-fold dimmer than miRFP670. Co-expression of heme oxygenase, producing biliverdin chromophore for NIR FPs from heme, either via IRES sequence or via T2A peptide, as well as incubation of cells with heme precursor,
decreased the cellular brightness of NIR FPs, however, in the latter case elevated cellular autofluorescence. Exogenously added chromophore substantially increased smURFP brightness but only slightly enhanced brightness of other three NIR FPs. mIFP showed an intermediate while monomeric miRFP670 and miRFP703 exhibited a high binding efficiency of the endogenous biliverdin chromophore. Thus, a quality of the initial molecular engineering rather than an insufficient amount of the endogenous BV chromophore is the key factor determining the NIR FP brightness in mammalian cells. This feature makes them easy to use as GFP-like proteins for spectral multiplexing with FPs of visible range.

P2747

Board Number: B23

Light-activated protein interaction with high spatial subcellular confinement.
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Methods to acutely manipulate protein interactions at the subcellular level are powerful tools in cell biology. Several blue-light-dependent optical dimerization tools have been developed. In these systems one protein component of the dimer (the bait) is directed to a specific subcellular location, while the other component (the prey) is fused to the protein of interest. Upon illumination, binding of the prey to the bait results in its subcellular redistribution. Here we compared the ability to control dimer occurrence in small, subcellular volumes, of three such tools: Cry2/CIB1, iLiD, and Magnets. We show that both the location of the photoreceptor protein(s) in the dimer pair and its(their) switch-off kinetics determine the subcellular volume where dimer formation occurs. Magnets, a system in which both bait and prey are photosensitive elements, can induce and maintain protein dimerization in the smallest volume, although this comes at the expense of the total amount of dimer. These findings emphasize the importance of choosing an optogenetic system with qualities matched to the desired application.

P2748

Board Number: B24

Optogenetic systems for regulation of cellular metabolism with near-infrared light.
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Light-mediated control of protein–protein interactions to regulate cellular metabolism is an important application of optogenetics. Earlier, we have developed an optogenetic system based on reversible light-induced heterodimerization of bacterial phytochrome BphP1 and its natural partner PpsR2 from R. palustris bacteria. Characteristics of the BphP1–PpsR2 optogenetic system include its sensitivity to near-infrared (NIR) light of 740–780 nm, ability to utilize an endogenous biliverdin in eukaryotes including mammals as a chromophore, and spectral compatibility with blue-light-driven optogenetic systems, such as LOV, CRY and channelrhodopsins.
We characterized the BphP1–PpsR2 interaction both in vitro and in mammalian cells. Structural and biochemical analysis of PpsR2 resulted in truncation of PpsR2 to 3-fold smaller fragment, termed Q-PAS1, which was able to bind BphP1. Q-PAS1 lacks the undesired PpsR2 oligomerization behavior and possesses faster kinetics of heterodimerization. BphP1 interaction with PpsR2 and Q-PAS1 was exploited to develop NIR light-controllable transcription activation and inhibition systems. We achieved 40-fold activation contrast in cultured cells, 32-fold in subcutaneous mouse tissue, and 5.7-fold in deep tissues in mice. The developed transcription regulation systems are compatible with reporter systems based on Tet response element and Gal4 upstream activation sequence and can be used to control non-invasively the expression of intracellular proteins. Moreover, PpsR2 and Q-PAS1 can be used to translocate target proteins to different cellular compartments upon NIR light-induced interaction with BphP1. We showed light-inducible control of cell morphology by targeting of DPH domain of Cdc42 to the plasma membrane. Simultaneous use of BphP1–Q-PAS1 and LOV-based optogenetic systems demonstrated their negligible spectral crosstalk. By further integrating Q-PAS1 and LOV domains in a single optogenetic tool, we achieved tridirectional protein targeting between nucleus, cytoplasm and plasma membrane controlled by NIR and blue light. This approach opens a possibility to engineer multicomponent optogenetic tools to regulate cellular metabolism by light-controlled targeting of metabolically active proteins to desired cellular compartments.

P2749
Board Number: B25
Development and application of designable RNA-binding protein for live-cell imaging and manipulation of authentic RNAs.
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RNAs do not only serve as the blue print for the protein assembly, but also play wide variety of essential functions in cells. Thus, visualization and manipulation of RNAs in living cells would beneficial for both basic and applied sciences. MS2 and PP7 systems are now widely used for this purpose. However, a tandem repeat of stem loop sequence should be introduced into the target RNA as the binding sites for MS2 or PP7 proteins, which could potentially affect the function, localization or stability of the target RNA. In situ hybridization can detect unmodified, authentic RNAs, but is difficult to apply to living cells. Here, we report the development of designable RNA-binding protein. We have first established an ELISA-like in vitro assay system using our bright bioluminescent protein, Nano-lantern (Takai et al., PNAS 2015). The proteins we have designed showed high affinity (1-10 nM) specifically to the target RNAs. We, then, designed the probes for beta-actin mRNA and examined their avidity to bind to the target mRNA in living cells. Immunoprecipitation of the probe followed by quantitative PCR analysis demonstrated that the target, authentic beta-actin mRNA is specifically recognized in vivo. We also showed that the same probe can be used for the visualization of the dynamics of the authentic beta-actin mRNA in living cells. Furthermore, manipulation of the localization of the beta-actin mRNA using the probe fused to constitutively active kinesin resulted in the neurite-like elongation of cellular processes. These data collectively suggests that our new probe for RNA would serve as a powerful tool for the imaging and manipulation of unmodified, authentic RNAs in living cells or organisms.
P2750
Board Number: B26
An improved methodology for determining migration defects after in utero electroporation by utilizing an internal randomized control.
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In utero electroporation is a powerful tool for introducing plasmid DNA into neurons and neuronal precursors that are located adjacent to ventricles. This technique traditionally relies on collecting matched sections from littermate controls to determine differential effects of knockdown or overexpression of desired proteins. Creation of knockout animals also requires matched sections to determine the effects of gene deletion. We have developed a new and improved methodology, allowing for expression of either a green fluorescent protein (GFP) or a red fluorescent protein (RFP) in addition to a gene or microRNA to a gene of interest (control and experimental respectively) within the same region of the same brain. This system uses a Cre/LoxP approach to stochastically express either GFP (control) or RFP coupled with overexpression or knockdown (experimental) in separate populations of neurons. We also show this system does not have any leakiness, which would result in expression of RFP and the gene of interest in the absence of Cre. Importantly, the eventual “fate” of a neuron as green or red is pseudo-randomly determined, avoiding complications that arise from some plasmids being more amenable to electroporation, and some cell types having a differential likelihood of being electroporated. As proof of principle, we demonstrate that misexpression of CIP4, an F-BAR protein that induces lamellipodia-like protrusions in neurons, results in migration defects of cortical neurons.

P2751
Board Number: B27
Intravital imaging of the Tumor Microenvironment using Endogenous Fluorescence.
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Breast tumors exist in a complex milieu of components that make up the tumor microenvironment, which is composed of the extracellular matrix, various stromal cells, blood vessels, and numerous biochemical signals. Using multiphoton and intravital imaging of human breast biopsies, our lab has found that collagen fibers surrounding tumors become aligned and reorient perpendicular to the tumor boundary, which creates a biomarker that can be utilized to accurately predict patient outcome. Local collagen alignment has been shown to recruit other players into the tumor microenvironment including immune cells. Macrophage infiltration and recruitment in breast tumors has been correlated with poor prognosis in breast cancer patients and has been linked to tumor cell dissemination. Much of our understanding comes from animal models in which macrophages are labeled by expression of an extrinsic fluorophore. However, conventional extrinsic fluorescence labeling approaches are not readily applied to human tissue and clinical use. Exploiting endogenous fluorescence from the metabolic co-
factors NADH and FAD with quantitation from Fluorescence Lifetime Imaging Microscopy (FLIM) is a novel means to non-invasively identify tumor-associated macrophages in the intact mammary tumor microenvironment. Macrophages were both FADHI and demonstrated an increase in NADH-FLIM signature, which was readily separated from the intrinsic fluorescence signature of tumor cells. This non-invasive quantitative technique provides a unique ability to discern specific cell types based upon their metabolic signatures without the use of exogenous fluorescent labels. Not only does this technique provide high resolution of temporal and spatial views of macrophages in live animal breast cancer models, this approach can be extended to other animal disease models where macrophages are implicated and has potential for clinical applications.

P2752
Board Number: B28
Single-cell fluorescence thermometry visualizing intracellular events associated with heat production in brown adipocytes.
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After the discovery of brown adipose tissue in adult humans, brown adipocytes (BAs) have attracted great interest to counteract obesity-related metabolic diseases. The strategies to promote their energy expenditure would benefit the ongoing efforts for the treatment of obesity and diabetes. BAs are characterized by a high metabolic capacity due to their high content of mitochondria. While mitochondrial pH is an important physiological factor that is regulated dynamically in response to stimulation and in return affects various cellular activities, how mitochondrial pH in adrenergically-stimulated BA is regulated is unknown. We have recently reported a thermosensitive dye targeted to the endoplasmic reticulum (ER), ERthermAC [Ex/Em; 560/580 nm (approx.)], to optically detect the thermogenesis in individual BAs upon stimulation [Sci. Rep., 7, 1383 (2017)]. The fluorescence intensity of ERthermAC decreases as the temperature increases, thus the heat production can be visualized under the fluorescence microscope. Here, we report our recent application of ERthermAC for dual-color imaging with a mitochondrial targeted pH-sensitive protein, mito-pHluorin [480/520] in BAs. Fluorescence imaging of mito-pHluorin with tetramethylrhodamine methyl ester (550/575 nm) showed a triphasic mitochondrial pH change in BAs upon adrenergic stimulation. ERthermAC revealed that phases 1 and 2 of the pH increase precede thermogenesis, while phase 3, characterized by a pH decrease, occurs during thermogenesis. The mechanism of pH increase was partially related to electron transport chain activity. Further analysis by using cells co-transfected with pHluorin and R-GECO (560/590 nm), a Ca\(^{2+}\) indicator, demonstrated that the mitochondrial pH increase occurs concurrently with an increase in mitochondrial Ca\(^{2+}\), and this Ca\(^{2+}\) increase was contributed by an influx from ER. Our results demonstrated that an increase in mitochondrial pH is implicated as an early event in adrenergically stimulated BAs, suggesting a role of this pH increase in the potentiation of thermogenesis [Mol. Metab., 6, 797-808 (2017)]. We will discuss the advantages, current limits, and future perspectives of the single-cell fluorescence thermometry.
P2753

Board Number: B29
Mechanisms of milk-lipid secretion in live mice unraveled by intravital subcellular microscopy (ISMic).
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Throughout lactation, the mammary epithelium transports and secretes diverse nutrients, including proteins, lipids and carbohydrates for the nourishment of the suckling neonate. To develop this system for mechanistic studies of trafficking and secretory processes we used intravital imaging techniques employing transgenic mice, which express fluorescently-tagged marker proteins. Glands were surgically exposed under anesthesia and the formation and secretion of BODIPY-stained neutral lipid droplets followed by intravital subcellular microscopy (ISMic) over periods of 1-2 h. Lipid droplets arose from basal regions and were transported on apparent tracks to the cell apex by superdiffusive motion. Transit was slow (0-2 micrometers/min), intermittent and marked by numerous fusion events, especially in apical regions. On occasion, droplets caught up with and fused with droplets further along the same track or pathway. Lipid expansion was most pronounced in apical nucleation centers and continued as the droplets were budding from the cell enveloped by apical membranes. Droplet release into luminal spaces required oxytocin-mediated contraction of the myoepithelium. Thus milk-lipid secretion is regulated. This novel approach will have broad application for investigating mechanisms of intracellular transport and secretion across the mammary barrier.

P2754

Board Number: B30
Live-cell and single-molecule imaging reveal contrasting localization and kinetics of Tet proteins in naive mouse embryonic stem cells.
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Epigenetic modification of chromatin, such as DNA methylation and histone modifications, constitutes a fundamental mechanism of gene expression regulation in embryonic development. The Ten-Eleven Translocation (Tet) family of dioxygenases erase DNA methylation by the catalytically oxydizing methylcytosine to further oxidized derivatives, such as hydroxymethylcytosine (hmC), and have been shown to act as both transcriptional activators and repressors, especially during stem cell pluripotency maintenance and exit. While genome-wide studies have shown average genomic localization of Tet proteins in embryonic stem cells (ESCs), their distribution and kinetics in living cells and at the single-molecule level have not been investigated.

Here, we use live-cell imaging, single-molecule tracking (SMT), and super-resolution microscopy to investigate the localization and mobility of Tet1 and Tet2 in mouse ESCs. We used Cas9-mediated genome editing to insert Halo-, SNAP-, or mNeonGreen tags at the endogenous Tet1 and Tet2 loci, ensuring physiological expression levels. We show that Tet1 forms small, mobile foci which colocalize with low-density euchromatin areas, and are generally excluded from dense heterochromatin areas. Live-cell, single-molecule imaging revealed that Tet1’s C-terminal catalytic activity locally influences residence time at chromatin loci, enriching for residence times on the tens-of-seconds timescale.

However, FRAP and super-resolution microscopy revealed that the global localization of Tet1 is heavily dependent on the large unstructured N-terminal domain, which contains multiple PTM, protein-
chromatin-binding sites. In stark contrast, Tet2 shows a more diffuse localization pattern, a larger mobile fraction in FRAP assays, and shorter residence times in SMT experiments compared to Tet1, suggesting different target search mechanisms and binding site stoichiometry.

Taken together, we demonstrate markedly different kinetics between two enzyme paralogs in ESCs, Tet1 and Tet2, which function to maintain gene expression homeostasis. These data are consistent with accumulating evidence for the non-catalytic roles of Tet proteins. Future work will help elucidate how these differences in kinetics underlie these enzymes’ functions with regards to the transition from naive to primed pluripotency.

**P2755**

**Board Number: B31**

Development of BiFC system based on a bright and photo-stable fluorescent protein for detecting a limited number of protein-protein interactions.

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Protein-protein interactions (PPIs) are essential for nearly all cellular processes. To understand the dynamic aspects of PPIs in action, it is important to visualize the PPIs in living cells. To achieve this purpose, bimolecular fluorescence complementation (BiFC) assay has been developed. To visualize a limited number of PPIs, it is required to utilize a bright and photo-stable fluorescent protein. In this study, we have developed a BiFC system based on a bright and photo-stable and fast-maturing green-yellow fluorescent protein, mNeonGreen. To determine split points of mNeonGreen, we based on the structural information of mNeonGreen, we estimated the positions of linker region without secondary structure. We used the known Net1-Sir2 interaction in nucleoli of budding yeast cells as a platform to evaluate the BiFC efficiency. The mNeonGreen fluorescence intensity in nucleoli of the budding yeast cells harboring Net1-mNeonGreen[N] and Sir2-mNeonGreen[C] was quantified. By use of this screening platform, we identified split points within the linker between the 8th and 9th beta-sheets of mNeonGreen for efficient BiFC. To demonstrate the capability of mNeonGreen BiFC system to detect a limited number of PPIs in living cells, we tried to detect the interaction between two Cse4 (CENP-A in budding yeast) incorporated in a centromeric nucleosome. Budding yeast has a point centromere on each chromosome. This means that a single budding yeast diploid cell has 32 chromosomes. Under the assumption that Cse4-ymNeonGreen[N] and Cse4-ymNeonGreen[C] are expressed at the same level, probabilistically 16 mNeonGreen molecules will be reconstituted by intranucleosomal BiFC. We confirmed that the Cse4-Cse4 mNeonGreen BiFC signal was detectable in living yeast cells. Also we confirmed that the Cse4-Cse4 mNeonGreen BiFC signal was co-localized with a kinetochore protein Mtw1 fused with a red fluorescent protein mRuby3. This results show that mNeonGreen BiFC is utilizable for detection of a limited number of PPIs in living cells. The Cse4-Cse4 mNeonGreen BiFC signal was bright and photo-stable sufficient for time-lapse live cell imaging covering a whole cell cycle of budding yeast. At bud emergence, the Cse4-Cse4 BiFC signal showed a transient disappearance. This phenomenon could result from cell cycle-dependent degradation of Cse4. To test this possibility we measured the dynamic change of Cse4-Cse4 BiFC signal in cells lacking Psh1, an E3 ubiquitin ligase required for degradation of artificially expressed excessive Cse4 proteins. In the cells lacking Psh1, a part of Cse4-Cse4 BiFC signal remained at bud emergence. This result indicates that Psh1 could be involved in degradation of the Cse4 proteins incorporated in endogenous centromeres.
**P2756**

**Board Number: B32**

Dissecting molecular determinants of signaling dynamics via optogenetic control of innate immunity.

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The innate immune system must prevent an infection without causing hyperinflammation; a balancing act that requires elaborate signaling networks to regulate gene activation. The Nuclear Factor-kB (NF-kB) network in particular, employs complex methods of gene regulation. Recent single cell studies reveal that the NF-kB pathway can regulate gene expression with temporal patterns of activity (i.e. oscillations). While it is clear that NF-kB signaling dynamics are receptor and stimuli specific, the molecular mechanisms that reinforce correct dynamics are not known. Using live single cell imaging we detected dampening of oscillations shared by the Toll Like and IL1 receptor branches of the pathway, indicating the presence of a negative feedback mechanism. To locate the source of the negative feedback we developed optogenetic tools that can activate the pathway at different nodes. Using combinations of light and cytokine stimuli, process of elimination revealed that Interleukin-1 Receptor-Associated Kinase1 (IRAK1) modification is correlated with negative feedback. Upon cytokine stimulation, fluoresently tagged IRAK1 forms transient clusters that are linked to both oscillations and tolerance. We find that in a kinase inactive mutant, IRAK1 post-translational modification and clustering are abolished. However, cells are still able to signal and show increased oscillations. Our work identifies IRAK1 activity to be dispensable for signaling but critical for dynamics.

**New Technologies in Cell Biology: General**

**P2757**

**Board Number: B33**

EXPLORING THE POSSIBILITY OF CO-CULTIVATING THE GREEN MOSS PHYSCOMITRELLA PATENS WITH HUMAN FIBROBLASTS.

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Plant-based biotechnologies represent a promising new strategy for the production of effective medical materials and pharmaceuticals. Biomolecule production in plants, including proteins, vaccines and antibodies, is a convenient, safe and cost effective alternative to expression systems based on microorganisms, animal tissue cultures or transgenic animals. A plant-based production has many advantages, such as the absence of human pathogens, viruses and prions, correct posttranslational modifications and sub-cellular localization, proper assembly and folding of recombinant proteins, increased stability and effective storage of expressed product in plant tissues without substantial losses in biological activity. One of such potential approaches is the introduction of a vital recombinant plant tissue expressing the target products directly into human cell cultures. The moss Physcomitrella patens is a well-established model system used in plant development, desiccation and drought tolerance research, as well as for the production of biopharmaceuticals. The aim of this work was to test the
feasibility of using the moss P. patens as a living supporting scaffold for mammalian cell culture in cocultivation experiments. Specifically, we tested the ability of P. patens cells and human skin fibroblasts to survive long-term in each other’s presence and the influence of moss protonema on cell culture characteristics. We observed that after co-cultivation of P. patens and human fibroblast at 37°C and 5% CO2 without light for 27 days human skin fibroblasts formed normal monolayer. At the same time, all moss cells remained green at the stage of protonema and gametophores were not formed. Overall, after co-cultivation with P. patens, the human cell culture retained fibroblast-like morphology and surface markers (CD 90, CD105, CD 44). Viability tests showed that human skin fibroblasts retained their proliferation ability after co-cultivation with moss protonema. Thus, our data indicate that prolonged co-cultivation of moss and human cells does not appear to induce detrimental changes in fibroblast culture. We suggest that our data may form the foundation for the future investigations aimed at the development of a new effective natural systems for stable pharmaceutical production based on live plant tissue from the model moss Physcomitrella patens. This study was conducted according to the Russian Government Program of Competitive Growth of Kazan Federal University.

P2758
Board Number: B34
Homoharringtonine enhances transdermal absorption in the skin by regulating epidermal tight junction barriers.
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Objectives: The tight junction (TJ) is responsible for the epithelial barrier function of the skin. A TJ is composed of membrane proteins such as claudins, occludin, and ZO-1. It has previously been thought that only the stratum corneum is responsible for skin barrier function; however, knock-out mouse analysis has shown that claudin-1 is essential for skin barrier function. Furthermore, other TJ components including claudin-4 and occludin also contribute to the skin TJ-barrier. Homoharringtonine (HHT) is an alkaloid derived from the evergreen tree Cephalotaxus harringtonia. HHT reduces intestinal epithelial barrier function by inducing a change in the expression and localization of TJ components. However, the effect of HHT on the skin TJ barrier remains unclear. In the present study, we investigated HHT’s influence on the skin TJ barrier and the compound’s potential to enhance transdermal absorption.

Methods: The effect of HHT on the skin epithelial barrier was analyzed by measuring its effect on transepithelial membrane electrical resistance (TEER) and paracellular tracer flux analysis in Normal Human Epidermal Keratinocytes (NHEK), a human skin model. The effect of HHT on the expression of TJ components was analyzed by immunoblot assay. To examine the effect of HHT on mouse skin permeability, a filter paper impregnated with HHT and fluorescein isothiocyanate (FITC) -dextran (with molecular weights of 4,000 (FD-4), 10,000, or 20,000) were applied to mouse skin after removal of stratum corneum by tape-stripping, and then a blood time-course was collected. Results: HHT decreased TEER values and enhanced the paracellular flux of FD-4 in NHEK cells in a dose-dependent manner. The protein expression of TJ components such as claudin-1, claudin-4, and occludin was decreased by HHT. Examination of the transdermal absorption-promoting activity of HHT in vivo showed that the amount of FD-4 absorbed over 24 h of treatment was increased by HHT in a dose-dependent manner. A time-course of FD-4 absorption indicated that HHT increased the amount of FD-4 absorbed starting after about 6 h of treatment. Analysis using model dextrans showed that compounds with molecular weights of up to 10,000 were absorbed. Conclusion: We evaluated the transdermal absorption-promoting effect of HHT both in vitro and in vivo. HHT exhibited a transdermal absorption-promoting effect for macromolecules up to 10,000 Da in size, and reduced the expression of TJ components that are

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important for construction of the skin TJ barrier, including claudin-1, claudin-4, and occludin. These findings indicated that HHT acts on the epidermal TJ barrier and may serve as a novel transdermal absorption-promoting agent that permits the passage of high-molecular-weight drugs such as biopharmaceuticals.

P2759

Board Number: B35

Development of a stretchable device for live-cell imaging.

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Cells are mechanically sensitive and respond to their physical environment by alterations in protein levels, function, and localization. Processes such as proliferation, locomotion, invasion and metastasis can affect the mechanobiology of a cell. In vitro studies that aim to simulate in vivo conditions have significantly helped our understanding of the mechanobiology underlying such cellular processes. The ability of cells to respond to mechanical changes in their environment, and to alter their own mechanical attributes is, at least in part, dependent on a physical link between the cytoskeleton and the nucleus, which is provided by LINC (Linker of Nucleoskeleton and Cytoskeleton) complex proteins. The LINC complex consists of an outer nuclear membrane (ONM) nesprin (Nuclear Envelope Spectrin Repeat Protein) protein, and an inner nuclear membrane (INM) SUN domain protein, which form a physical connection in the perinuclear space, the space between the ONM and INM. Studying LINC complex proteins in the context of mechanobiology can help elucidate the underlying biophysics of these proteins and provide insights into mechanotransduction mechanisms.

Our aim is to exert physical stress on cells by uniaxial stretching and observe in real time the cellular responses, in particular at the level of LINC complex proteins. Several cell stretching devices have been developed to investigate controlled stretch and the effects of mechanical stimuli on cellular behavior. For our work, an important factor in such devices is their compatibility with live-cell imaging, which is critical in understanding how physical forces affect the LINC complex dynamics and their role as mechanosensors/mechanotransducers. In addition, we sought to develop a device that would exert even, uniaxial force across the entire field of stretch to allow for more accurate quantitative analysis. In response to these requirements, we have developed a simple, 3D-printed, low-cost microscope stage insert that allows for live-cell imaging under controlled uniaxial stretching that can be continuous or intermittent with time-controlled intervals. As with previously published stretching devices, we used elastomeric polydimethylsiloxane (PDMS) as membrane material to plate and stretch the cells. The stretch parameters (such as amplitude and frequency) of this device are easily adjustable, allowing for intra- and inter-experiment variability. We will use this stretcher to look at changes in LINC complex proteins in response to mechanical stress.
P2760
Board Number: B36
Functional characterization of an in vitro generated 3-D nerve bundle using capillary alginate gel on a microelectrode array.
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Microelectrode arrays (MEAs) are a useful tool for monitoring the functional activity of electrically excitable cells. On an MEA, traditional 2-D neuronal cultures fail to provide detectable action potentials from individual axons, and instead, electrical readings are recorded largely from the neuronal cell body. Here, we show the functional characterization of an in vitro generated 3-D peripheral nerve bundle using a capillary alginate gel (Capgel™) from the dorsal root ganglion (DRG) of embryonic rats on an MEA. We demonstrate that, in the absence of neuronal cell bodies, bundling and outgrowth of axons within the gel lead to recordable action potentials. We also show that electrical readings can be recorded over several weeks and the well-wide mean firing rate of the 3-D nerve bundle is far superior to the 2-D control conditions. Further analysis of individual electrodes reveals the presence of multiple action potentials on the same electrode, indicating that several different axons within the same bundle are contributing to the electrical activity. This was further validated by treatment of the 3-D nerve bundles with capsaicin, a compound that elicits a response from nociceptive neurons. In response to capsaicin, we observe that some axons within the bundle have firing rates that are four times the basal readings, whereas other axons within the same bundle show no change in the firing rate. This supports the idea that the nerve bundles generated using Capgel™ are a heterogeneous population of axons which recapitulate the composition of in vivo nerve fascicles. Overall, we show here in the context of recording electrophysiological activity that a 3-D environment facilitates bundling of axons and yields enhanced recordable action potentials over longer periods of time. Clinically, hyperexcitability of the DRG is one of the important underlying mechanisms of neuropathic pain. We propose the use of this 3-D system for electrophysiological studies that may provide insight into cellular behaviors and serve as a platform for the development of new therapeutic targets.

P2761
Board Number: B37
Properties of Compressed Melanin Sheets.
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Melanin is an ancient biopolymer pigment which appeared shortly after the dawn of life and is present in most organisms. It is recognized as a unique nano-material and is being developed for a wide range of potential applications, including sensors, coatings, and electrical devices. We have fabricated the melanin in a novel form which may be practical for a range of uses, and we have surveyed some of the interesting material science properties of the melanin slabs created. This included the hardness, elastic modulus, failure stress, and radiation absorbing capacity of samples created with a range of hydration
and temperature parameters. We have also examined the fabricated samples with scanning electron microscopy. Sepia (cuttlefish) melanin was heated, dehydrated, milled and compressed into sheets 0.5-3 inches thick. The resulting material was rapidly vacuum packed, and remained sealed until the mechanical testing or radiation testing was performed. The x-ray testing was conducted using x-ray cabinet X-RAD 320 at 320 kV, 2 mA and 98 seconds. The mechanical testing (elastic modulus and failure stress) employed a Tinius Oslen 10000 tension compression machine at 5mm / min with test run until sample failure. Hardness testing used a durometer with a D2240 type A probe and set at 5 imprints with 0.24 inches apart. Electron microscopy employed the Agilent FESEM 8500, a compact field emission scanning electron microscope. Mechanical testing showed an elastic modulus range of the samples of 10.44 – 27.67 MPa and failure stress of 0.709 - 12.72 MPa. The hardness range was 48 - 75 HV. The x-ray absorption testing revealed that the melanin had 11% more x-ray absorption than water. Scanning electron microscopy demonstrated spherical melanosomes, which remained generally intact in spite of the compressive forces applied during the preparation of the samples. Basic material science properties of a novel formulation of melanin were successfully measured, and will serve as the basis for further studies to enhance its applicability to a variety of applications.

**P2762**

**Board Number: B38**

**Cell culture confluence estimation using embedded application on the InCellis® Smart Cell Imaging System.**

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In cell biology related studies, accuracy and efficiency in cell culture quality checks are crucial in order to avoid any potential complications in the downstream analysis. Usually, different cell parameters such as cell counting and cell size measurement are assessed and estimated visually in a subjective way. However, visual assessments are unreliable, a time consuming and often yielding inaccurate results which lead to incorrect conclusions and incorrect recommendations. This also adds to the end cost of the analysis with regards to consumables such as costly reagents. One of the most common and important cell culture parameters is cell culture confluence. As the name suggests, confluence is the proportion of the area that is covered by cells and is the key parameter for most cell biologists. Similar to cell counting, cell confluence estimation is also based on a visual cell line assessment. In this study, cell confluence application was used to accurately estimate the proportion of adherent cells in an automated manner using a cell culture confluence application available on the InCellis® Smart Cell Imaging System (Bertin Technologies). HeLa cell lines were plated on the petri dish and incubated at 37°C for in appropriate cell culture media (DMEM). Cell confluence application installed on the InCellis® was then used to calculate cell culture confluence every 24 hours for three consistent days. A series of images of the cell line were taken in phase contrast mode using 10x and 20x objectives on day-1, day-2 and day-3 at the same time in order to check the accuracy of cell confluence. The cell line confluence ranged from 24% on day-1 to 58% on day-3. The results obtained showed consistent and accurate percentage of confluence across images taken using different objectives without affecting the cell lines. The cell confluence application supplies robust results with a staining free method. The application ensured a rapid and efficient quality control of the cell lines before use in other analysis such as transfection, cell-based assays or any other cellular analysis. The automated estimation of the cell
confluency not only provides consistent results but also significantly reduces the hands-on time for all cell-based assays.

**P2763**
**Board Number: B39**
Efficient microscopy image visualization and cell tracking analysis of multi-gigabyte datasets. M. Jones¹, H. Lai¹, V.T. Chou², J.B. Long³, M. Arnes³, K. Obbad³, S.V. Alworth⁴, C. Huang⁵, L.A. Lucas⁶, D. Van Vactor⁶, J.S. Lee⁶;
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Characterizing the molecular and cellular migration behavior is critical for understanding the complex protein interactions that drive brain development and neurodegeneration. Light sheet microscopy coupled with modern sample clearing techniques enable the visualization of multi-millimeter samples (e.g., a mouse brain) with high spatiotemporal resolution. Efficient display and accurate analysis of multi-gigabyte datasets remain a major bottleneck. Traditionally, the analysis pipeline of these datasets (visualization followed by tracking) is accomplished using open-source tools (such as FIJI or ICY) with an off-the-shelf algorithm (such as Brownian motion) or a custom algorithm tailored to a specific application (such as plusTipTracker plug-in for Matlab). However, neither approach is suitable for tracking the wide range of motion types that exist in a developing neuronal circuit. We have developed a new volume rendering pipeline which is capable of handling and displaying data sets that can be hundreds of gigabytes (GBs) for a 3D image, and multiple terabytes (TBs) for a 3D time lapse. The new renderer leverages the advantages of using an internal multi-resolution, multi-block architecture. Moreover, we have optimized the memory management system which ensures real-time high definition rendering and interaction after the file is loaded. We have also developed a general-purpose 3D+time particle tracking algorithm that enables tracking objects with complex spatiotemporal features with good specificity. Our tracking algorithm combines the tracking stability of the Hungarian algorithm and the tracking sensitivity of the greedy algorithm for matchmaking. The algorithm is further enhanced with self-correction that updates track matchmaking using temporal information from previous frames. We have applied our tracking algorithm to analyze the dynamics of EB1-GFP labeled microtubules in *Drosophila* neuromuscular junctions and sensory dendrites, and to the developing *Drosophila* embryo reference dataset from the Cell Tracking Challenge 2015. This allowed us to validate the performance of our algorithm when tracking complex particle motions in both 2D and multi-GBs 3D time-lapses. Efficient real-time handling of multi-TBs 3D time lapse data sets coupled with accurate cell tracking of a range of motion types is critical for the analysis of microscopy images. The present work describes a pipeline which covers both visualization and analysis of very large multi-dimensional images. This pipeline can aid in the advancement of research in neurosciences, developmental biology, cancer biology as well as systems biology.

**P2764**
**Board Number: B40**
High-throughput open source analysis of 3D structures using CellProfiler.
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Increasingly, researchers create complex biological model systems involving three-dimensional structures, including organoids, tumor spheroids, and even whole organisms. Imaging this biology in 3D
creates datasets that are volumetric, high-resolution, and orders of magnitude larger than typical 2D images. High-content screening systems enable acquisition of 3D images in high-throughput, yet the software to analyze crucial metrics from these images has lagged behind. We have added 3D segmentation, registration, and measurement capabilities to CellProfiler, open-source software for high-throughput image analysis. We are testing these new features in several experimental situations including 3D images of live gene-edited human induced pluripotent stem cells labeled with cellular and nuclear dyes.

P2765
Board Number: B41
Assessing autophagic flux in 2D and 3D cell culture models with a novel plate-based assay.
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The importance of autophagy in cell health, both in normal and diseased states, has become increasingly clear. Although autophagy is a growing target of screeners in areas such as cancer, neurodegeneration, inflammation and immunology, there is a need for more convenient and effective assays for screening applications. Here we utilize NanoLuc® Binary Technology (NanoBiT™) to develop a homogeneous plate-based assay to measure autophagic flux in both 2D and 3D cell culture models. In this approach, LC3B (Atg8) protein was tagged on its N-terminus with an 11 amino acid peptide, HiBiT, as well as an intervening “spacer” sequence. When stably expressed at low to moderate levels in mammalian cell lines, this LC3-based reporter is subject to degradation by the autophagic pathway. Following compound treatment, cellular levels of this novel autophagy reporter is determined by addition of a lytic detection reagent containing Large BiT (LgBiT). LgBiT rapidly associates with HiBiT in the cell lysate, producing a bright, luciferase activity in the presence of substrate that is proportional to the amount of autophagy reporter present in the assay well (R² = 0.99). Assay signal following reagent addition is stable (T1/2 > 3h), allowing assay of multiple 96- or 384-well plates in the same experiment. In U2OS cells stably expressing this autophagy reporter, assay signal following mTORC inhibitor treatment decreased by 25% (rapamycin) to 60% (PP242, AZD8055), consistent with compound stimulation of autophagic flux and consequent degradation of autophagy reporter. In contrast, treatment with autophagy inhibitors (bafilomycin A1, chloroquine) produced a 70-80% increase in assay signal, consistent with accumulation of autophagy reporter following blockade of basal autophagy. Similar results were obtained in HEK293 cells stably expressing the reporter. Mechanism of action of autophagy modifiers was confirmed through blockade of effects by 50nM bafilomycin cotreatment. Multiplex with a cell necrosis detection agent allowed for same-well determination of cytotoxic effects that might undermine analysis of a compound’s discrete effects on autophagic flux. When assayed in 384-well plates with automation, U2OS and HEK293 autophagy reporter cells produced Z’ values of 0.6-0.7 in response to autophagy induction (PP242), and Z’ values of 0.7-0.8 by subsequent blockade of autophagy (PP242 + bafilomycin A1). In HEK293 reporter cell spheroids, both induction and inhibition of autophagic activity was easily observable following reference compound treatment. Therefore, using this novel plate-based assay system for the determination of autophagic flux, it is possible to screen test agents and quantitatively assess their effects in both 2D and 3D cell culture models.
P2766

Board Number: B42
Combining impedance-based viability measurements and flow cytometric analyte quantitation to evaluate effector cell killing of T lymphocytes.
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Cancer immunotherapy is increasingly being evaluated as an approach to cancer treatment by harnessing the immune system to attack cancer cells. Moving this research from the bench to the clinic is critically important but reliable tools are needed to translate in vitro protocols and results for use in vivo. Here, we have used a combination of impedance-based technology and flow cytometry to evaluate both the target and effector cells in an T-cell mediated B cell killing assay. Impedence based Real Time Cell Analysis (RTCA) technology with the xCELLigence® system provides a continuous readout of target cell viability while flow cytometry allows measurement of specific responses such as target cell viability and cytokine/cytolytic production by cells throughout the course of the assay. Here, we investigated the role of CD19-BiTE® to enhance the cytotoxic effect of T lymphocytes on a B-cancer cell line, Daudi cells. In conjunction, cytokine and cytolytic protein concentration measurements were determined to enhance our understanding of the T cell response. CD19-BiTE treatment dramatically enhances T cell-mediated B cell killing which can be assayed on both the xCELLigence RTCA and NovoCyte® flow cytometer. The addition of CD19-BiTE also leads to increases in the production of important cytokines and cytolytic proteins crucial for a robust cytotoxic T cell response such as IFNγ, IL-2, TNFα, sFASL, Granzyme B, and Perforin.

P2767

Board Number: B43
Expansion and Optimization of DIVA DNA Sequence Validation Services.
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Advances in DNA sequence validation technology has enabled the Joint BioEnergy Institute (JBEI) to offer high-throughput plasmid sequencing to its researchers. However, the types of compatible samples have not significantly expanded. This lack of compatibility leads to time and materials wasted on purifying or transforming a plasmid into E. coli. Here, we sought to update the current sequencing protocol to include the most utilized species and plasmid types, including E. coli cultures with medium and low copy plasmids, A. tumefaciens, P. putida, S. cerevisiae, and P. pastoris. Based on previous runs, the laboratory determined that a baseline coverage greater than 60x over the whole plasmid yielded the most accurate reads. This project showed that E. coli was the most robust species, with sequencing succeeding for all liquid cultures, regardless of copy number, and for resuspended colonies picked directly from agar plates. The remaining species experienced varying levels of coverage failure. However, minimal modification to the existing sequencing workflow, such as longer lysing times or the use of higher copy plasmids, could increase overall coverage. Overall, the sequence validation services provided by JBEI have numerous advantages over traditional Sanger sequencing with third-party companies. By expanding the compatible sample types, we are making this efficient, cost-effective sequencing service more accessible to JBEI’s research community.
P2768
Board Number: B44
Efficient Selection of Single-Domain Antibodies from a Naïve Synthetic Library Using Phage Display and Yeast Two-Hybrid Screening.
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High-affinity single-domain antibodies, like VHH, represent indispensable tools for research and clinical applications. To access VHH without Llama immunization, we have built a fully synthetic humanized naïve Llama VHH library containing 3x10^9 antibodies, based on a unique scaffold with random complementary determining regions (CDRs). Using phage display, we successfully selected antibodies from this library against a variety of antigens from large proteins to haptens, soluble proteins and receptors directly selected from cell surface expression. The affinity of the VHHs obtained from this library is similar to the affinity of antibodies selected after animal immunization. A combination with subsequent exhaustive yeast two-hybrid (Y2H) screening allows to further favor the selection of intrabodies – antibodies working inside living cells. The VHH clones are directly accessible and the recombinant antibodies can be produced as fusions to different Fc domain (human, mouse, etc.). We will present here the selection of single-domain antibodies from our synthetic library against p53, USP7 and a cell surface antigen.

P2769
Board Number: B45
Engineering Vero Cell Line to Enhance Vaccine Production.
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Worldwide, the ability to control infectious diseases requires improved vaccines, increasing their production capacity and reducing manufacturing costs. Using genome-wide small interfering RNA (siRNA) screens, we validated for NEU2, COQ9 and BTN2A1 genes in Vero cells as essential for rotavirus, dengue virus, enterovirus 71 and yellow fever virus replication. In this study, we used the CRISPR/Cas9 system to edit these genes in Vero cells. Following transection of the CRISPR/Cas9 plasmids, we observed average transfection efficiencies of 14.85%, 13.54% and 14.64% for the NEU2, COQ9, and BTN2A1 genes, respectively. Putative knockout clones will be validated through sequencing and testing of phenotypic increase of viral production. Results will demonstrate the applicability of genetic engineering in increasing the production output of vaccine cell lines.
P2770

Board Number: B46

A novel rapid cell ablation model for eliminating one or two maker-labelled cell populations in mice—a model for studying the pathogenesis of human diseases.
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Loss-of-function (LOF) studies using conditional targeted cell ablation have been widely used in vivo to study cell functions and interactions, tissue repair and differentiation though to date they can only target a single labelled cell population. Therefore, there is an unmet need to develop a tool that is able to specifically eliminate a subset cells that require more than one marker to appropriately identify in animals for LOF studies. To this end, we propose to generate an interleukin-12 (IL12)-mediated subset cell ablation model by targeting cell subsets with two markers. ILY, a toxin secreted by Streptococcus intermedius (SI), exclusively binds to the human cell membrane protein CD59 (hCD59), but not to CD59 of any other species. Once bound, ILY rapidly and potently lyses the cells. Taking advantage of these features, we recently established a Cre-inducible floxed STOP-hCD59 transgenic mouse (ihCD59) where hCD59 expression only occurs after Cre-mediated recombination (Feng D, et al. JCI 2016). Administration of ILY to various lines of Cre+ihCD59+ mice resulted in rapid and specific ablation of single marker immune, epithelial or neural cells without any off-target effects. Importantly, ILY has a large pharmacological window, which allows us to perform dose-dependent study. We also tested ILY/ihCD59-mediated cell ablation in several disease models, including inflammatory diseases, hepatocyte and/or biliary epithelial damage and regeneration. This line (ihCD59) targets only one marker-labelled cell population, and cannot be used for ablating subset cell populations. To further advance this tool towards subset cell ablation, using the well-established Cre-loxP and Flp-Frt systems, we have successfully developed a double inducible mouse strain: CAG-floxSTOP-floxP-Frt-STOP-Frt (LSL-FSF)-hCD59 (DihCD59) where the transgene (hCD59) expression occurs only following Cre- and Flp-mediated combinational events. To investigate whether Cre and Flp-mediated combination are required to induce hCD59 expression in DihCD59, we crossed DihCd59 with ROSA29Flp+/-, a germ line Flp expressing strain, and Foxp3CreER+/-, a Foxp3 (Treg marker) promoter-controlled Cre expressing strain to generate the triple transgene positive mice (DihCD59+/−-ROSA29Flp+/-Foxp3CreER+/-). We documented that hCD59 expression in Treg depends on both Cre and Flp-induced recombinational events to remove the two Floxed STOP cassette in triple transgene positive compound mice but not Cre or Flp-induced single recombination. ILY injection resulted in ablating the hCD59 expressing Treg cell population but not any other cells. Together, these results document we have successfully generated a novel rapid cell ablation model for eliminating one or two maker-labelled cell populations in mice.

P2771

Board Number: B47

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Antibiotic-resistance genes are now widely used as markers for gene transfer in many organisms and have greatly contributed to biological experiments. Antibiotic selection, such as puromycin, hygromycin and geneticin selection, has been applied to nematode Caenorhabditis elegans (C. elegans) to create
transgenic worms. However, from perspectives of efficiency, facility and cost, developing another choice is valuable and expected.

Nourseothricin (NTC) is an aminoglycoside glycopeptide antibiotic of Streptothromycin class. Because of its nephrotoxicity, NTC is not used for therapeutic purpose. However, it has already been reported as a marker for genetic modifications in yeast, plants and mammalian cells. By trying NTC resistance genes in *C. elegans*, we found one of the resistant gene *Sat2* works in *C. elegans* and established this system as a new transgenic marker for *C. elegans* genetics.

To determine the critical concentration of NTC for antibiotic selection, we added certain concentration of NTC solutions to NGM plates already seeded with OP50, then put adults or synchronized L1 worms on them. Control worms without a resistant gene died in the presence of NTC. The critical concentration of NTC was lower than Hygromycin. Next, we developed nematode transformation vector carrying *Sat2* under the control of universal promoter of *C. elegans* ribosomal protein gene *rps-27*. *Sat2* carrying worms could grow on NGM plates containing NTC. Now we are trying to combine this NTC-*Sat2* system with CRISPR/Cas9 system and establish new genetic methods.

As NTC has no cross-reactivity with other aminoglycoside antibiotics such as Hygromycin or Geneticin, resistance genes for these drugs can be used with *Sat2* simultaneously. The efficiency and cost of NTC selection is comparable to widely-used Hygromycin selection. Thus, combining with other drugs will enable the development of wider range of powerful applications. In this poster, I will show the method of NTC-*Sat2* screening and discuss its possible application.

P2772

**Board Number: B48**

**SUMO-TARGETING OF A STRESS-TOLERANT SUMO PROTEASE.**

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SUMO proteases of the SENP/Ulp family are master regulators of both sumoylation and desumoylation and regulate SUMO homeostasis in eukaryotic cells. SUMO conjugates rapidly increase in response to cellular stress, including nutrient starvation, hypoxia, osmotic stress, DNA damage, heat shock, and other proteotoxic stressors. Nevertheless, little is known about the regulation and targeting of SUMO proteases during stress. To this end we have undertaken a detailed comparison of the SUMO-binding activity of the budding yeast protein Ulp1 (ScUlp1) and its ortholog in the thermotolerant yeast Kluyveromyces marxianus, KmUlp1. We find that the catalytic UD domains of both ScUlp1 and KmUlp1 show a high degree of sequence conservation, complement a Ulp1Δ mutant in vivo, and process a SUMO precursor in vitro. Next, to compare the SUMO-trapping features of both SUMO proteases we produced catalytically inactive recombinant fragments of the UD domains of ScUlp1 and KmUlp1, termed ScUTAG and KmUTAG respectively. Both ScUTAG and KmUTAG efficiently bind a variety of purified SUMO isoforms. Specifically, they bound immobilized SUMO1 with nanomolar affinity. However, KmUTAG showed a greatly enhanced ability to bind SUMO and SUMO-modified proteins in the presence of oxidative, temperature and other stressors that induce protein misfolding. We also investigated whether a SUMO-interacting motif (SIM) in the UD domain of KmULP1 that is not conserved in ScUlp1 may contribute to the SUMO-binding properties of KmUTAG. In summary, our data reveal important details about how SUMO proteases target and bind their sumoylated substrates, especially under stress conditions. We also show that the robust pan-SUMO binding features of KmUTAG can be exploited to detect and study SUMO-modified proteins in cell culture systems.

Tuesday-30
P2773

**Board Number: B49**

Development and functional analysis of novel PEI-based mammalian cell transfection reagents.

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As novel research and therapeutic techniques are developed that require the delivery of nucleic acids into mammalian cells and tissues, the need for highly effective transfection reagents that display minimal toxicity is essential. Cationic polymers, whose architecture can be chemically altered to control their functionality, have become a particularly attractive target for further development. Among cationic polymers, polyethylenimine (PEI) has shown promising DNA complexation and transfection efficiency rates, but also exhibits relatively high cell toxicity. In this project, we have worked to develop easily-tailorable, non-toxic, nucleic acid delivery systems based on PEI functionalized with polyethylene glycol molecules. We have studied the toxicity and transfection ability of a pair of novel, chemically-related polymers in range of cell lines, including utilizing fluorescently-tagged polymer and plasmid DNA to enable the detailed monitoring of the DNA-delivering complexes in cells via confocal microscopy. These studies demonstrate that minor differences in the chemical architecture of functionalized PEI can have significant effects on cell toxicity, and point the way towards the development of effective, targeted cationic polymer-based reagents.

P2774

**Board Number: B50**

Matching refractive index of mounting media improves axial resolution and image quality in 3D biological samples.

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Axial resolution in light microscopy can be improved by matching the refractive index (RI) between the biological specimen and the microscope objective. The path of a photon emitted by a fluorophore in a biological sample passes through 1) the mounting media in which the sample is embedded, 2) a glass coverslip, 3) immersion oil, and finally 4) the microscope objective. While the optical path between the objective and coverslip (2-4) typically has a matched RI of 1.52, the majority of biological specimens are mounted in media of substantially lower RI, ranging from 1.42 - 1.47, causing a loss in axial resolution due to refractive index mismatch. Here, we use laser scanning confocal microscopy to demonstrate the deleterious effects of mismatched refractive index in conventional mounting media by measuring the point spread function (PSF) of sub-diffraction sized fluorescent microspheres. We then compare these results with specimens prepared in ProLong™ Glass mounting media, featuring an optimized refractive index of 1.52. Our data indicates that at depths beyond 20 μm, an RI-mismatched mounting media suffers significant losses in axial resolution, whereas the RI-matched mountant maintains optimal axial resolution throughout, enabling up to a 75% improvement in axial resolution at focal depths of 100 μm and beyond. By eliminating unwanted refraction, ProLong™ Glass also maximizes the light reaching the objective lens, thus providing higher sensitivity and a brighter image when imaging thick biological samples, such as spheroid cultures that mimic tumor microenvironments. Combined with photobleach protection antifade properties in the visible and near infra-red spectra, the optimized refractive index of ProLong™ Glass mounting media measurably increases fidelity and image quality for bright, high-resolution 3D analysis of any cell/tissue type.
P2775
Board Number: B51
Engineered Cell Penetrating Peptides for Molecular Delivery.
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Cell penetrating peptides (CPPs) are a unique non-viral vector for delivery of molecules into cells. The earliest CPPs were based on natural protein sequences such as TAT from HIV-1 and Antp from Drosophila. These peptide sequences contain many positively charged amino acids (i.e. arginine and lysine) which allow for interactions with the negatively charged plasma membrane of cells. Later, synthetic and designer CPP sequences were developed for specific applications such as drug delivery, imaging, and gene therapy. These synthetic sequences are typically amphiphilic (or amphipathic), containing a hydrophilic and a hydrophobic region. Our lab has developed a novel amphiphilic CPP, called PepB, which is able to internalize into various cell types including stem cells and cancer cells. Depending on the number of repeats PepB can form α-helices and the number of repeats will modulate the cell penetrating capabilities. In this presentation, uptake and cytotoxicity properties of PepB will be correlated to the peptide sequence lengths. We, in addition, will provide live-cell fluorescent microscopy data utilizing multiple mammalian cell types.

P2776
Board Number: B52
POST-STIM1 interactions modulate Ca²⁺ oscillation frequency via modulation of PMCA4 function.
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Ca²⁺ oscillations control both NF-kB and NFAT activity and are therefore essential for T cell activation. Ca²⁺ oscillations reflect a combination of ER Ca²⁺ release, Ca²⁺ entry and Ca²⁺ pumping; as a regulator of both Ca²⁺ entry via Orai1 and Ca²⁺ pumping via PMCA4, STIM1 would be expected to serve a critical role in the generation of Ca²⁺ oscillations. The ER membrane protein Partner of STIM1 (POST) was found to bind both PMCA4 and STIM1; how this affects oscillation frequency is less clear. To address this question, Jurkat T cells were transfected via electroporation with a POST plasmid for overexpression or siRNA for knockdown. These cells were then stimulated with activating antibodies directed against human CD3/CD28 and loaded with Fura-2 AM. Stimulated and unstimulated T cells were placed in a Ca²⁺ containing imaging buffer and oscillations in cytosolic Ca²⁺ were observed for ten minutes. The variance in cytosolic Ca²⁺ concentration under of each condition was compared. Surprisingly, Ca²⁺ oscillations were observed even in unstimulated Jurkat T cells. As expected, activation with anti-CD3/CD28 antibodies increased oscillation variance under all conditions. In unstimulated conditions, POST expression was correlated with the fraction of non-oscillating cells. In stimulated conditions, POST expression shows a Goldilocks effect with both POST overexpression and knockdown having smaller fractions of non-oscillating cells as compared to cells with endogenous levels of POST. Considered collectively with POST-mediated regulation of PMCA4 function, these findings reveal important roles for POST and STIM1 for control of PMCA4 activity in the generation of Ca²⁺ oscillations for control of T cell activation.
P2777

Board Number: B53

A Novel Approach for Characterizing the Cell-Implant Adhesion.

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Cell adhesion is essential to cell communication, cell regulation, and the development and maintenance of tissues. We have developed a non-invasive real-time approach based on the use of the quartz crystal microbalance with dissipation monitoring (QCM-D) for monitoring and characterizing the adhesion process of human epidermal keratinocytes to the titanium surface, a common material for medical implants. With this approach, we have investigated the effects of chemical, electrical and biochemical treatments on the adhesion process, particularly on the level of the cell adhesion. A further understanding of the cell adhesion process and the alteration of such process in responding to exogenous stimuli may provide leads on the development of therapies for enhancing adhesion of cell on medical implants and promoting wound healing and tissue regeneration.

Actin Nucleating Proteins

P2778

Board Number: B55

Arp2/3 complex- and formin-mediated actin networks tune actin-binding protein sorting in fission yeast.

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F-actin networks are diverse and specialized to carry out specific cellular processes, such as polarization, endocytosis, and cytokinesis. Each F-actin network is tightly regulated by a unique set of actin-binding proteins (ABPs) that initiates and maintains the organization and dynamics of actin filaments. However, the general mechanistic principles by which specific ABPs sort to particular F-actin networks remain largely unclear. By combining in vivo fluorescence microscopy and in vitro reconstitution, we discovered that two actin assembly factors, Arp2/3 complex and formin Cdc12, tune the binding of ABPs fimbrin and tropomyosin to specific F-actin networks in fission yeast. Disruption of F-actin networks by small molecule inhibitors or genetic manipulation revealed that fimbrin is preferentially recruited to Arp2/3-complex mediated actin patches, while tropomyosin is preferentially targeted to the contractile ring by Cdc12-mediated filaments. To investigate the role of Arp2/3 complex and Cdc12 in this sorting, we used four-color in vitro TIRF microscopy to reconstitute ABP sorting with purified proteins. We discovered that either fimbrin or tropomyosin alone binds similarly to Arp2/3 complex- and Cdc12-mediated actin filaments. Conversely, sorting of these ABPs to their preferred actin filaments occurs when Arp2/3 complex- and Cdc12-mediated filaments are assembled together in the presence of both fimbrin and tropomyosin. Under these conditions, fimbrin accumulates ~2-fold more at Arp2/3 complex branch points, while tropomyosin association is enhanced 3.5-fold on filaments assembled by Cdc12. This result suggests that competition between fimbrin and tropomyosin is necessary for their sorting to different F-actin networks. In summary, these findings reveal for the first time that the F-actin assembly factors...
Arp2/3 complex and formin Cdc12 facilitate the recruitment of specific ABPs, thereby tuning ABP sorting and subsequently establishing the identity of a given F-actin network.

P2779
Board Number: B56
Arp2/3 complex and the nucleation promoting factor Wash are involved in the formation of MTOC-TMA during Xenopus oocyte maturation.
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During maturation of Xenopus oocytes, the microtubule organizing center and transient microtubule array (MTOC-TMA) forms at the vegetal side of the nucleus and we have previously demonstrated that this formation requires ADF/cofilin dependent actin reorganization. However, the molecular mechanism that coordinates actin filaments with microtubule (MT) during oocyte maturation wholly remains to be clarified. Here we show that Arp2/3 complex and its nucleation promoting factor Wash are involved in the MTOC-TMA assembly. Overexpression of Arp2/3 complex subunits increased actin filament formation inside the yolk free region and generated additional MTOC-TMA- like structures along the edge of yolk free region. Wash depletion disrupted actin and MT alignments at the base of MTOC-TMA. In vitro co-sedimentation assay revealed that the Xenopus Wash VCA domain which is the actin and Arp2/3 complex binding domain directory bound to MT. However, the WHD2 domain that previously reported to be a MT-binding domain of mammalian Wash did not bind to MT. Interestingly, injection of the WHD2-deleted mutant into oocytes caused drastic accumulation of actin filaments in the cytoplasm and malformation of MTOC-TMA, suggesting that WHD2 domain negatively regulates the VCA domain activity and is responsible for precise localization of Wash during oocyte maturation. Our results suggest that Wash-Arp2/3 complex-nucleated actin filaments are involved in the assembly of MTOC-TMA during Xenopus oocyte maturation.

P2780
Board Number: B57
Abp1 stimulates Arp2/3 complex nucleation of actin filament branches and protects them from debranching by GMF.
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Abp1 (also known as mAbp1, HIP55, SH3P7, or Drebrin-like protein) is a multi-domain actin binding protein that is highly conserved from yeast to humans. Abp1 binds to actin filaments through its conserved N-terminal ADF (Actin depolymerization factor homology) domain and links endocytic machinery to the actin cytoskeleton through its C-terminal SH3 domain. Yeast Abp1 alone shows no effects on actin filament dynamics but has nucleation-promoting factor (NPF) activity on Arp2/3 complex despite lacking actin monomer binding affinity like other NPFs (Goode et al., 2001). Here, we examined Abp1’s effects on Arp2/3 complex-mediated actin assembly and actin filament branch turnover using single molecule TIRF microscopy. In contrast to its close relative ADF/cofilin, Abp1 interacted with actin filaments non-cooperatively and with a sub-second resident time. Abp1 also interacted dynamically with Arp2/3 complex in the absence of actin, using its two acidic (A) motifs. However, Abp1 bound strongly, with a residence time of tens of seconds, to Arp2/3 complex in filament
branch junctions. These properties could allow Abp1 to scan filaments and find branch junctions in vivo. Through direct visualization of fluorescently labeled Arp2/3 complex, we confirmed a longstanding hypothesis that Abp1 stimulates daughter filament nucleation by recruiting Arp2/3 complex to the sides of mother filaments. Further, single particle EM analysis revealed that two Abp1 molecules bind to Arp2/3 complex. We also observed a competitive relationship between Abp1 and GMF, another ADF-like protein that interacts with Arp2/3 complex. Abp1 directly competed with GMF for Arp2/3 binding, and attenuated GMF’s ability to prune branches. Taken together, this evidence points to a novel mechanism of stimulating daughter branch nucleation and branch stabilization, completely distinct from the canonical ‘VCA pathway’.

P2781
Board Number: B58
Arp2 Phosphorylation is Not Essential for Arp2/3 Complex Activity in Fission Yeast.
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A 2008 study of Arp2/3 complex proposed that three conserved amino acids of the Arp2 subunit must be phosphorylated to activate the complex in Acanthamoeba castellanii. (LeClaire et al. 2008) However, these sites are not phosphorylated in crystal structures of purified Bos taurus Arp2/3 complex, which is highly active and polymerizes branched actin filaments in vitro.

We tested whether Arp2 phosphorylation is necessary to activate the fission yeast (Schizosaccharomyces pombe) Arp2/3 complex. The proposed phosphorylation sites are conserved in S. pombe as Y198, T233, and T234, and mass spectrometry revealed that Y198 and T233 are phosphorylated in vivo. We replaced one, two, or three of these amino acids in the S. pombe genome with alanine, which cannot be phosphorylated. Arp2/3 complex activity is essential for viability in fission yeast, yet all generated mutants remained viable. Therefore, phosphorylation at these sites is not necessary for the essential activities of S. pombe Arp2/3 complex. Interestingly, when T233 was replaced with aspartic acid, which mimics constitutive phosphorylation, the resulting mutant displayed a growth defect at high temperature. Replacing both threonines with aspartic acid was lethal.

In S. pombe, the Arp2/3 complex continuously assembles branched filaments in actin patches, generating force to internalize endocytic vesicles. These patches are consistent in size and can be labeled by GFP-tagging the actin crosslinker Fim1, facilitating automated image segmentation and tracking. We used quantitative fluorescent confocal microscopy to observe the time course of actin patch assembly in vivo. Replacing all three proposed phosphosites of Arp2 with alanine slowed actin patch assembly by 42%, demonstrating that the mutations reduce Arp2/3 complex activity. However, replacing T233 with aspartic acid had a similar effect. Therefore, we propose that phosphorylation of Arp2 at Y198, T233 and T234 is not required for the Arp2/3 complex to be active in fission yeast. Instead, mutations at these sites may reduce the activity of the complex by altering its structure. If these mutations serve an important regulatory function in A. castellanii, it is not conserved in fission yeast. However, phosphorylation at other sites, such as the widely conserved residue Y218, may still play an important role in Arp2/3 complex regulation.
P2782
Board Number: B59
Multiscale Model of the Formin Homology 1 Domain Illustrates its Role in Regulation of Actin Polymerization.
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Formins are important regulators of actin polymerization. Formins bind profilin to the polyproline tracks of its believed flexible Formin Homology (FH) 1 domain. The FH2 domains wrap around the barbed end of the actin filament and elongate the filament processively. Profilin-actin complexes on the FH1 domain are modeled to transfer to the barbed end; however, the mechanism is not known. Previous models of the FH1 domain have not captured sequence-specific effects such as the length and distribution of the polyproline tracks and possible variety in mechanosensitivity and response to bound profilin/profilin-actin. To remedy this, we perform simulations of the FH1 domain of well-studied formins: the mouse formins mDia1 and mDia2, the budding yeast formins—Bni1 and Bnr1, and the fission yeast formins—Cdc12, Fus1, and For3. We perform all-atom molecular dynamics simulations of each of these FH1 domains and show that FH1 is a typical intrinsically disordered protein (IDP), with the polyproline tracks forming high propensity poly-L-proline helices. We develop an alpha-carbon coarse-grained model that retains the sequence-specificity of the FH1 domain which is consistent with the IDP notion of FH1, and use this to study the FH1 domain in the context of its biological role. We use the coarse-grained model to investigate the response of FH1 to force and bound profilin. We show how bound profilin/profilin-actin may extend the FH1. We show definitively that multiple profilin-actin complexes can simultaneously bind to the FH1, which may be biologically important given the relatively high concentration of profilin-actin in vivo. We examine the transfer mechanism in further detail. We show how the FH1 may be affected by being on the FH2-bound actin filament barbed end rather than in isolation and examine the geometry of this system with bound profilin-actin.

P2783
Board Number: B60
Force dependence of filopodia adhesion: involvement of myosin II and formins.
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Filopodia are dynamic membrane protrusions driven by polymerization of an actin filament core. This polymerization is mediated by formin molecules at the filopodia tips. Growing filopodia can adhere to the extracellular matrix and experience both external and cell generated pulling forces. The roles of such forces in filopodia growth and adhesion is however insufficiently understood. Here, we induced sustained growth of filopodia by applying pulling force to their tips via attached fibronectin-coated beads trapped by optical tweezers. Strikingly, pharmacological inhibition or knockdown of myosin IIA, which localized to the base region of the filopodia, resulted in cessation of filopodia growth and

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weakening of filopodia adherence strength. Similar effects were obtained by treating cells with a small molecular formin inhibitor SMIFH2. SMIFH2 caused deterioration of myosin X- VASP- and talin-enriched patches associated with actin filaments at the filopodia tips, followed by quick myosin II dependent retrograde movement of these components. Such dynamics can be explained by SMIFH2-induced detachment of actin filaments from formin molecules observed in in vitro experiments. Collectively our results suggest that myosin IIa generated centripetal force transmitted to the filopodia tips through interactions between formins and actin filaments is required for filopodia adhesion and growth. This force dependent adhesion behavior could play an important role in filopodia-mediated matrix rigidity sensing, durotaxis and tumor invasion.

P2784
Board Number: B61
The interaction of FHOD1 with nesprin-2G activates a cryptic actin binding site and stimulates potent actin bundling activity: implications for nuclear movement.
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The formin FHOD1 binds to specific spectrin repeats (SR11-13) in the outer nuclear membrane protein nesprin-2G and this interaction is required for formation of TAN (transmembrane actin-associated nuclear) lines and nuclear movement in fibroblasts (Kutscheidt et al, NCB, 2014). To understand the mechanistic basis for this, we tested how the interaction of nesprin-2G’s SR11-13 affected FHOD1’s actin polymerizing and bundling activities. Using purified recombinant proteins, we confirmed that full length (FL) FHOD1 alone did not stimulate actin polymerization by pyrene assays but rather weakly inhibited it. Addition of nesprin-2G SR11-13 activated the inhibitory effect of FL FHOD1 on actin polymerization to a similar degree as that of constitutively active FHOD1 ΔDAD. FHOD1 has reported F-actin bundling activity (Schonichen et al, JCS, 2013) and we confirmed that FHOD1 ΔDAD bundled F-actin and that this activity required both its FH2 domain and a putative second actin binding site (ABS). Low speed pelleting assays to detect actin bundling activity revealed that SR11-13 activated FL FHOD1’s bundling activity (SR11-13 had no activity alone). The level of bundling with FL FHOD1 and SR11-13 was similar to that of FHOD1 ΔDAD, yet SR11-13 further stimulated FHOD1 ΔDAD’s bundling activity to the low nM range making it more potent than that either fascin or α-actinin. TIRF microscopy of fluorescent actin filaments showed that actin bundles were formed by FL FHOD1 only when SR11-13 was present. To understand how SR11-13 stimulated bundling, we tested its effect on the ABS upstream of FHOD1’s FH1 and FH2 domains. Using an N-terminal (NT) FHOD1 construct containing the ABS and adjacent SR11-13 binding site, we found that NT FHOD1 alone did not bind to actin filaments, but did so in the presence of SR11-13. These results show that the nesprin-2G potently activates FHOD1’s actin bundling activity by activating a cryptic actin binding site and suggest that the nucleus plays an active role in strengthening it’s interaction with actin bundles during nuclear movement.

P2785
Board Number: B62
Understanding muscle cell size regulation by the Caenorhabditis elegans formin FHOD-1.
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Formins are best known for their ability to nucleate actin and to remodel actin organization within cells. A mammalian formin FHOD3 has been shown to be required for cardiac muscle to mature past
premyofibrils formation in mice and for sarcomere organization in cardiomyocyte cell culture. Activated FHOD3 induces hypertrophy in neonatal rat cardiomyocytes. However, we do not know how FHOD3 functions. To avoid the pleiotropic effects and embryonic lethality of the mouse model, we will examine a FHOD3-related formin of Caenorhabditis elegans, FHOD-1. FHOD-1 localizes to all muscle types within the worm, including the largest muscle group, the striated body wall muscles (BWM). Previous work has shown that knockout of fhod-1 in the worm will cause thinning of the BWM cells. These fhod-1(-) worms also have disrupted dense bodies, attachment complexes thought to be analogous to mammalian Z-lines, and have a reduction in muscle specific myosin heavy chain 3 (MYO-3) protein expression. We considered four plausible mechanisms of reducing MYO-3 expression in fhod-1(-) worms: 1) reduced transcription, 2) reduced mRNA stability, 3) increased protein degradation or 4) reduced translation. To determine if myo-3 mRNA level was reduced in fhod-1(-) worms, we performed qRT-PCR. Surprisingly, we discovered an increase in myo-3 mRNA levels upon loss of FHOD-1. This increase in myo-3 mRNA in fhod-1(-) worms removes transcription and mRNA stability as an explanation for the reduction in MYO-3 expression. We are currently exploring two alternative mechanisms. One possible mechanism is increased degradation of MYO-3 protein. We will test MYO-3 expression in fhod-1(-) worms after individually inhibiting each of the three known protein degradation pathways in BWM of C. elegans to determine if rescue occurs. The second possible mechanism is reduced translation of myo-3 mRNA. We will test levels of polyribosomes bound to myo-3 mRNA in wild-type and fhod-1(-) worms. Determining how FHOD-1 regulates protein expression might help resolve the role of FHOD3 in the hypertrophy of cardiac muscle.

P2786
Board Number: B63
The Drosophila Formin Fhod Nucleates Actin Filaments.
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Formins are a conserved group of proteins that nucleate and processively elongate actin filaments. Among them, the formin homology domain-containing protein (FHOD) family of formins contributes to contractility of striated muscle and cell motility in several contexts. However, the mechanisms by which they carry out these functions remain poorly understood. Unlike other formins, mammalian FHOD1 and FHOD3 do not accelerate actin assembly in vitro, and have instead been suggested to act as barbed end cappers or bundlers. Here, we show that purified Drosophila Fhod, in contrast with the mammalian homologues, potently accelerates actin assembly by nucleation. We found that Fhod binds tightly to barbed ends, where it slows elongation in the absence of profilin and allows elongation in the presence of profilin. Fhod protects barbed ends from capping protein, but dissociates from barbed ends relatively quickly. Finally, we used cosedimentation assays to determine that Fhod binds the sides of actin filaments and bundles filaments. This work establishes that Fhod shares the capacity of other formins to nucleate and bundle actin filaments, but is notably less effective at processively elongating barbed ends.
P2787
Board Number: B64
Intracellular zinc regulates actin nucleation during mouse oocyte maturation and fertilization via Spire.
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Zinc plays essential roles in mammalian oocyte maturation, fertilization, and early embryogenesis. Previously it has been known that depletion of zinc impairs various stage of oocyte maturation, including cell cycle controls, asymmetric division, and cytokinesis. But the exact mechanistic roles of zinc involved in these process is elusive. Here, we report that zinc is one of the essential cofactors in the actin cytoskeleton remodeling during mouse oocyte maturation, via actin nucleator Spire. Depletion of zinc in oocyte using chemical chelator or knockdown of zinc transporters impair cortical and cytoplasmic actin formation. Spire is colocalized with zinc-containing vesicle via C-terminal FYVE domain containing zinc finger motifs and it is essential for their localization and actin mesh formation in oocytes. Expression of truncation mutants of spire revealed that proper localization and activity of spire require all three major domains of Spire. After fertilization/parthenogenetic activation, oocyte zinc level is decreased by the release of zinc. The subsequent decrease of zinc content after parthenogenetic activation, Spire localization in cortex and vesicle is dramatically changed, indicating that zinc is an essential cofactor for Spire. Collectively, our data revealed the novel roles of zinc in the regulation of actin nucleator spire and controlling asymmetric division during mammalian oocyte maturation.
*Supported by a grant from the Next Generation Biogreen 21 Program (PJ011206), Rural Developmental Adminstration, Republic of Korea.

P2788
Board Number: B65
Epidermal specific knock out of N-WASP expression caused atopic dermatitis like inflammation in mice.
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Neural-Wiskott Aldrich Syndrome Protein (N-WASP) is a ubiquitously expressed protein which regulates the actin cytoskeleton by activating the actin nucleating activity of the Arp2/3 complex. In order to characterize the role of N-WASP in skin barrier function and their related disease such as atopic dermatitis (AD), we generated conditional N-WASP knockout mice (N-WASPK14KO; N-WASPF/fi; K14-Cre) using K14-cre and N-WASPF/fl mice to ablate expression of N-WASP in keratinocytes. The N-WASPF/fi; K14-Cre (N-WASPK14KO) mice were born following mendelian genetics suggesting that N-WASP expression in keratinocytes is not essential during development. Transepidermal water loss (TEWL) measurement showed a significant increased water loss in N-WASPK14KO mice compared to its control litter mates, N-WASPCtr suggesting a skin barrier defect. The skin barrier defect in N-WASPK14KO mice was confirmed by Lucifer Yellow (LY) penetration assay which revealed greater penetration of the LY in the skin of N-WASPK14KO compared to controls. Bacterial swab and subsequent culture test showed higher number of S. aureus colonization in N-WASPK14KO mice skin compared to control mice skin. The bacterial colonies were confirmed as S. aureus by PCR amplification of Staphylococcal enterotoxin B (SEB). Toluidine blue, Congo red and Immunostaining showed significant increased infiltration of mast cells, eosinophil and T- lymphocytes respectively in the skin of N-WASPK14KO mice compared to control mice. We found significantly higher levels of IL-1α, TNF-α, IL-6
and IL-17 in serum of N-WASP14KO mice compared to control mice. In conclusion, N-WASP knockout in keratinocytes caused atopic dermatitis like inflammation in the skin and activate local and systemic immune response in the mice, suggesting that N-WASP is involved in maintaining the skin barrier and homeostasis in adult mice skin. Thus we have identified N-WASP as one of the critical mediator in atopic dermatitis pathology.

**P2789**

**Board Number: B66**

**Identifying Functions of a *Chlamydomonas* Formin in Flagellar Assembly.**

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*Chlamydomonas reinhardtii*, a unicellular green alga, is used as a model organism for ciliary/flagellar studies, genetics of centrioles, cell-cell recognition, and cell cycle control. However, little is known about the function of the actin cytoskeleton within this cell. We previously showed that actin plays a role in assembly of the microtubule-based sensory organelle, the cilium. In order to test the role of a subset of the actin network on regulating flagellar length in *Chlamydomonas*, we used a compound, SMIFH2, that inhibits formin, an actin nucleator responsible for making linear filaments. These experiments showed that SMIFH2 has specific shortening effects on flagella. We hypothesized that genetic manipulation of a *Chlamydomonas* formin gene would partially recapitulate the inhibitor phenotype and affect flagellar assembly or maintenance. In contrast to the inhibitor result, we found that the formin mutant had longer flagella and a faster regeneration rate following deflagellation. In a classical experiment to assay the quantity of limiting flagellar precursor protein (the excess protein available for incorporation into flagella without additional protein synthesis), we found that the formin mutant contained a larger precursor pool. Following deflagellation, these mutants also had increased protein synthesis relative to wild-type cells. In pool depleted conditions, new flagellar protein incorporation was initially delayed in the formin mutant but eventually was faster in formin mutants the regenerating flagellum. Together, these data suggest a subset of linear actin filaments play an inhibitory role in the synthesis and incorporation of flagellar proteins. Additional functions for other *Chlamydomonas* formins targeted by SMIFH2 and branched actin may account for the discrepancies between these results and the aggregate effects of actin inhibitors/null mutants on flagellar assembly.

**P2790**

**Board Number: B67**

**Screening for function-altering INF2 mutants using a live-cell actin polymerization assay.**

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The inverted formin-2 (INF2) is a vertebrate formin (1249 Amino Acids in length) that can both polymerize and depolymerize actin filaments. In cells, INF2 exists in 2 forms - namely the CAAX form, which localizes to the endoplasmic reticulum (ER); and the non-CAAX form, which is found in the cytoplasm. INF2 stimulation in multiple cell types induces a transient burst of actin polymerization in the cytosol, which peaks within 1 min and depolymerizes by 4 min (Shao et al (2015) PNAS, Ji et al (2015) eLife, Wales et al, (2016) eLife). Recent discoveries showed that mutations near the N-terminus Diaphanous Inhibitory Domain (DID) region correlated with two diseases: focal segmental
glomerulosclerosis (FSGS) and Charcot-Marie Tooth disease (CMTD). Conversely, little is known about how C-terminus mutations affect the function of INF2 biochemically or in mammalian cells. Using genomic sequence and proteomic information from the Broad Institute, UniProt.org, and PhosphoSite.org - we generated a library of INF2 mutations predicted to disrupt post-translational modifications such as ubiquitination (K966N) and phosphorylation (S1192A, T1199A). We screened these mutants for recovery of the ionomycin-induced “actin burst” in INF2-KO U2OS cells by live-cell microscopy. Through our initial screen, we found that these mutations led to subtle but significant differences in actin polymerization and depolymerization. The actin burst for K966N exhibited a lower amplitude (8% decrease) with a shorter recovery time (~40 seconds difference). Both phosphorylation mutants INF2-CAAX induced bursts with slightly lower amplitudes compared to WT (3% for S1192A and 4% for T1199A) but with significantly longer recovery times (~120 seconds delay for both). We also tested the ionomycin-stimulated increase in mitochondrial calcium, which is also INF2-dependent (see poster from Chakrabarti et al), and found 7.7% decrease for K966N compared to WT, mirroring the observations of the actin "burst". Taken together, these results highlight the importance of the C-terminal region of INF2 in its regulation and function. Further studies will probe effects on ER morphology and mitochondria dynamics.

P2791

Board Number: B68

Actin nucleation factors that control autophagy are important for zebrafish organ development.

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Autophagy is a process of intracellular degradation that is crucial for removing unwanted cytoplasmic material, maintaining cellular homeostasis, and enabling embryonic development. The actin cytoskeleton, which plays key roles in numerous intracellular trafficking pathways, has recently been shown to significantly impact autophagy. In mammalian cells, the homologous actin nucleation factors JMY and WHAMM promote autophagosome biogenesis, but the importance of these proteins in animal development is not known. To address this question, we used the model vertebrate organism Danio rerio (zebrafish) to examine the functions of JMY and WHAMM. We found that the zebrafish orthologs of JMY and WHAMM contain motifs known to be important for binding actin, the Arp2/3 complex, and autophagosomal membranes. Consistent with mammalian JMY activities, zebrafish JMY nucleated actin in the presence and absence of the Arp2/3 complex in vitro, but it lacked the microtubule binding ability of human WHAMM. Unexpectedly, zebrafish JMY appeared to be a more potent Arp2/3 activator than mammalian JMY. In vivo, zebrafish embryos treated with morpholinos for depleting JMY exhibited changes in body morphology, alterations in heart and pronephros development, and pericardial edema. Treatment with a combination of morpholinos to JMY and WHAMM caused more pronounced morphological defects than JMY knockdown alone. However, these phenotypes were not as severe as those found in embryos treated with a morpholino to Atg5, a core component of the autophagy machinery. These results indicate that JMY and WHAMM are important for organogenesis during embryonic development and suggest that actin nucleation plays a key role in this process.
P2792
Board Number: B69
Actin filament elongation factors formin and Ena/VASP trigger the transition from lamellipodia- to filopodia-like networks.
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Cells simultaneously assemble and maintain different actin filament (F-actin) networks that are involved in diverse processes such as cell motility and cell division. Filopodia are finger-like membrane protrusions composed of long F-actin bundles that have been proposed to sense extracellular chemical and mechanical signals to steer the cell. Two actin elongation factors, formin and Ena/VASP, have been found at filopodia tips and induce specific architectures. It has been hypothesized that filopodia can be generated from F-actin networks that consist of densely packed, branched actin filaments that are kept short by capping protein.

How can filopodia emerge from this high density of capped filaments, and do different assembly factors facilitate the formation of diverse filopodia?
We propose that, in the presence of capping protein, filopodia formation is triggered by the combined action of the actin bundling protein fascin and the actin elongation factors formin mDia2 or Ena/VASP. By combining micropatterning and bead motility assays with three-color Total Internal Reflection Fluorescence (TIRF) microscopy, we reconstituted the formation of filopodia-like F-actin networks from densely-branched networks in vitro. We show that in the presence of saturating concentrations of capping protein, filopodia-like network formation is inhibited. In contrast, it is rescued by inclusion of the processive actin filament barbed-end elongator formin mDia2, and to lesser extent by Ena/VASP. Interestingly, formin-generated networks are longer and free of capping protein, unlike the short Ena/VASP-generated networks. In addition, the F-actin bundler fascin facilitates filopodia-like network formation by inhibiting Arp2/3 complex-mediated branching up to 50 percent. Finally, we found that saturating concentrations of Ena/VASP increase the dissociation rate of formin mDia2 from F-actin barbed ends.

Our reconstituted in vitro system reveals that the combination of the F-actin bundler fascin with the actin elongation factors formin or Ena/VASP is sufficient to trigger a transition from a dense branched network of capped F-actin to filopodia-like networks. Additionally, formin and Ena/VASP generate specific protrusion morphologies resembling those that have been observed in vivo.

P2793
Board Number: B70
Characterization of Formin-2 Functions using in vitro Approaches.
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The cytoskeleton plays an indispensable role in the cellular morphogenesis, endocytosis, cell division and other cellular processes. Orchestration of actin and microtubules is central to the dynamicity of the cell cytoskeleton. Formins are cytoskeleton modulators and play an essential role in the development process of cell cytoskeleton by modulating both microfilaments and microtubules. Formin-
2 (Fmn2) is characterised as a cytoskeleton regulator involved in organising the actin filaments. Fmn2 has an essential role in spindle positioning and migration in oocytes. Fmn2 interacts with FilaminA to regulate endocytosis and with p21 to control cell cycle. Recently our lab has shown that Fmn2 is required for spinal commissural connectivity in chick neurons. Fmn2 belongs to typical formin family characterized by the presence of known FH1FH2 domains in their C-terminal end. In order to understand Fmn2 function and its regulation, we have undertaken a detailed characterization of chick Fmn2 using in vitro reconstitution assays. Chick Fmn2 is capable of potently binding F-actin. Using fluorimetric assays, we show that chick Fmn2 is able to nucleate, elongate actin filaments and also replace CapZ from the filament tip. Co-sedimentation assays revealed that Fmn2 can cross-link and bundle F-actin. The latter was also confirmed by TIRF microscopy. Our data suggest that Fmn2 also binds to microtubule and we are currently conducting detailed structure-function analysis to uncover the domains mediating the actin microtubule cross talk of Fmn2. These studies offer important insights into Fmn2 function, especially the co-regulation of actin and microtubule dynamics. Detailed characterization of these activities is likely to reveal key modalities of Fmn2 regulations and its ability to coordinate the microtubule and actin cytoskeletons.

P2794

Board Number: B71

Adenomatous polyposis coli (APC) nucleates actin assembly to drive cell migration and microtubule-induced focal adhesion turnover.

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Adenomatous polyposis coli (APC) is a tumor suppressor protein (310 kDa) that functions in Wnt signaling and in cytoskeleton rearrangements that govern cell migration, cell protrusions and adhesion. APC is also the ‘gatekeeper’ gene in human colorectal tumorigenesis, with C-terminal truncations of APC leading to >80% of all colorectal cancers. The C-terminal ‘Basic’ domain of APC binds to microtubules and actin, and potently nucleates actin assembly in vitro. APC also governs microtubule organization and dynamics in vivo. The lack of clean separation-of-function mutants in APC has made challenging to disentangle APC’s effects on actin versus MTs, and to rigorously assess the in vivo importance of its specific interactions and activities. With the goal of uncoupling APC’s effects on actin nucleation from its direct interactions with MTs, we mapped the sequences in APC required for actin nucleation, and then generated the APC-m4 mutant, which alters only two residues but abolishes actin nucleation activity without altering in vitro interactions with MTs. Cells expressing full-length APC carrying the m4 mutation (APC-m4) complemented in vivo cellular functions in microtubule organization, microtubule dynamics, and mitochondrial distribution caused by RNAi depletion of endogenous APC, but failed to support directed cell migration. APC-WT and APC-m4 proteins both localized to focal adhesions, but expression of APC-m4 led to diminished actin assembly at focal adhesions and severe defects in microtubule-induced focal adhesion disassembly/turnover. Using the APC-m4 mutant, we have demonstrated the in vivo importance of the actin assembly-promoting activity of APC and discovered that it is critical for directed cell migration. Our observations further suggest that these functions stem from a requirement for APC-mediated actin assembly at FAs - in a role that is required for MT-induced FA disassembly. To our knowledge, this is the first evidence of an actin assembly requirement in the MT-induced FA turnover process, and given the dual interactions of APC with MTs and actin, suggests that APC may directly coordinate MT and actin dynamics in stimulating FA turnover, which is a critical step in directed cell migration.
Actomyosin and Contractility

P2795
Board Number: B72
A novel interaction between NMIIB and Survivin is essential for proper cell division.
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Cell division is necessary for proper growth and development of many organisms. It ends with the physical separation of the two daughter cells, a process known as cytokinesis. The primary motor protein responsible for cytokinesis is non-muscle myosin II (NMII), and together with F-actin it assembles the cleavage furrow forming the contractile ring. The site of the contractile ring assembly and the timing of its constriction are closely coordinated with chromosome segregation to allow accurate partitioning of the genome and formation of the two daughter cells. The chromosomal passenger complex (CPC) plays an important role in coordinating and regulating these processes through its functions in central spindle formation, regulation of furrow ingestion, and abscission. The CPC is composed of Aurora B kinase, Borealin, INCENP, and Survivin. When one of the CPC proteins is perturbed, the other components do not localize properly during cell division, leading to disruption in cell division. The mechanism by which CPC localizes to the cleave site and regulates the formation of the contractile ring to eventually achieve cytokinesis is unknown. The CPC member, Survivin is believed to play a dual role in cell cycle progression and apoptosis. We recently found that Survivin and NMII form a complex in vivo through direct interactions. This interaction is NMII isomorph specific and is regulated by Survivin phosphorylation. Survivin inhibits NMIIB filament assembly, which may indicate that it prevents the premature formation of the contractile ring. Identification of Survivin binding site to NMIIB provides a molecular explanation for this inhibition. During telophase and cytokinesis, NMII and Survivin co-localize to the cleavage furrow and midbody. These results may indicate that Survivin and NMIIB interaction is essential for cell division.

P2796
Board Number: B73
Graded activation of ROCK and MLCK tunes regional stress fiber formation and mechanics via preferential myosin light chain phosphorylation.
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Stress fibers (SFs) are prominent force-generating cytoskeletal structures chiefly comprised of actin and non-muscle myosin II (NMMII). NMMII motor activity is strongly promoted by phosphorylation of the NMMII light chains (MLCs) by MLCK and Rho-associated kinase (ROCK). MLCs can be phosphorylated once (p-MLC) or twice (pp-MLC), with each phosphorylation state distinctly influencing myosin ATPase activity. Prior work suggests MLCK and ROCK regulate SF formation in the cell periphery and cell center respectively, and both MLC phosphorylation states have been observed throughout the cell. However, it is unclear how MLCK and ROCK differ in their regulation of MLC phosphorylation state, how these kinases regulate these two pools of SFs, and how these parameters influence SF mechanics. Here, we address these questions by combining inducible genetic expression of MLCK or ROCK with mechanical measurements of single SFs in live cells and phosho-specific immunoanalysis. Immunoblots
reveal that constitutive activation (CA) of MLCK preferentially increases whole-cell levels of p-MLC while CA of ROCK increases pp-MLC. Quantitative immunofluorescence analysis demonstrates that MLCK preferentially increases recruitment of p-MLC to peripheral SFs while ROCK recruits pp-MLC to central SFs. To relate kinase activity to SF mechanical properties, we used ablation to sever single SFs in live cells and tracked the retraction of the severed ends as tension dissipated. We found MLCK increased the effective viscosity and total stored elastic energy in peripheral SFs but not central SFs, while ROCK preferentially increased these same parameters in central SFs. To directly link MLC phosphorylation state and stress fiber mechanics, we overexpressed phosphomimetic mutants of p-MLC and pp-MLC and found that phosphomimetic p-MLC influenced only peripheral SF viscoelastic parameters while phosphomimetic pp-MLC influenced only central SF viscoelastic parameters, phenocopying our ablation findings. Finally, by varying the concentration of the small-molecule inducer in the culture medium, we could establish gradations in kinase expression and concomitant p- and pp-MLC levels, thereby enabling quantification of these relationships. Application of this strategy revealed surprising and previously unappreciated nonlinearities between MLC phosphorylation state and SF viscoelastic properties. This work supports a model in which MLCK regulates peripheral stress fiber viscoelasticity via monophosphorylation of MLC while ROCK regulates central stress fiber viscoelasticity via di-phosphorylation of MLC.

P2797
Board Number: B74
Cdc42 GEF Gef1 coordinates actomyosin ring constriction and septum ingression during cytokinesis.
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Cytokinesis is a temporally organized multi-step process leading to the physical separation of daughter cells during division. In fission yeast, cytokinesis involves the assembly of an actomyosin ring that constricts concurrently with septum ingression and furrow formation. The septum provides the force required to overcome internal turgor pressure in the cell. It is not clear how a cell spatiotemporally coordinates simultaneous ring constriction and septum ingression. We have reported that the conserved GTPase Cdc42 is activated in a unique spatiotemporal manner during cytokinesis depending on the localization of its activators, Gef1 and Scd1. Gef1 specifically promotes the recruitment of the septum-synthesizing enzyme Bgs1 at the ring to allow timely onset of ring constriction and septum ingression. It is not clear how Gef1 promotes the recruitment of Bgs1 to the division site. Previous reports suggest that Bgs1 localization at the ring depends on the F-BAR protein Cdc15. Here we show that mutants with reduced recruitment of Cdc15 at the assembled ring and also lacking gef1 display defects in ring constriction. In addition, these mutants also display uneven distribution of Cdc15 at the ring. Interestingly, these mutants do not show any defect in ring assembly, suggesting that the constriction defects observed here are due to impaired events after ring assembly. Cells with uneven Cdc15 distribution along the ring display abnormally long constriction times. Regions of the ring with reduced Cdc15 show delayed constriction rates and non-concentric furrowing. To understand how Gef1 promotes Cdc15 distribution and timely Bgs1 recruitment along the ring, we developed a computational model to describe how proteins are evenly organized in an actomyosin ring. Our model predicts that protein-protein interaction along with robust protein recruitment and recycling along the ring leads to even protein distribution at the ring. To test if Gef1 promotes protein-protein interactions, we investigated the distribution of other proteins along the ring in a Gef1-dependent manner. We find that
loss of gef1 leads to defects in the distribution of the type II myosin Myp2, but not that of the ring assembly myosin, Myo2. Previous reports have shown that Myp2 is recruited to the ring after ring assembly and is required for normal ring constriction. Based on these findings, we posit that Gef1 at the assembled actomyosin ring coordinates Bgs1-dependent septum ingression and Myp2-dependent ring constriction, leading to concentric furrow formation. Thus the Cdc42 GEF Gef1 allows the actomyosin ring to act as a landmark for the proper organization of proteins at the division site to ensure efficient septum ingression, ring constriction, and furrow formation.

P2798
Board Number: B75
Compartmentalized regulation of myosin light chain phosphatase and myosin light chain kinase during cell spreading.
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Non-muscle myosin II (NMMII) is critical for cell migration; phosphorylation of the regulatory light chain (RLC) controls myosin motors binding to actin filaments and drives cell contraction. Myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) are the primary regulators of RLC phosphorylation. However, how the activities of these two regulatory proteins coordinate to promote cellular movement are not well understood. To investigate the distribution of MLCP activity in live cells, we developed a FRET-based MLCP biosensor. REFS2 fibroblasts transfected with the MLCP sensor were introduced to nitric oxide (NO), which induced MLCP activity. Chemical generation of NO inhibited cell spreading on a fibronectin matrix and was associated with MLCP activation. Similarly, modifying the potential MLCP phosphorylation sites to Alanine at (T694A) and (T852A) also increased activity. To determine the distribution of MLCK and MLCP, cells were co-transfected with CFP/YFP kinase FRET sensor and OFP/RFP phosphatase FRET sensor. Multi-parametric measurements determined the localization of active MLCP and MLCP during spreading. Differential regulation of MLCK and MLCP was observed along the moving membrane, and enhanced MLCK activity and decreased MLCP activity was observed adjacent to the leading edge. Additionally, presence of ROK inhibitor Y27632 reduced MLCK activity and enhanced MLCP activity during migration. These results suggest the importance of compartmentalized MLCP and MLCK activity during cell movement.

P2799
Board Number: B76
Actomyosin contractility maintains the integrity of the Drosophila testis niche during gonadogenesis.
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Proper formation of niches is essential to maintain the balance of self-renewal and differentiation within associated stem cells. We use the Drosophila testis as a model to study these interactions. In this organ, the niche makes intimate contacts with the germline stem cells it regulates. Thus, the efficiency of signaling depends on proper morphogenesis of the niche; however, how the niche achieves its final architecture remains unknown. Using live imaging techniques, we have identified two phases during niche formation: 1. Assembly, in which specified cells migrate and coalesce at one end of the organ, and 2. Compaction, during which the assembled niche cells condense further, finalizing their associations.
with germ cells. Here, we show that actomyosin contractility is implicated to maintain the integrity of the niche along its germ cell interfaces during compaction. Live imaging showed that compaction was correlated with the enrichment of non-muscle myosin II and increase of f-actin protrusive activity along niche-stem cell interfaces. This suggests that forces are acting during compaction. Therefore, we explored the relative magnitude and direction of these forces. We used a laser to cut niche-stem cell interfaces and measure the retraction velocity as a proxy for tension. We found that these interfaces were under greater tension later during compaction than at the onset of this process when germ cells and niche cells first associate. Furthermore, retraction velocities were decreased in the presence of a Rho Kinase inhibitor, suggesting that actomyosin contractility was implicated in producing tension. We additionally observed a change in the direction of the net forces comparing early versus late compaction. After an early cut, the interface deformed such that the germ cell encroached into niche cell space. This did not occur upon later cuts. The data suggests that germ cells apply a force orthogonal to the niche during compaction, and normally the niche cells offer resistance to that force. Later in morphogenesis, forces are re-directed, as tension is aligned with niche-stem cell interfaces. Our findings show that the behavior of stem cells and their relationship with the niche are integral to niche morphogenesis.

P2800
Board Number: B77
Profilin Directly Enhances Microtubule Growth Through Residues Mutated in Amyotrophic Lateral Sclerosis.
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Profilin is one of the most abundant and central actin regulatory proteins in eukaryotic cells. Profilin also decorates the sides of microtubules and indirectly influences cellular microtubule dynamics through interactions with Formins. Here, we investigated whether Profilin has direct regulatory effects on microtubule dynamics. We show that human Profilin-1 binds to microtubules in vitro and enhances the growth rate of microtubules several-fold. These microtubule effects are conserved in budding yeast and Drosophila Profilin homologs, and are unaffected by mutations in its canonical actin monomer— or poly-L-proline—binding sites. Instead, microtubule regulation depends on several residues mutated in patients with amyotrophic lateral sclerosis (ALS). The enhanced microtubule dynamics elicited by Profilin are attenuated by increasing concentrations of actin monomers. This suggests a competitive relationship between microtubules and actin for Profilin binding and this agrees with the close proximity of the known actin and microtubule binding surfaces. Consistent with these biochemical results, a two-fold increase in expression of wildtype Profilin accelerates the growth rate of microtubules in cells, and cells expressing similar levels of each of the ALS-associated Profilin mutants did not. These results demonstrate Profilin directly interacts with and enhances the growth rate of microtubules in vitro and in cells, and indicate that Profilin coordinates cellular actin and microtubule dynamics. Further, cells harboring ALS-linked Profilin mutations may ultimately leads to the motor neuron degeneration through defective microtubule regulation.
**P2801**  
**Board Number: B78**  
**Composition of LAT clusters regulates their movement within actomyosin networks.**  
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In activated T cells, phase separated LAT clusters move from the edge to the center of the immune synapse. Recent studies have used super-resolution microscopy to describe two networks of actin within the immune synapse; a region of actin retrograde flow that exists near the synapse edge and a region of formin-generated actin bundles that exists adjacent to the region of retrograde flow, near the center of the synapse. LAT clusters move through these two regions at velocities that are tightly correlated with actin movement, suggesting that cluster movement is directly linked with actin movement. However, the molecular mechanism that regulates cluster movement through the different actin cytoskeletal networks within activated T cells was unknown. Using Jurkat T cells that fluorescently expressed components of LAT clusters, we observed that the molecular composition of LAT clusters in activated Jurkat T cells changes as clusters move from the edge to the center of the synapse. We hypothesized that this composition change may alter the ability of clusters to interact with the surrounding actin cytoskeletal network. To test our hypothesis, we reconstituted LAT phase separated clusters within two types of active actomyosin networks on supported lipid bilayers. Within a steady-state actomyosin network, in which LAT clusters of varying compositions formed amid a constantly moving actin network, we observed that cluster composition regulated the mechanism by which clusters were actively moved by the actomyosin network. Within a contractile actomyosin network, in which LAT clusters of varying compositions were formed within an existing actin network prior to Myosin II-induced actin network contraction, cluster movement was tightly correlated with actin movement depending on the composition of LAT clusters. Using our reconstituted system, we discovered a molecular clutch that links LAT cluster movement with actin filament movement in active actomyosin networks. The biochemical results from our reconstituted system explain how the movement of LAT clusters is correlated with the movement of both actin retrograde flow and contractile actomyosin bundles within different regions of an activated Jurkat T cell.

**P2802**  
**Board Number: B79**  
**Optimal adhesion stability is required for proper sarcomere assembly in cardiomyocytes.**  
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Hypertrophic and dilated cardiomyopathies affect 1 in 500 and 1 in 250 individuals in the US, respectively. ~70% of the causative mutations in patients are in genes that encode either adhesion or contractile proteins. A balance of adhesion and contraction is required in healthy cardiomyocytes (i.e., heart muscle cells), loss of which leads to detrimental phenotypes and disease states. Treating these diseases will require reconstructing the delicate balance between adhesion and contraction found in healthy cardiomyocytes. Unfortunately, how the adhesive and contractile systems assemble in cardiomyocytes is not well understood. Classic work has shown that inhibiting contraction (i.e., beating)
of cultured primary cardiomyocytes results in a loss of adhesion. This indicated that there is feedback between the adhesive and contractile systems. To characterize this potential relationship, we studied iPSC-derived human cardiomyocytes (hCM) in culture during adhesion and sarcomere assembly. To investigate the role of adhesion stability in hCM contractility, we modulated adhesion stability with several different techniques. Inhibition of focal adhesion kinase (FAK, an integral component of focal adhesion turnover) led to stabilized adhesions in hCM. Surprisingly, FAK inhibition also caused precocious sarcomere formation. Stabilization of adhesions by plating on increased fibronectin concentrations also caused precocious sarcomere formation, and plating on decreased fibronectin concentrations reduced sarcomere formation. However, precocious sarcomere formation resulting from increased adhesion area led to aberrant beating characteristics and sarcomere disorganization. siRNA mediated knockdown of FAK led to decreased adhesion stability, and disrupted sarcomere formation. Similarly, inhibition of contraction rapidly breaks down sarcomeres. To quantify these observed changes, we performed computer-assisted analysis of the borders of sarcomeres (i.e. Z-lines) to quantify metrics of sarcomere assembly over hundreds of hCM. These techniques ensure quick, unbiased, and quantitative measurements and specifically detect minute changes in sarcomere structure. We have shown that decreasing adhesion in turn decreases sarcomere assembly, and similarly, that increasing adhesion leads to precocious sarcomere formation in a disorganized manner. These results suggest there is an optimal adhesion amount for proper sarcomere assembly and homeostasis. Understanding this relationship helps us better understand the mechanisms underlying cardiomyopathy pathology.

P2803
Board Number: B80
How is apical constriction triggered? Possible roles for afadin and zyxin.
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Apical constriction is a commonly used mechanism to change a cell's shape and initiate morphogenesis in broad range of animal systems, including the vertebrate neural tube. Apical constriction occurs as a result of actomyosin contractions at the apical surface, which shrink the exterior face of the cell as the cells move to the interior of the embryo. This project seeks to understand the mechanisms used by cells and tissues during development to spatially and temporally regulate the forces that generate shape changes using Caenorhabditis elegans gastrulation as a model for studying the mechanisms of apical constriction. Gastrulation in C. elegans involves two endodermal precursor cells (E cells) that shrink their apical surfaces and move from the embryo's surface to the interior at the 26-28 cell stage. Surprisingly, data suggests that both actomyosin contractions and strong apical tension precede shrinking of the apical surface. This suggests that a temporally regulated link allows for the coordination of the contracting apical actomyosin networks with the apical cell-cell junctions to trigger apical constriction. We pursued both a proteomic as well as a bioinformatic approach to identify proteins that could act as a link between α-catenin in the adherens junction and the actin cytoskeleton. We identified genes of interest that show enrichment in the E cell lineage prior to gastrulation and are evaluating whether or not they are required for normal development. These approaches have identified two major candidates that could function in a clutch mechanism – afd-1/afadin and zyx-1/zyxin. Both candidates display gastrulation defects when knocked down by dsRNA injection and have known functions consistent with the idea that they could form a link between adherens junctions and the actin cytoskeleton. So far we have determined that afd-1 is required for normal gastrulation and that the protein is expressed and colocalizes with the cadherin-catenin complex. Also, knockdown of zyx-1 appears to result in defective coupling of actomyosin contractions to cell junctions during apical constriction. I am currently working to characterize the mechanisms through which afadin and zyxin are functioning in vivo. Both afadin and
zyxin are broadly conserved genes and these data could apply to mechanisms that regulate apical constriction in other systems.

**P2804**

**Board Number: B81**  
The pre-metazoan origin of animal cell contractility.  
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Actomyosin-mediated cell contractility has been fundamental to the evolution of animal complexity. During development, morphogenesis is mediated by embryonic cell contractility and movements of adult organisms rely on the specialized contractility of muscle cells. Even though embryonic and adult cell contractility have been documented in all major animal lineages (sponges, ctenophores, cnidarians, bilaterians) and thus likely date back to the last common animal ancestor, their pre-metazoan evolutionary origins remain unclear. Genomic data indicate that choanoflagellates genomes encode homologs of the main metazoan contractility proteins: actin, myosin, their upstream regulators (myosin kinases, calcium sensors, voltage-gated calcium channels), and even transcription factors that specify animal muscle cells (Mef2, Myocardin and SRF). Immunostainings have confirmed the presence of actomyosin at the basal pole of the cell (which shows contractile activity during filopodia-mediated settlement), pharmacological assays and live imaging have shown that its activity is necessary for proper settlement, and traction force microscopy allows quantification of contractile forces. By comparing the cellular modules and transcriptional circuits involved in choanoflagellate contractility to their animal counterparts, we infer the presence in choanozoan ancestors of an ancient mechanosensory-effector arc controlling contractile behavior that paved the way for the later evolution of animal contractile cells, including muscle cells.

**Regulation of Actin Dynamics 2**

**P2805**

**Board Number: B82**  
Septins as modifiers of actin dynamics.  
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Controlled dynamic rearrangements of the actin cytoskeleton underlie essential changes to cell shape, steps of cell division and cell motility. The septins, a group of GTP-binding filament forming proteins, constitute a relatively new class of cytoskeletal proteins and were initially discovered as participants in yeast cell division. Septins are known to interact with the actin cytoskeleton, but neither the detailed mechanism of septin interactions with actin nor their effect on actin dynamics are well understood. A number of septin families exist in vertebrates and are thought to interact with each other to form discrete hetero-oligomeric complexes, which can then assemble into filaments. One complex is well established, the Sept2/6/7 hexamer, which is two subassemblies of Sept7 bound to a Sept6 family member, bound to a Sept2 family member, held together by Sept2-Sept2 interactions, and is supported by a crystal structure. The Sept3 family is thought to add at the ends of this complex to produce an octamer. Increased expression of the Sept9 member of the Sept3 family is associated with a number of invasive cancer phenotypes, suggesting that the hexameric and octameric assemblies may have distinct functions. Given this observation, and the necessary role of actin dynamics in altered cell motility, we
asked whether septins directly alter actin dynamics in vitro. We find that both the Sept2/6/7 complex and Sept9 have effects in Arp2/3 complex mediated actin polymerization assays. Intriguingly, actin polymerization in the presence of Sept9 is accelerated, while in the presence of the Sept2/6/7 complex polymerization is delayed. From this we conclude that distinct Septin assemblies likely have distinct functional roles in modifying cellular actin dynamics.

P2806
Board Number: B83
BMW is an exceptionally potent actin assembly factor from a human parasite.
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Assembly, organization and turnover of filamentous actin structures are essential for cell function. The initial and rate-limiting step in actin filament formation is the assembly of actin nuclei onto which additional monomers are subsequently added by filament elongators. As yet three types of actin nucleators have been established: the Arp2/3 complex, formins and tandem-monomer-binding nucleators. Here were characterized a novel and powerful tandem nucleator from a parasitic nematode causing lymphatic filariasis in man. *Brugia malayi* WH2-domain containing protein (BMW) harbors four WH2 domains preceded by proline-rich stretches at its N-terminus suggesting that it could recruit four actin monomers to form a polymerization nucleus. In vitro BMW nucleates actin polymerization at low nanomolar concentrations in a dose-dependent manner and does not require profilin for activity. A short N-terminal fragment encompassing the WH2 domains is sufficient for nucleation, but the nucleation activity is markedly enhanced in longer constructs suggesting synergy with other functional domains for full activity. In line with this notion, but in contrast to other tandem nucleators, BMW contains multiple F-actin binding sites which are required to induce the formation of massive bundles with mixed polarity in vitro as assessed by TIRF imaging. The bundles increase by growth on both ends and by fusion with neighboring bundles. Consistently, ectopic expression of BMW in commonly used cell types also leads to formation of massive bundles composed of hundreds of filaments and is associated with drastic depletion of the cellular G-actin pool arguing that most actin is consumed by BMW. Multi-color TIRF imaging at low BMW concentrations further revealed that the protein primarily nucleates actin at filament barbed ends and is subsequently either released to the filament sides or back into solution. Surprisingly, however, BMW can also associate with the pointed ends of the filaments. Finally, we explored the requirement of the WH2 domains for nucleation and bundling by analyses of deletion mutants in vitro and in vivo. Unexpectedly, nucleation and bundle assembly is markedly enhanced in constructs lacking the first two WH2 domains and is still seen with constructs lacking the first three WH2 domains in biochemical assays and transfected cells. Since BMW is a monomer as assessed by analytical ultracentrifugation, we conclude that BMW must utilize a novel nucleation mechanism, which will be discussed.
Sialic acids are negatively charged nine carbon atom sugar present in N-glycan, O glycan and gangliosides. GNE (UDP-GlcNAc 2-epimerase/ ManNAc kinase) is the key regulatory enzyme for biosynthesis of sialic acid that catalyzes epimerization of UDP-GlcNAc to ManNAc followed by phosphorylation to ManNAc-P. Mutations in GNE lead to rare genetic disorders: sialuria characterised by excretion of excess sialic acid in urine due to loss of feedback control and GNE related myopathy characterised by muscle weakness and atrophy due to hyposialylation. The pathomechanism of disease is poorly understood as GNE has been found to affect other cellular functions beside sialic acid synthesis such as apoptosis and cell adhesion. GNE interacts with α-actinin 1 and 2 and affects integrin signalling leading to focal adhesion formation. In the present study, we aim to decipher role of GNE in actin dynamics to regulate cytoskeletal organization for muscle cell integrity. For this purpose, L6 rat skeletal muscle cell line was knocked out for GNE gene either at exon 2 or exon 9 using pSEPT vector and rAAV mediated homology recombination technology. However, GNE knockout resulted in 50% cell death that could not be rescued after sialic acid supplementation. Alternately, the study was carried out in HEK cell based model overexpressing pathologically relevant GNE mutations (D176V-epimerase mutant and V572L-kinase mutant) that showed hyposialylation and reduced GNE enzymatic activity. Reduced G- and F-actin levels were observed in GNE mutant cells compared to wild GNE or vector control by immunoblotting and phallolidin staining. However GNE neither directly binds actin nor affect its in vitro polymerization activity rather RhoA and Cofilin levels were reduced in GNE mutant cells that may contribute to reduced G- and F-actin levels. Our study indicates that mutation in GNE affects actin levels that may contribute to cytoskeletal disruption and inhibit structural integrity of the cell. Molecules for Upregulation of actin levels may offer therapeutic tools for diseases associated with deficiency of GNE.

RACK1 (Receptor for Activated C Kinase 1) is critical for fundamental cellular activities and has a central role in the activation and response of immune cells. Mast cell activation via the high affinity IgE receptor (FceRI) is accompanied by a reorganization of the actin cytoskeleton and ultimately results in degranulation and release of inflammatory mediators. However, the role of RACK1 in mast cell activation has not been previously investigated. Using proteomic analysis of lipid raft fractions, we identified, for the first time, the presence of RACK1 in RBL-2H3 mast cells. By immunostaining, in non-stimulated mast cells, RACK1 was distributed in a punctate manner throughout the cytoplasm both in vivo and in vitro. Following RBL-2H3 mast cell stimulation via FceRI for 15 s and 1 min, the majority of RACK1 was localized in regions adjacent to the plasma membrane. To knockdown RACK1, RBL-2H3 mast cells were transduced with lentiviral particles encoding shRNAs against RACK1. The transduced cells
showed an approximately 54% reduction in RACK1. To analyze if actin distribution is affected by RACK1, the ShRNA control and RACK1 knockdown mast cells were stimulated or not via FcεRI, stained with phalloidin-Alexa 488, and analyzed by confocal microscopy. The non-stimulated ShRNA control mast cells were fusiform in shape and the cortical actin formed a continuous layer adjacent to the plasma membrane. In contrast, non-stimulated RACK1 knockdown mast cells were rounded or ovoid and the cortical F-actin was fragmented with spaces in the subcortical areas devoid of actin. Following stimulation via FcεRI, cortical F-actin was dramatically reduced in RACK1 knockdown mast cells in comparison with ShRNA control cells. RACK1 knockdown resulted in a significant increase in basal and FcεRI-stimulated β-hexosaminidase release. The increased basal degranulation may be related to the fact that CD63+ cytoplasmic granules were localized in the cortical F-actin-free regions in RACK1 knockdown mast cells, thus facilitating release at the plasma membrane. Furthermore, single cell calcium imaging analysis showed that thapsigargin and FcεRI-stimulated Ca2+ entry in RACK1 knockdown mast cells was decreased relative to ShRNA control cells. Additionally, in the absence of extracellular Ca2+, RACK1 knockdown mast cells showed a defective Ca2+ store depletion when stimulated via FcεRI. Thus, the present investigation indicates that RACK1 is a critical regulator of actin dynamics in mast cells, a novel negative regulator of mast cell degranulation, and may have a significant impact on the regulation of calcium signaling.

P2809  
Board Number: B86  
Morphodynamics of cell edge protrusion in Lamellipodin (Lpd) knockout cells.  
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Lamellipodia constitute actin networks at the cell periphery built by Arp2/3 complex and accessory proteins and are driving efficient migration on plane substrates in various cell types and conditions. Lamellipodin (Lpd) localizes to the very edge of lamellipodia. Lpd was identified as a ligand of Ena/VASP proteins, which promote actin filament elongation. In addition, Lpd binds to the WAVE regulatory complex, the latter of which mediates Arp2/3 complex activation in lamellipodia. Lpd is considered to be important for lamellipo-dia formation, positively regulates cell migration and has been implicated in promoting cancer cell invasion in 3D. However, a precise understanding of Lpd functions in protrusion is so far lacking. Here, we describe the first characterization of B16-F1 cell lines, in which detectable Lpd protein levels are eliminated by CRISPR/Cas9 technology. Interest ingly, formation of lamellipodia was not inhibited upon deletion of Lpd, although defects in their dynamics were observed: Video microscopy combined with quantifications of various protrusion parameters employing a method involving semi-automatic, dynamic cell-edge detection followed by MATLAB analysis revealed that Lpd knockout B16-F1 cells display a decreased average rate of protrusion and an increase of retraction speed as well as of cell numbers exhibiting fluctuating protrusions. In addition, average rates of random cell migration were also moderately reduced in Lpd knockout clones. These phenotypes, surprisingly, were independent of lamellipodial actin filament densities, Arp2/3-complex distributions or rates of actin network polymerization, all of which remained unchanged in Lpd knockout cells as compared to controls. Current efforts to uncover the molecular mechanisms underlying observed phenotypes in Lpd-deficient B16-F1 cell lines include determination of localization and average residency times at lamellipodia tips of key components driving the formation of these structures. In conclusion, this study provides the first
systematic, software-aided analysis of cell edge morphodynamics of cells in the absence and presence of Lpd, laying the foundation for clarifying its function in cell edge protrusion.

**P2810**

**Board Number: B87**

Characterization of cell lines lacking ubiquitous WAVE complex.
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Mesenchymal cell migration is frequently accompanied by protrusion of thin leaflets of plasma membrane called lamellipodia or membrane ruffles. We are interested in the mechanistic dissection of these actin networks formed downstream of the key Rho-family GTPases Rac and Cdc42. We recently showed that Cdc42 operates through FMNL2 and FMNL3 formins to generate a subpopulation of lamellipodial actin filaments essential for homogenous protrusion and appropriate force development. However, FMNL2/3 functioned independently from Arp2/3 complex-dependent filament branching, also revealing that the latter alone is not sufficient for productive lamellipodial protrusion. Moreover, although Arp2/3 complex is essential for the generation of these structures, it remains unclear how tuning of its activity by its lamellipodial activator WAVE complex, operating downstream of the Rho-GTPase Rac, affects critical protrusion parameters. To address this, we have recently developed mammalian, non-haematopoietic cell lines genetically deficient for distinct subunits of the pentameric WAVE complex using CRISPR/Cas9. We have used highly motile B16-F1 melanoma cells, constituting an excellent, well-studied and widely used model system for lamellipodia protrusion and lamellipodia-dependent cell migration. Clonal cell lines genetically disrupted for the Specifically Rac-associated protein 1 (Sra-1) and its isogene PIR121 are completely devoid of lamellipodia and membrane ruffles, and display severely diminished cell migration, as expected from previous RNAi studies. Moreover, re-expression of wildtype Sra-1 but not of a Rac binding-deficient mutant fully restores lamellipodia formation, confirming lamellipodia deficiency to be solely caused by disruption of functional WAVE complex downstream of Rac. In contrast to Sra-1/PIR121 double knockout, genetic disruption of the NCK associated protein 1 (Nap1), so far considered to be an essential WAVE complex subunit in the non-hematopoietic system, causes much more variable results, with clones ranging from being almost devoid of lamellipodia to one clone displaying cells with lamellipodia at a frequency of 70% (11 Nap1 KO clones in total). Importantly, the ability to form lamellipodia in these clones correlates with spontaneous, compensatory expression of the hematopoietic Nap1 parologue Hem1. Moreover, the level of spontaneous Hem1 expression defines the density of Arp2/3-complex as well as F-actin in the lamellipodia of distinct cell lines, thus allowing for the first time to quantitatively assess how the extent of WAVE complex-mediated Arp2/3 complex activation at the protruding edge of lamellipodia contributes to rates of actin network polymerization and protrusion as well as lamellipodial force development.
P2811
Board Number: B88
Building and burning bridges: Adaptor protein Bbc1 regulates endocytic actin patch assembly by disrupting interactions of Wsp1/Vrp1 with Myo1.
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Arp2/3 complex-mediated branched actin nucleation is important across species for cell motility and endocytosis. Branched actin assembly is stimulated by Nucleation Promoting Factors (NPFs), such as Wiskott-Aldrich Syndrome protein (WASP). WASp works in complex with WASp Interacting Protein (WIP), which may enhance its NPF activity. An important unresolved question is how the actin assembly machinery is positioned to efficiently drive endocytic invagination and internalization. We investigate mechanisms of branched actin assembly in a favorable model system, endocytic actin patches in fission yeast S. pombe. We have determined that the fission yeast WIP homolog verprolin Vrp1 acts as a transient bridge between myosin-1 Myo1 and WASp homolog Wsp1, helping these two proteins stay in actin patches longer. Here we investigate the role of an adaptor protein Bbc1, which is often classified as a member of the WASP-Myosin-1 module but whose physiological role remains unclear. We knocked out the bbc1 gene through homologous recombination (bbc1Δ), combined bbc1Δ with actin patch markers labeled with fluorescent proteins, and examined the effects of deleting bbc1 on actin patch dynamics by quantitative spinning disk confocal microscopy. We found that Bbc1 interacts with the Myo1 SH3 domain in vivo, depends on the Myo1 SH3 domain for localization to patches, and competes with Vrp1 for binding the Myo1 SH3 domain. Deletion of bbc1 appears to lengthen the transient interaction of Wsp1-Vrp1 with Myo1. Normally Myo1 and Wsp1-Vrp1 exhibit distinct localization: Myo1 remains at the plasma membrane while Wsp1-Vrp1 internalize with the endocytic vesicle. In contrast, in bbc1Δ cells a portion of Wsp1-Vrp1 remains at the membrane with Myo1, splitting Wsp1-Vrp1 puncta. The splitting depends on an interaction between Myo1 and Wsp1-Vrp1 because splitting is reduced partially or completely when bbc1Δ is combined with either vrp1 deletion or deletion of the Myo1 SH3 domain, respectively. Strikingly, bbc1Δ cells feature endocytic invaginations that are twice as long, which we propose is due to the increased population of Wsp1 and Vrp1 at the base of invagination. Additionally, Bbc1 acts as an inhibitor of actin assembly, but this function is redundant with the adaptor protein Sla1. Our data provide a novel mechanism for regulation and positioning of the actin assembly machinery at endocytic sites. We propose that Bbc1 disrupts the transient Myo1-Vrp1-Wsp1 bridge. In the absence of Bbc1 a greater fraction of Wsp1-Vrp1 remains at the membrane, focusing new branches towards the membrane thus providing an increased number of tracks for Myo1 to increase its pull on the membrane, which results in increased tubule size.

P2812
Board Number: B89
Viable mice and primary cells in the absence of beta-actin protein.
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The highly homologous beta (ACTB) and gamma (ACTG1) cytoplasmic actins are ubiquitously expressed in all cell types and differ by only 4 functionally similar amino acids within the N-terminus. Despite the
remarkable conservation between the ACTB and ACTG1 sequences, previous \textit{in-vitro} and \textit{in-vivo} data suggest that cytoplasmic actins support unique functions. Mice homozygous for severely hypomorphic or null Actb alleles are early embryonic lethal. In contrast, Actg1 null mice survive to term but display significant perinatal lethality, stunted growth, and decreased survival into adulthood. Ablation of ACTB in primary mouse embryonic fibroblasts (MEFs) resulted in severe growth impairment not observed in ACTG1 null MEFs. To determine if the striking phenotypic differences between ACTB and ACTG1 null models were due to the 4 N-terminal amino acid variances, we used transcription activator-like effector nuclease (TALENs) and a single stranded donor oligo sequence to edit the endogenous mouse Actb locus. We altered the ACTB nucleotides that coded for the 4 beta-actin amino acids to instead code for the 4 gamma-actin amino acids by editing the wobble base in each codon. The resulting mice express a chimeric Actb/Actg1 (\(\beta\gamma_{\text{chimera}}\)) mRNA transcript expressed under control of the endogenous ACTB regulatory elements and translated into gamma-actin protein. Crosses of heterozygous mice yielded wildtype (WT), heterozygous and homozygous \(\beta\gamma_{\text{chimera}}\) mice born at Mendelian ratios that live to at least 3 months of age. Additionally, primary MEFs generated from heterozygous and homozygous \(\beta\gamma_{\text{chimera}}\) mice displayed growth rates not different from WT MEFs. Furthermore, qRT-PCR data from primary MEFs, brain and lung tissue demonstrate transcript synthesis at both the endogenous Actg1 and the \(\beta\gamma_{\text{chimera}}\) loci, which corresponded with a 2-fold increase in gamma-actin protein expression and no beta-actin protein expression as assessed by Western blot. Total actin levels remained constant across all three genotypes. The surprising viability of \(\beta\gamma_{\text{chimera}}\) mice and primary MEFs is in stark contrast to what we and others have previously published. Our data suggest that cells and tissues can sense the specific absence of beta-actin protein while regulating total actin expression to maintain a constant cellular protein level. The dispensability of beta-actin protein in cells and mice further suggests that the severe cell and organismal phenotypes observed with gene knockout are due to factors beyond simple loss of beta-actin protein expression.

P2813

\textbf{Board Number: B90}

\textbf{Vav2-RhoG–mediated cytoskeleton and nuclear skeleton remodeling.}

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The oncoprotein Vav2 is a target of tyrosine kinases that are often overexpressed in cancer. As a member of the Dbl family of the guanine nucleotide exchange factors (GEFs), Vav2 activates the Rho GTPases (Rac1, Cdc42, RhoA, and RhoG); the molecular switches regulating cytoskeleton dynamics, cell shape, growth, adhesion, migration as well as gene expression. By mass spectrometry (MS) and immunoprecipitation, we have previously shown the association of Vav2 with tyrosine kinase-type receptors: Human epidermal growth factor receptor 2 and 4 (HER2 and HER4) and insulin-like growth factor I receptor (IGF-IR) in human MCF-7 breast cancer cells. In the presence of HER2/4 and IGF-IR signaling, Vav2 localized to the plasma membrane, and its overexpression caused massive lamellipodial protrusions fringed by F-actin. Upon inhibition of IGF-IR’s kinase, Vav2 overexpression induced prominent stress fibers, traversing the cell body and interconnecting focal adhesions. This distinct phenotype was accompanied by the nuclear deformation and decrease in the nuclear matrix protein lamin A/C. The specific domains of the Vav2 protein responsible for the regulation of the cyto- and nuclear skeleton were examined through the mutational analysis of the Vav2 protein. The expression of oncogenic (delta 1-183) Vav2 and a partially activated Y172FVav2 increased the number of cells with the misshapen nuclei, whereas the catalytically inactive mutant (L217A Vav2) and the SH2 domain mutants (W673RVav2 and G693RVav2) significantly reduced the number of cells with the structural defects in the nuclei. Analysis of Vav2 mutants uncovered the importance of (i) Vav2 interactions with HER tyrosine

\textsuperscript{Tuesday-56}
kinases via the SH2 domain and (ii) Vav2’s GEF activity in cyto- and nuclear skeleton remodeling. Through MS/MS spectra search with 1% of false discovery search, we identified the small GTPase RhoG (Ras homology growth-related) protein in the Vav2 interactome. Although RhoG has been reported to interact with Vav2, its cellular functions remain poorly characterized. We posited that Vav2 --RhoG regulated cyto- and nuclear skeleton. To begin to test the hypothesis, we examined the organization of F-actin and DNA by immunofluorescence microscopy and lamin A/C expression by fluorescence-activated cell sorting (FACS) in the control and RhoG knockdown breast cancer cells. Silencing of RhoG reduced stress fibers buildup and partially protected the cell nuclei from distortion and loss of lamin A/C that would otherwise be caused by Vav2 overexpression. These analyses uncovered the role of Vav2 and RhoG in the cyto- and nuclear skeleton remodeling. Altered nuclear organization by oncprotein Vav2 may affect genome integrity in cancer and it warrants further investigation.

P2815
Board Number: B92
MRTF/SRF transcription promotes cell-in-cell invasion for entosis through Ezrin-dependent bleb-dynamics.
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Entosis is a nonapoptotic cell death process involving homotypic cell-in-cell invasion that occurs in human tumors or malignant fluids and has been associated with tumor suppressive as well as promoting effects. We previously demonstrated the importance of formin-mediated polarized actin dynamics and the essential role of nonapoptotic plama membrane blebbing in this cellular motile process. Although the contractile actin cortex involved in bleb-driven motility is well characterized, a role for transcriptional regulation has not been addressed. Serving as a paradigm for actin-controlled transcription, we explored the impact of the actin-regulated MRTF/SRF transcriptional pathway for sustained blebbing and subsequent entotic invasion. Our findings reveal a remarkably tight association between cortical blebbing and rapid MRTF nucleocytoplasmic shuttling resulting for SRF transcriptional activity. Specifically, we show that SRF-dependent up-regulation of the metastasis-associated ERM protein Ezrin is critical for bleb dynamics to promote cell-in-cell invasion. Our results highlight the essential role of the MRTF/SRF transcriptional pathway for bleb-associated invasive motility.

P2816
Board Number: B93
The Role of Actin Trails in Mediating Bulk Axonal Actin Transport.
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Classic pulse-chase radiolabeling studies have shown that actin – a critical cytoskeletal protein – is conveyed in axons via slow axonal transport; but the mechanistic basis for this movement is unknown. Recently, using filamentous actin (F-actin) probes and low-light imaging, we found that axonal actin was surprisingly dynamic, with local assembly/dis-assembly events (“hotspots”) and polymers elongating.
along the long-axis of the axon-shaft (“actin trails”). Though the frequency of anterogradely-elongating polymers was greater, it’s unclear how hotspots and trails can lead to axonal transport. To understand axonal actin organization at a molecular level, we generated a biophysical model starting with first principles of monomer/F-actin assembly; simulating axonal actin dynamics including hotspots and trails. Interestingly, our model predicted a slow, biased transit of the actin population, which we also confirmed experimentally. Our data suggest that actin is conveyed in axons by an unprecedented mechanism involving local assembly and biased polymerization – kinetics that ultimately lead to its slow transport. We posit that other dynamic cellular structures might use similar mechanisms for conveyance.

P2817
Board Number: B94
Nanotopography biases cell migration, cytoskeletal dynamics, and focal adhesion distribution.
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Many biological and physiological processes depend upon directed migration of cells, which is typically mediated by chemical or physical gradients or by signal relay. Here we show that cells can be guided in a single preferred direction based solely on local asymmetries in nanotopography on subcellular scales. These asymmetries can be repeated, and thereby provide directional guidance, over arbitrarily large areas. The direction and strength of the guidance is sensitive to the details of the nanotopography, suggesting that this phenomenon plays a context-dependent role in vivo. We demonstrate that asymmetric nanotopography guides the direction of internal actin polymerization waves, and that cells move in the same direction as these waves. The conservation of the asymmetric shape of many natural scaffolds suggests that actin-wave-based guidance is important in biology and physiology. Furthermore, nanoridges/grooves impact the focal adhesion distribution and cytoskeletal orientation in epithelial cells. Both the focal adhesion and the cytoskeleton behaviors can be tuned by changes in nanotopographies. Overall, the excitable systems character of the cellular scaffolding, controlled via nanotopography, provides a simple, universal mechanism for guiding a range of migratory behaviors in many living systems.

P2818
Board Number: B95
Exploring actin dynamics of thin filaments in Caenorhabditis elegans striated muscle.
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Actin is the major component of thin filaments, which form part of the core structure of muscle sarcomeres. Actin and various other sarcomeric proteins have been shown to undergo dynamic turnover without affecting the overall structure of the muscles in different model systems. The actin filaments, in particular, have been found to be dynamic in both immature and mature muscle cells. This dynamic nature of actin might help in the maintenance of thin filaments. It was observed in chick cardiomyocytes that injected rhodamine-labeled actin was incorporated at both ends of filaments with greater amounts incorporated at the pointed ends (Littlefield et al; 2001). This leads us to believe that the actin dynamics at the filament ends play a major role in maintaining the length of the thin filaments.
Caenorhabditis elegans is a powerful system to study for studying the protein interactions in sarcomeres. The actin dynamics in the thin filaments of Body Wall Muscles (BWMs) in C. elegans has not been characterized. We are using a GFP-actin expressing worm strain to perform Fluorescence Recovery After Photobleaching (FRAP) experiments. By staining F-actin with phalloidin we have confirmed that GFP-actin localized similar to wild-type actin in the BWMs. We performed FRAP experiments on immobilized live worms and observed 40-50% recovery of total GFP-actin within 25 minutes. This is the first experimental evidence that actin filaments are dynamic in the BWMs of C. elegans. In order to assist in resolving actin recovery with respect to barbed and pointed ends, further FRAP analysis will be done in worms co-expressing Z-line associated α-actinin fused to m-Cherry with GFP-actin. With this, we should be able to quantify the actin dynamics at the barbed and pointed ends of the thin filaments. Capping proteins and tropomodulin help in the stabilization of barbed and pointed ends of the thin filaments respectively, whereas the barbed end regulators that aid the incorporation of G-actin from the G-actin pool are not entirely clear. Formins are a conserved family of proteins that nucleate and elongate actin filaments in vitro. We observed the formins FHOD-1 and CYK-1 localized near/at the Z-lines in sarcomeres. The mutants of these two formins had reduced BWM width. We hypothesize that formins play a role in the regulation of thin filament dynamics. We will further continue our analysis of actin dynamics with the worms bearing mutations in fhod-1 and cyk-1 genes.

P2819
Board Number: B96
Characterization of the cytoskeleton of *Porphyra umbilicalis* and comparison to other red algae based on analysis of the completed *Porphyra* genome.

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Recent assembly of the nuclear genome (87.7 Mb) of the red alga *Porphyra umbilicalis* (Bangiophyceae) made it possible to characterize the cytoskeleton of this economically important and evolutionarily significant species and to gain insight into the cytoskeletons of red algae more broadly (Brawley et al. 2017. *PNAS* www.pnas.org/cgi/doi/10.1073/pnas.1703088114). The red algae are a founding lineage of the Archaeplastida. Multicellular red algae are known from 1.6 billion year old fossils, and the oldest taxonomically-resolved fossil of a multicellular eukaryote is *Bangiomorpha* (1.2 billion years old, Bangiophyceae). Both red algal nuclear genes and a red algal chloroplast are found in diatoms, haptophytes, apicomplexans, and many dinoflagellates due to the evolution of these groups through ancient secondary endosymbioses involving red algae. Using bioinformatic tools including BLAST and PSI-BLAST, we searched for key filament, motor, and regulatory proteins, and found that the complement of cytoskeletal components in *Porphyra* is significantly reduced relative to other multicellular organisms. For example, while the actin and tubulin filament proteins are present, as expected, a number of key regulatory proteins are absent, and the number of motors is especially reduced. In particular, the actin motor myosin is entirely absent from *Porphyra* and other sequenced members of the most species-rich classes of red algae (Bangiophyceae, Florideophyceae; BF). Only a small set of kinesins and no dynein motors of the canonical microtubule-based motors are present. These observations raise questions about how *Porphyra* cells accomplish intracellular transport. We have extended our cytoskeletal analyses to other red algae with sequenced genomes and find that the only universal motor proteins are a small number of kinesins. Some species in a sister clade (SCRP) of mesophilic red algal classes and the extremophiles *Galdieria* (Cyanidiophyceae) have a single myosin that resembles the myosin-27 of apicomplexans. Another surprising observation is the lack of the actin-
nucleating complex ARP2/3, which raises questions about how *Porphyra* neutral spores accomplish amoeboid movement. Formins are present, and may support this movement. The array of nuclear actin-related proteins is also reduced, suggesting that *Porphyra* might have a reduced ability to regulate chromatin-remodeling as compared to other eukaryotic lineages. Overall, we suggest that the reduced cytoskeletal capabilities and complexity of *Porphyra* and other red algae may explain apparent limitations to cell size, the ability to form complex tissues, and to achieve large individual stature in comparison to other multicellular organisms.

**P2820**  
**Board Number: B97**  
**Mechanosensory role of alpha-actinin 4 in pancreatic cancer cell migration.**  
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Alpha-actinin 4, a cytoskeletal actin bundling protein, is upregulated and implicated in the metastasis of a variety of cancers, including pancreatic ductal adenocarcinoma (PDAC). The alpha-actinin family consists of four members, alpha-actinin 1 through 4. In dissemination assays using 3-dimensional tissue spheroids consisting of a heterogeneous mixture of alpha-actinin 4 overexpressing cells and wild-type, overexpression of alpha-actinin 4 increased dissemination compared to wild type cells. Cellular behavior of the two isoforms is similar in randomly migrating cells and dual expression of mCherry-ACTN1 and GFP-ACTN4 show similar localization patterns at the edges of lamellapodia and protrusions in migrating cells. Alpha-actinin 4 alters cell mechanics as determined by micropipette aspiration experiments. Additionally, upon applied mechanical stress, alpha-actinin 1 and 4 exhibit distinct behaviors in cells, notably alpha actinin 4 displays robust accumulation at sites of cortical dilation, while alpha-actinin 1 does not mechanorespond. We hypothesize that this response is mediated both in part by the catch-bond behavior of alpha actinin 4 as well as differential actin binding affinities between the two isoforms. The actin binding domains of alpha-actinin 1 have a >100x higher affinity towards actin than alpha-ctinin 4 (0.36 μM and 34 μM, respectively). Purified alpha-actinin 1 and alpha-actinin 4 proteins exhibit similar behaviors in actin bundling assays, producing thick and ordered actin bundles. However, mutation of the alpha-actinin 4 lysine residue at position 255 to glutamic acid (K255E) results in a 5-fold increase in actin binding, but leads to a loss of localization to leading edge protrusions, as well as a loss of accumulation at sites of dilational stress. The actin bundles formed with the K255E mutant are thinner and create erratic meshed networks. These data suggest that the dynamic turnover necessary for proper actin bundle formation is driven by both actin affinity as well as the catch-bond behavior of alpha-actinin 4.

**P2821**  
**Board Number: B98**  
**Determining Concentration-Dependent Effects of Thymosin β4 in Living Cells through Quantitative Protein Delivery.**  
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The ability of actin to contribute to essential cellular functions such as motility, cell division, and vesicle trafficking is dependent on the precise control of its polymerization from monomers (G-actin) into filaments (F-actin) and the depolymerization of filaments back into the monomer pool. To support the dynamic nature of the actin cytoskeleton, the cell keeps a large reserve of actin monomers through G-
actin binding proteins such as Thymosin β4 (Tβ4). Tβ4 sequesters actin monomers and prevents their polymerization. However, in addition to its main function in inhibiting actin assembly, Tβ4 can also localize monomers to specific types of actin networks by regulating their release from a non-polymerizable sequestered state. The majority of our understanding of Tβ4 is from in vitro biochemical assays, which can be difficult to directly translate to the more complex environment of a living cell. Using electroporation, we are able to introduce known quantities of purified protein into cells, thus potentially bridging the gap between bulk solution assays and cellular experiments. With electroporation, we can introduce purified protein as large as 50 kDa across a wide range of concentrations. Also, millions of cells can be electroporated simultaneously with high cell viability and nearly 100% expression. The goal of this project is to determine the in vivo role of Tβ4 in regulating the G-actin pool and the assembly of F-actin networks. We have introduced fluorescently-labeled, purified Tβ4 into cells in defined amounts and measured its effects on both actin monomers and filaments, on the organization of F-actin throughout the cell, and on the localization of Tβ4 and actin monomers. In addition to establishing the concentration-dependent relationship between Tβ4 expression and actin dynamics, this study provides a template for quantitative delivery of protein into living cells as a means for determining its cellular role.

Kinesins 2

P2822
Board Number: B100
Inhibitable kinesin motors to study intracellular trafficking.
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The kinesin-2 motor KIF3A/KIF3B/KAP is the essential driver of intraflagellar trafficking (IFT) in mammalian cells and interference with its function results in the absence of cilia. In contrast, KIF17 appears to be an accessory IFT motor, as knockout of KIF17 alone has little effect in mice, but results in synthetic effects when combined with other knockouts. The lack of fast and specifically acting kinesin inhibition methods currently precludes the direct analysis of kinesin-2 function in cilia. Here we adapt a chemical-genetic kinesin inhibition approach that we originally developed for kinesin-1 (Engelke et al., 2016, Nat. Commun.) and demonstrate that this approach yields functional kinesin-2 motors that can be abruptly inhibited by small, cell-permeable molecules. To do this we pursued two strategies: I) We inserted the six amino acid tetracysteine tag into surface loops of the motor domain such that binding of biarsenic dyes conformationally distorts and thereby inhibits motility. II) We fused DmrB dimerization domains to the motor heads such that addition of B/B homodimerizer cross-links the motor domains and inhibits processive motor stepping. Using cellular assays, we show that the engineered kinesin-2 motors are able to transport artificial cargo similarly to the wild type motor, but cargo transport is efficiently inhibited by the addition of inhibitor. Future studies will enable us to deploy the inhibitable kinesin-2 motors and for the first time abruptly inhibit KIF3A/KIF3B/KAP and monitor its function in the genesis, maintenance, and resorption of primary cilia. For KIF17 this approach will allow us to determine the direct involvement of KIF17 in IFT, before compensatory mechanisms can attenuate the resulting phenotype.
Regulated autoinhibition of kinesin-1 is essential to polarized dendritic transport.
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Neuronal polarity relies on the selective localization of cargo to axons or dendrites by molecular motors. Kinesin-1 has a critical role in transportation of cargo into axons, but is also known to be active in dendrites. This raises the question of how kinesin-1 activity is regulated to maintain the polarized distribution of cargo within neurons. Our in vivo structure-function analysis of endogenous kinesin-1 in Drosophila reveals a novel role for autoinhibition in polarized dendritic transport. Mutations that disrupt kinesin-1 autoinhibition result in the mislocalization of Golgi outposts (GO) to axons. Our findings show that disruption of autoinhibition is distinct from mutations that inhibit ATP hydrolysis; axonal mislocalization of GO is not found when kinesin-1 enzymatic activity is impaired. Autoinhibition also functions to control kinesin-1 localization within the neuron. Uninhibited kinesin-1 accumulates in axons and is depleted from dendrites, resulting in dendrite growth defects. Confirming an essential role for kinesin-1 in dendrite development, dendritic morphology is also altered when kinesin-1 levels are depleted through RNAi. Genetic interaction tests reveal that a balance of kinesin-1 inhibition and dynein activity is necessary to localize GO to dendrites and keep them from entering axons. Lowering both kinesin-1 and dynein levels is not sufficient to drive GO into axons. In contrast, relieving kinesin-1 autoinhibition when dynein levels are reduced is sufficient to disrupt the polarized transport of outposts. We propose a mechanism in which dynein is required to carry outposts into dendrites, yet kinesin-1 activity must be precisely regulated by autoinhibition to achieve the selective dendritic localization of outposts. Finally, we have evidence that localization of other dendritic cargo may rely on the tight regulation of kinesin-1 autoinhibition. This suggests that autoinhibition as a mechanism of regulating kinesin-1 activity may be broadly important for compartment-specific localization of cargo in neurons.

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Kinesin-5 mitotic motors play essential roles in mitotic spindle assembly and dynamics by cross-linking and sliding apart antiparallel microtubules emanating from the spindle pole bodies (SPBs). Recently, we found that localization to the spindle of S. cerevisiae kinesin-5 Cin8 is regulated by phosphorylation at three Cdk1 sites in its catalytic motor domain. We also determined that each of these Cdk1 sites contribute to phospho-regulation of Cin8 in a unique manner. The mechanism of this phospho-regulation remains unclear. It has been previously suggested that phospho-regulation may occur by two modes; (1) contest-specific which constrains the phospho-regulation events to specific sites and will not recapitulate at a different site, or (2) plastic in which phospho-regulation is spatially flexible within a specific domain and may occur at distant sites. Here we tested the plasticity of phospho-regulation of
Cin8, and examined whether novel synthetic Cdk1 sites created by a single amino acid replacement can mimic the known phospho-regulation or create new phenotypes. For this purpose, we systematically generated Cin8 mutants carrying a novel Cdk1 site as a sole source for Cdk1 phospho-regulation and examined them by in vitro phosphorylation assay, quantitative and qualitative in vivo microscopy and in vivo yeast viability assay. The majority of the sites resulted in phosphorylation independent phenotypes. We found that out of 29 novel sites that we have generated, only one site in the motor domain, adjacent to a native Cdk1 site, recapitulated the phospho-regulation of the adjacent native site, although several sites were created nearby. This result indicates that phospho-regulation of Cin8 by Cdk1 at this site is rigid and undergoes phospho-regulation in highly context-specific manner. Two additional sites resulted in novel phospho-regulation of Cin8; however they resulted in a less optimized phospho-regulation. Interestingly multiple-sequence-alignment revealed that one of these sites is present in other organisms, suggesting that phospho-regulation at this site is flexible throughout evolution and may occur according to the functional requirements in these organisms.

P2825
Board Number: B103
Macromolecular crowding modulates intracellular transport by teams of kinesin-1 motors.
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The cytosol is crowded with a high concentration of macromolecules. Crowding can alter protein conformation, binding rates, and reaction kinetics, yet it is not known how crowding affects intracellular cargo transport by molecular motor proteins. We report on the consequences of crowding on cargo transport by kinesin-1 motors both in Drosophila embryos and in vitro. Surprisingly, we find that crowding significantly slows transport by teams of motors, while having no effect on single motor velocity. By applying controlled forces on single motors using an optical trap we find that this emergent property of kinesin teams results from the individual’s increased sensitivity to hindering load in a crowded medium. The consequences of this single motor property shed light on the long observed variability of cargo transport velocity in different cellular contexts. Based on the data, we further propose a model by which entropic forces in the crowded medium lead to the motor’s altered detachment characteristics, and suggest that crowding can be used as an additional control parameter in the quest for a better understanding of kinesin’s mechanochemical cycle.

P2826
Board Number: B104
Aplip1 (Drosophila JIP1) regulates myonuclear positioning and muscle stability.
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During muscle development myonuclei undergo a complex set of movements that result in evenly spaced nuclei throughout the muscle cell. In Drosophila two separate pools of Kinesin and Dynein govern this process. Although these two pools of motors work in synchrony, how these two pools are specified is not known. Here, we investigate the role of Aplip1 (a JIP1 homolog), a known regulator of both Kinesin and Dynein, in myonuclear positioning. Aplip1 localizes to the myotendinous junction where it can extend from the muscle pole. Additionally, Aplip1 has independent roles in myonuclear
positioning and muscle stability. In Aplip1 mutant embryos there was an increase in the percentage of embryos that had both missing and collapsed muscles. Via a separate mechanism, we demonstrate that Aplip1 is required to position and dynamically move nuclei within muscle. Through genetic interaction experiments, we saw that Aplip1 interacted with the Dynein anchor, Raps, and Kinesin to correctly position nuclei within muscle cells. We propose that Aplip1 is important for normal myonuclear movement via the regulation of Dynein at the cell cortex and the regulation of Kinesin activity at the nuclear envelope.

**P2827**

**Board Number: B105**

**BORC regulates the axonal transport of synaptic vesicle precursors by activating ARL-8.**

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Axonal transport of synaptic vesicle precursors (SVPs) is essential for synapse development and function. The conserved ARF-like small GTPase ARL-8 is localized to SVPs and directly activates UNC-104/KIF1A, the axonal-transport kinesin for SVPs in C. elegans. It is not clear how ARL-8 is activated in this process. Here we show that part of the BLOC-1 related complex (BORC), previously shown to regulate lysosomal transport, is required to recruit and activate ARL-8 on SVPs. We found mutations in six BORC subunits--blos-1/BLOS1, blos-2/BLOS2, snpn-1/ Snapin, sam-4/Myr lysin, blos-7/Lyspersin and blos-9/MEF2BNB cause defects in axonal transport of SVPs, leading to ectopic accumulation of synaptic vesicles in the proximal axon. This phenotype is suppressed by constitutively active arl-8 or unc-104 mutants. Furthermore, SAM-4/Myr lysin, a subunit of BORC, promotes the GDP to GTP exchange of ARL-8 in vitro and recruits ARL-8 onto SVPs in vivo. Thus, BORC regulates the axonal transport of synaptic materials and synapse formation by controlling the nucleotide state of ARL-8. Interestingly, the other two subunits of BORC essential for lysosomal transport, kxd-1/KXD1 and blos-8/Diakedin, are not required for the SVP transport, suggesting distinct subunit requirements for lysosomal and SVP trafficking.

**P2828**

**Board Number: B106**

**Motility and kinetic properties of the kinesin-4 members KIF27 and KIF7.**

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The kinesins are a large superfamily of ATP-dependent motor proteins that can walk along microtubules to transport cargo. However, the kinesin-4 motor KIF7 appears to lack the ability to undergo processive motility. Thus, the motility and enzymatic properties of the COS2/KIF7/KIF27 subfamily of kinesin-4 motors remain poorly understood. In this study, we demonstrate that whereas mammalian KIF7 and its Drosophila homolog Coastal2 (COS2) show no motility at the single molecule level, the mammalian parologue KIF27 is a slow processive motor. To test whether these kinesin-4 motors can drive processive motility when working in teams, we carried out microtubule gliding assays in vitro and peroxisome transport assays in living cells. In both assays, KIF27 showed slow motility whereas KIF7 failed to
transport cargo, consistent with their motility in single molecule assays. Both KIF7 and KIF27 showed defective mechanochemical coupling, existing in a strongly microtubule-bound state regardless of nucleotide condition. The mechanistic basis of immotile KIF7 behavior was determined to arise from an inability to release ADP in response to microtubule binding. The mechanistic basis of slow processive KIF27 behavior was determined to arise from its slow ATPase rate and high affinity for both ATP and microtubules. Our results demonstrate that sequence changes to the core kinesin motor domain can result in dramatic differences in kinetic and motility properties across the kinesin-4 family of motors.

P2829

Board Number: B107

Analysis of plus end directed motor proteins Cin8 and Kip3 reveals their unique role in maintaining the integrity of the chromosome specifically during meiosis.

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Kinesin motors provide the molecular forces that are required at the kinetochore-microtubule interface and thereby control the process of chromosome segregation. Despite elucidation of their functions in mitosis, their roles in meiosis are poorly explored. Given the fact that at least in budding yeast, there are temporal and mechanistic differences between mitosis and meiosis in kinetochore maturation, establishment of kinetochore-microtubule attachment and orientation of the sister kinetochores with respect to the spindle poles, we found a temporal variation in the distribution pattern of Cin8, Kip1 and Kip3 from prophase I to metaphase I between centromeres and SPBs suggesting their distinct roles in the early meiotic events. Consistent to this we observed an erroneous homolog pairing during meiosis I in the \(cin8\Delta\) as compared to \(kip1\Delta\) or \(kip3\Delta\) and consequently a defect in the homolog bi-orientation on meiosis I spindle in the former mutant but not in the latter two. These defects may account for very poor spore viability in \(cin8\Delta\) but not in \(kip1\Delta\) or \(kip3\Delta\). Further we checked the mitotic role of Cin8 and Kip3 in the maintenance of kinetochore integrity during meiosis and interestingly we found that especially in meiosis Cin8 is predominantly required for the stable localization of outer kinetochore protein Ndc80 as compared to Kip3 and as a result reduction in the localization of Ndc80 at the centromere leads to the destabilization of the inner kinetochore protein, Ndc10 in case of \(cin8\Delta\) but not in case of \(kip3\Delta\). Owing to the compromised localization of Ndc80 at the centromere and functional redundancy among the motors we studied meiosis in \(cin8\Delta\;kip3\Delta\) double mutant and we observed a drastic reduction in the spore viability. Further analysis revealed that both Cin8 and Kip3 act at the downstream of the FEAR pathway. As a result, the way essentiality of the FEAR components changes between mitosis and meiosis similarly it does in case of \(cin8\Delta\;kip3\Delta\) double mutant. Examining the meiosis undergoing \(cin8\Delta\;kip3\Delta\) cells, an unusual aneuploidy was detected in the meiosis II cells which surprisingly later proved to be an outcome of the chromosome breakage verified by different experimental techniques. Albeit the mechanism behind the loss of chromosome integrity is still under investigation, overall our study indicates that the kinesin motors may influence the kinetochore-microtubule interface and thus the gross chromosome segregation significantly in meiosis perhaps more than they do so in mitosis.
P2830
Board Number: B108
VLDL Secretion from Hepatocytes is Controlled by Phosphatidic Acid.
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Lipid droplets (LDs) are catabolized in hepatocytes to provide lipids for assembling VLDL particles, which are then secreted to peripheral tissues. This requires extensive physical interactions between LDs and endoplasmic reticulum (ER). We found that this interaction requires LDs to be transported towards the smooth ER by motor proteins, resulting in lipid and protein exchange between these two organelles. How do metabolic signals control the activity of motors on LDs to facilitate catabolism of lipids? Here, using targeted lipidomics, in-vitro reconstitution, organelle motility and biochemical assays, we have attempted to solve this puzzle. We found that insulin via phospholipase D activity increases the phosphatidic acid level on LDs in fed state of an animal. This recruits the small GTPase Arf1 and the kinesin motor to LDs, which are transported to the ER followed by LD catabolism. Phosphatidic acid levels on LDs are reduced after fasting. This removes Arf1 and kinesin from LDs to reduce ER-LD physical contacts, and in turn decreases lipid availability for VLDL lipidation. This mechanism appears to control triglyceride secretion from the liver across feeding/fasting cycle and ensures that serum triglyceride levels are maintained approximately constant.

P2831
Board Number: B109
The unique N-terminal extension of the oncogenic KIF14 kinesin motor domain is an F-actin bundler elongater, and nucleator.
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Introduction: Kinesins are widely known as regulators of microtubule dynamics and as microtubule-based transporters of vesicles and chromosomes in eukaryotes. The KIF14 kinesin is unique in that it possesses a ~350 amino acid N-terminal extension of its motor domain (named the NTE domain) that helps it associate with, and trigger assembly of actin filaments and actin filament bundles1. These abilities help explain the importance of KIF14 in mitosis and cytokinesis, which are microtubule and actin-based functions, respectively. They could also have an important correlation with the overabundance of KIF14 in many aggressive cancers2,3. Our dissection of the human KIF14 NTE into its separate functional subdomains has begun to establish the mechanistic basis of actin polymer assembly by this motor.
Results: Assessment of the ability of different length NTE protein constructs to stimulate F-actin assembly in vitro revealed that the C-terminal region of the NTE was essential for this activity; even though shorter NTE constructs lacking this region could still bind F-actin. TIRF microscopy showed that F-actin assembly involves a combination of actin nucleation and elongation from both ends of these actin nuclei. Further NTE dissection identified actin-binding site(s) between residues 133-260, and showed that the actin filament bundling motif(s) resides within this region. Analytical size-exclusion chromatography of the truncated NTE proteins demonstrated that the C-terminal-inclusive constructs are multimeric, while C-terminally truncated proteins are monomers. This implies a necessity for a...
higher-order arrangement of KIF14 to stimulate F-actin formation. We also provide evidence to suggest that this higher-order NTE assembly involves association of NTE regions that are largely disordered, and possibly dynamic, in the absence of actin.

Conclusion: The structural details of the KIF14 NTE-actin interaction are still under investigation and are occurring in parallel with studies of its motor domain with microtubules. This work will inform on how KIF14 influences actin dynamics at the midzone during cytokinesis, perhaps as a mediator of contractile ring formation, and could help identify a strategy to inhibit KIF14 activities for the benefit of cancer sufferers.


P2832
Board Number: B110
Geometry Matters for Cargos Navigating 3D Microtubule Intersections.
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Eukaryotic cells transport organelles and other cargos along microtubules to control their distribution within the cell and deliver them to distant locations. While we understand how molecular motors can transport cargos along individual microtubules, the cell’s microtubules are usually arranged in a complex 3D network. While traversing this network, cargos need to navigate intersections where microtubules cross at a wide variety of separation distances and angles. To gain insight into how cargos navigate these intersections, we have used a recently established 3D construction technique based on holographic optical trapping to build single 3D microtubule intersections in vitro with relevant nanoscale precision. We then used these fully suspended microtubule structures to perform motility assays on kinesin-1 coated cargos. We find that some intersection geometries influence cargos to pass along their current microtubule, while other geometries influence them to switch to the intersecting one. To understand how, we use a 3D Brownian dynamics simulation of cargo transport to investigate the mechanisms which give rise to the observed switching probabilities across separation distances and angles. Using these stochastic simulations, we find that switching probability is often determined by a competition between a stronger motor team on the primary microtubule and the intersecting microtubule sterically hindering that team’s progress. This understanding of the basic mechanisms of switching at single intersections in 3D helps lay a foundation for understanding how the cell may regulate switching to control how cargos navigate the MT network and ultimately their spatial organization.
The mitotic spindle is microtubule (MT)-based machine that segregates a replicated set of chromosomes during cell division. Many chemotherapeutics target the spindle by altering or disrupting microtubules, the polymer that forms the spindle. While these drugs are efficacious, microtubules are a major component of all cells and their disruption can have deleterious effects on cell types that rely on MTs for function, such as neurons. In addition to tubulin, MT-dependent motors that function during mitosis are logical targets for drug development. Eg5 (Kinesin-5) and Kif15 (Kinesin-12), in particular, is an attractive pair of motor proteins to pharmacologically target since they work in concert to drive centrosome separation and promote spindle bipolarity. Kinesin 5 inhibitors (K5Is) have been extensively studied since their discovery, with many advancing to both Phase 1 and 2 clinical trials. Despite the initial excitement for K5Is due to their promising results in cell and mouse tumor models, they have largely failed in the clinic. Since Kif15 over expression has been shown to overcome K5I treatment in tissue culture cells, a potential explanation for K5I clinical failure may be due to the cell’s ability to utilize a Kif15 dependent spindle assembly pathway. Recently, our laboratory discovered that the emergence of K5I resistance, a phenomenon commonly observed in tissue culture cells, depends on the expression of Kif15. This result underscores the hypothesis that a combinatorial drug approach to target spindle assembly, by inhibiting both Eg5 and Kif15, will cripple rapidly dividing cancer cells. Therefore, we set out to perform a small molecule screen on a focused group of known kinase inhibitors, with the goal of identifying lead chemical scaffolds that inhibit Kif15. Using an in-vitro ATPase assay, the Published Kinase Inhibitor Set (distributed by GSK) was screened in duplicate and two compounds, both containing oxindole cores, significantly inhibited Kif15’s MT stimulated ATPase activity. The activities of both compounds were confirmed in a MT gliding assay as well as a second ATPase assay. Concentration response curves were performed in triplicate and IC50s were calculated for each. VU0482674 became our lead compound, exhibiting an IC50 of 800nM. Similarly, VU0482674’s IC50 in the MT gliding assay was calculated to be 734nM. Furthermore, treatment with VU0482674 on K5I resistant cells (KIRCs), whose ability to form bipolar spindles relies on Kif15, results in nearly 100% monopolar spindles. VU0482674 has no effect on mitotic progression in normal RPE-1 cells, suggesting that the compound primarily inhibits Kif15 during cell division. Structure Activity Relationship (SAR) analysis of VU0482674 is currently underway.
Members of the kinesin motor superfamily are subject to post-translational modifications, which are hypothesized to tune kinesin motors for specific cellular functions. The kinesin-5 motor Eg5 (Kif11) forms homotetramers that function to crosslink and slide anti-parallel microtubules during spindle formation. Eg5 is subject to a number of post-translational modifications. Here we focused on understanding the role of acetylation at lysine 146, a residue in the α2 helix of the Eg5 motor domain. Acetylation at this site disrupts the formation of a salt bridge with aspartate 91. We have assessed the activity of an acetylation mimic mutant Eg5 (K146Q) at the single molecule and cellular levels. Time resolved kinetics assays indicate that Eg5 K146Q displays enhanced coupling of switch 1 and the neck linker. In single molecule optical trapping assays, Eg5 K146Q dimers are more likely than wild type (WT) dimers to stall rather than dissociate from the microtubule under load. Based on these results, acetylated Eg5 motors would be predicted to stall microtubule sliding during spindle formation, acting as a brake and slowing spindle pole separation. To test this prediction, mCherry (mCh) tagged Eg5 WT and K146Q motors were expressed at low levels in HeLa cells. Both WT and K146Q mCh-Eg5 localized to the mitotic spindle, with similar distributions along the spindle length. To compare the functional activity of WT and K146Q Eg5, cells expressing GFP-tubulin and equivalent levels of mCh-Eg5 WT or mCh-Eg5 K146Q were treated with the Eg5 inhibitor monastrol, resulting in mitotic arrest and the formation of monopolar spindles. Bipolar spindle formation following monastrol washout was then imaged and measured. While spindle lengths at the completion of pole separation were similar in cells expressing mCh-Eg5 WT or K146Q (11.02 ± 0.29 μm WT, 10.87 ± 0.26 μm K146Q, mean ± SEM), pole separation occurred at a significantly slower velocity in cells expressing mCh-Eg5 K146Q (0.84 ± 0.07 μm/min) than in cells expressing mCh-Eg5 WT (1.35 ± 0.15 μm/min). This velocity difference is consistent with Eg5 acetylated at K146 stalling rather than dissociating from the microtubule, and acting as a brake during pole separation.

**P2835**

**Board Number: B113**

**Membrane-mediated Motor Reorganization in Microtubule-based Transport.**

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Transport of membranous organelles, such as mitochondria and endosomes, are of vital importance for the survival and proper operation of cells. These organelles are transported by kinesin and dynein motors that walk towards the plus-end and minus-end of microtubules, respectively. Despite considerable understanding in the chemomechanical cycle of single motors, how multiple motors coordinate with each other when connected through a membrane is not well understood. During transport, membrane-bound motors are expected to slip in the plane of the membrane, which should diminish their transport abilities, but also are expected to diffuse and accumulate on the microtubule, which should increase the number of active motors pulling the cargo. To visualize these processes, we set up a 2D system in which kinesin-1 motors are transporting microtubules while linked to a lipid bilayer supported on a glass surface. At high motor density, kinesin-1 quickly accumulated to the microtubule upon its landing and the accumulation process reached steady-state in seconds. At steady-state, motor intensity along a microtubule increased from the minus- to the plus-end. These phenomena indicated that the fluid lipid bilayer allows motors to freely diffuse and reorganize along the...
microtubules during vesicle transport. This reorganization of motors may serve as a strategy that cells use to maintain vesicle transport in the highly-crowded cytoplasm.

P2836
Board Number: B114
Understanding the roles of kinesin-1 during axon degeneration.
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Axons are the longest cellular processes in animals which often have to be maintained for an organism’s lifetime, despite the fact that their delicate structure renders axons vulnerable to degeneration. Mutations of the MT-based motor protein kinesin-1 have prominent links to axon degeneration, but the underlying pathomechanisms remain unclear. Normally, axons contain parallel bundles of microtubules (MTs) which form their structural backbones and tracks for long distance transport, whereas kinesin-1 mutant axons show swellings with disorganised MT bundles. We propose that these disorganised MTs are not a consequence but a potential cause of axon degeneration. We test this hypothesis in Drosophila primary neurons which we have developed into a powerful model for studying axonal cytoskeletal machinery (Prokop et al., 2013, J. Cell Sci. 126, 2331ff.). In fly axons, kinesin-1 depletion leads to reduced axon growth and areas of disorganised MTs, which we propose as a potential model for axon swellings. Using point mutations in kinesin-1 or genetic depletion of specific interaction partners, we assessed the contribution of three sub-functions of kinesin-1 towards MT regulation: (1) MT sliding, (2) cargo transport or (3) mitochondrial dynamics. We found that impaired cargo or mitochondrial transport, but not MT sliding cause MT disorganisation. Furthermore, we found that kinesin-3 contributes to those two functions, and that it strongly interacts with kinesin-1 during MT disorganisation, suggesting that the observed phenomenon might link to a wider pool of axonal transport motors.

We will discuss our latest investigation results into the underlying pathomechanisms, focussing on mitochondrial ROS and functional inhibition of other classes of MT regulators, such as MT stabilisers or severers. We believe that an understanding of the roles of motor proteins in MT regulation will provide fundamental new understanding of axon biology, and that Drosophila is the most useful tool to achieve this goal.

P2837
Board Number: B115
Transport Properties of Molecular Motor Ensemble with Bi-Directional Motors : A Computational Approach.
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Intracellular transportation involves multiple molecular proteins working together in coordination. These proteins are of different types, i.e. they have different chemical as well as physical properties. Understanding transport properties of these molecules individually as well as of their ensembles help understand the underlying mechanism of genesis of neurodegenerative diseases. Analytical approaches and Monte-Carlo simulations used to study how motor-proteins coordinate the transportation of a cargo are either simplistic in nature and require high computational power, while being unable to capture the rare events. To overcome these challenges, semi-analytical approaches have proven to be quite effective for studying biologically relevant quantities while enabling us to observe the occurrence
of rare events. So far, semi-analytical approaches have been developed for studying the transportation of a shared cargo by a team of unidirectional motors. Here, the movement of cargo is restricted to only one direction on microtubule lattice. Further work has been done to extend this approach to the case when some of the motors are mutated while still being restricted to one directional motion of cargo. These approaches, while being less computationally intensive compared to Monte-Carlo simulations, produce comparable results to any other techniques available for simulating single species, unidirectional transportation. They also give insights about some of the rare events occurring which other techniques fail to capture. This provides the motivation to extend the existing semi-analytical framework for bi-directional intracellular transportation, where transport is enabled by a team of multiple kinesin and dynein motors.

We develop a more generalized approach which enables us to study the transportation of cargo by an ensemble of motors travelling in opposite directions on the mitorubule. This enables us to study biologically relevant quantities such as average run-length and average velocity of the cargo molecule while taking into account bi-directional motion of ensemble. Added direction of motion and forces gives rise to the change in behavior of ensemble and makes the extension of the single species, unidirectional case to a two species, bi-directional case a non-trivial task. We begin by defining the transition probabilities of change in absolute configurations of ensemble. We determine an equivalence class of various absolute configuration by using the relative configurations defined by the relative distances of the motors. We prove that the number of such relative configurations are finite. We further calculate these quantities for an ensemble of motors consisting of Kinesin and Dynein and some of the key results are presented.

Myosins 2

P2838
Board Number: B116

Myosin 18A-alpha targets the Rac GEF Beta-PIX to the dendritic spines of cerebellar Purkinje neurons and is required for normal spine morphology.

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Dendritic spines are signaling microcompartments that serve as the primary site of synapse formation in neurons, and that house the machinery underlying memory formation. Actin plays a vital role in the generation and maintenance of spines, and changes in spine actin organization underlie memory formation (Hotulainen and Hoogenraad, 2010). Not surprisingly, therefore, non-muscle myosin 2 (NM2) also plays a critical role in spine structure and function (Ryu et al., 2006). Myosin 18A (M18A) is a NM2-like myosin expressed from flies to man that co-assembles with NM2 to make mixed filaments (Billington et al., 2015). Importantly, M18A is alternatively spliced to create many versions that contain unique N- and C-terminal extensions harboring both recognizable (e.g. PDZ domains, SH3 domain binding sites) and uncharacterized protein: protein interaction domains. For example, M18A-alpha possesses a 300-residue N-terminal extension that contains a PDZ domain, a binding site for F-actin, and a binding site for the Rac GEF Beta-Pix, which has been shown to play a key role in controlling spine actin assembly (Zhang et al., 2005; Saneyoshi et al., 2008). Current thinking is that M18A’s primary biological function is to present these protein: protein interaction domains on the surface of mixed NM2 bipolar filaments to dramatically increase their functional diversity. Here we show that endogenous and exogenous GFP-tagged M18A-alpha are concentrated along with NM2 in the dendritic spines of
cerebellar Purkinje neurons, and that M18A-alpha’s N-terminal extension drives its spine localization. miRNA-mediated knockdown of M18A-alpha results in significant defects in the number of primary dendrites, spine number and spine length, all of which are rescued with an RNAi-immune version of M18A-alpha. Intriguingly, preliminary data shows that Beta-PIX co-localizes with M18A-alpha in spines, and that it’s spine localization is lost upon M18A-alpha knockdown. Taken together, these data suggest that M18A-alpha and Beta-Pix form a complex in spines that plays a role in establishing normal cell polarity upon initial neurite outgrowth and normal spine morphology.

P2839
Board Number: B117
Genomic knockout of Myosin-X in mouse results in semi-lethality and decreased filopodia.
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Myosin-X (Myo10) is an unconventional myosin best known for its striking localization to the tips of filopodia and spindle assembly and positioning. Despite the broad expression of Myo10 in vertebrate cells and tissues, its functions at the organismal level remain largely unknown. We report here the generation of KO-first (Myo10tm1a/tm1a), Floxed (Myo10tm1c/tm1c), and KO mice (Myo10tm1d/tm1d) that target full-length and headless Myo10. Complete knockout of Myo10 (both Myo10tm1a/tm1a and Myo10tm1d/tm1d) results in semi-lethality and a 60% incidence of exencephaly, a severe defect in neural tube closure that is incompatible with survival after birth. Myo10 KO embryos that were not exencephalic were able to develop into adults, but exhibited several defects. 100% of Myo10 KO mice exhibited a white belly spot, ~50% had webbed digits, and virtually all had a small kink near the tip of the tail. 100% of Myo10 KO mice exhibited persistent hyperplastic primary vitreous (PHPV) containing the hyaloid vasculature, a fetal vasculature that perfuses the developing eye and that normally regresses as the adult retinal vasculature forms. Myo10 KO mice that survived birth could breed with one another to produce litters of KO embryos, demonstrating that Myo10 is not strictly essential for adult survival, meiosis, or mitosis. Heterozygous KO mice appeared normal, demonstrating that one copy of Myo10 is sufficient for normal development. Homozygous KO-first mice exhibited the same phenotype as homozygous KO mice. An independent mouse mutant containing a spontaneous deletion in full-length and headless Myo10 (Myo10tm1/m1) exhibited the same core phenotype, conclusively demonstrating that complete loss of Myo10 results in a high frequency of embryos with exencephaly and adults with a white belly spot, persistent hyaloid vasculature, and a high incidence of webbed digits. During retinal angiogenesis, the tip endothelial cells of the vascular fronts exhibited 60% fewer filopodia in Myo10 KO compared to controls, demonstrating that Myo10 is required to form normal numbers of filopodia in vivo. The generation of Myo10 KO-first, Floxed, and KO mice provides key tools for the field and reveals the organismal phenotype resulting from complete loss of Myo10. It also reveals that Myo10 has important functions in mammalian development in neural tube closure, pigmentation, and regression of the hyaloid blood vessels.
P2840

Board Number: B118

The proteolysis of Myo10, by Calpain, under low calcium conditions is activated by its FERM domain Acyl-CoA Binding Protein Motif.

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Myosin-X (Myo10) is a ubiquitously expressed motor protein involved in the formation of filopodia and tunneling nanotubes (TNTs), as well as in the invasiveness of cancer and the spread of pathogens. Its structure is unique, containing a Motor Head domain, 3 IQ motifs, an α-helix, a coiled coil, 3 PEST domains, 3 PH domains, a MYTH4 domain, and a FERM domain. Under physiological conditions, full length Myo10 (FLMyo10) is expressed at low levels and proteolyzed into three distinct bands by gel electrophoresis. We hypothesized that Myo10 overexpression may overwhelm the proteolytic machinery thereby allowing more FLMyo10 to reach the plasma membrane where it induces TNT formation.

Previously, Berg et al. hinted at a role for calpain in Myo10 proteolysis. Wang et al. suggested that calpain binds to PEST sites, in a Ca2+ dependent manner, and cleaves its target proteins at their IQ motifs. Proteolysis can be inhibited by Calmodulin (CaM) binding to the IQ motif. Myo10 possesses each of these domains, and the calmodulin-like protein, CALML3 can bind to Myo10, increasing both FLMyo10 expression and the number and length of filopodia.

Here, we show that KCI a disrupter of CaM complex formation, and the calmodulin inhibitor J8, decrease FLMyo10 *in cellulo*. Surprisingly, calpain inhibitors have no effect on Myo10 proteolysis, and Ca2+ chelators increased proteolysis. Interestingly, Myo10 contains an Acyl-CoA Binding Protein (ACBP) motif, a potent activator of Calpain-2 (CAPN2) under low Ca2+ requirements. Thus, we examined whether Myo10 ACBP motif could bind with CAPN2 and serve as an activator of Myo10 proteolysis under physiological Ca2+ conditions. Our *in silico* docking experiments show that Myo10 ACBP motif is predicted to bind to a specific pocket of CAPN2. Furthermore, binding to this pocket is disrupted by the loss of the calpain small subunit (CAPNS1), and completely lost upon the formation of a complex with calpastatin (endogenous calpain inhibitor).

Next, we used UCSF Dock Blaster drug discovery tools to find compounds that could disrupt the ACBP/calpain binding. One of these compounds in CAD cells lowered the proteolysis of Myo10 by 40% and a cell line with a CAPNS1 deletion, expressing long, thin filopodia-like structures, displays a striking absence of the Myo10 proteolytic bands.

Overall, the Myo10 ACBP motif, under low Ca2+ levels, specifically targets Myo10 for proteolysis. This proteolysis is disrupted by increased Ca2+ through the activation and binding of CaM and CALML3 to the IQ domains, and/or by the loss of the CAPNS1 small subunit. This work shows that calpain can be activated under physiological Ca2+ levels enhancing our understanding of how calpain targets its substrates and could lead to a novel method of TNT induction.

P2841

Board Number: B119

Mitochondria distribution to filopodia by the actin-based motor Myo19.

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Eukaryotic cells are exposed to many environmental cues and stress conditions which have profound effects on mitochondria dynamics. The role of the actin cytoskeleton in relation to mitochondria...
function and dynamics is only now beginning to emerge, revealing new functions for actin-based motors. Here, we focus on the recently discovered actin-based motor, Myo19, which is associated with the mitochondria and on its effects on mitochondrial biology. We show that Myo19 localizes with mitochondria to filopodia tips in response to glucose starvation, ROS and EGF. However, how Myo19 localizes to mitochondria, how its enzymology allows the translocation of mitochondria to filopodia, and what is the function of mitochondria at these filopodia is not known. First, we reveal that Myo19 is integrated to the outer mitochondrial membrane (OMM) through a previously unidentified binding motif in its tail domain, ensuring a highly stable interaction between Myo19 and the OMM. Point mutations within the 30 amino acids of this motif inhibit localization of Myo19 to the OMM. Secondly, using time-lapse fluorescent microscopy we show that Myo19 undergoes both anterograde and retrograde movements in filopodia, which are coupled to their extension and retraction, respectively. Thirdly, by studying the enzymology of Myo19 we provide a detailed reaction mechanism of its ATPase cycle. Both the slow ADP isomerization and ADP release prolong the time Myo19 spend in the strong actin binding state and hence contribute to its relatively high duty ratio. However, the predicted duty ratio based on our measured rate and equilibrium constants is lower than required to support motility as a monomer. Thus, we predict that an ensemble of Myo19 motors is required to efficiently propel mitochondria movement on actin filaments. Finally, we provide a model explaining how Myo19 translocation may be regulated by the local ATP/ADP ratio. Interestingly, local mitochondria concentration in neurons is correlated with increased branching of actin-based protrusions of the dendritic spines. Thus, we suggest that Myo19 acts as an ATP sensor, and translocates mitochondria to regions that demand high local levels of ATP for processes such as actin polymerization.

**P2842**

**Board Number: B120**

**Uncovering a Myosin XI-Mediated Transport Mechanism Conserved between Physcomitrella patens and Arabidopsis thaliana.**

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The diverse morphologies observed across plants and animals require a coordinated asymmetry of intracellular components organized by the cytoskeleton. Tip growth of filamentous protonemata in the moss Physcomitrella patens presents an experimentally robust platform to investigate polarized growth driven by the molecular motor myosin XI (homologous to myosin V). This machinery continuously elongates the cell by trafficking secretory vesicles without actively transporting large organelles as observed in flowering plants. Therefore, by coupling vesicles to myosin XI, an unidentified moss myosin XI-cargo complex may function as an essential and conserved component of growth. Here we exploited P. patens’ only two functionally redundant myosin XIs, compared to the 13-member A. thaliana myosin XI family, to elucidate putative cargo-binding interfaces. We hypothesized that A. thaliana myosin XI isoforms of higher sequence identity and similar subcellular (apical) localization to moss myosin XIs will functionally complement growth in P. patens myosin XI-RNAi knockdown background. Although A. thaliana and P. patens diverged approximately 450 million years ago, their myosin XI proteins share approximately 60-70% identity within the cargo-binding domain. We generated chimeric myosin XIs through fusions of the P. patens head domain with three isoforms of the cargo-binding tail domain of A. thaliana (K,E, and F). Chimeric myosin XIs K and E reconstituted polarized growth in moss in an RNAi complementation assay, whereas isoform F failed to complement. Following this result, we attempted to identify the heretofore unknown myosin XI vesicle receptor through an extensive yeast two-hybrid
screen. This approach identified a RabE subfamily member (Sec4 in yeast, Rab8 in human) as a putative binding partner. Preliminary data with in-house directed yeast two-hybrid and bimolecular binding assays with purified myosin XI and RabE suggests a direct interaction. These results support the notion of sequence-level functional conservation of myosin XI transport across the plant kingdom.

P2843
Board Number: B121
Drosophila myosin 7a in Phagocytosis and Eye Development.
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Drosophila myosin 7a (Dm7a) is an unconventional myosin required for the maintenance of the Johnston’s organ (the auditory center in Drosophila) and the morphology of the bristle structures on the thorax. Both of these structures are formed by bundled arrays of actin. Here we show that Dm7a is involved in the process of engulfment of bacteria particles in the Drosophila larval hemocytes. Using the UAS/GAL4 system, we express GFP-Dm7a in the larval hemocytes where it localizes to the cortical regions of the cell and induces the formation of filopodia that are also enriched in Dm7a. We imaged the phagocytosis of fluorescently-tagged bacteria by hemocytes expressing GFP-Dm7a. When a bacterium contacts the cell surface of a hemocyte, the latter sends out a filopodium which wraps around the bacterium followed by engulfment. Occasionally an extended filopodia was seen to contact a bacterium and transport it back to the cell surface. Phagocytosis assays revealed that hemocytes from Dm7a mutant flies are defective in their ability to engulf bacteria compared to wildtype hemocytes, although bacteria do bind to their cell surface. Additionally, a lethality assay whereby larvae were challenged with bacteria show that 21% of wildtype larvae eclosed into adult flies, but less than 10% of Dm7a mutant larvae eclosed under these conditions. These results suggest a role for Dm7a in phagocytosis and in the defense mechanism of the fly. Additionally, immunostaining of adult eyes with Dm7a showed its localization in the pigment cells. In electron microscopy sections of the adult eyes of Dm7a mutants the pigment granules around the primary pigment cells and at the base of the rhabdomeres of the photoreceptor cells were absent. We are investigating the functions of Dm7a in the development of the eye.

P2844
Board Number: B122
The mechanome of asymmetric cell division.
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Asymmetric cell division (ACD) generates cellular diversity and is an important process during development. Stem cells in particular utilize ACD in order to self-renew the stem cell yet generate differentiating siblings. Some stem cells undergo both physical and molecular ACD and it is unknown how biophysical parameters, such as cortical tension, stiffness or osmotic pressure affect the formation of sibling cell size asymmetry. We use Drosophila neural stem cells (neuroblasts) to study the contribution of biophysical parameters on ACD. We are combining fluorescence microscopy with atomic force microscopy (AFM), particle image velocimetry (PIV) and genetically encoded stiffness sensors to measure the dynamics of the actomyosin network and cortical stiffness of cultured neuroblasts. The
combination of these measurements allow us to propose a model, explaining how dynamic changes in physical parameters contribute to the establishment of sibling cell size differences during mitosis.

**P2845**

**Board Number: B123**

The cytokinetic localization of Sid2p and Mob1p of the fission yeast Hippo like pathway requires both formin and type V myosin.

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The Hippo tumor suppressor pathway is an evolutionarily conserved signaling pathway that regulates cell proliferation from yeast to human. At its core, the Hippo pathway is a kinase cascade consisting of two kinases Mst1 and LATS1, in addition to their respective activators Salvador and MOB1. The actin cytoskeleton has been identified as a key regulator of the Hippo pathway but the molecular mechanism remains unclear. Here we examined whether actin binding proteins regulate the localization of the Hippo pathway proteins in fission yeast. Schizosaccharomyces pombe possesses a Hippo like pathway, Septation Initiation Network (SIN), which includes Sid2p and Mob1p, the homologues of LATS1 and MOB1 respectively. Our study focused on five actin binding proteins, For3p, Myo51p, Myo52p, Adf1p (cofilin), and Fim1p. We counted the number of Mob1p and Sid2p molecules in either the spindle pole bodies (SPBs, the yeast equivalent of centrosomes), or the contractile ring during cell division with quantitative fluorescence microscopy. On the SPBs, the number of both Mob1p and Sid2p gradually increase starting at metaphase until reaching ~1200 at +76 minutes after the separation of SPB. On the contractile ring, these two molecules start to accumulate during anaphase B, ~33 minutes after the separation of SPBs. Their numbers reach a peak of ~1600 when the contractile ring initiates constricts. Because the ratio of these two molecules in either the SPBs or the contractile ring remain 1:1 throughout cell division, we only counted only the number of Mob1p molecules in five mutants for3Δ, myo51Δ, myo52Δ, adf1-M3A, and fim1Δ. Only the deletion of either For3p or Myo51p resulted in ~20% decrease in the number of Mob1p molecules in the contractile ring. In contrast, the numbers of Mob1p molecules in the SPBs were not affected by any of the five mutations. For3p is a formin required for assembly of the actin cables in fission yeast and Myo51p is a type V myosin essential for the intracellular transportation along the actin cables. To determine whether Mob1p is transported to the contractile ring by Myo51p along the cables, we quantified the number of Mob1p molecules in the contractile ring in a myo51 mutant that is missing its cargo binding domain. We found that the number of Mob1p-GFP molecules in the contractile ring decrease by ~30% in this myo51 truncation mutant. In conclusion, we discovered that in fission yeast the actin cytoskeleton regulates the localization of the Hippo pathway proteins Sid2p and Mob1p through myosin V mediated intracellular transportation along bundled actin filaments assembled by formins. Our study provides a potential mechanism for the regulation of the Hippo pathway by the actin cytoskeleton in other organisms including humans.

**P2846**

**Board Number: B124**

Dissecting the Molecular and Cellular Basis of Familial Cardiomyopathies.

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Familial cardiomyopathies, affecting up to 1 in 500 individuals, are the leading cause of sudden death in young people. The most common forms, dilated and hypertrophic cardiomyopathies, are characterized
by pathological remodeling of the heart tissue and increased fibrosis, which can lead to heart failure and arrhythmias. In dilated cardiomyopathy (DCM), the ventricular wall thins while in hypertrophic cardiomyopathy (HCM), it thickens. Both diseases are caused by mutations in the proteins that regulate heart contraction, a prominent one being troponin-T. Interestingly, mutations within the same protein can lead to either dilation or hypertrophy and different prognoses depending on the site of the mutation. To understand how molecular changes lead to differing disease phenotypes, we have studied the contractile effects of two mutations in troponin-T, R92Q and delta K210, that cause HCM and DCM, respectively, in patients. Using purified recombinant proteins, we see that R92Q causes hypercontractility in the in vitro gliding assay while delta K210 causes hypocontractility. Using transient kinetic and steady state biochemical measurements, we show that these effects are due to alterations in the positioning of tropomyosin along the actin filament during muscle activation. Moreover, we show that these molecular-based changes lead to alterations in the contractility of stem-cell derived cardiomyocytes that have undergone genome editing to introduce the disease-causing mutations. Taken together, our results reveal new insights into the disease pathogenesis.

P2847
Board Number: B125
HSPB1 protein increases actomyosin ATPase activity of myofibrils fraction.
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HSPB1 is one of the small heat shock proteins (HSPBs). HSPB1 is predominantly expressed in skeletal muscle, and forms a complex between HSPBs according to cellular stress response. HSPB1 protein is suggested to various functions of skeletal muscle in stressed or unstressed condition. Although HSPB1 has been suggested to be involved in various functions of skeletal muscle under stress or unstressed conditions, its detailed molecular mechanism is not clear. Therefore, we performed a covalent immunoprecipitation (Co-IP) assay with anti-HSPB1 antibody against the lysate of skeletal muscle and searched for HSPB1 binding protein. As a result of the nanoLC/MS/MS analysis, we identified MLC1/3 protein which is one kind of myosin light chain of myofibrils as candidate of novel HSPB1 binding protein. In this study, in order to elucidate the role of the interaction between HSPB1 and MLC1/3, we analyzed the influence of HSPB1 protein to actomyosin ATPase in myofibrils activity by molybdenum blue colorimetric method. Adding purified recombinant HSPB1 protein to myofibrils fraction prepared from mouse hind limb muscle, a significant increase of actomyosin ATPase activity was confirmed depending on the concentration of HSPB1. The highest value of actomyosin ATPase activity was observed when HSPB1 protein was added to myofibrils fraction in a molar ratio of 1: 1 to myosin heavy chain (MHC). On the other hands, the increase in actomyosin ATPase activity was not confirmed by addition of HSPB1 (S135F) which is a point mutation related to Charcot-Marie-Tooth disease. Furthermore, in immunofluorescence staining using paraffin section of mouse gastrocnemius muscle, HSPB1 proteins were co-localized with MLC1/3 and MHC proteins near A-bands in sarcomere of myofibrils. These results suggested that HSPB1 regulates muscle contraction between actin and myosin bands in muscle cells.
P2848
Board Number: B126
Normal cardiac physiology of mice with one allele of mouse α-cardiac myosin replaced with human β-cardiac myosin.
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Switching of cardiac myosin heavy chain (MyHC) expression between α and β isoforms is important in development and disease. It also differs substantially in direction and timing between species. Homozygous null mice lacking α-MyHC die in utero, while heterozygotes are haploinsufficient with cardiac dysfunction (1). To determine the effect of human β-MyHC on this haploinsufficient phenotype, we have replaced the entire coding and intronic regions of the mouse Myh6 gene with those of the human MYH7 gene. Heterozygotes express human β-MyHC at about 50% of the total myosin. Mice homozygous for this replacement exhibit embryonic lethality, but heterozygotes are surprisingly indistinguishable from homozygous wild-type controls in baseline echocardiographic and histological measurements, thus rescuing the cardiac deficits caused by hemizygosity for Myh6. The heterozygotes showed a significant hypertrophic response to isoproterenol, suggesting that these mice can be a good model system to study the effects of cardiomyopathy-causing mutations in human MYH7.

P2849
Board Number: B127
Dictyostelium Myosin Heavy Chain Kinase D Phosphorylates Myosin II and Localizes to Leading Edge Structures.
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The assembly of myosin II bipolar filaments results from interactions between the heavy chain “tails” of myosin II monomers and leads to the formation of filaments that can contract actin filaments and drive cell shape change. In Dictyostelium cells, myosin II filament assembly is inhibited by phosphorylation of the tail region of the myosin II heavy chain (MHC) and is catalyzed by at least three MHC kinases (MHCKs -A, -B, and -C) that share homologous alpha-kinase catalytic and WD-repeat domains. In the current study, we examined the cellular and enzymatic properties of fourth Dictyostelium heavy chain kinase, MHCK-D. We found that overexpression of MHCK-D resulted in a severe reduction in Dictyostelium growth in suspension culture that was accompanied by a marked increase in the number of multinucleated cells. Further studies revealed that overexpressing MHCK-D leads to a nearly complete loss of myosin II bipolar filaments associated with the cytoskeletal fraction of the cell. These results are significant since they demonstrate that MHCK-D can indeed function as an effector of myosin II filament turnover in the cell. Complementary studies of cells lacking MHCK-D expression revealed that MHCK-D, which is only expressed during Dictyostelium multicellular development, is required for normal disassembly of myosin II filaments. Truncation analysis revealed that the WD-repeat domain of MHCK-D is required for the overexpression phenotype and indicates that the WD-repeat domain of MHCK-D plays a similar substrate-targeting function as the WD-repeat domains of the other MHCKs. Studies of GFP-MHCK-D localization in live cells revealed that the kinase translocates from a mainly cytosolic
distribution to cortical patches when aggregation-competent cells are stimulated with cyclic-AMP. In migrating cells, the kinase localizes to leading edge structures, suggesting that MHCK-D plays a role in the turnover of myosin II filaments specifically at the front of the cell, and indicates that localization is an important means by which the kinase’s function is regulated. Studies examining the catalytic activity of affinity-purified Flag-tagged MHCK-D showed that the kinase exhibits robust activity toward a peptide substrate MH-1 and toward myosin II filaments, providing further direct evidence of MHCK-D’s role in controlling myosin II filament turnover. The kinase activity of MHCK-D also appears to be activated by autophosphorylation. Truncation analysis revealed that the WD-repeat domain is required for phosphorylation of myosin II, but not for phosphorylation of MH-1 peptide, indicating a substrate-targeting role for the WD-repeat domain.

P2850
Board Number: B128
Actin and microtubule crosstalk mediates persistent polarized growth.
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A dynamic actin cytoskeleton is essential for polarized growth in mosses. Without actin, cells swell at their apexes and growth is severely inhibited. During growth, wild type cells have an accumulation of actin just below the cell tip. While this apical actin accumulation is persistent over long time periods, imaging with high temporal resolution reveals that this population of actin filaments is highly dynamic constantly turning over. In the absence of microtubules, the apical actin accumulation loses the longer temporal persistence and instead stochastically forms and reforms in various regions of the cell, often leading to cell expansion at other sites.

In wild type cells, cytoplasmic microtubules are focused with their plus ends just below the apical actin accumulation. We find that in a mutant lacking all five myosin VIII genes (Δmyo8), the microtubule focus is lost and the actin accumulation loses the long-term persistence similar to loss of all microtubules. Interestingly directional persistence is impaired in this mutant. We also find that myosin VIII localizes at the junction between the focused microtubule plus ends and the apical actin accumulation. This suggests that myosin VIII is the physical link between microtubules and actin. These data so far suggest that microtubules are required for the formation and maintaining of actin structures. When microtubules are disrupted, burst of actin filaments randomly form in the cytoplasm, associated with strong accumulation of actin nucleator For2A. This suggests that proper distribution of For2A depends on intact microtubule network, and For2A greatly influenced actin structure formations. Interestingly, in Δmyo8 mutant cells, we observed waves of actin bundles moving along the cell cortex. We found that For2A localization in the cytoplasm is only slightly altered compared to cells with disrupted microtubules, suggesting there are other factors contributing to the actin phenotypes we observed in Δmyo8 mutant. Interestingly, we found that the population of For2A at the cell cortex is hyperactive, producing more actin filaments compared to wild-type cells. These hyperactive For2A potentially results in more actin bundle formation at the cell cortex.

In conclusion, we found that myosin VIII mediated actin-microtubule crosstalk is important for maintaining persistent directional growth in moss protonemal cells. We also found For2A to be another player coordinating the functions of the two cytoskeletons. In addition, myosin VIII modulates For2A activity at the cell cortex in some fashion. We don’t know how myosin VIII modulates For2A activity at this point, but this observation will provide us a window to investigate the interplay among these molecules.
**P2851**

**Board Number: B129**

**Functional relationship of MyTH-FERM myosin and VASP during filopodia initiation.**

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Cells use filopodia, slender actin-filled membrane protrusions, to sense the environment, aid directed migration and serve as the first points of contact for cell-cell interactions. Filopodia initiation requires re-organization of the cortical dendritic actin network into actin bundles oriented perpendicular to the membrane, but how proteins assemble into initiation sites and reorganize the cortical actin network during this process is not well understood. The conserved mechanism of filopodia formation in metazoan and amoebozoan cells, such as Dictyostelium, requires a MyTH-FERM myosin and VASP, however, it is not known how these two proteins are recruited to the membrane, whether they cooperate during filopodia initiation and what role the myosin motor activity plays in initiation. The Dictyostelium filopodial MyTH-FERM myosin, DdMyo7, is localized to the cell cortex prior to filopodia initiation in wild type cells but not in a vasp null mutant, revealing a role for the actin regulator VASP in DdMyo7 localization. Using a bypass approach, lipid anchored DdMyo7 was found to localize to the cortex in vasp null cells, but did not significantly promote filopodia formation. The role of the DdMyo7 motor activity in filopodia initiation was investigated by introducing mutations in the catalytic domain of DdMyo7 that confer either weak or strong actin binding by disrupting ATP hydrolysis or ATP binding, respectively. Neither DdMyo7 mutant was capable of supporting filopodia formation, revealing that full ATP hydrolysis activity is required for its function. The strong actin binding rigor mutant DdMyo7 is enriched at the cell cortex but, in contrast, the weak actin binding mutant (i.e. non-hydrolyzer) has significantly reduced cortical localization. Introduction of mutations in conserved sites in the DdMyo7 tail required for autoinhibition rescues localization by the hydrolysis mutant. Altogether, the data support a model whereby DdMyo7 is recruited to the cortex by VASP, then actin binding releases motor auto-inhibition by the tail, allowing DdMyo7 and VASP to organize the actin network into parallel bundles to initiate filopodia. This work was supported by a GIA from the AHA and a grant from the Minnesota Medical Foundation.

**P2852**

**Board Number: B130**

**UNC-45A oligomerizes at the neck.**

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UNC-45A is a highly conserved member of the UCS protein family (UNC-45/CRO1/She4p). Each the product of a unique gene, vertebrates have two isoforms, UNC-45A which is detectable as at least RNA in all cells, and UNC-45B which is expressed in muscle cells only. We and others have recently shown that UNC-45A is required for a variety of NMII-mediated functions in mammalian cells, including motility, adhesion, exocytosis, and cytokinesis (1-4). Mechanistically, we have recently proposed a model whereby UNC-45A acts like a negative regulator of Rho activity upstream of ROCK (5).
Studies conducted in C. elegans have revealed that UNC-45 has four domains, an N-terminal domain known to bind to Hsp90, a C-terminal domain, whose functions and binding partners are still unknown, a neck domain that is required for UNC-45A oligomerization, and a C-terminal domain which binds to non-muscle myosin II (NMII). We set forth to determine whether the mammalian form of UNC-45, UNC-45A, oligomerizes in vivo and in vitro. Moreover, we sought to determine the region of UNC-45A required for oligomerization. Cell lysates from cancer cells were treated with increasing concentrations of the cross-linking agent disuccinimidyl suberate (DSS). We confirmed the presence of UNC-45A oligomers by Western blot analysis. Our data shows that endogenously expressed UNC-45A does indeed form high molecular weight oligomers in human cancer cells. Additionally, full-length, deleted N-terminal, deleted central or deleted C-terminal recombinant proteins were treated with DSS. Our Western blot analysis shows that none of the deletions abrogated the ability of UNC-45A to form oligomers suggesting that the neck domain may be required for oligomerization.


Microtubules Nucleation and Organization 2

P2853
Board Number: B132
XMAP215 is a microtubule nucleation factor that functions synergistically with the gamma-tubulin ring complex.
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Microtubules form a variety of cytoskeletal structures vital to the function of the cell, including the mitotic spindle. The first step in building these structures is the generation of microtubules, yet little is known about how microtubules are nucleated in the cell. For several decades, γ-tubulin has been accepted as the universal microtubule nucleator of the cell. Although there is evidence that the γ-tubulin ring complex (γ-TuRC) might not be the sole microtubule nucleator, identification of other nucleation factors has proven difficult. We developed novel tools to visualize and quantify individual microtubule nucleation events in meiotic Xenopus egg extracts. Using these assays, we discovered that XMAP215, the well-characterized microtubule polymerase, is essential for nucleation of microtubules. XMAP215 promotes microtubule nucleation in a concentration-dependent manner and requires γ-TuRC for this function. We demonstrate that distinct domains of XMAP215 are required for its polymerization and nucleation activity, and that TOG domains of XMAP215 are essential for mediating its microtubule

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nucleation activity as well as binding to γ-TuRC. Finally, we reconstituted microtubule nucleation in vitro using purified γ-TuRC and XMAP215. While XMAP215 and γ-TuRC possess minimal nucleation activity individually, together these factors synergize to stimulate microtubule nucleation. In sum, XMAP215 is a novel MT nucleation factor that cooperates with γ-TuRC to generate the microtubule cytoskeleton of the cell.

P2854
Board Number: B133
Characterization of a plant-specific microtubule-nucleating protein MACET.
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In plants, rigid oligosaccharide cell walls impose restrictions on the mechanism of cytokinesis. Whereas animal cells constrict along the division plane and abscise, plant cells initiate a partition at the center of the cell that then expands centrifugally. The animal cytokinetic microtubule array, the midbody, is static and stationary; in contrast, the plant cytokinetic array, the phragmoplast, is dynamic and mobile. The phragmoplast delivers vesicles containing cell wall material to the partition site. The vesicles coalesce into a continuous cell plate which, guided by the phragmoplast, eventually fuses with the mother cell plasma membrane. During cell plate construction, the phragmoplast can expand directionally through the cytoplasm for hundreds of microns, as is the case in highly anisotropic vascular cells in the xylem and phloem. Although most known phragmoplast proteins are also essential for midbody functions, the distinct mechanisms of these two microtubule arrays necessitates that phragmoplast expansion involves plant-specific regulators of microtubule dynamics. Identification of novel regulators will advance our understanding of plant cytokinesis and enable better modeling of this unique process. Here we have identified a land plant-specific microtubule-associated protein MACERATOR1 (MACET1), which directly binds microtubules through two novel microtubule-binding domains located at the N- and C-termini. In vitro turbidimetric and co-sedimentation assays reveal that MACET1 promotes microtubule polymerization. Interestingly, MACET1 induces formation of asters similar to known microtubule-nucleating proteins and reduces average microtubule length in vitro. Microtubule nucleation assays in vitro demonstrated that MACET1 facilitates de novo nucleation of microtubules. Time-lapse imaging of individual microtubules in vitro showed that MACET1 inhibits rescue following depolymerization, but does not affect polymerization or depolymerization rates. MACET1 expresses only in mitotic cells and delineates the site of the future cell plate position in prophase, and localizes to the phragmoplast distal zone during cytokinesis, which is the zone where microtubules are thought to be nucleated. MACET1-GFP also localizes to microtubule-nucleation sites during interphase in transiently transfected cells. Mutant analysis revealed the importance of MACET1 for root growth rate, implicating MACET1 in regulation of cell division. Our data demonstrate that MACET1 plays a role in cytokinesis by promoting microtubule nucleation and restricting their elongation.

P2855
Board Number: B134
Spectraplakin Shot anchors a perinuclear MTOC in Drosophila polyploid cells.
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Microtubule-organizing centers (MTOCs) are subcellular sites capable of growing and arraying microtubules. Apart from centrosomes in dividing cells, non-centrosomal MTOCs (ncMTOCs) are
widespread in nature. It is not known how uncoupling of the centrosome cycle from the cell cycle is achieved in polyploid cells, arising from repeated genome replication without cell division. In a screening for genes affecting Collagen secretion in Drosophila, we found absence of the spectraplakin Shot caused defective Collagen release by fat body adipocytes, a polyploid cell type in the larva. In trying to understand Shot function, we discovered that in adipocytes and other polyploid cell types the nuclear envelope organizes a dense perinuclear microtubule cortex and therefore functions as a ncMTOC. This perinuclear microtubule organizing center (pnMTOC) is responsible for nuclear size, nuclear position and organelle distribution. Anchoring of microtubules to the nuclear membrane requires Shot, as loss of Shot causes collapse of the perinuclear microtubule cortex into a single centrosome-like microtubule organizing center (clMTOC) with profound effects in cellular organization. The ectopic clMTOC does not seem to form through failure of centrosome inactivation during diploid-to-polyploid transition, since conditional knock down of Shot after polyploidization still evokes the phenotype. To better understand this phenomenon, we conducted a second screening for cytoskeleton-related genes required for the formation of the clMTOC. Through this screening, we found several genes involved in formation of the pnMTOC, including nespin Msp-300 and microtubule severing enzyme Katanin. We also found that microtubule minus-end binding protein Patronin, which normally localizes to the pnMTOC, is required for nucleation of the ectopic centrosome-like structure. We finally show that formation of both the pnMTOC and the ectopic clMTOC is dependent on cell cycle progression, and that the clMTOC can direct growth of a membrane cone akin to a monopolar mitotic furrow, to which both the actin and microtubule cytoskeletons contribute.

P2856
Board Number: B135
Adapting proximity labeling techniques to identify novel non-centrosomal MITOC proteins in C. elegans.
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Microtubules orchestrate critical processes such as intracellular transport and mechanical support. Microtubules have inherent structural polarity, with a dynamically growing plus end and a comparatively stable minus end which associates with microtubule organizing centers (MTOCs). MTOCs play a critical role in nucleating, stabilizing, and/or anchoring microtubules. The centrosome is the primary MITOC during cell division, but in differentiated cells, MITOC function is often reassigned to new sites called non-centrosomal MTOCs (ncMTOCs). Although centrosome composition has been studied thoroughly, ncMTOC composition is relatively poorly understood. Additionally, compared to proteins that associate with plus ends or the entire length of microtubules, the number of minus end proteins that have been identified is relatively small, suggesting that our understanding of the protein composition of ncMTOCs is far from complete. We aim to define a more complete picture of ncMTOC protein composition by identifying proteins proximal to the ncMTOC-specific microtubule minus end protein PTRN-1/Patronin. The technique BioID (proximity-dependent protein biotinylation labeling) offers the unique advantage of screening for proximal proteins in an in vivo context by fusing a promiscuous mutant biotin ligase (BirA*) to a bait protein and identifying proximal proteins that were biotinylated. As a model, C. elegans has been very useful for studying ncMTOCs in many tissues, with the intestine being the best characterized. We are adapting BioID for use in the C. elegans intestine by testing several recently-identified fast acting BirA mutants for biotinylation activity in vivo. Expression of BirA(11mut) in the intestine resulted in significantly increased cytoplasmic biotinylation, indicating that endogenous biotin is sufficient for
BirA(11mut) activity. To enrich BirA(11mut) at the ncMTOC, we fused BirA(11mut) to PTRN-1. These fusion proteins properly localized to the ncMTOC and enriched biotinylation to the ncMTOC. We are currently optimizing biotinylation activity, and testing other BirA mutants. We will use these tools in conjunction with mass spectrometry to identify novel proteins that compose the ncMTOC. Additionally, we will probe the localization of candidate proteins and use reverse genetic tools to determine the role of the candidate proteins for ncMTOC function. Overall, adapting BioID to C. elegans will provide an additional screening tool in a living multicellular animal for the discovery of novel proximity proteins in vivo.

P2857
Board Number: B136
Non-random γ-TuNA-dependent spatial patterning of microtubule nucleation at the Golgi.
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Non-centrosomal microtubule (MT) nucleation at the Golgi generates MT network asymmetry in motile vertebrate cells. Investigating Golgi-derived MT (GDMT) distribution, we find that MT asymmetry arises from non-random nucleation sites at the Golgi (hotspots). Using computational simulations, we propose two plausible mechanistic models of GDMT nucleation leading to this phenotype. In the “Cooperativity” model, formation of a single GDMT promotes further nucleation at the same site. In the “Heterogeneous Golgi” model, MT nucleation is dramatically upregulated at discrete and sparse locations within the Golgi. While computationally both models are equally probable, GDMT nucleation leans toward simultaneous rather than sequential, supporting Heterogeneous --Golgi model. Investigating the molecular mechanism underlying hotspot formation, we have found that hotspots are significantly smaller than a Golgi subdomain positive for scaffolding protein AKAP450, which is thought to recruit GDMT nucleation factors. We have further probed potential roles of known GDMT-promoting molecules, including γ-TuRC-mediated nucleation activator (γ-TuNA) domain-containing proteins and MT stabilizers CLASPs. While both γ-TuNA inhibition and lack of CLASPs resulted in drastically decreased GDMT nucleation, computational modeling revealed only γ-TuNA inhibition suppressed hotspot formation. We conclude that clustered GDMT nucleation is a result of γ-TuNA-dependent local activation of γ-TuRC at the Golgi.

P2858
Board Number: B137
Non-centrosomal microtubules and not the centrosome control endothelial cell polarity and sprouting angiogenesis.
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Microtubules regulate cell polarity during diverse processes such as directional migration. In dividing mammalian cells, microtubules are traditionally believed to be organized into a radial, centrosomally-anchored network where the relative positioning of the centrosome and the nucleus has been long considered to play a pivotal role in setting up asymmetry. Here, by using sprouting angiogenesis as a paradigm of a polarised physiological process, we deciphered the contribution of centrosomal and non-centrosomal microtubules to controlling cell asymmetry. In strong contradiction with the crucial role
attributed to the centrosome in setting up polarity, we showed that the loss of centrosomes had no effect on the ability of endothelial cells to polarize and move in 2D and 3D environments. In contrast, by silencing the microtubule minus-end-stabilizing protein CAMSAP2, we uncovered a key function for non-centrosomal microtubules in establishing endothelial cell polarity during the process of sprouting angiogenesis. Non-centrosomal microtubules controlled Golgi positioning and trafficking, 2D directional migration and formation of large persistent protrusions in 3D. Moreover, by interfering with CAMSAP2 recruitment to the Golgi apparatus, we showed that although Golgi-tethered microtubules are important for regulating polarity, other non-centrosomal microtubules could also contribute to polarized cell sprouting in 3D. Importantly CAMSAP2 was also required for persistent endothelial cell sprouting during in vivo zebrafish vessel development. In the absence of CAMSAP2, cell polarization in 3D could be partly rescued by centrosome depletion, indicating that the centrosome acts as an inhibitor of cell polarity. We propose that CAMSAP2-protected non-centrosomal microtubules are needed for breaking the symmetry imposed by the radial centrosome-anchored microtubule array to enable microtubule enrichment in a single cell protrusion.

P2859
Board Number: B138
Stability and function of a putative microtubule organizing center in the human parasite Toxoplasma gondii.
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The organization of a microtubule-based cytoskeleton is dictated by microtubule nucleators or organizing centers. Toxoplasma gondii, an important human parasite, has an array of 22 regularly spaced cortical microtubules stemming from a hypothesized organizing center, the apical polar ring. We have examined the functions of the apical polar ring by characterizing two of its components, a putative microtubule-associated motor, KinesinA, and a second protein, apical polar ring protein 1 (APR1). Parasites that lack both of these proteins are capable of generating 22 cortical microtubules with wild-type like arrangements, indicating that these proteins do not template and are not “rulers” dictating the spacing between the microtubules. However, the apical polar ring is fragmented in live ΔkinesinAΔapr1 parasites and is undetectable by electron microscopy after detergent extraction. Other cytoskeletal structures such as the conoid, preconoidal rings, intra-conoid microtubules and cortical microtubules are not affected, highlighting the modularity of these substructures within the same framework. Mechanical instability of the apical polar ring is associated with detachment of microtubules from the apical end of the parasite, and linked to an overall diminished ability of the obligate intracellular parasite to move and invade host cells, as well as decreased secretion of effectors important for these processes. Together, the findings demonstrate the importance of the structural integrity of the apical polar ring and the microtubule array in the Toxoplasma lytic cycle, which is responsible for massive tissue destruction in acute toxoplasmosis. Efforts are currently underway to identify the protein(s) responsible for nucleating and templating the 22 cortical microtubules by biochemical and proteomic methods, as well as to reconstitute activity of the putative organizing center in vitro.

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P2860

Board Number: B139

A novel perinuclear non-centrosomal MTOC in Drosophila fat body cells maintains nuclear positioning through pericentriolar material proteins and the LINC complex.

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Many differentiated cell types establish non-centrosomal microtubule organizing centers (MTOCs) to achieve critical cellular, developmental, and physiological functions. Here we identified a novel non-centrosomal MTOC localized in the perinuclear region of Drosophila fat body, a differentiated cell type with uniform nuclear spacing. The fat body MTOC generates radial perinuclear microtubules. We show that nuclear spacing and MTOC assembly is mediated by pericentriolar material (PCM) proteins and LINC complex proteins. This fat body perinuclear MTOC is composed of at least ten PCM proteins with perinuclear localization, but lacks non-PCM centriolar proteins. Two PCM proteins, centrosomin (Cnn) and Sas-4 cooperate in the assembly of perinuclear microtubules ring and the recruitment of other PCM proteins including the microtubule anchor Ninein to the perinuclear region, as revealed by cnn sas-4 double mutant. The perinuclear assembly of the MTOC in fat body cells requires the LINC complex proteins, including SUN domain protein Koi, which we found critical for proper nuclear positioning in fat body cells. In koi mutant, the perinuclear localization of Cnn, Nin and microtubules are impaired, which may account for the uneven nuclear spacing in the fat body cells of koi mutant. Thus, our study uncovers a novel non-centrosomal perinuclear MTOC in Drosophila fat body cells that is required for proper nuclear positioning through PCM proteins and the LINC complex.

P2861

Board Number: B140

Nesprin-1α-dependent microtubule nucleation from the nuclear envelope via Akap450 is necessary for nuclear positioning in muscle cells.

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Microtubules are a major component of the cytoskeleton and mediate important cellular functions including organelle positioning, intracellular trafficking and mitotic spindle formation. In most proliferating cells, microtubules are nucleated radially from a classical microtubule organizing center, the centrosome. By contrast, a variety of differentiated cells nucleates and organizes microtubules from other sites than the centrosome, such as the Golgi, the plasma membrane or the nucleus. In 1985, Bornens and colleagues described the relocalization of pericentriolar material at the nuclear envelope (NE) of muscle nuclei. Since then, the mechanism and the proteins involved in this event remained unknown. We performed a siRNA screen against 300 NE proteins in differentiating muscle cells and follow relocalization of pericentrin, a major centrosomal protein, and microtubule nucleation.
Interestingly, we found the LInker of Nucleoskeleton and Cytoskeleton (LINC) complex to be involved in these processes. Myotubes depleted of different LINC components showed mislocalization of pericentrin to the cytoplasm and failed to nucleate microtubules from the NE. Using the proximity-dependent biotin identification (BioID) method, we found several centrosomal proteins, including Akap450, Pcm1, and Pericentrin, whose association with Nesprin-1α is increased in differentiated myotubes. We show that Nesprin-1α recruits Akap450 to the NE independently of kinesin and that Akap450, but not other centrosomal proteins, is required for MT nucleation from the NE. Furthermore, we demonstrate that this mechanism is disrupted in congenital muscular dystrophy myotubes from a patient carrying a nonsense mutation within the SYNE1 gene (23560 G>T) encoding Nesprin-1. Finally, using computer simulations and cell culture systems, we provide evidence for a role of MT nucleation from the NE on nuclear spreading in myotubes. Our data thus reveals a novel function for Nesprin-1α/Nesprin-1 in nuclear positioning through recruitment of Akap450-mediated MT nucleation activity to the NE.

P2862
Board Number: B141
Distinct roles of pericentriolar proteins in acentriolar MTOC of mouse meiotic spindle formation.
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Mammalian oocytes lack a centriole that acts as a microtubule organization center (MTOC) in most somatic cells. During oocyte maturation, MTOCs undergo remodeling processes including decondensation, fragmentation, and self-organization. However, the underlying mechanisms of MTOC remodeling in mouse oocytes are not well-understood. We showed that two pericentriolar proteins, Cep192 and Cep152, play crucial roles during MTOC remodeling in mouse oocytes. Cep192 is present in MTOCs at all stages of oocyte maturation, and its depletion induces ablation of MTOC, delay in spindle formation, and abnormal chromosomal alignment in spindles. In the case of Cep152, its localization on MTOC is limited at the germinal vesicle (GV)-stage and then disappears from the MTOC after germinal vesicle breakdown (GVBD)-stage. Cep152 exclusion from MTOC is involved in the fragmentation of MTOC, and it is regulated by CDK1 activity. Together, our results demonstrate the different roles of Cep192 and Cep152 in MTOC remodeling, and a novel regulatory mechanism during meiotic spindle formation in mouse oocytes. *Supported by Grant from Next Generation Biogreen 21 Program (PJ011206), Rural Developmental Adminstration, Republic of Korea.

P2863
Board Number: B142
Golgi-derived microtubules in pancreatic β-cells are regulated by glucose through cAMP and EPAC2.
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Insulin release from pancreatic β-cells is tightly regulated. In response to evenly sustained high glucose only a small fraction of insulin granules are released. Previous work from our lab has shown that microtubules (MTs) act to negatively regulate insulin release and in response to high glucose are destabilized to help with the release of granules. The formation of MTs in cells occurs primarily through templated nucleation, which uses the γ-tubulin ring complex as a template and can occur at the
centrosome, from the side of previously formed MTs (augmin-dependent), or at the Golgi. Successful MT formation also requires stabilization of newly-forming MT seeds, a process that is known to require CLASP2 for Golgi-derived microtubules (GDMTs). Previous work has shown that GDMT nucleation in pancreatic β-cells is increased upon a high glucose stimulus. Now, we have addressed the function of these newly formed GDMTs. Our data indicate that they likely play a role in trafficking of newly synthesized proinsulin through the membrane networks from the ER to the Golgi and further into nascent secretory granules, and might affect proinsulin to insulin processing, potentially affecting glucose-stimulated insulin release (GSIS). We were also interested in what processes in the cell cause the increase in GDMTs in response to high glucose. Here we show that GDMT nucleation increases in two waves in response to high glucose in an isolated β-cell line, MIN6, which mirror the well-described two waves of GSIS. The first wave doubles to amount of GDMTs and occurs rapidly, peaking by 5 minutes. This increase proceeds the expansion of the Golgi that occurs during glucose stimulation, indicating it involves a change in regulation and not an increase in available sites for nucleation. We decided to further explore what is causing this increase during the first wave. We have found that the cAMP pathway, which is also involved in GSIS, controls the increase in GDMT nucleation. Addition of a cAMP analog in low glucose leads to a doubling of GDMT nucleation. Out of the two major cAMP effectors, EPAC2, a paralog of the more ubiquitously expressed EPAC1, rather than protein kinase A (PKA) cause this increase. Interestingly, EPACs interact with the light chain, LC2 and LC1, of MAP1A and MAP1B, respectively, which are known to bind and stabilize MTs. We propose that EPAC2 in β-cells is acting though LC1 or LC2 to stabilize newly nucleated GDMTs. When activated upon glucose stimulation, EPAC2 stabilizes these newly formed GDMTs allowing them to grow out from the template, therefore increasing the number of GDMTs in high glucose. To better understand this process, we are currently testing this hypothesis.

P2864
Board Number: B143
Comparison of migration rates and microtubule nucleation in MDA-MB-231 and MCF-7 breast cancer cells.
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Golgi derived microtubule (MT) arrays have been shown to be essential for directionally persistent cell migration and vesicle transport in some cells. This mechanism works through the recruitment of a number of proteins, including a MT precursor γ-tubulin ring complex (γ-TuRC), to the cis-side of the Golgi. This study seeks to determine if Golgi derived MT arrays are an important defining characteristic of highly invasive breast cancer cells. Two breast cancer cells lines, MDA-MD-231 and MCF-7, were compared for migration rates in scratch assays and co-localization of Golgi proteins with MT and MT recruitment proteins using both microscopic co-localization and co-immunoprecipitation (co-IP). In scratch assays, MDA-MB-231 cells display a significantly different (P < .05) mean (n=9) migration rate of 18 x 103 μm2/hr compared to MCF-7 cells with a mean (n=9) migration rate of 4.7 x 103 μm2/hr. In microscopic co-localization experiments, the overlap of immunofluorescence of components required for microtubule formation were compared using calculated Mander’s Co-localization Coefficient values. Co-localization of a cis-Golgi membrane protein (GM130) and the microtubule subunit, α-tubulin, has a higher incidence (p <0.0001) after microtubule disruption in MDA-MB-231 cells (0.82±0.11, n=5) than in MCF7 cells (0.08±0.06, n=5). Co-localization of GM130 with γ-tubulin, a component of the γ-TuRC, was also considered. This co-localization was compared between cell lines and between cells with and without an environment conducive to migration, through the treatment of cells with epidermal growth
factor (EGF). These components more readily co-localize (p<0.0001) within MDA-MB-231 cells in a migratory environment (0.47±0.23, n=10) than in either cell type without treatment (MDA: 0.06±0.04, n=10; MCF7: 0.03±0.02, n=10) or MCF7 cells treated with EGF (0.10±0.06, n=10). These findings suggest a marked increase in Golgi derived microtubule activity in breast cancer cells that are highly invasive. However, preliminary experiments to co-IP GM130 and γ-tubulin have not supported the hypothesis. Experiments are ongoing to more precisely characterize any co-immunoprecipitation of relevant proteins.

**Microtubules Dynamics and Its Regulation**

**P2865**

**Board Number: B144**

*Septin-microtubule interplay enables initiation of branching morphogenesis.*

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Many organs, such as kidney, lungs and mammary gland, are formed by a process of branching morphogenesis which begins with the formation of cytoplasmic extensions from the basal surface of polarized cells in an epithelial sheet. We have shown that the microtubule (MT) cytoskeleton is necessary for the formation of these extensions, and now we have established that septins also play a key role in extension formation as well. Septins have previously been shown to play an essential role in a variety of morphogenic changes and are also necessary for epithelial cells to switch to a motile phenotype. Septins can interact directly with MTs and indirectly with the MT network via MAPs. Here, we sought to determine if the interaction between septins and the MT cytoskeleton plays a role in this earliest stage of branching morphogenesis -- the formation of primary extensions from single epithelial cells. We induced morphogenesis in spheroids of polarized epithelial cells grown in 3D culture with hepatocyte growth factor (HGF), which resulted in the formation of cytoplasmic protrusions extending from the basal surface of single cells. These early stage extensions are filled with prominent MT bundles, and disruption of the MT cytoskeleton inhibits extension formation. Septins are also recruited to the base of the protrusions, where they colocalize with the MT bundles. Additionally, septins colocalize with MT bundles in the body of mature extensions. Septin disruption before morphogenesis leads to a decrease in extension formation. When septin inhibitors were added after morphogenesis was initiated, we observed extensions containing disorganized, curved and splayed MTs as opposed to tight bundles of parallel MTs. We hypothesize that septins interact with MT bundles and this interaction may provide the mechanical stiffening and cytoskeletal reorganization necessary for the initiation and stabilization of HGF-induced extensions in morphogenesis.

Supported by NIH-NIGMS grant #5R01GM098619.

**P2866**

**Board Number: B145**

*Effects of Microtubule Stabilizers in Neurodevelopment and Injury.*

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Microtubules (MTs) are structural components vital for important neuronal functions such as neurite outgrowth and maintenance, as well as for axonal trafficking and synaptic remodeling. Neuronal MTs
are particularly stable compared with those in other cell types and often exist for years before they get degraded and recycled. They are mostly synthesized in the somatodendritic compartment and have to be transported along axons as much as a meter or more in humans to reach the terminals where synaptic proteins are delivered and synaptic transmission occurs. A significant portion of neuronal MTs stay polymerized when challenged with MT destabilizers in test tubes and various non-neuronal cell lines, suggesting that baseline MT stability is high in neurons. Such stability increases during development and maturation, but decreases with axonal injury and neurodegeneration. This leads to an intriguing question: can stabilizing MTs facilitate neurite outgrowth during early development or restore axonal integrity and function upon injury? To address these questions, we have characterized a group of MT stabilizing drugs (Epothilone D, Epothilone B, Ixabepilone, Taxol and Synstab) regarding their binding affinity to MTs in vitro using biochemical assays and fluorescent live imaging; and evaluated their effects on neurite growth during normal differentiation and regrowth after axotomy. Dose and time dependent drug treatments were performed on ReNcell VM cells (an immortalized human neural progenitor cell line), and differentiated VM cells that exhibit neuronal morphology and electrophysiology, followed by high-content live-cell imaging and automated imaging analysis. We found that these drugs showed bimodal effects on initial neurite extension and regeneration after injury. To understand the molecular mechanisms underlying regulation of MT stability by these drugs and their biological effects, we have used multiplex proteomics to study dose-dependent changes in signaling pathway components and MAPs over time. These results may provide useful information for understanding not only neuronal MT dynamics and stability in health and disease, but also for determining the therapeutic value of MT stabilizers in axonal injury and neurodegeneration where loss of neuronal MT integrity may exacerbate disease pathology.

P2867
Board Number: B146
The microtubule plus-end-tracking protein TACC3 promotes persistent axon outgrowth and mediates responses to axon guidance signals during development.
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Precise neuronal connection requires proper axon guidance. Microtubules (MTs) of the growth cone are the driving force to navigate the growing ends of axons. Pioneering microtubules and their plus-end tracking proteins (+TIPs) play integrative roles during this navigation. Recently, we introduced the protein TACC3 as a member of the +TIP family regulating microtubule dynamics in Xenopus laevis growth cones and show that manipulation of TACC3 levels affects axon outgrowth by regulating axon outgrowth velocity and the frequency of axon retraction. Additionally, we show that over-expressing TACC3 mitigates nocodazole-induced reduction in MT dynamics parameters suggesting that TACC3 could play a protective role against nocodazole induced MT depolymerization. Moreover, we find that TACC3 and its partner XMAP215, a well-characterized MT polymerase, cooperate to promote axon outgrowth and rescue axon growth defects. Finally, we show that reduction of TACC3 levels causes pathfinding defects in axons of developing spinal cord motor neurons in Xenopus laevis in vivo and increased TACC3 levels interfere with the growth cone response to the axon guidance cue Slit2. Currently we are investigating whether TACC3 could be a potential target of Abelson kinase downstream of the Slit2 guidance cue. Together, our results suggest that by regulating MT behavior, the +TIP TACC3 is involved in axon outgrowth and pathfinding decisions of neurons during embryonic development, and that TACC3 phosphorylation events, which remain to be elucidated, could be important regulators of this involvement.
P2868
Board Number: B147
Trim9 participates in a microtubule quality control pathway that ensures local nucleation in dendrites does not disrupt uniform polarity.
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In Drosophila neurons, minus-ends of microtubules (MTs) are oriented away from the cell body in dendrites. One contributor to generating this non-centrosomal MT array is local nucleation in dendrites. g-Tubulin, the core MT nucleation protein, is concentrated at dendrite branch points where it can generate new microtubules. If these new microtubules were generated randomly in all directions, then local nucleation would disrupt uniform minus-end-out polarity. Here, we investigated how local nucleation is coupled to maintenance of uniform polarity. First, we closely monitored the behavior of MT plus-ends originating within dendrite branch points using EB1-GFP. Roughly one third of these new plus ends grow away from the cell body in a plus-end-out orientation. However, on average only 10% of microtubules between branch points are plus-end-out. This suggests that some kind of checkpoint prevents plus-end-out microtubules from leaving the branch point. Indeed when we examined the behavior of microtubules as they exited the branch point, the success rate was twice as high when they exited towards the cell body as opposed to away from it. We hypothesized that the polarity of pre-existing MTs might distinguish the two exits from one another. Consistent with this hypothesis, in genetic backgrounds where stable microtubules have mixed polarity, newly polymerizing MTs exited in all directions with a high success rate. To identify proteins required for this checkpoint, we carried out a candidate screen and identified Trim9 as a positive regulator of exit success. Without Trim9, new growing MTs had the same low success rate exiting all directions. Surprisingly, when expressed in Drosophila S2R+ cells, Trim9 colocalized with over-expressed EB1 on the MT lattice. To determine where Trim9 localized in neurons, we expressed mNeonGreen-Trim9 in Drosophila neurons. It strongly localized to dendrite branch points, in line with where it functions. We propose that Trim9 is part of a quality control mechanism that ensures that microtubules generated at dendrite branch points contribute to maintenance of uniform polarity, rather than its disruption.

P2869
Board Number: B148
TACC3, a microtubule plus-end tracking protein, regulates neural crest cell motility in vitro and in vivo.
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Coordinated cell migration is critical during embryogenesis. During development, cells from multiple lineages delaminate from their point of origin to travel through the embryo, providing foundations for the heart, gut, vasculature, & nervous system. Cell motility relies on dynamic coordination of the F-actin and microtubule (MT) cytoskeletons. These systems function together to respond to chemical cues, directionally polarize, and generate force and traction for motility. Therefore, cytoskeletal proteins that impact motility are critical during embryogenesis, and disruption of these genes can give rise to developmental disorders. Our work focuses on how MT plus-end regulators impact migration of cranial neural crest (CNC) cells, a multipotent cell that differentiates to form muscle, cartilage, bone, & nerves of the face. We identified one MT regulator, transforming acidic coiled-coil 3 (TACC3), as a putative
effector of CNC motility. We previously showed that TACC3 functions as a MT plus-end tracking protein and regulates MT growth velocities in CNCs. Interestingly, TACC3 is one of 5 genes deleted in Wolf-Hirschorn syndrome, a genetic disorder that presents craniofacial defects consistent with disrupted CNC migration. Using in situ hybridization, we show that TACC3 is highly-expressed in motile CNCs in X. laevis. Manipulation of TACC3 protein levels is sufficient to alter CNC velocity in vitro. Overexpression (OE) drives increased single-cell migration velocities and explan dispersion. TACC3 KD results in marked motility defects, with fewer cells able to migrate persistently. In order to assess how TACC3 manipulation impacts cell velocity, we use confocal microscopy to examine localization of GFP-TACC3 in live cells, & effects of TACC3 manipulation on MT stabilization, adhesion turnover, and chemotaxis. Finally, KD but not OE of TACC3 significantly impacts pharyngeal arch morphology in vivo. Together, these data support a role for TACC3 in embryonic cell motility.

P2870
Board Number: B149
EB1 and EB3 regulate microtubule minus end organization and Golgi morphology.
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End binding proteins (EBs) are key regulators of growing microtubule plus ends. Recent work demonstrated that microtubule minus ends can also grow in cells, and that minus end dynamics are important for shaping non-centrosomal microtubule networks. However, the role of the EBs in controlling the organization of microtubule minus ends is poorly understood. Here, by using CRISPR/Cas9 technology, we have generated cell lines that lack EB2, EB3 and the C-terminal partner binding part of EB1. These cell lines show only mild defects in mitosis and microtubule plus end dynamics, but, surprisingly, they display severely perturbed microtubule minus end organization. In the studied cell lines, microtubule minus ends that are not attached to the centrosome are stabilized by CAMSAP2, and many of them are tethered to the Golgi apparatus. Disruption of EB1 and EB3 leads to shortening of CAMSAP2-decorated microtubule minus ends, their detachment from the Golgi and their relocation to the cell periphery. EB1 or EB3 are recruited to the Golgi through an interaction with myomegalin, and our data suggest that the weak binding of Golgi-associated EB1 and EB3 to stable microtubule lattices contributes to microtubule tethering to Golgi membranes and counteracts compaction of Golgi stacks. Furthermore, we find that disruption of EB1 and EB3 affects vesicle transport, cell motility on 2D substrates and cell invasion in 3D matrices. Our results demonstrate that EB proteins control diverse aspects of interphase cell architecture and have an unexpectedly profound impact on the organization of microtubule minus ends.
During angiogenesis, endothelial cells (ECs) utilize signaling molecules and microtubule (MT) associated proteins (MAPs) to control the dynamic and coordinated remodeling of the actin and MT cytoskeleton to become polarized. Rac1, a signaling molecule of the Rho GTPase family, promotes cell protrusion and enhances MT plus-end assembly into protrusions, thereby driving EC polarization. As ECs polarize, actin filaments assemble and become linked to focal adhesion (FA) complexes that mature at the positions where the cell interfaces with the extracellular matrix. Recent investigations have identified that a subset of elongating MTs undergo “capture” at FAs, and that FA-captured MTs promote directed cell migration, yet how MTs are designated for FA capture is not known. CAMSAPs are MAPs that associate with MT minus-ends, where they inhibit MT disassembly and promote plus-end MT growth. Septins are MAPs that promote actin stress fiber-mediated maturation of FAs and that spatially guide MT plus-end dynamics. Collectively, these data point to a potential mechanism used by ECs to delineate MT functions via association with CAMSAPs and septins. Here, we tested the hypothesis that FA capture of MTs is controlled by signaling from Rac1 to promote septin-mediated guidance of CAMSAP-associated MTs for FA capture. Live-cell fluorescence imaging of ECs revealed that MTs captured at FAs are predominantly nucleated at the Golgi-apparatus rather than the centrosome. Expression of a constitutively active Rac1 promoted increased CAMSAP stretch length on Golgi-derived MT minus-ends, while a dominant negative Rac1 had the opposite effect. Analysis of MT growth events revealed that CAMSAP-associated MTs consistently grew into septin stretches that localized adjacent to FAs, resulting in FA capture of MTs in almost all cases. Pharmacologic inhibition of myosin-II, to relieve contractile forces on FA-associated actin filaments, resulted in a significant reduction of both FA-associated septin and FA capture of MTs, and this effect was dependent on Rac1 activity. Expression of either CAMSAP or septin, or simultaneous expression of both proteins, increased EC migration in a wound-healing assay. These results suggest that Golgi-derived, CAMSAP-associated MTs represent a distinct population of MTs that are guided by peripheral septin for FA capture. Moreover, these data support a model in which a balance of Rac1 and myosin-II activity promotes CAMSAP-association with Golgi-derived MTs and increases septin localization to peripheral FAs to promote persistent and directional EC migration. Future investigations will mechanistically determine how CAMSAP-associated MTs are distinguished by septin, and how septin-mediated FA capture of MTs contributes to enhanced EC migration.

Oligodendrocytes are specialized glial cells in the central nervous system that produce myelin, the fatty layers of insulation that wrap around axons to facilitate efficient action potential conduction. Unlike Schwann cells in the peripheral nervous system that ensheathe a single axonal segment, one
Oligodendrocyte can ensheath multiple axonal segments and consequently extends multiple processes. These microtubule-rich processes are elaborate and highly branched, yet it is unclear how they are organized and how they form. We now show using live-cell imaging that microtubules in oligodendrocytes have uniform polarity, with growing EB3-labeled plus ends directed away from the cell body. Interestingly, though polarity is consistent throughout oligodendrocyte differentiation, speeds of polymerization vary at different developmental time points. In addition, we now show by immunostaining that oligodendrocyte processes contain Golgi outposts, which may act as a source of acentrosomal microtubule nucleation at sites that are far from the cell body. Previous experiments in Drosophila neurons have demonstrated roles for Golgi outposts in microtubule nucleation and dendrite branching. In order to screen for candidate Golgi outpost interactors, we used our lab’s RNA-Seq database to identify microtubule-associated proteins that are highly and specifically expressed in oligodendrocytes. We identify TPPP (tubulin polymerization promoting protein) and show that it selectively localizes to Golgi outposts but not to Golgi bodies in the cell body. Knockdown of TPPP results in aberrantly mixed microtubule polarity and increased branching in oligodendrocytes, but does not alter the speed of EB3-labeled plus ends, suggesting that contrary to its name, TPPP does not actually mediate microtubule polymerization in cells. An alternative possibility is that TPPP stabilizes microtubules by binding at the minus end and we are currently addressing this by using polarity marked microtubules and in vitro kinesin motility assays. In addition, we are using mass spectrometry to identify the Golgi outpost proteome, by purifying Golgi from rat pup brains followed by immunoprecipitation against TPPP. Finally, preliminary data from mice indicate that TPPP knockout may lead to sensorimotor deficits and anxiety-like behavior. Together, our data demonstrate that TPPP is required for uniform microtubule polarity and process branching in oligodendrocyte development.

**P2873**
**Board Number: B152**
**Mechanism of Catalytic Microtubule Depolymerization via KIF2-tubulin Transitional Conformation.**
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Microtubules (MTs) are dynamic structures that are fundamental for cell morphogenesis and motility. MT-associated motors work efficiently to perform their functions. Unlike other motile kinesins, KIF2 catalytically depolymerizes MTs from the peeled protofilament end during ATP hydrolysis. However, the detailed mechanism by which KIF2 drives processive MT depolymerization remains unknown. To elucidate the catalytic mechanism, the transitional KIF2-tubulin complex during MT depolymerization was analyzed through multiple methods, including atomic force microscopy, size-exclusion chromatography, multi-angle light scattering, small-angle X-ray scattering, analytical ultracentrifugation, and mass spectrometry. The analyses outlined the transitional conformation in which KIF2core domain binds tightly to tubulin dimers in the middle pre-hydrolysis state during ATP hydrolysis, a process critical for catalytic MT depolymerization. The X-ray crystallographic structure of the KIF2core domain displays the activated conformation that stabilizes the transitional KIF2-tubulin complex.
Little is known about how the crowded environment of the cytoplasm affects molecular processes within the cell. The cell often encounters changes in cytoplasmic density, for instance in the course of the normal cell cycle as well as in response to environmental changes. For instance, changes in osmolarity lead to loss of intracellular volume through loss of water. In vitro studies predict that molecular crowding may speed up processes such as microtubule polymerization. Here, we have probed the effects of cytoplasmic density on microtubules and actin in the fission yeast S. pombe. Addition of sorbitol, an osmotic agent, causes large reversible changes in cell volume and cytoplasmic density. Sorbitol causes a dose dependent decrease microtubule and actin dynamics. Strikingly, a high osmotic shock completely freezes microtubule and actin dynamics, while at intermediate dosage, microtubule polymerization and shrinkage are decreased. Potential explanations for these effects include steric crowding, effect on regulators, or a requirement for sufficient water molecules. These studies introduce a new system to study the effects of cytoplasmic density and a method to rapidly and reversibly tune cytoskeletal dynamics.

In order for the microtubule cytoskeleton to function in diverse processes, the dynamic property of cellular microtubules must be tightly controlled in time and space. The microtubule motor protein, kinesin, can transport cargo, regulatory proteins and other microtubule filaments along microtubules. Additionally, some kinesins directly control the dynamic behavior of their cellular track. Thus, kinesins are major regulators of the organization and function of the microtubule cytoskeleton. Kinesin-8 is a conserved subfamily of motor proteins and an important class of microtubule regulators in diverse species. Kinesin-8 from several species are known to be multifunctional proteins, and the budding yeast kinesin-8, Kip3, combines plus-end directed motility with microtubule stabilizing, destabilizing, and anti-parallel cross-linking activities in a single molecule. Moreover, Kip3 regulates numerous aspects of microtubule dynamics during distinct cellular processes. It is known that the N-terminal motor domain is sufficient for microtubule depolymerization, while the C-terminal tail is needed for microtubule cross-linking and spatially induced microtubule rescue. Yet, how Kip3 achieves multiple activities, and how they are selectively deployed to differentially control specific microtubules within the cell remains largely unknown. We performed a functional analysis of the Kip3 tail. Interestingly, the regions proximal and distal to the motor domain confer opposite sensitivities to microtubule destabilizing compounds. We show that the proximal region is sufficient for Kip3 localization to microtubule plus ends and the general regulation of astral microtubules within the cytoplasm. It also functions to regulate microtubule dynamics at the bud tip during early spindle positioning. Notably, the proximal region is sufficient to mediate the rescue of astral microtubules spatially within the bud compartment. In contrast, the distal tail region is required for proper spindle function during anaphase. Kip3 lacking this region is a gain of...
function for controlling spindle length; spindles do not reach full length. We show that the distal tail is a negative regulator of spindle disassembly during anaphase. This distal tail mediates the antagonism between the microtubule polymerase Stu2, and the destabilizing activity of Kip3 within the spindle. Furthermore, we reveal that the function of this molecular antagonism is to confer structural robustness to the dynamic spindle midzone. Overall, our data demonstrate that the distal tail region of Kip3 functions to temporally regulate the stability of the spindle midzone during anaphase.

P2876

Board Number: B155

Investigating the Structure and Functional Role of the C-Terminal Domain of the Drosophila XMAP215 Protein Family Member Minispindles.

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Microtubules (MTs) are cytoskeletal polymers responsible for multiple cytoplasmic activities: enabling intracellular transport, stabilizing the cell’s shape, and forming the mitotic spindle. MT-associated proteins (MAPs) localize to MTs where they modulate MT dynamics through molecular and structural changes to the MTs. Members of a subgroup of MAPs that specifically localize to the MT plus end are referred to as +TIPs.

A group of highly conserved +TIPs, known as the XMAP215 family, accelerate MT assembly and promote MT growth. XMAP215 family members promote polymerization via TOG (tumor over expressed gene) domains that bind to tubulin heterodimers. TOG domains contain tandem HEAT repeats with highly conserved intra-HEAT loops essential for interacting with tubulin. Studies of XMAP215 family members in multiple species show that they play an essential role during interphase and mitosis, as their depletion leads to decreased MT growth rates and short spindles or defects in spindle architecture. Members of the XMAP215 family contain a varying number of N-terminal TOG domains, however, the structure and functional role of the C-terminal domain (CTD) remains unknown. Using bioinformatics, we show that the highly conserved central region of the CTD of XMAP215 family proteins contains HEAT repeats that strikingly resemble what is known to form a TOG domain. We hypothesize that the structural elements of the conserved CTD of the XMAP215 family of proteins are responsible for regulating MT dynamics by properly localizing the full-length molecule to MT plus ends. We show that the CTD predicted TOG domain, of the Drosophila XMAP215 family member, Msps, is required to properly localize Msps in Drosophila S2 cells. Previous studies have shown that dsRNA induced depletion of Msps leads to reduction of MT growth velocities and decreased mitotic spindle length. We show that constructs lacking the entire CTD or the CTD TOG domain fail to rescue MT growth rates and spindle length. Using secondary structure predictions and multiple sequence alignment analyses of the CTD TOG domain we identified conserved predicted surface exposed charged residues. We systematically mutated these residues and determined that particular small sets of residues (2-3 amino acids) within this region are required to localize Msps to MT plus ends and rescue growth rates. Previous studies show that the plus-end accumulation of Msps is severely reduced upon depletion of Sentin, a plus-end tracking protein. Therefore, we hypothesize that the CTD TOG domain interacts with Sentin. We are performing in vivo and in vitro assays to elucidate the Msps CTD TOG:Sentin interaction. Further experiments are being performed to elucidate the structure of this domain and its interaction with Sentin.
P2877

Board Number: B156

Structure, biochemistry, and activity of a CLASP family TOG.
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Microtubule (MT) dynamics are tightly regulated by associated proteins. Two conserved regulatory factors, XMAP215 family polymerases and CLASP family rescue factors, use tubulin-binding TOG domains to control MT dynamics. In S. cerevisiae, the polymerase STU2 and rescue factor STU1 produce different effects on MT dynamics but have similar architectures: two TOG domains followed by a basic region and a dimerization domain. The minimal functional unit for a MT polymerase appears to be two flexibly-linked TOGs that preferentially bind unpolymerized αβ-tubulin (curved conformation) plus a lattice-binding basic region. The minimal requirement for a rescue factor has not been defined. Our working hypothesis is that the divergent functions of the two families arise from differences in the structural and biochemical properties of CLASP TOGs and/or how they are linked. To provide a structural context for subsequent biochemical and functional studies, I determined the structure of the TOG2 domain from Stu1. The overall structure of this domain resembles that of another CLASP TOG and is characteristically distinct from that of polymerase TOGs in a way that suggests a change in the conformation-selectivity of TOG-tubulin interactions. The structure of STU1:TOG2 also reveals that part of the sequence that would normally link TOG1 to TOG2 forms a helix that docks onto the TOG2 domain in a novel way, indicating that the Stu1 TOGs are more rigidly coupled than the polymerase TOGs. Also in contrast to the polymerase TOGs, STU1:TOG2 shows appreciable affinity for both curved/unpolymerized αβ-tubulin and straight/polymerized αβ-tubulin. STU1:TOG1, on the other hand, does not show appreciable affinity for either polymerized or unpolymerized αβ-tubulin. These interactions with curved and straight αβ-tubulin both depend on the same conserved positions that mediate tubulin binding in polymerase TOGs. To begin investigating conformation-selectivity of interactions between STU1:TOG2 and αβ-tubulin, I measured the effects of the domain on MT polymerization dynamics in vitro. These assays revealed that STU1:TOG2 by itself – without a basic region or a dimerization element - suppresses catastrophe and promotes rescue. These results expand our understanding of TOG structure, biochemistry, and mechanism. They also indicate that in contrast to polymerases where two TOGs are required, a single CLASP-family TOG is sufficient to suppress catastrophe and promote rescue.

P2878

Board Number: B157

Reconstitution of Microtubule Dynamics from Budding Yeast Lysate.
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Microtubules (MTs) are important for cellular structure, transport of cargoes, and segregation of chromosomes and organelles during mitosis. The stochastic growing and shrinking of MTs, known as dynamic instability, is necessary for these functions. The parameters of MT dynamic instability, such as the rates of growth and shrinkage and the frequencies of rescues and catastrophes have been shown to be cell cycle-dependent and tuned by MT-associated proteins (MAPs) when measured in cells. Previous work to characterize individual MAP effects on MTs has been performed either through in vivo studies, which vary widely by cell type, or in vitro studies that typically focus on individual protein activity but lack cellular context and can even display different dynamics due to cross-species components. In order to investigate the coordinated activity of all native MAPs on MT dynamics, we have developed a cell-free
assay using TIRF microscopy and whole cell lysate from the budding yeast, *Saccharomyces cerevisiae*. Here, we take lysate from GFP-tubulin-expressing yeast strains and observe MT polymerization off of pre-assembled seeds adhered to a coverslip. This allows observation of single MTs whose behavior may normally be obscured by cellular structures or other MTs in the mitotic spindle. Through use of cell-division cycle mutants (cdc), we have found that MT polymerization and dynamic instability are dependent upon the cell cycle even *in vitro*. Additionally, we have found that the two known yeast MT depolymerases, Kip3 and Kar3, heavily contribute to the overall dynamic instability of MTs. However, through combining the cdc and depolymerase mutations, we have learned that these dynamics are ultimately limited by the cell cycle of the harvested yeast. Kar3 is especially interesting because its function and localization are controlled by mutually exclusive binding partners, Cik1 and Vik1. Past studies have alluded to differences in the function of these two complexes and our work has expanded to show very distinct activities exclusive to each.

**P2879**  
**Board Number: B158**  
**Behaviors of Microtubules and other Steady-State Polymers Depend on Multiple Critical Concentrations.**  
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The concept of critical concentration is a central idea in the understanding of microtubules and other steady-state (energy-utilizing) biological polymers. Classically, the critical concentration (CC) is the concentration of subunits necessary to obtain polymer. However, the classical theory used to explain and predict CC fails to account for dynamic instability or energy utilization. It is as yet unclear how the classical theory should be adjusted to incorporate dynamic instability or how the behavior of an individual dynamically unstable filament relates to that of its population.

To address these questions, we used our previously established simulations to follow at multiple scales the behavior of systems of computationally modeled dynamic microtubules (MTs). We show that polymers like MTs that exhibit dynamic instability have not one but at least two experimentally relevant critical concentrations: one above which individual filaments can grow transiently (CC\textsubscript{indgrow}), and another above which a population of filaments will grow steadily (CC\textsubscript{pogrow}). Normal steady-state dynamic instability occurs between these two CC values. Most experimental approaches to measuring critical concentration yield CC\textsubscript{pogrow}. A surprising implication of this revised understanding is that the stereotypical steady-state microtubule dynamic instability behavior occurs at tubulin concentrations below the value traditionally believed to be the lower limit required for polymer assembly. We propose that whether a steady-state polymer is like MTs and displays dynamic instability or is like actin and can be modeled as an equilibrium polymer depends on how far apart these two critical concentrations are.

While aspects of this framework conflict with common assumptions, it helps explain a range of otherwise confusing experimental observations.
P2880

Board Number: B159

Site-occupancy calibration of taxane pharmacology. J.J. Pineda¹, M.A. Miller², R. Weissleder³, T.J. Mitchison⁴; ¹Dept. Systems Biology, Harvard Medical School, Boston, MA, ²Center for Systems Biology, Mass General Hospital, Boston, MA

Taxanes and epothilones are important anti-cancer drugs whose tumor-regressing mechanisms are poorly understood. They bind to an overlapping site in the microtubule lumen and stabilize the lattice. In cells, this inhibits dynamic instability and promotes ectopic nucleation and bundling of microtubules. At low concentration, they disrupt chromosome segregation with minimal mitotic arrest, and at high concentrations they promote strong mitotic arrest. Most taxane-site pharmacology is based on measurement of empirical IC50 values, and is has been unclear what fraction of the microtubule lattice must be taxane-bound to cause different biological effects. We developed a method for measuring taxane site occupancy in living cells using competitive displacement of the SirTb fluorescent probe quantified by automated microscopy. We then measured different readouts of taxane activity as a function of site occupancy in RPE1 cells. Perturbation of plus end dynamics was first observed at relatively low site occupancy (<20%), micronucleation occurred over a broad range, and mitotic arrest required high (>80%) site occupancy. When dose-response data for 3 epothilones and paclitaxel were normalized to site occupancy, their effects were very similar. By comparison with intravital imaging data in a mouse tumor model we estimate that therapeutic efficacy of paclitaxel requires ~60% site occupancy shortly after drug administration. Our assay will be useful for structure-activity characterization during drug development. We speculate that efficient formation of micronuclei at intermediate site occupancy is an important cellular activity of taxanes with a central role in promoting tumor regression.

P2881

Board Number: B160

Mechanisms to localize and regulate katanin activity. G.M. Burkart¹, R.V. Dixit²; ¹Biology, Washington University in St.Louis, St. Louis, MO

The microtubule severing protein katanin, a heterodimer of a p60 catalytic subunit and a p80 regulatory subunit, regulates the organization and turnover of the microtubule cytoskeleton by the localized destruction of microtubule polymers. In higher plants, katanin activity is essential for the formation of linearly organized cortical microtubule arrays, which determine the axis of cell expansion. Live imaging studies have shown that even though p60 binds to the sidewalls of cortical microtubules, severing activity is restricted to microtubule crossover and nucleation sites. We have hypothesized that both targeting and protective mechanisms are needed to precisely localize katanin severing activity. We are using genetic and biochemical approaches to investigate whether p80 plays a role in targeting p60 to particular microtubule locations. There are four p80 orthologs in Arabidopsis thaliana, and we found that all interact with p60 with p80-4 consistently shows the strongest interaction in yeast-two-hybrid studies. In addition, we discovered that severing activity is regulated by phosphorylation of three serine residues in the N-terminal domain of p60. Mutating all three residues to aspartate to mimic phosphorylation completely inhibits severing activity in vitro, whereas single site phosphomimetic mutants did not alter severing activity compared to wild-type p60. Furthermore, mutating these residues to alanine did not alter severing activity of p60. Both the phosphomimetic and phosphonull
mutants of p60 interact normally with all four p80 subunits and we are currently investigating whether microtubule binding and/or ATPase activity is affected in these p60 mutants. Using in vitro severing assays, we found that the microtubule bundling protein MAP65-1, a plant homolog of human PRC1, is a potent inhibitor of katanin-mediated microtubule severing. At low MAP65-1 concentrations, severing is inhibited at bundled microtubule segments and the severing rate of non-bundled microtubules is reduced. At higher MAP65-1 concentrations, severing is nearly completely inhibited, even on non-bundled microtubules, thus providing a mechanism to protect the sidewalls of linearly ordered cortical microtubules against katanin attack. Our ongoing biochemical and cell biological experiments seek to determine whether inhibition of p60 by MAP65-1 requires microtubule bundling activity and whether it involves sterically blocking p60 binding to microtubules. Together, these experiments are advancing us toward our ultimate goal of reconstituting targeted microtubule severing in vitro.

P2882
Board Number: B161
Aurora B kinase modulates an extended conformation of lattice-bound Kinesin-13 MCAK.
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Kinesin-13 motors regulate precise microtubule dynamics and limit microtubule length throughout metazoans. The Kinesin-13 MCAK is an unconventional kinesin, which depolymerizes microtubules ends. Recently, MCAK has been proposed to undergo large conformational changes during its catalytic cycle, as it switches from solution to bound state and during regulation by phosphorylation. However the nature of the structural changes and how they are coupled with MCAK depolymerization activity remain unclear. Here, we show that MCAK has a compact conformation in solution using crosslinking and electron microscopy. When MCAK is bound to the microtubule ends, it adopts an extended conformation with the N-terminus and neck region of MCAK interacting with the microtubule. Interestingly, the region of MCAK that interacts with the microtubule is the region phosphorylated by Aurora B and contains an EB-binding motif. Phosphorylation of this region decreases the affinity of MCAK for the lattice, which results in a decrease in microtubule depolymerase activity. Therefore the N-terminus of MCAK form a platform to integrate downstream Aurora B kinase signals and in response fine-tunes its depolymerase activity.

P2883
Board Number: B162
Monte Carlo simulations of the dynamic microtubule cytoskeleton: The critical roles of the cell boundary and rescue transitions in shaping the array.
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The role of specific proteins that modulate dynamic instability and the molecular mechanisms responsible for tubulin polymerization have been extensively studied, and yet we currently lack a comprehensive method to explore how the combined parameters of dynamic instability function together to shape an entire array of microtubules, or how changes in dynamic parameters influence the array's organization over time and space. Here we describe a relatively simple Monte Carlo model to simulate a cell's microtubule array from parameters including the radius of the cell, total tubulin
concentration, microtubule nucleation rate from the centrosome, and plus end dynamic instability. The model does not simulate molecular mechanisms underlying these parameters, but is accurate in accounting for the amount of tubulin assembled into polymer. The model includes two possible cellular states, which allows the system to switch to a new parameter set (e.g. interphase to mitosis). Outputs from the simulation include the free tubulin concentration, average microtubule lengths, microtubule length distributions, and examples of individual microtubule length history over time. Using this platform and published parameters measured in interphase LLCPK1 epithelial cells, we find that differences in the frequency of rescue, where microtubule polymers switch from shortening to growth phases, made the largest contribution to microtubule number, average length and free tubulin concentration. Simulating the switch from interphase to prophase plus end dynamics demonstrated that the decreased rescue frequency at prophase was the critical factor needed to rapidly clear the cell of interphase microtubules prior to mitotic spindle assembly. Simulations also predict that lowering the total tubulin concentration by ~ 15 - 20% results in microtubule plus ends that are distributed across a wider area and not confined to the cell boundary, indicating that sequestering modest amounts of tubulin could reshape the distribution of microtubule ends across the cell radius. The model and simulations are useful for predicting changes to the entire microtubule array after modification to one or more parameters, including predicting the effects of tubulin-targeted chemotherapies.

P2884
Board Number: B163
Investigating the role of the GTP hydrolysis rate in regulation of microtubule stability.
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Dynamic instability of microtubules, the switching between phases of microtubule growth and shrinkage, enables the microtubule network to remodel throughout the cell cycle. A growing microtubule end incorporates GTP-bound beta-tubulin subunits, which are subsequently hydrolyzed, resulting in a microtubule lattice consisting of GDP-bound beta-tubulin. The growing end maintains a cap of GTP-tubulin, thought to protect the microtubule against transitions from growth to shrinkage (known as ‘microtubule catastrophe’). Recent in vitro studies found that microtubule tip-tracking protein EB1 is sensitive to the nucleotide state of tubulin in the microtubule lattice, and can therefore be used as a marker for the stabilizing nucleotide cap (‘GTP cap’). Fluorescent EB1 reveals that EB1 localization resembles a ‘comet’ shape, decorating the end of a growing microtubule, consistent with the expected shape of the GTP cap. The size of the GTP cap is defined by microtubule growth and GTP hydrolysis rates, and thus modulation of either of these parameters has the potential of producing a more or less stable microtubule. Indeed, increasing microtubule growth rate, as achieved by increasing tubulin concentration in vitro, results in a linear increase of EB1 comet lengths. At the same time, increase in tubulin concentration leads to a moderate suppression of microtubule catastrophe. Here, we aim to perturb the rate of GTP hydrolysis, using a number of agents that have been implicated in modulating the GTP hydrolysis rate of tubulin, including glycerol, magnesium, and phosphate analogues. We use an in vitro biochemical reconstitution assay with total internal reflection fluorescence (TIRF) microscopy to investigate the effect of these perturbations on both EB1 comet size, as well as the corresponding rates of microtubule catastrophe. Our goal is to elucidate how the properties of the GTP cap influence microtubule stability.
P2885
Board Number: B164
H⁺ and Na⁺ elicited rapid changes of the microtubule cytoskeleton in the biflagellated green alga Chlamydomonas.
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Although microtubules are known for dynamic instability, the dynamicity is considered to be tightly controlled to support a variety of cellular processes. Yet diverse evidence suggests that this is not applicable to Chlamydomonas, a biflagellate fresh water green alga, but intense autofluorescence from photosynthesis pigments has hindered the investigation. By expressing a bright fluorescent reporter protein at the endogenous level, we demonstrate in real time discreet sweeping changes in algal microtubules elicited by rises of intracellular H⁺ and Na⁺. These results from this model organism with characteristics of animal and plant cells provide novel explanations regarding how pH may drive cellular processes; how plants may respond to, and perhaps sense stresses; and how organisms with a similar sensitive cytoskeleton may be susceptible to environmental changes.

P2886
Board Number: B165
A chemical synthetic lethality screen identifies a new pharmacological agent that sensitizes cells to paclitaxel.
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Microtubule stabilization by chemotherapy is a powerful weapon in the war against cancer. Paclitaxel (PTX) has been used successfully for the treatment of solid tumors for decades. Several features, including side-effects and resistance of some cancers make this drug, although very useful, not always effective. The search for next generation microtubule stabilizing drugs with increased efficacy as well as for drugs sensitizing cancer cells to PTX is still intense.

With the aim to identify new chemical compounds that sensitizes cell to paclitaxel we screened a library of 8,000 compounds, to select those not toxic for cell cultures when applied alone, that became toxic when applied in combination with a non-toxic dose of paclitaxel.

This cytotoxicity screening, was performed in parallel on cells treated with the compounds alone and matched cells treated with a non-toxic low dose of PTX. Such an approach, using a combination of chemicals is conceptually similar to the screens based on synthetic lethality, but replacing the genetic perturbation by a chemical perturbation (PTX).

Among compounds active on cellular microtubule, we focused on a carbazole derivative the "T4" molecule. When applied in conjunction with PTX, a non-toxic dose of T4 with no effect on cell proliferation decreased by 2.5 fold the GI50 (50% of growth inhibition) of PTX.

To gain insight in the perturbation produced by T4 we studied its effect on microtubule dynamics and cell cycle. We were able to show that T4 slows down microtubule dynamics. This effect was enhanced when T4 was applied in conjunction with a non-toxic dose of PTX. Toxic doses of T4 blocks cells in mitosis before a complete metaphase and induces abnormal mitotic spindles with chromosomes...
Synergy, growing function promotes MMP14 trafficking, and is associated with MMP14 activity at focal adhesions.

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Angiogenesis is a process by which new vasculature forms from existing vessels, using cues from the extracellular matrix (ECM) to stimulate endothelial cells (ECs) to extend branches, polarize, and migrate. Microtubules (MT) are polymers of the protein tubulin that undergo bouts of polymerization and depolymerization, a process termed dynamic instability. In addition to their structural role in defining EC morphology, MTs also function as an intracellular roadway along which molecular motor proteins traffic cargo. One major group of cargo trafficked along MTs are matrix metalloproteases (MMPs), which function to guide EC migration by locally degrading the ECM to promote directional cell movement. MMP14 is a unique metalloprotease that is membrane-bound and has been implicated in cell invasion in cancers. However, it is not known if, or how, MMP14 trafficking and localization is spatially regulated by MT dynamics. Additionally, MMP14 is known to localize to focal adhesions (FA), however it is not known how ECM density affects FA organization and MMP14 activity. We hypothesized that MMP14 trafficking to the cell surface would be increased in regions of the cell where MT growth is enriched. Further, we hypothesized that MT growth would be locally enriched near FAs, on ECMs that promote an increase in FA number. To test this hypothesis, ECs were cultured in low, intermediate, or high-density 3D collagen ECMs, and live-cell imaging of fluorescent MMP14 and EB3, a marker of MT growth, was performed. Experiments revealed that MMP14 was bi-directionally transported along MTs within cytoplasmic vesicles, and this was inhibited by nocodazole or paclitaxel, suggesting that MT dynamics are required for MMP14 trafficking. Additionally, EC engagement of intermediate density ECMs promoted increased MT growth speed and growth lifetime as well as enhanced MMP14 transport velocity. To monitor MMP14 function in living ECs, we developed a biosensor capable of measuring MMP14 activity with spatio-temporal specificity. MMP14 biosensor data showed that MMP14 activity is spatially restricted to the leading edge and within branched protrusions, where MMP14 activity appears co-localized with growing MT plus-ends. Biosensor data also revealed that active MMP14 was detected as discrete puncta localized at or directly adjacent to FAs, suggesting that MMP14 activity is influenced by proximity to FAs. Taken together, these data identify that EC engagement of intermediate density collagen ECMs promotes fast and long-lived MT assembly and increased MMP14 transport to regions of the cell where FAs are found. Ongoing studies are focused on investigating how MT and FA dynamics communicate to control MMP14 activity, and the consequences for ECM degradation and directional EC migration.
P2888
Board Number: B167
Analysis of Cytoskeletal Filament Bending via Curvature.
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Cytoskeletal filaments such as microtubules and actin filaments play important roles in mechanical signal transduction allowing cells to respond to their environment. Measuring the mechanical properties of cytoskeletal structures is crucial for gaining insight into intracellular mechanical stresses and their role in regulating cellular processes. One of the ways to characterize such bio-filaments is by measuring their persistence length, the average length over which filaments stay straight. Here, we show how curvature distributions can be used as a tool to quantify bio-filament deformations, and investigate how the apparent stiffness of filaments depends on the resolution and noise of the imaging system. We present analytical calculations of the scaling curvature distributions as a function of filament discretization, and test our predictions by comparing Monte Carlo simulations to results from Fourier and tangent correlation analysis. We also apply our approach to microtubules and actin filaments obtained from in vitro gliding assay experiments with high densities of non-functional motors. Finally, the effect of motor density on the curvature of the filaments is tested with Brownian dynamics simulations to confirm the accuracy of experimental results. We discuss how curvature analysis improves accuracy of filament bending characterization in vivo, and provide a freely available easy-to-use ImageJ Plugin.

Ciliary/Flagellar Motility

P2889
Board Number: B169
Multiple Functions of the Striated Rootlet Proteins of the Paramecium Basal Body.
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Paramecium ciliary basal bodies align in straight rows from posterior to anterior. Each basal body is connected to three rootlets (Post Ciliary, Transverse and Striated (SR)). The SR, the longest, projects from the basal body toward the anterior past several more anterior basal bodies. We previously found that the depletion of Meckelin (MKS3) misaligns SRs, disorganizes basal body rows (Picariello et al., 2014) and makes the SRs appear ragged and serpentine. In our study of the connection of MKS3 to the SR, we found that depletion of some SR proteins by RNA interference (RNAi) produced similar basal body mis-alignments. Therefore, we examined genes and proteins of the SR. The SR of Chlamydomonas is the best studied. Using the single SR Chlamydomonas gene SF Assemblin to search, we found thirty Paramecium genes in 13 paralog groups with one to eight genes in each. Proteins from 12 paralog groups were confirmed to be in the SR structure using immunofluorescence. MS analyses of density fractions from SR isolation show all 30 SR members are within the same density fraction as intact SRs. Cells were treated with RNAi for each paralog group; groups with more than one member were treated with RNAi to silence all the group members concurrently. We grouped the SR genes: 1, 2-6, 7-8-10, 11-13. Depletion of some individual SRs (e.g. 1, 3, 9) and groups (2-6; 8-10 and 11-13) showed distortions
of the basal body rows and misalignment of the SRs. Others showed a normal phenotype. Interestingly, RNAi for 8, 9 or 10 alone did not show a phenotype, but when all three groups within the larger group were targeted, there was a strong phenotype. Others like 1 or 7 with two genes in their paralog groups showed extreme phenotypes when reduced by RNAi. Isolated SRs show a striated pattern that matches that of Hufnagel et al., 1969. SRs from RNAi treated cells for SR 2, 3 or 4 are of control length and periodicity. RNAi for others (SR 1, 4 or 7) caused abnormal shapes and periodicity. There is a correlation between the RNAi SR surface misalignment phenotype and the isolated SR RNAi phenotype for shape and periodicity of the SR. Perhaps when lacking specific SR proteins, the rest fail to interact properly with each other through heptad repeats of coiled coil domains to maintain the rootlet structure and directionality toward the anterior.

NIGMS grant P20GM103449; P20 GM103446.
P2890
Board Number: B170
The generation and sensation of fluid flow by cells: roles in development and disease.
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Like classical signal transduction cascades, the microscopic flow of fluids within biological systems provides cues critical for development, growth, and homeostasis. Organisms are permeated with networks of fluid-filled tubes while embryos contain cavities within which flows convey patterning information. Flows are critical for kidney morphogenesis, heart and vascular development, the migration of neurons, and the establishment of left-right asymmetry. However, we know very little about how flows are generated and sensed by cells. This remains a pressing concern given the increasing number of diseases associated with flow abnormalities including structural birth defects, polycystic kidney disease, heart disease, and cancer progression. Cilia, microtubule-based organelles that project from the cell surface, are central players in fluid biology. Motile cilia on specialized cells beat to generate fluid flow across epithelial sheets, while primary cilia, which are found on nearly all vertebrate cells, respond to external cues including flow forces.
We use forward and reverse genetics, as well as a range of cell biological approaches, in zebrafish to interrogate the mechanisms of flow generation and sensation by cells and the roles of these processes in development and disease. I will first report data which demonstrates a molecular link between the pathways that control cilia motility, cilia polarization, and the planar cell polarity of multi-ciliated cells centering on the human disease-associated protein C21ORF59.
Next, I will discuss our recent work that demonstrates a requirement for cilia motility in the brain/spine of zebrafish in maintaining spine straightness during growth. We used a thermogenetic approach to modulate protein function at distinct developmental times which identified a critical window when cilia motility is required for spine maintenance. Absence of cilia motility during this window results in the development of severe spinal curves. We are currently systematically ablating distinct cell populations within the brain and spine to ascertain which motile ciliated cells are involved in preventing spinal curves.
Our zebrafish work has led us to hypothesize a novel cell biological mechanism to explain the human disease Idiopathic Scoliosis (IS), a common spinal curvature that impacts up to 3% of the world's children. I will discuss the implications of our zebrafish data in the context of IS and suggest potential avenues for future work and disease treatments.
P2891
Board Number: B171
Biophysical interactions between cilia and mucus underlie directed fluid transport in the ventral epithelium of the planaria Schmidtea mediterranea.
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Directional flow is required for physiology and development of many organ systems; ranging from the respiratory tract, where flows are responsible for mucus clearance, to the brain, where they transport cerebrospinal fluid. Ciliated epithelia organize spatio-temporally, across different scales, to produce these large scale fluid flows. Here we use the planaria Schmidtea mediterranea as a model system to study how interactions between cilia and the extra-cellular environment, give rise to large scale fluid flows. Planarians secrete mucus and glide over this layer using motile cilia that move the mucus over a less dense periciliary layer (PCL). Using live imaging and techniques from fluid mechanics we characterize the dynamics of this system at different scales, ranging from individual cilia to tissue-scale mucus flows. We observe that only a subset of the cilia in the epithelium are required to be active during planaria gliding motility. This subset changes as the worm crawls and impacts the direction of overall motility and the surrounding fluid flow. Similarly, changes to the properties of the mucus influence the speed of the surrounding flow, ciliary and worm motility. Our results indicate that the ability to achieve directional flows depends both on cilia and the surrounding fluid. Studying cilia and mucus as a unit, with a focus on describing the biophysical properties that control mucus flow at a tissue scale, will provide a holistic understanding of how individual components integrate in ciliated epithelia to achieve directed fluid transport.

P2892
Board Number: B172
FBB18, a homologue of C21orf59, regulates cytoplasmic preassembly of outer and inner dynein arms in Chlamydomonas.
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Dynein arms were preassembled in the cytoplasm, transported into cilia by intraflagellar transport, and docked onto specific sites on the doublet microtubules. Assembly factors are needed to assist stability and preassembly of dynein arm subunits into a functional complex. Knockdown of C21orf59 in zebrafish and planaria induces loss of outer dynein arms. C21orf59 mutations cause primary cilia dyskinesia in human. However, whether C21orf59 functions in the motile cilia or in the cytoplasm is not clear. Furthermore, the molecular mechanism of C21orf59 is not known. Here, we show that Chlamydomonas homologue of C21orf59, FBB18, is a preassembly factor for both outer and inner dynein arms (ODAs and IDAs). Most cells of a null mutant of FBB18 exhibited no or very short flagella of 2-4 mm long. ODAs and IDAs were partially or complete lost in the mutant cells shown by electron microscopy. This data is consistent with the immunoblotting results from the mutant flagellar samples, where outer arm dyneins DHCa and DHCb, and inner arm dyneins dynein b, c and d were not detected or greatly reduced. In the cytoplasm, these proteins were barely detectable. These data suggest that FBB18 is involved in preassembly and stability of the dynein arms in the cytoplasm. Deletion of the N-terminal coiled-coil
domain or the C-terminal sequence that contains a DUF2870 domain generated similar phenotype as the null mutants. Interestingly, deletion of the C-terminal 252-277 amino acids that mimics a truncation mutation in a human patient restored the flagellar length but not motility. Compared to DHCb, dynein c and e, DHCa and dynein b were lost in the flagella as well as in the cell body. DHCa, dynein b and c were co-immunoprecipitated with FBB18. These data suggest that FBB18 forms a large complex with subunits of dynein arm and involved in stability and preassembly of the dynein arms (This work was supported by the the National Natural Science Foundation of China (31330044, and 31671387) and Sino-German Science Center (GZ990) to JP).

P2893
Board Number: B173
Repetitive buckling of microtubules driven by axonemal dynein arrays reconstituted on a microtubule.
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The complexity of the eukaryotic flagellar axoneme is derived from 200-600 types of modular building blocks assembled hierarchically. Nine doublet microtubules surround a pair of singlet microtubules. On each of nine doublet microtubules cyclically arrayed in an axoneme, dyneins are aligned in two rows, outer- and inner-arm dyneins. Each of the heavy chains is reported to have different mechanical properties. They are precisely arranged along doublet microtubules and regulated in a coordinated fashion to produce periodic flagellar beating. The coordination among these building block under strict spatio-temporal regulation makes flagella beat in an organized manner. To reveal the mechanism of coordination and regulation, we have carried out in vitro reconstitution of axonemal structures in a bottom-up manner and have compared mechanical properties of the reconstituted axonemes with those of intact ones. Tubulins were polymerized into microtubules from fragmented axonemes working as seeds. To these microtubule bundles with the same polarity, we added crude outer-arm dynein extract from Chlamydomonas axonemes. These dynein arms formed regular arrays (24nm-repeat) on the microtubules in a self-organized manner and made stiff microtubules bundles. On addition of ATP, a pair of microtubules occasionally displayed association and dissociation cycles. The repetitive buckling lasted a few minutes and the frequency of each buckling ranged from 1 to 3 Hz in the presence of 1 mM ATP. Occasionally, we found the microtubule bundles with both ends of the bundle were clamped. In these bundles, when the shear between microtubules at the middle part occurred, the microtubules were looped out and formed characteristic S-shaped bending and then recoiled. Force generated at the middle part was estimated using Euler formula to be ca. 1 pN per dynein arm. These cyclic interactions between dynein and microtubules were derived from the intrinsic nature of dyneins and were probably regulated by the degree of microtubule bending. These features could be the essence of the beating mechanism working in an axoneme. This work was supported by Grant-in-Aids for Scientific Research (C), the Japan Society for the Promotion of Science (JSPS, grant numbers 26440089 and 17K07376 to K.O.), and the Takeda Science Foundation (K.O.).
P2894

Board Number: B174

Conserved complexes regulating ciliary motility and waveform asymmetry.
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Ciliary motility requires the coordination of multiple axonemal dyneins by regulatory complexes located within the 96 nm axoneme repeat, but little is known about the polypeptides that alter the waveform in response to internal or external stimuli. Several mutations have been identified in *Chlamydomonas* that alter flagellar motility, including some that convert the waveform from an asymmetric, ciliary type stroke to a symmetric, flagellar type stroke. Many motility mutations affect well-known axonemal structures such as the outer and inner dynein arms, central pair microtubules, radial spokes, and regulatory complexes located at the base of radial spokes (e.g., Heuser et al., 2009, 2012a, b). These structures are also highly interconnected (Nicastro et al., 2006; Heuser et al., 2012a, b). However, the polypeptides identified thus far represent less than half of the axonemal proteome. In addition, cryo-electron tomography (cryoET) has revealed the presence of several uncharacterized substructures in the 96 nm axoneme repeat. We are using improved proteomic approaches to reanalyze several motility mutants to identify polypeptides associated with these less-explored substructures and to probe their role in the regulation of motility. The *mbo* (move backwards only) mutants were first identified as cells that are unable to swim forwards with an asymmetric waveform, even when calcium is reduced below micromolar levels (Segal et al., 1985). Instead *mbo* mutants swim backwards using a symmetric waveform, similar to the photoshock response of wild-type cells triggered by a transient increase in intraflagellar calcium. iTRAQ based proteomics has identified a subset of ~19 polypeptides that are missing or reduced in different *mbo* mutants, including one inner arm dynein. Eleven polypeptides are algal specific, but eight are conserved in other species with motile cilia and flagella. Most MBO-associated polypeptides contain coiled coil domains, but three contain EF-hand or IQ motifs potentially involved in calcium/calmodulin binding. CryoET of *mbo2* mutant and *MBO2-SNAP*-tagged axonemes has revealed defects in a novel structure located within the 96 nm axoneme repeat. The further characterization of regulatory components in *Chlamydomonas* should provide insight into new regulators and the function of related proteins in other species and potentially identify new loci involved in primary ciliary dyskinesia (supported by NIH grants to M.E. Porter and D. Nicastro).

P2895

Board Number: B175

Comparative proteomics reveals candidates for novel ciliary central apparatus components.
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Normal ciliary and flagellar motility requires the integrity of the “9 + 2” axoneme, which is composed of 9 outer doublet microtubules with associated structures and a central apparatus (CA) consisting of two microtubules plus projections. To date, 22 proteins have been characterized as components of the CA, of which 19 are specific to the CA. However, cryo-EM analysis has revealed that the CA has a highly complex architecture, and it is likely that it contains many more proteins than have been localized to it. In an effort to identify novel CA components, we have compared the proteomes of isolated *Chlamydomonas* wild-type axonemes with that of the CA-less mutant pf18 by label-free quantitative proteomics. Comparative analysis reveals novel candidates for novel CA components, including a calcium/calmodulin-binding module. These findings provide new insights into the structure and function of ciliary and flagellar CA.
mass spectrometry. In two independent experiments, we identified a total of about 1100 proteins. Nearly all known subunits of the inner and outer dynein arms and radial spokes were identified and found to be present in a 1:1 ratio in the two proteomes, indicating that the approach detected most axonemal proteins and accurately reported their relative amounts in the two proteomes. 21 of 22 known intraflagellar transport (IFT) particle proteins were identified and found to be elevated 10-100X in the pf18 axonemes, consistent with the previously reported trapping of IFT particles in the lumen of CA-less mutants. In contrast, all 19 of the previously known CA-specific proteins were greatly reduced in the mutant proteome. Of uncharacterized proteins present in the wild-type proteome in amounts comparable to the known CA proteins, 54 were greatly reduced in the mutant proteome; these are good candidates for being CA components. Seventeen of these are highly conserved in humans. We have selected six of the conserved proteins for characterization by mutant analysis, super-resolution light microscopy, thin-section EM, and immuno-gold EM to confirm their location in the CA and to determine how their loss affects flagellar motility and axonemal structure. A more complete knowledge of the ensemble of CA components will be an important step toward understanding how the CA performs its essential functions in motile cilia and flagella. Moreover, the human genes encoding CA proteins are likely to cause primary ciliary dyskinesia when mutated, but patients would be expected to have normal situs solitus because nodal cilia, which initiate left-right asymmetry, lack the CA and thus would be unaffected. The availability of additional candidate genes will facilitate identification and diagnosis of such patients.

P2896
Board Number: B176
Antioxidant treatment prevents alcohol-induced ciliary dysfunction in Chlamydomonas reinhardtii.
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Excessive alcohol consumption has been shown to result in pulmonary dysfunction, in part, by compromising the motility of cilia in the upper airway that are critical for mucociliary clearance. The ciliary slowing effect of alcohol has been termed “Alcohol-Induced Ciliary Dysfunction” (AICD). We have previously demonstrated that AICD is conserved from mammals to the powerful model protist, Chlamydomonas reinhardtii (Yang et al., 2015). We determined that alcohol exposure reduces the activity of the outer dynein arm motors and, for the first time, identified specific ciliary proteins that show altered phosphorylation in response to alcohol. In mammalian models, AICD involves the generation of reactive oxygen and nitrogen species (ROS/RNS) and antioxidant treatment prevents AICD (Simet et al., 2013). However, the specific downstream targets that are modified by alcohol-induced changes in redox potential are unknown. Here, we demonstrate that antioxidant treatment prevents AICD in Chlamydomonas cells demonstrating that the protective role of antioxidants in AICD is conserved. Pretreatment of Chlamydomonas cells with two antioxidant compounds, N-acetylcysteine and Tempol, prevents the reduction in swimming speed induced by alcohol exposure. Of interest, Wakabayashi and King reported that altering redox conditions results in changes in ciliary beat frequencies, mediated by the ODA, which correlates with changes in protein-protein interactions of two ODA light chains and one ODA-docking complex subunit via mixed disulfides (Wakabayashi and King, 2006). How these changes ultimately alter ciliary beat frequencies is unclear, and the specific downstream targets of alcohol that are modified by ROS/RNS have yet to be identified. We will continue to utilize the exceptional experimental advantages of Chlamydomonas to determine if alcohol
exposure induces nitro-oxidative stress that targets redox-sensitive proteins that control outer dynein arm activity.

P2897
Board Number: B177
EFHC1 and EFHC2 are necessary for motile cilia function and A-tubule MIP recruitment.
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Motile cilia are essential cellular structures that beat to move extracellular fluid in a unified direction. Disruption of ciliary beating has dire consequences on a number of essential processes including the generation of cerebral spinal fluid movement, mucus clearance from the lungs and movement of the egg down the fallopian tube during ovulation. The structural organization of thousands of motor proteins and regulators along a microtubule-based scaffold is critical for the function of motile cilia. They are composed of nine sets of modified doublet microtubules arranged radially around a central pair of microtubules. Ciliary doublet microtubules are unique in that they are extremely stable and resistant to the mechanical forces associated with ciliary beating. Recent advances in cryo-electron tomography have made it possible to map the structure of doublet microtubules with molecular resolution. We now know that there are a number of densities on the luminal surfaces of the doublet microtubules termed Microtubule Inner Proteins (MIPs), however, their composition and function remain unexplored. We have identified the role of two proteins, EFHC1 and EFHC2, in motile cilia function and MIP formation. We find that Tetrahymena thermophila homologs of EFHC1 and EFHC2, Bbc73 and Bbc60, respectively, localize to the doublet microtubules of both basal bodies and cilia. Strains with single or double null alleles of Bbc73 and Bbc60 exhibit motile cilia beating and assembly defects. Interestingly, when axonemes are visualized using cryo-electron tomography, knockout of either Bbc73 or Bbc60 results in independent loss of a number of A-tubule MIPs. Furthermore, we find that introduction of Bbc60-GFP into Bbc60 knockout cells rescues the loss of MIPs associated with Bbc60 absence and imparts and extra density in the region of MIP4. We hypothesize that this extra density corresponds to the additional density of the GFP tag on Bbc60 and represents the first evidence of the identification of a MIP protein. To identify other MIPs associated with EFHC proteins we used mass spectrometry to identify axoneme proteins lost in EFHC mutant Tetrahymena cells. CAPS was identified as a potential MIP protein and Tetrahymena cells that are heterozygous for CAPS show swimming defects that are consistent with deficiencies of motile cilia beating. Overall, our data reveal a major role for EFHC proteins in the function of motile cilia through the recruitment of a complex network of MIPs to the A-tubule of doublet microtubules.
P2898
Board Number: B178
FMG-1B glycoprotein is necessary for expression of force at the Chlamydomonas flagellar surface.
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When there is insufficient water to allow swimming via flagellar beating, Chlamydomonas cells initiate an alternative form of motility in which cells glide along a surface in a flagella-dependent manner. Bidirectional movements of microspheres along the flagellar surface are thought to be a proxy for gliding motility and thus can be used to reveal the activity of the motor used for gliding motility. While flagellar beating requires outer doublet-associated axonemal dynein, force transduction for gliding motility and microsphere movement is dependent upon Intraflagellar Transport (IFT) and cytoplasmic dynein 1b/2 (Shih et al. 2013). An extensive literature (mostly from the Bloodgood lab) provides circumstantial evidence that the mechanical coupling of the intraflagellar force transduction machinery to the surface of a microsphere or a planar substrate for gliding is mediated by the transmembrane glycoprotein FMG-1B, which comprises most of the protein of the flagellar membrane. Using strains obtained from the Chlamydomonas Library Project (CLiP; https://www.chlamylibrary.org/) having insertions in the 5’ or 3’ UTRs of FMG-1B, we have demonstrated that FMG-1B is required for microsphere movement. First, we determined that strains containing an insertion in the 5’ UTR, but not the 3’UTR, fail to synthesize FMG-1B, consistent with disruption of ribosome binding due to an insertion in the 5’ UTR. Immunoblot analysis of whole flagella showed no detectable FMG-1B in the 5’-UTR mutant, while the 3’UTR mutant expressed WT levels of FMG-1B. Second, immunofluorescence microscopy showed a reduction in flagellar staining in the 5’-UTR mutant relative to the 3’UTR mutant and WT strains. Third, TEM analysis of the mutant lacking FMG-1B showed a dramatic reduction of the prominent glycocalyx normally observed on the flagellar surface suggesting that this glycocalyx is composed of the large ectodomain of FMG-1B; the 3’UTR mutant had a WT appearance. All three strains had normal length flagella, suggesting that FMG-1B is not necessary for flagellar membrane assembly or flagellar stability despite contributing most of the protein in the flagellar membrane. Importantly, microsphere motility was greatly reduced in the 5’UTR mutant, demonstrating that FMG-1B is necessary for the expression of force at the flagellar surface. We are currently assessing the precise position of the insertions in these mutant strains, as the data from CLiP on the position of the insertions are provided with only 75% accuracy. We are also in the process of assessing the effect of these mutations on gliding motility.

P2899
Board Number: B179
Exploring the role of FoxJ1 transcription factor in a new species of planarian “Dugesia guanajuatiensis”.
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Cilia are cell surface associated, microtubule-based, filamentous organelles with wide spread distribution among protozoans and most metazoan phyla. The transcription factor FoxJ1(DNA-binding
forkhead) is the master regulator of motile cilia. Here, we focus on the diploid mexican planarian, *Dugesia guanajauiensis* (*Dgua*), and the role *Dgua* - *Foxj1* plays in the function of its cilia. In stark contrast to other planarian species, *Dgua* consistently moves by peristaltic waves of whole body contractions or inchworming rather than a canonical gliding motion. *In situ* hybridization showed that *Dgua-foxj1* is widely expressed in the ventral and dorsal motile ciliated cells, and is also expressed in the pharynx, auricles and in the brain. Immunofluorescence and scanning electron microscopy revealed that *Dgua* has patchy cilia on its ventral epithelium, whereas a naturally gliding species, *Schmidtea mediterranea*, has a complete lawn of cilia. We examined the role of *Dgua-foxj1* in the function of ventral cilia by RNAi and treated animals experienced a further loss of motility. Contributions of *Dgua-foxj1* to the maintenance of cilia will be discussed.

**P2900**

**Board Number: B180**

A phase separated organelle for dynein arm assembly in ciliated cells.

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Motile cilia are responsible for a multitude of functions in the human body, including mucociliary clearance in the lung, cerebrospinal fluid circulation in ventricles, gamete propulsion, and laterality determination during early development. The wavelike motion of cilia is the result of dynein arm complexes that exert force to bend microtubules within cilia through coordination of multiple motor protein subunits. The cytoplasmic preassembly and stabilization of these dynein arms has been shown to involve a group of proteins known as axonemal dynein assembly factors (DNAAAFs) working in conjunction with chaperone machinery to fold and complex subunits prior to ciliary transport and docking. However, the mechanism by which this process occurs remains unclear. Here, we describe that many known DNAAAFs colocalize with one another at in multiciliated cell-specific cytosolic granules, which we term DynAPs (Dynein Assembly Particles). DynAPs display compositional similarity to known liquid-liquid phase separated organelles, including enrichment of stress granule components. Physical properties of DynAPs are akin to those observed in other cytoplasmic phase separated organelles, including fusion and rapid exchange of material with the cytosol. Further, we demonstrate that human disease-related molecular changes in DNAAAFs are associated with alteration of these physical properties and lead to downstream defects in dynein assembly and ciliary beating.

**Ciliopathies**

**P2901**

**Board Number: B181**

Altered centrosomal trafficking disrupts cilia assembly and signal transduction in Down syndrome.

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Ciliopathies share substantial phenotypic overlap with Down syndrome (DS; trisomy 21), including the presence of congenital heart defects, craniofacial abnormalities and altered cerebellar development. Whether these similarities result from cilia defects in DS cells is not known. To begin to address this...
possibility, we investigated cilia formation and ciliary signaling in DS cells. Trisomy 21 increases the expression of the chromosome 21 gene Pericentrin. At normal expression levels, Pericentrin localizes ciliary assembly and signaling proteins to the centrosome via a trafficking network that traverses cytoplasmic microtubules. In DS, excess Pericentrin disrupts the spatial organization of this ciliary trafficking network. This is because excess Pericentrin increases gamma-tubulin levels at the centrosome, which causes increased and disorganized cytoplasmic microtubules. In turn, the altered microtubule network disrupts dynamic Pericentrin trafficking events to and from the centrosome. Pericentrin is required to localize the cilia assembly factor, IFT20, to the centrosome for eventual loading into the ciliary compartment. In DS, Pericentrin trafficking defects reduce the centrosomal level of IFT20, which, ultimately, decreases cilia formation. When cilia do form in the presence of excess Pericentrin, they do not properly transduce Sonic hedgehog (Shh). These results demonstrate that increased gene dosage of ciliary trafficking proteins disrupts the dynamic recruitment of ciliary assembly and signaling proteins. We propose that altered ciliary trafficking is a potential disease mechanism that may contribute to ciliopathy-like phenotypes in Down syndrome and other aneuploid disorders.

P2902
Board Number: B182
Cardiac valve abnormalities in PKD are due to defects in ciliogenesis, and ciliogenic programs involving the exocyst are conserved across organs.
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Cardiac valve abnormalities are associated with polycystic kidney diseases including: ADPKD, Joubert and Meckel’s syndromes. Our recent work showed that primary cilia are present on developing cardiac valves, but are reduced in adulthood, and that normal valve development requires functional cilia. Mutations in members of the highly-conserved eight protein exocyst complex result in Joubert and Meckel’s syndromes. We previously showed in Madin Darby canine kidney (MDCK) cells that shRNA-induced knockdown of Exoc5, a central exocyst component, inhibited ciliogenesis, led to increased cell proliferation, and resulted in low intracellular calcium levels that did not increase in response to fluid flow. Cell polarity was not grossly affected. Exoc5 overexpression in MDCK cells resulted in longer cilia, with normal intracellular calcium levels that showed an enhanced response to fluid flow. The exocyst appears to be necessary for ciliogenesis by trafficking vesicles from the Trans-Golgi network carrying ciliary proteins such as polycystin-2. We showed in zebrafish that exoc5 knockdown, with antisense morpholinos, resulted in altered ciliogenesis, nephrogenesis, and cardiac edema. We recently generated Exoc5+/− mice, and kidney-specific knockout of Exoc5 resulted in a nephropathosis phenotype in mice surviving to 30 days. We also showed that Cdc42 localizes the exocyst to the nascent cilium, and that Cdc42 and the exocyst genetically interact in zebrafish. We generated Cdc42 kidney-specific knockout mice, which also resulted in a nephropathosis phenotype. Here, we report that homozygous mutation of exoc5 in zebrafish leads to a non-functional protein and reproduces the morphant phenotype, with defects that include cardiac edema and severe cardiac valvular stenosis. Rescue of exoc5 mutants with human EXOC5 mRNA reverses the phenotype. In exoc5 mutant zebrafish, the Hippo pathway is turned on with significantly higher levels of active Mob1. The Hippo pathway has previously been linked, by us and others, to PKD. In mice, cardiac valve specific knockout of Exoc5, using a Nfatc1 driver line, leads to bicuspid aortic valve disease that is highly penetrant. In summary, cardiac
valvulogenesis is dependent on normal ciliogenesis, and ciliogenesis in cardiac valves, in turn, is dependent on the exocyst. These data show that ciliogenic programs are conserved across organs and species, which helps explain the association of ciliogenic abnormalities and PKD. In addition, these animal models may be useful for testing therapeutic compounds.

P2903

Board Number: B183

Two-Color STORM Reveals that Disruption of Ciliary Transition Zone Architecture Causes Joubert Syndrome.

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Diverse human ciliopathies, including nephronophthisis (NPHP), Meckel syndrome (MKS) and Joubert syndrome (JBTS), can be caused by mutations affecting components of the transition zone, a ciliary domain near its base. The transition zone controls the protein composition of the ciliary membrane, but how it does so is unclear. To better understand the transition zone and its connection to ciliopathies, we defined the arrangement of key proteins in the transition zone using two-color stochastic optical reconstruction microscopy (STORM). This mapping revealed that NPHP and MKS complex components form nested rings comprised of nine-fold doublets. The NPHP complex component RPGRIP1L forms a smaller diameter transition zone ring within the MKS complex rings. JBTS-associated mutations in RPGRIP1L disrupt the architecture of the MKS and NPHP rings, revealing that vertebrate RPGRIP1L has a key role in organizing transition zone architecture. JBTS-associated mutations in TCTN2, encoding an MKS complex component, also displace proteins of the MKS and NPHP complexes from the transition zone, revealing that RPGRIP1L and TCTN2 have interdependent roles in organizing transition zone architecture. To understand how altered transition zone architecture affects developmental signaling, we examined the localization of the Hedgehog pathway component SMO in human fibroblasts derived from JBTS-affected individuals. We found that diverse ciliary proteins, including SMO, accumulate at the transition zone in wild type cells, suggesting that the transition zone is a waypoint for proteins entering and exiting the cilium. JBTS-associated mutations in RPGRIP1L disrupt SMO accumulation at the transition zone and the ciliary localization of SMO. We propose that the disruption of transition zone architecture in JBTS leads to a failure of SMO to accumulate at the transition zone, disrupting developmental signaling in JBTS.

P2904

Board Number: B184

Primary Ciliary Deficits in the Dentate Gyrus of the Fragile X Syndrome Mouse Model.

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The primary cilium is the non-motile cilium present in most mammalian cell types, extending from the cell surface and functioning as an antenna for cells to sense signals. Although primary cilia are well-known to be important in regulating sonic hedgehog (Shh) signaling in developing brains, the function of the primary cilium in the adult brain, especially in neurons, has not been fully understood. Evidence has shown that the ablation of primary cilia in adult-born neurons of the dentate gyrus (DG) results in reduced dendritic arborization, synaptic strength, and adult neurogenesis. Moreover, losing cilia in adult
mouse brains demonstrated hippocampal-dependent learning and memory deficits, which often are phenotypes of neurodevelopmental disorders. Fragile X syndrome (FXS), a neurodevelopmental disorder, is a common form of inheritance for intellectual disabilities with a high risk for autism spectrum disorders, and is known to show cellular and behavioral phenotypes in FXS patients, which are recapitulated in Fmr1 knockout (KO) mice. Although Fmr1 KO mice show the abnormal spine morphology, reduced dendritic arborization of neurons, and reduced neurogenesis in the DG, the cellular phenotypes of primary cilia in the neurons of FXS have not been studied yet. To investigate the primary cilia in FXS, we analyzed the number of primary cilia in the hippocampus and the cortex of adult Fmr1 KO mice. As a result, Fmr1 KO mice showed reduced numbers of primary cilia in the DG but not in the CA1 or CA3 of the hippocampus, nor in the cortical regions of brain. Loss of primary cilia in the DG of Fmr1 KO mice is age-dependent as we observed the reduced number of primary cilia both in the later stages of development and in adults, but not in the early developmental stage in the DG of Fmr1 KO mice. Moreover, the reduction in cilia numbers in the DG of Fmr1 KO mice is mainly from neuronal cells and not from non-neuronal cells. Here, we demonstrate for the first time that the structural deficit of the neuronal primary cilia is observed in the mouse model of FXS.

P2905

Board Number: B185

A Comprehensive Portrait of Cilia and Ciliopathies from a CRISPR-based Screen for Hedgehog Signaling.

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The primary cillum is a signaling center that shapes embryonic development and is the unifying cause of developmental disorders known as ciliopathies. However, our understanding of how the cilium functions as an essential organizing center for Hedgehog signal transduction remains incomplete. We have conducted a functional genomic screen for Hedgehog signaling by engineering antibiotic-based selection of Hedgehog-responsive cells and applying genome-wide CRISPR-mediated gene disruption. The screen robustly identifies factors required for ciliary signaling with few false positives or false negatives. Functional characterization of hit genes uncovers novel components of key ciliary structures (including the EvC zone and the transition zone), a ciliopathy-associated ciliary protein required for Hedgehog signal transduction, and a tetrameric protein complex containing ε- and δ-tubulin that is required for centriole maintenance. The screen also provides an unbiased tool for classifying ciliopathies, revealing several poorly characterized syndromes to be ciliopathies ‘in hiding’ and indicating that many forms of congenital heart defects are likely ciliopathies. Collectively, this screen enables a systematic analysis of ciliary function and of ciliopathies and, more broadly, the platform presented here offers a versatile tool for dissecting diverse biological processes through CRISPR-based functional genomics.
Leber Congenital Amaurosis (LCA) is the most severe type of retinal dystrophies causing a very rapid and severe loss of vision in human and related experimental models. Like many of the other pathologies causing visual impairment, LCA causes an evident dysfunction at the photoreceptor level, leaving the other neuronal components in the vision network seemingly unaffected. There have been 18 identified genes associated with LCA, several of these encode for proteins related to ciliary function. We investigated an autosomal recessive form of LCA, Lebercilin (LCAS), which is specifically involved in the regulation of the intraflagellar trafficking in ciliated cells. Lebercilin protein has been best characterized in photoreceptors, and has been shown to be critical for intracellular transport of rhodopsin through the connecting cilium to the outer segment (OS) layer. Loss of lebercilin results in excess protein accumulation in the ONL leading to cell death. Loss of photoreceptors can be documented even before the outer segments have fully developed, suggesting that this early onset retinal dystrophy (EORD) could involve an additional cell compartment proceeding photoreceptor maturation. We hypothesize that ciliation in retinal pigmented epithelial (RPE) cells would be affected by loss of LCAS in murine and human experimental models leading to early pathologic consequences. Although it is known that RPE cells play an essential role in epithelial transport, protection against oxidative stress, secretion and phagocytosis for proper functionality and morphology of the photoreceptors, its role in retina degeneration, paradoxically, has been just recently appreciated. In order to investigate the pathophysiology of LCAS and understand how the RPE cells play a role in this EORD, we used patient iPSC-derived RPE cells and RPE flat mount from LCAS knockout mice. We observed a severe impairment of ciliogenesis and related alteration in RPE size, distribution and maturation. This new, so far unreported, phenotype could help to explain the severity of the LCAS depletion in the visual system, by affecting not only the retina directly but also by profoundly altering the physiology of its supporting cells. This complex mechanism of action, could lead to additional insight into the pathology of other EORDs where developmental impairments of the visual system effects neurodegeneration of the retina.

Multi-subunit molecular motors of the dynein family power the essential beating of cilia and flagella. Defective ciliary motility can lead to a severe congenital human ciliopathy called primary ciliary dyskinesia (PCD, OMIM: 242650) characterised by respiratory distress in new-borns which can progress to life-threatening complications. A major cause of PCD is a failure in dynein motor function or assembly. The majority of PCD causing mutations are found in genes encoding structural sub-units of the outer dynein arm (ODA) motor complex, the most common of these disease genes is Dynein Axonemal
Heavy Chain 5 (DNAH5). Human DNAH5 is the largest of the axonemal dynein genes and encodes a peptide 4624 amino acids long. Given their length and complex secondary structure, axonemal dyneins require a cytoplasmic chaperone relay both for folding of individual subunits as well as multimeric assembly. While the precise molecular mechanism of these chaperones remains unclear, these Dynein Axonemal Assembly Factors (DNAAFs) are required for the correct localisation and assembly of the Outer Dynein Arms (ODAs). These DNAAFs are also mutated in PCD, as demonstrated by our disease mouse model, which has a null mutation in Zmynd10, where rapid clearance of axonemal dyneins from the cytoplasm of motile ciliated mouse epithelia cells results from the PCD mutation. In order to characterise the process that results in the correct folding, trafficking and localisation of axonemal dynein heavy chains in ciliated mammalian cells, we have undertaken an endogenous tagging approach with a highly versatile SNAP tag in both wild type and mutant backgrounds. In parallel, we are investigating translational control of axonemal dyneins using RNA-FISH and the SunTag, and ask whether this is perturbed in the Zmynd10 null mouse model.

P2908
Board Number: B188
New insight on the TTBK2-dependent Sonic hedgehog activation from a CRISPR-based screen.
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Primary cilia are evolutionarily conserved, microtubule-based organelles that protrude from the surface of most differentiated cells. These specialized antennas act as a signaling hub critical for the development and the function of many tissues and organs. The primary cilium senses and translates extracellular cues into cellular responses. Ciliary dysregulations lead to a wide range of severe developmental abnormalities that include brain malformations, skeletal defects, retinal degeneration and polycystic kidney disease. Cilia-related diseases are commonly called “ciliopathies”, which comprise a spectrum of rare genetic disorders, such as Bardet-Biedl and Joubert Syndromes. The Sonic hedgehog (Shh) pathway is one of the major developmental pathways that is tightly controlled at the primary cilium. In a previous work, our lab discovered a novel link between cilia and a neurodegenerative disease by demonstrating that a kinase mutated in a hereditary cerebellar ataxia, Tau Tubulin Kinase 2 (TTBK2), is essential for primary cilia assembly, and for the activation of Shh signaling. TTBK2 mutants, lacking neural primary cilia, display drastic disruption of the Shh-dependent patterning in neural tube and limb. To investigate the molecular mechanism by which TTBK2 regulates Shh pathway, we performed a CRISPR/Cas9 screen based on the Shh activation. We used a mouse kinome CRISPR library that targets 713 mouse kinase genes. Our results focus on mapping the enrichment or depletion of sgRNA in hypomorphic Ttbk2 cells versus control cells. From these studies, we expect to unveil new mechanisms involved in cilia formation, and specifically to identify proteins that cooperate with TTBK2 to regulate cillum assembly.
The primary cilium is an organelle present on many cell types in mammals. In epithelial cells, it typically extends from the apical surface of cells into a lumen, serves as a sensor for a broad variety of molecular environmental stimuli, and contributes to cell polarity and polarized secretion. In autosomal dominant polycystic kidney disease (ADPKD), inherited mutations in polycystins 1 or 2 (encoded by PKD1 and PKD2), which function as a heterodimeric signaling complex at the cell cilium, abnormally activate signaling pathways regulating cell proliferation, migration, and response to environmental cues. By contrast, in cancers such as adenocarcinomas, which arise from mucus-secreting glands, ciliary dynamics are often affected, in parallel with multiple changes in cell signaling and metabolism. Intriguingly, studies of the signaling defects associated with ADPKD have increasingly identified parallel defects to those seen in cancer, in spite of the very different presentation of ADPKD and solid tumors. We have been evaluating targeted signaling inhibitors in ADPKD models to probe the biological differences between this disease and cancer, and to determine whether advanced cancer drugs are effective in ADPKD. Given recent models for ciliary dependence of defective polycystin signaling in ADPKD, we have also been assessing whether signaling inhibitors that impact cysts also impact ciliary biology. In published and ongoing work, we have found that small molecule inhibitors of heat shock protein 90 (HSP90; with ganetespib or STA-2842) or the epidermal growth factor receptor (EGFR; with erlotinib) in Pkd1-/− mice reduces initial renal cyst formation and slows the progression of these phenotypes in mice with pre-existing cysts. Conversely, inhibition of Aurora-A (AURKA; with alisertib) exacerbates cyst formation. Treatment of kidney epithelial cells with HSP90 inhibitors leads to the fast resorption of primary cilia. In contrast, treatment of Pkd1-/− mice with the Aurora-A kinase inhibitor MLN8237 (alisertib) caused defective ciliary resorption and morphological abnormalities of cilia. Surprisingly, erlotinib also increased ciliary length and stabilized cilia from resorption. These results indicated that 3 of 3 assessed cancer drugs had striking effects on cell ciliation, which identifies new protein regulators of ciliary assembly and disassembly, and could account for some of the clinical profile of these compounds in treatment of adenocarcinomas and other tumors. Based on this work, we have developed and applied an in vitro system allowing us to screen a larger panel of anti-cancer drugs to evaluate changes in ciliary dynamics and signaling.

Cytokinesis 2

At late mitosis, the mother cell divides leaving two daughter cells connected by a thin intercellular bridge (ICB). During abscission of the ICB, the ingestion of the cleavage furrow is formed, and the
central spindle microtubules are compacted into the structure known as midbody (MB). MBs are tubulin-rich structures that are known to play a key role in coordinating membrane transport and cytoskeleton re-arrangements during abscission by recruiting known abscission regulators such as ESCRT complex as well as Rab11 and Rab35 containing endosomes. In this study we have set out to identify and characterize novel abscission regulators. To that end we purified intact midbodies from HeLa cells and completed their proteome analysis to identify the proteins that associate with MBs during late telophase. Proteomic analysis identified over 600 proteins including many known MB resident proteins. Here we focused on Rab GTPases since their have been proposed to mediate actin dynamics and endosome targeting at the abscission site. Proteomic analysis of MBs identified 18 post-Golgi Rabs as putative MB associated proteins. Among them were Rabs that were previously shown to be present at the MB and to mediate abscission, namely Rab11a, Rab11b and Rab35. To further characterize whether all identified Rabs function during abscission we next overexpressed dominant-negative mutants of all identified new Rabs and tested the effect of these mutants in completing cytokinesis. We found that over-expression of Rab14 dominant-negative mutant significant increased the number of cell that failed to complete cytokinesis. Next we used shRNA as well as CRISPR/Cas9 approaches to down-regulate or knock-out Rab14. Consistent with the involvement of Rab14 in regulating abscission depletion of Rab14 led to increased multi-nucleation as well as arrest of cells in late telophase. All Rab GTPases function by recruiting and/or activating various effectors proteins. Thus, next we immune-precipitated Rab14 from late telophase cells and identified proteins that directly interact with Rab14 in GTP-dependent fashion. Finally, we then tested the involvement of Rab14-interactions proteins in mediating abscission. Based on all these data, we propose that Rab14 is a novel regulator of cytokinesis that function by regulating the targeting of endosomes to the ICB during late telophase and consequently affecting the abscission of daughter cells.

P2911
Board Number: B192
Oxidoreduction of F-actin controls the timing of cytokinetic abscission.
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At the end of each division, the mother cell is physically cleaved into two new daughter cells by a mechanism known as cytokinesis. Failure in cytokinesis leads to genetic instability and can promote tumorigenesis in vivo. Cytokinetic abscission, the final stage of cytokinesis, corresponds to the severing of the intercellular bridge connecting the two daughter cells. The mechanism mediating the final scission event crucially depends on the local constriction of ESCRT-III helices after complete cytoskeleton disassembly. While the ESCRT-associated enzyme Spastin cuts the microtubules of the intercellular bridge, the mechanism that actively clears F-actin at the abscission site is unknown. We found that oxidation-mediated depolymerization of actin by the redox enzyme MICAL1 (Molecules Interacting with CasL) is key for cytokinetic abscission. We observed that the depletion of MICAL1 delays cytokinetic abscission in human cells, due to a dramatic increase of F-actin within the intercellular bridges that impairs ESCRT-III recruitment to the abscission site. MICAL1 is recruited to the abscission site by the Rab35 GTPase through a direct interaction with a flat three-helix domain found in MICAL1 C-terminus. Mechanistically, in vitro single filament assays show that MICAL1 is activated by Rab35. We propose that MICAL1 oxidation weakens longitudinal interactions between actin subunits within the filaments, making the filaments more fragile and leading to enhanced F-actin depolymerization rates. We will also present unpublished data revealing the mechanisms that counteract F-actin oxidation during cytokinesis. Altogether, the levels of oxidized vs. reduced actin controls local F-actin depolymerization vs. stabilization and thus the timing of cytokinetic abscission.
P2912
Board Number: B193
Actin isoform-specific array organization during cytokinesis is differentially controlled by the formins DIAPH1 and DIAPH3.
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Cytokinesis is the final stage of cell division that divides one cell into two new daughter cells, each with a complete genome. The acute spatio-temporal control of actin cytoskeleton re-organization drives cytokinetic furrow ingression. However the actin cytoskeleton at different sites on the plasma membrane may have different roles during cytokinesis. Actin at the poles moves towards the ingression furrow in a process called cortical flow whereas de novo production of actin occurs at the site of the furrow. Interestingly these functional differences correlate with the different distribution of β-actin, at the furrow and γ-actin at the cortex. We demonstrate β-actin production at the furrow is driven by the actin isoform specific nucleation activity of the formin DIAPH3, a process that requires both RhoA and anillin. In contrast, DIAPH1 localizes to the mitotic cell cortex and is required for correct γ-actin organization at the cortex of dividing cells. DIAPH1 is activated by a comparable molecular mechanism as DIAPH3, but instead utilizing RhoA and IQGAP1. Our data suggest differential localized activation of formins by activators and enhancers is required for the production and function of isoform specific actin arrays required for the successful completion of cytokinesis.

P2913
Board Number: B194
Hold Me Tight! PIP2 and Sktl Are Required for Association of Plasma Membrane with the Contractile Ring During Cytokinesis.
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Cytokinesis, the process by which the cytoplasm is divided between daughter cells at the end of mitosis, is essential for growth and development, and defects in cytokinesis lead to genomic instability and cancer progression. Successful cytokinesis requires precise coordination and control of plasma membrane and cytoskeleton reorganization, including formation and maintenance of an actomyosin-based contractile ring at the cleavage furrow. Phosphoinositides, essential membrane lipids involved in diverse biological processes, are required for successful completion of cytokinesis. In addition, adaptor molecules including anillin and septins are likely involved in anchoring the contractile ring to the plasma membrane. However, the precise role of the phosphoinositide PIP2 during cytokinesis is not well established and little is known about the dynamic localization of contractile ring components at the cleavage site and their association with the plasma membrane. Here, we further dissect the role of PIP2 in cytokinesis using live imaging as well as pharmacological and genetic studies. We show that the PIP5-kinase Sktl localizes to the contractile ring and that PIP2 synthesis by Sktl at the cleavage furrow is required for successful cytokinesis. In addition, we demonstrate that normal PIP2 levels are required for maintenance of contractile ring-associated proteins myosin, actin, anillin and septins at the cleavage furrow and for linking the contractile ring to the plasma membrane. Contractile ring components were displaced from the cleavage furrow upon reduction of PIP2 levels by ectopic expression of a PIP2
phosphatase or mutating sktl. Increasing PIP2 levels by inhibiting PIP2 hydrolysis or overexpressing Sktl led to defective localization of septins. Upon furrow and plasma membrane regression during cytokinesis failure, septins remained associated with the contractile ring, which detached from the plasma membrane. In addition, experiments using a myosin light chain kinase inhibitor indicate that dynamic association of actin and septins with the furrow is regulated by myosin activation. Overall, our results suggest that local synthesis and turnover of PIP2 play crucial roles in establishing successful cell division by maintaining association of contractile ring complexes with the plasma membrane at the equator of dividing cells.

P2914
Board Number: B195
The Aurora kinase A activator TPXL-1 mediates aster-based clearing of contractile ring proteins from the cell poles during cytokinesis.
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At the end of cell division the mother cell splits into two daughter cells in a process called cytokinesis. During cytokinesis a contractile actin-myosin ring assembles between the two segregating chromosome masses beneath the plasma membrane. The position of the contractile ring is determined by signals from the mitotic spindle and thereby chromosome segregation is tightly coupled to cleavage furrow formation. A stimulatory signal from the mitotic spindle promotes cortical contractility at the cell equator. In parallel, an inhibitory signal from the centrosomal microtubule asters prevents the accumulation of contractile ring components at the cell poles. Attempts to reveal the molecular basis of the inhibitory signal have been hindered by the absence of a robust assay. To identify the inhibitory signal from the centrosomal asters, we established a microscopy assay in the one-cell C. elegans embryo. Using this assay, we identified TPXL-1, the homologue of human TPX2, to be required for clearing the contractile ring component anillin from the cell poles. TPXL-1 is an established activator of aurora A kinase and localizes to the centrosome and the astral microtubules. In tpxl-1 mutant embryos the kinetochoore microtubules are extremely short resulting in a small mitotic spindle at anaphase onset. To determine whether the short mitotic spindle or TPXL-1 depletion itself causes defects in aster-based suppression, we increased spindle length in tpxl-1 mutants by depleting the kinetochore component hcp-4. Rescuing spindle length in tpxl-1 mutants did not rescue the defects in aster-based suppression, suggesting that TPXL-1 has a direct role in this process. Next we tested whether aster-based clearing of anillin depends on the ability of TPXL-1 to activate aurora A kinase. To this end we generated wild type (TPXL-1\textsuperscript{WT}) and Aurora A binding-defective (TPXL-1\textsuperscript{RD}) RNAi-resistant TPXL-1 transgenes. We find that in the absence of endogenous TPXL-1, TPXL-1\textsuperscript{WT} but not TPXL-1\textsuperscript{RD} supports clearing of anillin from the cell poles. Our findings suggest that aurora A kinase activation by TPXL-1 is essential for the removal of contractile ring components from the cell poles. In summary, we identified TPXL-1 and aurora A kinase as the first molecular components of the aster-based mechanism that inhibits the accumulation of contractile ring proteins at the cell poles during cytokinesis.
Phosphorylation of a linker region masks Cdc15’s F-BAR domain to regulate its membrane and protein binding capacity, oligomerization, and contractile ring localization.

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F-BAR proteins coordinate actin cytoskeleton rearrangements at membranes using membrane-binding F-BAR domains coupled to protein-binding or signaling domains. Appropriate location and timing of F-BAR protein activity is vital to ensure the success of many processes, such as endocytosis, cell migration, neurite outgrowth, and cytokinesis. Various regulatory mechanisms have been proposed, including phosphorylation and autoinhibition. Here we determined the molecular mechanism by which phosphorylation inhibits the S. pombe cytokinetic F-BAR protein Cdc15. Cdc15 binds the plasma membrane at the division site through an N-terminal F-BAR domain and scaffolds critical components of the cytokinetic ring (CR) through a C-terminal SH3 domain. An intrinsically disordered region between the F-BAR and SH3 domains is phosphorylated on >35 sites during interphase. Hyperphosphorylation correlates with Cdc15 localization to the cytoplasm and inability to bind protein partners or oligomerize, while dephosphorylation is required for Cdc15’s stable association with the plasma membrane, oligomerization, and interaction with CR proteins.

Our data indicate that phosphorylation of the disordered linker region enables direct interaction between the phosphorylated linker and the F-BAR domain, which obstructs membrane binding. First, intramolecular fluorescence resonance energy transfer (FRET) assays of Cdc15 with acceptor and donor fluorophores at opposite termini reveals that interphase Cdc15 exhibits a FRET signal indicative of a “closed” conformation with nearby N- and C-termini. Additionally, a phosphomutant with abolished phosphorylation on 27 sites loses FRET signal throughout the cell cycle, indicating the protein’s conformation is “open” and that conformation is controlled by phosphorylation. Next, the F-BAR domain binds directly to the phosphorylated linker; the SH3 domain is neither necessary nor sufficient for this interaction. Finally, when bound by the phosphorylated linker, Cdc15’s F-BAR domain cannot interact with synthetic membranes.

We are currently investigating if a phosphothreshold exists for Cdc15’s conformational change using a series of intermediate phosphomutants. Preliminary results indicate that increasing the number of dephosphorylated residues (i.e. by abolishing phosphorylation via mutation) promotes the open conformation. Ongoing studies will attempt to pinpoint this threshold using intramolecular FRET analysis of phosphomimetics and in vitro phosphorylation of Cdc15. Together our findings provide a detailed model of F-BAR protein regulation by phosphorylation-dependent intramolecular inhibition. Since many F-BAR proteins are phosphorylated and contain disordered regions, this may be a common mechanism of regulation within the F-BAR family.
P2916
Board Number: B197
CCDC11 is Essential for Cytokinesis and Cell-cell Adhesion via Regulation of RhoA Protein Stability.
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Spatiotemporal regulation of RhoA is critical for cytokinesis and cell-cell adhesion. Here we show that the heterotaxy gene CCDC11 is a novel regulator of cytokinesis and cell-cell adhesion. In multiple heterotaxy patients, mutations in CCDC11 were identified, and CCDC11 depletion in zebrafish and Xenopus lead to left-right (LR) patterning defects. LR patterning is established by monocilia function at the LR organizer (LRO). Interestingly, we found that CCDC11 knockdown led to multiple cilia per cell, due to cytokinesis failure. We show that loss of CCDC11 results in failure of contractile ring ingestion. In addition, CCDC11 loss leads to defec-tts in cell-cell adhesion with reduced junctional localization of key adhesion molecules like β-catenin and cadherin. Abnormalities in cytokinesis and cell-cell adhesion can occur with the misregulation of RhoA, and CCDC11 co-localizes with RhoA at the contractile ring during cytokinesis. We found that CCDC11 is essential for proper total RhoA protein levels (GDP and GTP bound forms), as total RhoA is reduced upon CCDC11 depletion. Notably, RhoA transcript levels are unchanged, suggesting that CCDC11 affects RhoA protein stability in the cell. Finally, using IP-Mass Spec of CCDC11, we have identified promising candidates that interact with CCDC11 and may help unravel how CCDC11 regulates RhoA at the molecular level.

P2917
Board Number: B198
The F-BAR domain of Cdc15 simultaneously scaffolds protein partners and binds membrane to promote cytokinesis.
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Cytokinesis is the final step in cell division in which two daughter cells are physically separated. Many eukaryotic cells, including the model organism *Schizosaccharomyces pombe*, divide using an actin- and myosin-based contractile ring (CR) that is physically linked to the plasma membrane. In *S. pombe*, the F-BAR protein Cdc15 binds the membrane at the division site through its F-BAR domain and recruits additional proteins that help to link the plasma membrane to the CR. While most protein partners bind Cdc15’s SH3 domain, Cdc15’s F-BAR domain binds directly to Cdc12, the sole essential cytokinetic formin that nucleates the actin of the CR. To better understand this novel F-BAR domain-protein interaction, the Cdc12 binding site on Cdc15 was mapped to a negatively charged surface patch on the F-BAR domain’s cytosolic face (opposite of the plasma membrane binding face). Consistent with membrane- and Cdc12-binding occurring on opposite faces of the F-BAR, Cdc15 F-BAR binds both membrane and a Cdc12 N-terminal peptide simultaneously in vitro. Surprisingly, although Cdc12 mutants that cannot bind Cdc15 display mild cytokinetic defects in cells, mutating the Cdc15 F-BAR patch (cdc15-3A) to disrupt Cdc12 interaction results in severe morphological and cytokinetic defects. *cdc15-3A* mutant cells have a branched and elongated morphology and are delayed in building and
constricting the CR. We found no evidence of structural defects in circular dichroism and electron microscopy studies of purified mutant F-BAR and observed that Cdc15-3A is produced at wild-type levels and localizes properly in cells. Thus we hypothesized that the Cdc15 F-BAR patch interacts with additional proteins. While many CR proteins are not affected by the cdc15-3A mutant, we find that CR localization of the conserved calcineurin phosphatase is eliminated. These results indicate that the cytosolic faces of F-BAR domains can serve as landing pads to facilitate complex cytoskeletal rearrangements and signaling events at the plasma membrane.

P2918
Board Number: B199
Modeling contractile ring dynamics in the Caenorhabditis elegans zygote.
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Cytokinesis is required for cell proliferation with failures potentially leading to aneuploidy and cancer. The actomyosin contractile ring is a dynamic structure responsible for driving cytokinesis. Proper cytokinesis requires assembly of contractile ring components at the cell equator, in the cortex. Actin, myosin, crosslinkers, and regulators then reorganize as the ring matures from a wide band to a tight cable, becoming contractile. Previous work has provided insight into the changes that occur in ring and cell structure. However, less is known about changes on the mesoscopic and molecular scales due to imaging limitations. Several models have given insight to mechanisms of contractility, including actin treadmilling and minifilament adaptive response to force-load, but the predictive power of these is limited by the simplification of myosin minifilament and actin dynamics. Herein we set out to further bridge the gap between the models of cytokinetic ring components and quantitative cell biology, by first establishing a model of contractility where myosin minifilaments are modeled. We developed this model of cytokinesis using the software Cytosim, which provides unparalleled resolution on a molecular scale. In our model actin treadmilling and shortening, both of which have been implicated in models of contractility, provide for a dynamic actin meshwork. We built upon previous models depicting the catch-slip bond nature of myosin II motors binding to actin filaments and built full minifilaments as multiple motor heads protruding off either end of 300nm rods. To further refine our model we queried the changes in protein concentration of several components of the ring including myosin, actin, anillin, and septin. Previous methods for visualizing cytokinesis result in uneven illumination and detection of structures across the cytokinetic ring, with the illumination plane orthogonal to the contractile ring plane; making quantitative analysis of the ring less precise. To this end we used custom chambers to position C. elegans zygotes such that the entire contractile ring forms in the illumination plane of a focused light sheet. This setup allowed us to quantify the contractile ring densities of components over the length of cytokinesis. Using this system, we generate contractile rings, on the scale of C. elegans zygote contractile rings, that exhibit protein density dynamics like those measured in our in vivo data and show a model for how these rings may contract. Our initial estimates for all major structural components yield simulated rings that close at biologically-relevant timescales, exert force that constricts a “membrane,” and form mesoscopic contractile foci.
P2919

Board Number: B200

Regulation of Abscission by Class 1 Rab11-Family-Interacting-Proteins.
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Introduction: Cytokinesis occurs at the end of mitosis/meiosis wherein the cytoplasms of daughter cells are separated in two giving rise to two independent daughter cells. Evidently, this process needs to be highly regulated as deficient abscission, (the final cutting process) can lead to diseases such as Lowe-Syndrome and cancer. Importantly, the modulation of both the lipid composition and the actin cytoskeleton at the site of ingression and abscission needs to be tightly regulated to ensure this process is completed correctly. We recently identified an axis involving recruitment of class 1 Rab11-Family-Interacting Proteins (Rab11FIPs), the small GTPase Rab35 and its effector OCRL, an inositol polyphosphate 5-phosphatase, to the intercellular bridge in order to modulate the lipid composition and promote abscission. Methods and Results: Using immunofluorescence/time-lapse microscopy, we found that Rab11FIP1 is recruited to the intercellular bridge (ICB). Moreover, siRNA-mediated Rab11FIP1 knockdown induces binucleation, abscission timing delay and accumulation of actin at the ICB. This phenotype is reminiscent of the loss of Rab35 or OCRL function. It can be rescued through the re-expression of the isoforms Rab11FIP1B and Rab11FIP1C but not Rab11FIP1A. Furthermore, the observed cytokinesis defects can also be rescued through the removal of over-accumulated actin in the ICB by treating cells with the actin-depolymerizing drug LatrunculinA. Finally, we detected an interaction between Rab11FIP1B and Rab35 in cells using CoIP assays. We were also able to show that both OCRL and Rab35 levels at the ICB decrease when cells are treated with siRab11FIP1. Conclusion and Relevance: Our work shows that Rab11FIP1 is involved in the spatio-temporal regulation of OCRL at ICB, which is necessary for the progression to abscission. Overall, this project will aid in better understanding cytokinesis, a fundamental cellular mechanism that impacts virtually every cellular process.

P2920

Board Number: B201

The recruitment and organization of ESCRT-III abscission machinery is spatiotemporally regulated by septins.
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Cytokinetic abscission is the final step of mitosis and involves the constriction and fission of membrane connecting the two daughter cells, which is mediated by the endocytic sorting complex required for transport (ESCRT). Assembly of the ESCRT machinery is spatially and temporally regulated, but the mechanisms underlying this regulation are not well understood. Septins are GTP-binding proteins that interact with the cytoskeleton and cell membranes. Septins are required for the completion of cytokinesis, but their roles in abscission are unclear. Here, we show that following cleavage furrow ingression, septins localize to distinct microtubule and membrane domains of the emerging midbody. High- and super-resolution microscopy show that in mitotic MDCK epithelial cells, SEPT2/6/7/9 complexes are organized as two membrane-bound rings that flank the center of the midbody (stem body). Time-lapse microscopy reveals that ESCRT-III (CHMP2A/CHMP4B) rings assemble flanking the stem body, in between and adjacent to the two septin rings. As CHMP2A/CHMP4B fluorescence increases, septin ring fluorescence decreases. Strikingly, septin ring disassembly is temporally coincident
with the remodeling and expansion of CHMP2A/CHMP4B rings into nested helical spirals that appear as cones at the site of membrane fission. Knock-down of SEPT9, which has been previously shown to disrupt abscission specifically, prevents the recruitment of an early ESCRT-III component (CHMP6) and results in ESCRT-III (CHMP4B/CHMP2A) ring disorganization and diminished recruitment. In later stages of cytokinesis, SEPT9 depletion abrogates the remodeling the ESCRT-III rings into cones and interferes with the completion of abscission. We have found that SEPT9 interacts with the early ESCRT component TSG101 (ESCRT-I) but SEPT9 depletion does not affect TSG101 recruitment and localization. We hypothesize that SEPT9-TSG101 interaction is critical for recruitment and proper assembly of ESCRT-III components. On-going work aims to investigate this hypothesis in order to gain a better understanding of how ESCRT-III assembly is spatiotemporally and functionally linked to septins.

P2921
Board Number: B202
Adaptor proteins are essential for Septin-mediated cytokinesis.
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Cytokinesis is a carefully orchestrated process which drives the division of the cytoplasm following the equal separation of genetic material into the daughter cells. The successive events leading to cytokinesis can be divided into four stages; appearance of the cleavage plane, ingestion of the cleavage furrow to the cell middle, formation of the intercellular microtubule-based bridge and the midbody, and the final separation of daughter cells, referred to as abscission. Despite extensive studies on cytokinesis, understanding of the key mechanisms that regulate abscission is still limited. We are particularly interested in the Septin family of GTP binding proteins, known to be essential for cytokinesis in yeast and animal systems. Nevertheless, the precise role of Septins in cytokinesis regulation has yet to be determined. We have demonstrated that SEPT9 is indispensable for the successful completion of cytokinesis, where SEPT9 deficiency causes defects in midbody abscission and constitutively necessitates the dividing cells to halt at the telophase stage. Septins have been identified as molecular scaffolds, as these proteins have protein-binding motifs that connect to protein-binding partners and recruit proteins to a particular site in the cell. In order to unravel the mechanism of cytokinesis in mammalian cells, we are interested in identifying the downstream signaling proteins that are involved in septin-mediated cell abscission. Thus, we hypothesized that SEPT9 may recruit specific factors that are critical for abscission to the midbody, hence regulating cell division. Vesicle transport to the abscission site, mediated by the exocyst complex, is necessary to proper completion of cytokinesis. SEPT9 has been previously identified as an essential factor for targeting exocyst complex to the midbody, providing mechanistic insight into the role of septins during abscission. Similar to scaffolds, adaptor proteins contain protein-binding motifs that mediate protein-protein interactions, thus influencing the signal transduction pathways. In confocal micrographs, we have recently observed adaptor proteins Cin85 and CD2AP, which are associated with the regulation of actin cytoskeleton, to be localized to the midbody in dividing cells. The western blot analysis revealed that the N-terminus of SEPT9 recruits these adaptor proteins to the abscission site, and we further revealed that the depletion of either of these two adaptor proteins results in abscission defects and causes the formation of a persistent connection between the two daughter cells. Interestingly, our novel collective data can shed light on the downstream signaling pathways regulating cytokinesis and may lead to potential therapeutic approaches for diseases caused by cell division failure.
Formation of the mitotic spindle lies at the heart of cell division, and it is necessary for correct and faithful chromosome segregation. Generation of the mitotic spindle is highly dynamic, and it requires tight spatio-temporal control of microtubule (MT) nucleation. Centrosome mediated microtubule nucleation has been well-characterized; however, other MT nucleation pathways exist. The process of branching MT nucleation requires the augmin complex and the γ-tubulin ring complex (γ-TuRC). The augmin complex recruits γ-TuRC to spindle MTs during mitosis and it has been proposed that branching MT nucleation contributes to spindle midzone MT-density and the efficiency of cytokinesis. To better understand how MT branching contributes to the efficiency of cytokinesis and to the establishment and maintenance of the cleavage furrow, we employed multi-color, live-cell TIRF microscopy to visualize MTs as well as augmin complex and γ-TuRC components during cytokinesis (C-phase) in Drosophila S2 cells. MT branching from equatorial astral microtubules is prevalent during late anaphase and possibly contributes to the robustness of cleavage furrow positioning by increasing the abundance of MT tips in the vicinity of the furrow. Live-cell imaging allowed us to observe and quantify numerous characteristics of MT branching nucleation during C-phase. Nascent branch sites on mother MTs exhibit an increase in α-tubulin fluorescence followed immediately by the appearance of a polymerizing daughter MT. Live-cell TIRF imaging of stable cell lines co-expressing α- and γ-tubulin, to identify bona-fide branch points, revealed that daughter MTs branch at an average angle of ~35°. The lag time between γ-tubulin binding to a mother MT and daughter MT birth is ~12 sec, and the mean life-time of daughter MTs is ~20 sec. Three-color TIRF imaging of DgtS (augmin complex subunit), γ-tubulin (γ-TuRC), and α-tubulin revealed that the augmin complex associates first with mother MTs and a subset of augmin complexes recruit γ-TuRC from the cytosol to nucleate daughter MTs. Upon daughter MT depolymerization γ-TuRC at the branch point dissociates from the mother MT while the augmin complex often remains and can support another round of branching MT nucleation. This work presents the first direct visualization in animal cells of branching MT nucleation and its central constituents in real-time.

In nonmuscle cells, actin is organized into either branched or linear networks, whose polymerization is mediated by the Arp2/3 and formin families of actin nucleators, respectively. Arp2/3 acts downstream of Rho family GTPases to form dendritic networks of actin that are crucial for lamellipodial cell protrusions. But while the role of Arp2/3 in two-dimensional cell motility is well characterized, less is known about its roles in large cells such as oocytes and embryonic blastomeres. During the early cleavages, Arp2/3 is recruited to the cortical cytoskeleton, where it is cleared from the cleavage furrow during cytokinesis. This removal of branched actin networks is critical for embryonic cleavage, as misregulation of Rac signaling blocks cytokinesis in an Arp2/3-dependent manner. In contrast, in highly
atypical cleavages such as those seen during oocyte meiosis, Arp2/3 is enriched in the polar body and is required for polar body extrusion. Arp2/3 may also play a role in polar lobe formation in mollusk embryos, where a transient constriction perpendicular to the cleavage plane sequesters vegetal cytoplasm during the first two embryonic cleavages. Arp2/3 is highly enriched at the vegetal pole prior to- and during polar lobe formation, with a clear boundary visible between the Arp2/3-enriched vegetal pole and the myosin II-mediated polar lobe constriction. Vegetal Arp2/3 is retained between the first and second cleavages, only to dissipate after the reabsorption of the polar lobe into the D blastomere. While it remains unclear how the polar lobe constriction is activated, Arp2/3 enrichment at the vegetal pole and initiation of lobe constriction is mechanistically distinct from cytokinesis, as Aurora inhibitors block cytokinesis without effecting lobe initiation. Together, these results suggest that while the selective exclusion of Arp2/3 may be important for the generation of cortical tension and contractility (e.g. contractile ring), the active recruitment of Arp2/3 may promote more extrusive forms of cytokinesis by affecting the local biophysical properties of the cortex.

P2924
Board Number: B205
Cytokinesis in plants involves exquisitely choreographed intracellular transport.
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Cell division in plant cells requires the deposition of a new cell wall between the two daughter cells. The assembly of this plate involves exquisitely coordinated movement of cargo-vesicles whose size is below the diffraction limited resolution of the optical microscope. We combined high spatial and temporal resolution confocal laser scanning microscopy with advanced image processing tools and fluorescence fluctuation methods to distinguish three distinct phases during the formation of the cell plate: Massive and highly choreographed delivery of vesicles first generates a disk shaped cell plate region at the equatorial plane of the cell. Subsequently, a primary rapid expansion phase is fuelled by intracellular transport mediated by the microtubular cytoskeleton followed by a secondary, slow expansion phase during which the extremity of the circular plate seeks contact with the mother wall and brings about the separation of the two portions of cytoplasm. Different effects of pharmacological inhibition emphasize the distinct nature of the assembly and expansion mechanisms characterizing these phases. The biphasic pattern is proposed to serve to optimize both efficiency and precision of cell plate formation.

P2925
Board Number: B206
Precise tuning of cortical contractility regulates mechanical equilibrium during cell division.
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The animal cell cortex comprises a thin network of actin filaments underneath the plasma membrane. Contractile force generation by myosin II (MII) in the cortex drives shape changes during cell division, cell migration and tissue morphogenesis. During the cytokinetic (C)- phase of cell division, large
contractile forces and cortical remodeling are required at the equatorial cortical network to drive furrow ingression. Outward pressures created by furrow ingression must be balanced by MII contractility at the polar cortical network. The roles for the two MII isoforms, MIIA and MIIB, in the establishment of a mechanical equilibrium between these two cortical networks are not understood. We found MIIA depletion resulted in slower cleavage furrow ingression, and a loss of both MIIA and MIIB filaments in the furrow, with no changes at the polar cortex. MIIB depletion, in turn, resulted in intense blebbing at the polar cortex, with no changes in furrow ingression. We predicted the cortex of MIIB depleted cells should be softer; however, AFM measurements revealed no changes in stiffness. This suggested molecular scale behaviors may be driving cortical mechanics, which may not be reflected in macroscopic measurements such as cell stiffness. We therefore sought to develop a robust coarse-grained mathematical description of cortical behavior, incorporating experimentally measured kinetic parameters. Measurement of localization patterns and lifetimes of MII isoforms during C-phase revealed MIIA had faster turnover and more transient kinetics compared to MIIB. Modeling the cortex based on active gel theory surprisingly revealed no significant differences in outward pressure and cortical tension in the two knockdown conditions. Instead, our model predicted differences in the duty ratios of MII isoforms and total contractility must account for the observed differences in cortical stability in the two knockdown conditions. We verified these predictions using both MII chimeras and biophysical assays. We further found Rho kinase and Myosin Light Chain Kinase regulated the recruitment and turnover of MII isoforms, respectively. Our working model posits that in the unperturbed state, competition between MIIA and MIIB regulates cortical turnover, while competition between MLCK and ROCK regulates the turnover of MII itself. Depletion of either isoform leads to compensation at the polar cortex, driving the cell to extremes of contractility, with MIIA and MIIB depletion leading to hypo- and hyper-contractility, respectively, leading to cytokinetic failure. Therefore, our data support a model where an intermediate level of contractility leads to efficient cytokinesis, allowing MIIA driven ingression at the equator balanced by MIIB driven tension generation at the poles.

P2926

Board Number: B207

An optogenetic tool for studying asymmetric cell division.

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Asymmetric cell division (ACD) is an evolutionarily conserved mechanism used by stem and progenitor cells to form differentiating progeny while maintaining a self-renewed stem/progenitor cell. Drosophila neuroblasts (NBs), the precursors of the fly’s central nervous system, provide a good model system to study ACD. Recently it was shown that two different pathways – the spindle-dependent and polarity-dependent pathways - co-regulate cleavage furrow positioning during ACD of NBs. The polarity pathway initiates non-muscle Myosin II (Myosin; Myo, hereafter) relocalization on the apical pole, whereas spindle-dependent cues are necessary for Myo clearing on the basal NB cortex. This spatiotemporal regulation of Myo allows NBs to generate a large apical neuroblast and a small differentiating ganglion mother cell (GMC) on the basal side. Spindle cues are delivered to the cellular cortex by the centraspindlin complex, composed of the kinesin-like Pavarotti (Pav) and the Rho family GAP Tumbleweed (Tum). It is assumed that spindle cues activate RhoGEF, which will in turn activate RhoA and will ultimately lead to Myo phosphorylation and therefore, activation. In order to understand the role of spindle cues, their downstream signaling cascade and their link to polarity, we created i) photorecruitable versions of Tum (LOVTum),
corresponding to the different structural domains of Tum (Pav binding domain, RhoGEF binding domain, RhoGAP domain), ii) a photorecruitable version of active RhoGEF (LOVGEF), iii) a photorecruitable version of active RhoA (LOVRhoA) and iv) a photorecruitable version of the polarity protein Miranda (LOVMira).

We sought to understand the cortical response to the recruitment of local spindle cues from the different LOVTum versions and their ability to create furrowing during the cell cycle. We found that physical cell size can be perturbed by local LOVTum recruitment and also found a spatiotemporal dependency in the ability of local LOVTum recruitment to create furrows. Additionally, we observed similar responses with LOVGEF and LOVRhoA, and found that apical recruitment of LOVMira induced ectopic furrowing in telophase.

P2927
Board Number: B208
Study of cisplatin induced ROS using C. elegans as a post-mitotic cell model.
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Cisplatin is a platinum based chemotherapeutic widely used for cancer treatment. However, tumor resistance to cisplatin limits its use. The ability to reverse resistance could significantly improve cisplatin therapy in patients. To enhance the efficiency of cisplatin mediated death more information on the molecular mechanisms and targets is needed. We study the targeting of ASNA-1 as a way to increase cisplatin sensitivity. Knockdown of the human ASNA1 causes cisplatin sensitivity in melanoma and ovarian tumour cell lines and knockdown in C.elegans has the same effect. Cisplatin mediates killing via effects on nuclear and non-nuclear targets in mitotic and post-mitotic cells. Heightened ER stress and induction of ROS levels are among the proposed killing mechanisms. Most studies of cisplatin in mammalian cells are performed in mitotic cells and a model for the effects of cisplatin on post-mitotic tissues is needed since these cells comprise a significant part of every tumor. Somatic cells in adult C.elegans are exclusively post-mitotic and provide a useful model to study the phenomenon. In normal conditions mammalian cells control reactive oxygen species (ROS) levels by protection systems. Cancer cells exhibit greater ROS than normal cells and cisplatin mediated death is thought to act in part by increasing ROS levels that consequently trigger cell death or DNA damage. How important that role is in post-mitotic cells remains an open question.

We found robust activation of oxidative stress response genes and ROS mediated damage upon cisplatin exposure. Moreover cisplatin induced lethality that could be blunted by antioxidant treatment. The DAF-16 and SKN-1/NRF2 pathways mediate oxidative defence in worms, but only the SKN-1 pathway appears to be important in the process. Mutants in upstream activators of SKN-1 are sensitive implying that oxidative stress regulation is important for cisplatin mediated killing. Curiously, the skn-1(zu67) mutants are much more resistant than mutants of the upstream kinases. Moreover, DAF-16 mutants which express high levels of asna-1 mRNA, are resistant to cisplatin even in high dosages. Survival of daf-16 mutants could be dependent on overexpression of asna-1. However mutants with high levels of ROS do not display increased sensitivity. Unexpectedly, in the asna-1 supersensitive mutants oxidative defence response appears to be intact. Taken together this indicates that oxidative stress defence is necessary but not sufficient for resistance of post-mitotic cells to cisplatin.
P2928
Board Number: B209
CedA mediated mechanism of cell division in E. coli under chromosomal over replication condition.
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In Escherichia coli, the process of cell division and chromosome replication occurs with high precision, coordination and regulation. This systemic and highly coordinated process is imprecise in DnaAcos cell, a cold sensitive mutant of E. coli. When these cells are grown at 30°C, chromosomal over-replication occurs due to multiple initiation signals at origin of replication site resulting into their filamentous morphology. Cell division in these cells is induced by over expression of cell division activator protein known as CedA. CedA is a 12 KDa, 87 amino acid long protein which is structurally homologous to other double stranded DNA binding proteins and known to interact with RNA polymerase multisubunit enzyme complex. The preferential DNA binding sites of CedA have been reported by Abe et al. in 2015. The regulatory action of CedA that initiates cell division without affecting the chromosomal over-replication in DnaAcos cells follows an unknown mechanism yet to be deciphered. In our present study, CedA is cloned, expressed, purified and characterized by mass spectrometric analysis (MALDI ToF/MS). CedA interacting proteins were identified by pull down assay using purified recombinant His6-tagged CedA. We identified nine CedA-binding proteins (CBPs) viz. PDHA1, RL2, DNAK, LPP, rpoB, G6PD, GLMS, RL3 and YBCJ, characterized by mass spectrometric analysis (MALDI ToF/MS and ESI nLC/MS). These CBPs are mainly associated with energy metabolic pathways and transcriptional regulation in E. coli suggesting multifaceted role of CedA in cell cycle regulation. The interaction of CedA with RNA polymerase subunit β (rpoB), one of the identified CBPs, is of major significance based on previous reports. The interplay between CedA, rpoB and DNA may provide a clue to decipher the mechanism behind CedA mediated cell division in E. coli. DNA sequence to which CedA preferentially binds is custom synthesized to study its interaction with CedA and rpoB by fluorescence spectroscopy and BioLayer Interferometry. The binding constants of these interactions clearly indicate that rpoB binds to DNA with higher affinity as compared to CedA. The biophysical results are well supported by in silico docking analysis that shows more intermolecular hydrogen bonds formation in rpoB-DNA complex as compared to CedA-DNA complex. Based on our findings we hypothesize a mechanism in which CedA recruit rpoB to specific DNA site(s), that initiates transcription of key cell division regulatory elements in E. coli cells under chromosomal over-replication conditions.

P2929
Board Number: B210
The regulation of Z ring dynamics in bacterial cytokinesis.
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In bacteria, cell division is carried out by the divisome, a set of proteins that localize to midcell and are required for cytokinesis. Decades of research have elucidated the identities of these proteins, including
FtsZ, a bacterial homolog of tubulin, as well as enzymes that synthesize cell wall at the division site. Like eukaryotic cells, filaments of FtsZ assemble into a ring structure, called the Z ring, that constrains as the cell divides. Unlike in eukaryotes, however, there are no known molecular motors in the division complex. The molecular mechanisms that underlie force generation and cytokinesis in bacteria have thus remained a mystery. Recently, it has become clear that protein dynamics in the divisome play a key role in the cytokinesis process. Using Total Internal Reflection Fluorescence (TIRF) microscopy, we have shown that FtsZ filaments treadmill around the division site, and elucidated the significance of these dynamics in bacterial cell division. We conclude that the Z ring consists of treadmill FtsZ filaments, that these dynamics power the motion of the essential cell wall synthesis enzyme Ppb2B, and that FtsZ treadmillig is required to distribute cell wall synthesis at the division site as an essential part of the bacterial cytokinesis process. Now, we are investigating whether and how FtsZ filament assembly and dynamics are regulated. Given that artificial perturbations to FtsZ’s treadmill dynamics can have dramatic effects on cytokinesis in bacteria, we reasoned that these dynamics might be regulated by the cell to ensure proper division. Additionally, there are proteins in the divisome whose proposed function is to bind to FtsZ filaments and alter their stability, which could alter both filament structure and treadmill dynamics. First, we introduce single molecule lifetime measurements as a quantitative metric of FtsZ’s treadmill dynamics which is independent of both spatial resolution and filament density, allowing us to sensitively measure treadmill in the Z ring even as it constricts. We combine these measurements with 3D Structured Illumination Microscopy (3D-SIM) imaging to probe the structure of the Z ring with high spatial resolution. Finally, we use these assays to ask whether and how proteins that bind to FtsZ control filament structure and stability during the bacterial cell cycle.

P2930

Board Number: B211

Screening gene deletion collection of Cryptococcus neoformans to elucidate the role of septin proteins in cytokinesis.

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Cryptococcus neoformans is a basidiomycetous yeast, able to infect immunocompromised individuals due to its ability to grow at host temperature. Septins are conserved filament forming GTP-binding proteins that assemble as a complex at the site of cell division and contribute to cytokinesis. In C. neoformans, septins are not required for cytokinesis at 24°C but are essential at the host temperature of 37°C. Moreover, septins become essential at 24°C when the calcium-dependent protein phosphatase calcineurin is inhibited. While the role of septins in cytokinesis is conserved in most eukaryotes, their exact contribution to this process remains elusive. Additionally, mechanisms of cytokinesis in C. neoformans are largely unknown. To define the roles of the septin complex and to identify other proteins involved in cytokinesis in C. neoformans, we performed a screen for genes necessary for growth at 37°C based on a collection of ~4,500 strains containing single deletions of non-essential genes. We searched for strains that at 37°C or in the presence of calcineurin inhibitor exhibited elongated bud morphology, a phenotype characteristic of septin mutants and indicative of cytokinesis failure. Our screen revealed 12 candidate genes, including 2 that were previously associated with cytokinesis and/or stress response (non-muscle myosin II and chitin synthase regulator), and 2 that encode hypothetical proteins. We tested candidate genes identified in the screen for synthetic interaction with the septin deletion to probe for the potential involvement in septin pathway. In addition, we examined if the candidate genes are involved in septin complex assembly and dynamics by introducing fluorescently
tagged septin to the identified deletion strains. Our research provides a better understanding of cytokinesis in C. neoformans and may lead to identification of novel antifungal drug targets against cryptococcosis.

**Kinetochoore Assembly and Functions 3**

**P2931**

**Board Number: B212**

Molecular delineation of CENP-T deposition in mammalian cell division cycle.

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The landmark determinant of chromosome inheritance, the centromere, is specified in many eukaryotes by an epigenetic mark. A two-step mechanism has been proposed to account for the epigenetic specification of centromere identity and function centered on CENP-A. Our early study demonstrated that define a novel molecular mechanism underlying the temporal regulation of CENP-A incorporation into the centromere by accurate Mis18β-HJURP interaction (Wang et al., 2014. J. Biol. Chem. 289, 8326-36). However, it has remained elusive as to other histone-fold containing proteins such as CENP-S/X and CENP-T/W in centromere specification and plasticity control. Here, we show that HJURP interacts with CENP-T and HJURP knock-out abrogates centromere localization of CENP-T. The CENP-T-interacting domain was mapped to C-terminal domain of HJURP which physically binds to the histone-fold of CENP-T. Interestingly, expression of HJURP-deficient CENP-T mutant perturbs accurate chromosome segregation, suggesting that precise loading of CENP-T to centromere is essential for achieving metaphase alignment. Currently, we are using super-resolution imaging to conduct precise localization of CENP-A and CENP-T and assess their temporal order of assembly in cell division cycle.

**P2932**

**Board Number: B213**

Microtubule sliding in the bridging fiber pushes kinetochore fibers apart to segregate chromosomes in human cells.

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During cell division, microtubules of the mitotic spindle segregate chromosomes by exerting forces on kinetochores, protein complexes on the chromosomes. The central question is what forces drive chromosome segregation. The current model for anaphase in human cells includes shortening of kinetochore fibers and separation of spindle poles. Both processes require kinetochores to be linked with the poles. Here we show, by combining laser ablation, photoactivation and theoretical modeling, that kinetochores can separate without any attachment to one spindle pole. This separation requires the bridging fiber, which connects sister kinetochore fibers. The number of bridging fibers per spindle correlates with the variable chromosome numbers in HeLa cells, suggesting a one-to-one relationship. Bridging microtubules in intact spindles slide apart together with kinetochore fibers, indicating strong crosslinks between them. We conclude that sliding of microtubules in the bridging fibers drives pole separation and pushes kinetochore fibers poleward by the friction of passive crosslinks between these
fibers. Thus, sliding in the bridging fiber works together with the shortening of kinetochore fibers to segregate chromosomes.

P2933
Board Number: B214
Multimerization of NDC80 kinetochore complexes is essential for efficient microtubule force-coupling.
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During mitosis, chromosomes connect to microtubules in the mitotic spindle through kinetochores, multi-protein complexes that self-assemble on the centromeric chromatin. The key connection is formed by the microtubule-binding NDC80 complex (NDC80C). Microtubule-kinetochore interfaces across species contain multiple copies of NDC80C, but the relevance of this multimerization for the kinetochore’s ability to follow growing and shrinking microtubules, and support faithful chromosome segregation, remains elusive. We reconstituted outer kinetochore modules with a precisely controllable number of NDC80 complexes and characterized their interaction with microtubules at a single-microtubule level. Step-wise addition of NDC80 complexes exponentially increased microtubule-binding times and the combination of three NDC80 complexes rendered a kinetochore unit able to follow depolymerizing microtubule ends. We used optical tweezers to show that such trivalent NDC80C assemblies stalled and rescued microtubule depolymerization in a force-dependent manner. Our measurements allowed us to distinguish NDC80C binding to depolymerizing microtubule ends from NDC80C binding to the microtubule lattice or to the stable ends. This revealed that the NDC80C-induced stalling of microtubule depolymerization is mediated by a unique connection that forms between the oligomerized NDC80C and the end of a shrinking microtubule. We thus conclude that the multimerization of NDC80C results in kinetochore modules that can hold on to depolymerizing microtubules and modulate microtubule dynamics. Our findings provide a mechanistic explanation for the importance of NDC80 multimerization in vivo.

P2934
Board Number: B215
Characterizing the role of BuGZ in mitosis.
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BuGZ is a mitotic protein recently identified through two independent RNAi screens: one targeting putative human transcription factors to isolate essential regulators of glioblastoma stem cell expansion (Toledo et al., 2014. Dev Cell 28, 282-294) and one targeting spindle matrix proteins in mouse embryonic stem cells (Jiang et al., 2014. Dev Cell 28, 268-281). We have previously shown that BuGZ is a kinetochore component that binds to and stabilizes the mitotic protein Bub3. Bub3 plays a key role in spindle assembly checkpoint signaling: it is responsible for recruiting BubR1 to kinetochores, and together with Mad2 and Cdc20, this group of proteins forms the Mitotic Checkpoint Complex (MCC), which binds to and inhibits the Anaphase Promoting Complex in the presence of unattached kinetochores. Additionally, Bub3 is responsible for recruiting Bub1 to kinetochores, which has roles in both the checkpoint and in regulating kinetochore-microtubule attachment and chromosome alignment. BuGZ binds Bub3 through a highly conserved GLEBS domain, which is found in the other mitotic Bub3
binding partners, BubR1 and Bub1. Inhibition of BuGZ compromises chromosome alignment and results in defects in chromosome segregation, but which functions of BuGZ contribute to this process currently remain unclear. In the current study, we have investigated how BuGZ and Bub3 are loaded onto kinetochores and the mechanism by which BuGZ contributes to chromosome alignment. Using a series of mutants, we have mapped the domain requirements for BuGZ kinetochore recruitment and determined how BuGZ kinetochore-association affects the recruitment of BubR1 and Bub1. We are currently determining which domains of BuGZ are required to rescue the mitotic defects observed upon depletion of the full length BuGZ protein, including increased inter-kinetochore distances, a weakened spindle assembly checkpoint, and chromosome alignment defects. In addition, we have carried out size exclusion chromatography to analyze BuGZ- and Bub3- containing complexes in different cell cycle phases and our results point to a model in which BuGZ stabilizes Bub3 protein levels and facilitates Bub3 loading onto kinetochores in early mitosis, which, in turn, facilitates Bub1 and BubR1 kinetochore loading.

P2935
Board Number: B216
Distinct surfaces of the microtubule-binding domain of the Ska1 kinetochore complex facilitate microtubule tip-tracking.
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Interactions between kinetochore complexes and dynamic microtubule plus ends are crucial for proper chromosome segregation during mitosis. The spindle- and kinetochore-associated multi-subunit Ska1 complex is essential for linking kinetochores to microtubules in mammalian cells. Although prior work has analyzed the association of the Ska1 complex with the ends of depolymerizing microtubules in vitro, its behavior on elongating microtubules has not been determined. To investigate whether Ska1 can autonomously track the polymerizing microtubule ends, we have used an in vitro assay with dynamic microtubules and two-color TIRF imaging. We observed binding and diffusion of GFP-labeled Ska1 complex on both the GDP-containing and GMPCPP microtubule lattices. Ska1 complex was enriched on the GMPCPP microtubules, suggesting a higher affinity of Ska1 for the GTP-containing tubulin structures as are found at the tip of a polymerizing microtubule. Indeed, we now demonstrate that the Ska1 complex can autonomously track the polymerizing microtubule ends in vitro, reinforcing the model that it helps mitotic chromosomes to associate processively with dynamic microtubule ends. This tip-tracking activity for the Ska1 complex is likely to be driven by a combination of affinity and diffusion-based mechanisms. In addition to binding to the microtubule lattice and curved protofilament structures, we demonstrate that Ska1 binds to soluble tubulin and can promote its oligomerization. We hypothesize that Ska1 interacts with diverse tubulin structures in multiple modes via several interaction surfaces on the microtubule binding domain of Ska1, and that this multimodal binding may contribute to Ska1 microtubule tip tracking. To test these hypotheses, we generated mutants on distinct surfaces of the Ska1 microtubule binding domain that disrupt binding to soluble tubulin, but do not prevent its binding to the microtubule walls. Interestingly, all these mutants failed to track the ends of elongating microtubules. Thus, presence of multiple positively charged tubulin-binding patches in the Ska1 microtubule binding domain is essential for its tip-tracking activity. In summary, our work establishes a molecular requirements for Ska1 microtubule-dependent functions in mammalian cells by

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demonstrating that interactions between multiple surfaces of Ska1 and tubulin substrates underlie its ability to track the dynamic microtubule end.

P2936
Board Number: B217
A role of Kinesin-5 in controlling Ndc80 functions at kinetochore.
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In eukaryotes, chromosome segregation machinery mediated by the dynamicity of spindle microtubules (MTs) is an essential and highly conserved process. There are two kinesin-5 family members called Kip1 and Cin8, in budding yeast. Both Kip1 and Cin8 are responsible for crosslinking antiparallel MTs and pushing spindle poles apart. We found the kinetochore localization of both Kip1 and Cin8 was greatest at metaphase and significantly reduced in anaphase. In addition, Kip1 and Cin8 localized close to the MT binding domains of the kinetochore protein Ndc80. The biochemical experiments found that Cin8 directly interacts with Dam1 complex. We confirmed that Dam1 complex is essential for the kinetochore localization of Cin8 in vivo. Depletion of either Kip1 or Cin8 induced a major delay in mitotic progression. In addition, it also induced significant reduction of tension at the MT binding domains of Ndc80 measured by a tension biosensor without changing the stoichiometry of core-kinetochore proteins. These phenotypes observed in kinesin-5 depletion were similar to observed phenotypes when the N-terminal tail was deleted from Ndc80. Interestingly, there was no additional phenotype when either Kip1 or Cin8 was depleted in Ndc80 tailless mutants. These results indicate that the mitotic delay induced by either kinesin-5 deletion or Ndc80 tailless lies within the same pathway. To achieve proper force production at the MT binding domains of Ndc80, the N-terminal tail of Ndc80 needs to be de-phosphorylated properly by PP1 (Protein Phosphatase 1). Biochemical studies showed that Cin8 directly binds PP1, and Cin8 mutated at PP1 binding sites induces a prolonged mitosis like Cin8 deletion. These results indicate that yeast kinesin-5 controls the MT binding affinity of Ndc80 for proper mitotic progression through the interaction with PP1 and Dam1 complex.

P2937
Board Number: B218
Centromeric Protein Dynamics Change in Response to Physiological Changes of Early Embryogenesis in C. elegans.
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Centromeres in eukaryotic cells carry out fundamental functions during all mitotic cell divisions; they are required for kinetochore assembly, microtubule attachment, and chromosome segregation. The histone-3 variant CENtromeric-Protein-A (CENP-A) is required for establishing the centromere. CENP-A is a relatively unique mitotic protein, as its subcellular localization does not change throughout the cell cycle. Embryogenesis requires millions of cell divisions in vertebrates, however, the dynamics of CENP-A in early development are not known. Here we report the dynamic behavior of CENP-A during early embryogenesis of C. elegans. In order to understand if CENP-A (HCP-3 in C. elegans) dynamics are consistent during the early cell cycles of the C. elegans embryo like they are in cell cultures, we temporally aligned and measured 4D images of early embryos whose only copy of CENP-A is labeled
with GFP. This allowed us to create representative profiles of HCP-3 dynamics throughout each early cell cycle, and throughout entire cell lineages; lineages are fixed in C. elegans as opposed to chaotic development in other organisms. Using this analysis method, we found that HCP-3 levels within each nucleus do not return to their original pre-mitotic levels in response to mitotic dilution during early development, in opposition to that found in somatic growth. In fact, the quantity of HCP-3 loaded onto chromatin each subsequent cell cycle decreases throughout early embryogenesis. We also find that HCP-3 levels rise nearer the end of the cell cycle, occurring after the rise observed for canonical histones during the S-Phase of each cell cycle. In order to resolve these two observations, we assessed protein turnover rate and stability by measuring fluorescence recovery after photobleaching (FRAP) in early embryos. From these data, we were able to ascertain how a cell lineage is able to modulate its amount of nuclear HCP-3 in each cell cycle by changing protein stability and nuclear import levels.

P2938

Board Number: B219

Mad1 promotes tumor progression through destabilization of p53.

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Mitotic arrest deficient 1 (Mad1) plays a well-characterized role in the mitotic checkpoint. However, interphase roles of Mad1 that do not impact mitotic checkpoint function are still largely uncharacterized. We have found that overexpression of Mad1, which is common in human breast cancer, results in decreased protein stability of the tumor suppressor p53 in multiple cell types. To gain mechanistic insight into this process, we first determined whether increasing expression of Mad1 alters its localization. Upregulated Mad1 localizes to puncta within interphase nuclei in both breast cancer tissue and cultured cells. We found that upregulated Mad1 localizes to ProMyelocytic Leukemia Nuclear Bodies (PML NBs), which have been implicated in the stabilization of p53. Immunoprecipitation results indicate that Mad1 and PML interact and that the C-terminal domain (CTD) of Mad1 and the N-terminal domain of PML are required for this interaction. Within the CTD of Mad1, the SUMO-Interacting Motif (SIM) is critical for the localization of Mad1 to PML NBs. MDM2 is an E3 ubiquitin ligase that targets p53 for degradation. In response to DNA damage, PML sequesters Mdm2 to the nucleolus, which stabilizes p53. In cells with elevated levels of Mad1, Mad1 interrupts the interaction between PML and MDM2. Mad1, rather than MDM2, is sequestered to the nucleolus after DNA damage, suggesting that Mad1 displaces MDM2 from PML. The displaced MDM2 is no longer sequestered in the nucleolus, freeing it to ubiquitinate p53, resulting in p53 destabilization. Upregulation of Mad1 accelerates growth of orthotopic mammary tumors, which show decreased levels of p53 and its downstream effector p21. In addition to causing a low rate of chromosome missegregation, our results show an unexpected interphase role for Mad1 in promoting tumor formation and progression by destabilizing p53.

P2939

Board Number: B220

Identification of drivers of Chromosome Instability in Breast Tumors.

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Numerical Chromosomal instability (CIN) is a form of genomic instability characterized by an increased rate of chromosome missegregation. It is unclear how cancer cells acquire the CIN phenotype. We have
developed a simple computational method that measured the degree of aneuploidy or structural rearrangements of large chromosome regions of >500 human breast tumors from TCGA data to identify the drivers of CIN. RNA analysis demonstrates that aneuploid tumors overexpressed regulators of mitosis, including proteins in the centromere signaling network and key kinetochore proteins. Aneuploid tumors also overexpress regulators of the DREAM complex that regulates the transcription of mitotic genes. Overexpression of the DREAM regulators E2F1, MYBL2 and FOXM1 was sufficient to increase the rate of lagging anaphase chromosomes in a nontransformed vertebrate epithelial tissue validating the results of our computational analysis of human tumors and connecting our aneuploidy measurements to CIN. Highly aneuploid human breast tumors were also highly enriched in TP53 mutations. There was strong co-association of DREAM regulators with TP53 mutations in human breast tumors suggesting that tumors require both events to gain evolutionary fitness. Our data suggest a two-step model for the generation of CIN in breast tumors. First, the overexpression of DREAM complex regulators overexpresses key mitotic regulators to generate anaphase lagging chromatids at higher rates. Second, loss of p53 function allows cells that have missegregated chromosomes to escape the resulting induction of senescence or cell death allowing cells with aneuploidy to undergo selection within the tumor.

P2940
Board Number: B221
Spatial architecture of the biochemical pathways that recruit the Ndc80 complex in human kinetochores.
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The human kinetochore is a multi-protein machine that binds ~20 spindle microtubules (MTs) to drive chromosome segregation during cell division. Although the biochemical bases of the functions that the kinetochore performs to segregate chromosomes are well-understood, defining the underlying mechanisms has proven to be challenging. This is because the kinetochore is highly complex in composition (hundreds of copies of eight protein components), and large in size (a ~200 nm diameter disk-shaped surface). To understand the molecular mechanisms underlying kinetochore functions, its spatial architecture must be defined. Specifically, the nanoscale architecture of its protein components relative to the 25 nm diameter of a MT, and sub-micron scale organization of ~20 such MT attachments over its ~200 nm wide surface must be determined to reveal how kinetochore components interact with one another, with regulatory proteins, and with the MT lattice. Here we elucidate the nanoscale architecture of the human kinetochore, using FRET to reveal the distribution of its components both around the circumference of the MT (by measuring FRET between multiple copies of the same protein) and along its axis (by measuring FRET between two different proteins). We initiated this analysis with the MT-binding Ndc80 complex, the linchpin of kinetochore-MT attachment. FRET measurements along Ndc80’s 50 nm length reveal its configuration relative to the MT lattice. We find that the MT-binding domains of adjacent Ndc80 molecules are clustered, whereas the centromere-anchored ends are more sparsely distributed. Furthermore, Ndc80 molecules are staggered along the MT lattice by 15-30 nm. Surprisingly, in unattached kinetochores, this Ndc80 architecture remains relatively unchanged except at its MT-binding domains, which separate from one another. We next probed the spatial architecture of the two parallel Ndc80 recruitment pathways initiated by the centromeric proteins CENP-C and CENP-T. Surprisingly, we detect little to no FRET between the Ndc80 recruitment domains of CENP-C or CENP-T, nor between Mis12 complexes, which link Ndc80 to these
recruitment domains. These measurements reveal that Ndc80 recruitment pathways are spatially separated by ≥ 10 nm, in stark contrast to budding yeast kinetochores. Interestingly, using RNAi-mediated protein knockdown of either CENP-C or CENP-T, we find that FRET at the MT-binding end of Ndc80 is not abolished. Thus, despite being spatially separated, Ndc80 molecules recruited by the two parallel pathways converge at the MT lattice. Our data elucidate the spatial architecture of the two biochemical pathways that organize the human kinetochore, and establish a foundation for defining a complete picture of its MT-binding machinery.

P2941
Board Number: B222
HPV oncoproteins cause specific types of chromosomal instability in head and neck cancer.
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Aneuploidy, a karyotype that differs from a multiple of the haploid, is a hallmark of cancer. Aneuploidy can be caused by an ongoing rate of chromosome missegregation during mitosis, known as whole chromosomal instability (CIN). Work from our lab and others has shown that while low rates of CIN promote tumorigenesis, high levels of CIN can be tumor suppressive. Human papillomavirus (HPV), specifically HPV subtype 16, is a growing cause of head and neck cancer worldwide. In cervical cancer, HPV is associated with CIN, which has been attributed largely to the expression of its E6 and E7 oncoproteins. HPV+ head and neck cancer patients show improved responses to radiation therapy than HPV- head and neck patients, potentially due to an increased basal rate of CIN. Here, we tested the ability of HPV E6, E7, or E6+E7 expression to induce CIN in normal oral keratinocytes (NOKs). Expression of E6 alone robustly induced misaligned chromosomes and multipolar spindles with supernumerary centrosomes, while E7 expression alone produced more moderate increases. Combined expression of E6 and E7 exacerbated the phenotypes observed in NOKs expressing E6 alone. Interestingly, HPV+ patient-derived xenograft (PDX) models of head and neck squamous cell carcinoma showed less evidence of mitotic defects than NOKs expressing E6 alone. A subset of HPV+ PDX tumors showed an increase in misaligned chromosomes when compared to HPV- tumors, but no increase in lagging chromosomes was observed. Together, these data suggest HPV infection causes an incompletely penetrant increase in specific types of CIN in head and neck cancer.

P2942
Board Number: B223
Ndc80 complex as an intrinsic regulator of molecular friction at mitotic kinetochores.
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The microtubule wall–binding Ndc80 complex serves as the primary molecular glue connecting mitotic kinetochores to spindle microtubules. During metaphase chromosome oscillations, Ndc80 is thought to form mobile diffusive bonds that enable sister kinetochores to glide repeatedly toward the plus- and minus-ends of the microtubules without detaching. To investigate the biophysical underpinning of this striking behavior, we adopted a highly sensitive dual-trap three-bead assay employing ultrafast force-
clamp spectroscopy in vitro. Microtubule-wall gliding of single Ndc80 molecules was examined under controlled forces, imitating the forces and motions the Ndc80 experiences during metaphase. Unexpectedly, we find that the character of Ndc80 motility and its velocity depend strongly on the direction of force. When pulled toward the microtubule minus-end, Ndc80 obeys the typical prediction for a diffusive MAP: it moves smoothly with little molecular friction, in correspondence with the relatively fast rate of its unloaded diffusion. However, Ndc80 strongly resists forces pulling it toward the microtubule plus-end, exhibiting frequent pauses that are indicative of catch-bond-like behavior. Strikingly, the strongly-bound Ndc80 retains the ability to glide continuously on the microtubule lattice, translocating almost 10-times slower than under the same force acting in the opposite direction. These results strongly suggest that Ndc80 has the ability to serve as an intrinsic direction-specific regulator of molecular friction at human kinetochore. We propose that low internal friction at the force-generating, leading sister kinetochore maximizes the useful work available from the microtubule depolymerization motor to transport the chromosome. The increased friction at the trailing kinetochore should help to prevent its slipping from the microtubule plus-ends, as this kinetochore is dragged passively in this direction by its sister.

P2943

Board Number: B224

Investigating in vivo variation in the strength of the spindle assembly checkpoint.

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The spindle assembly checkpoint (SAC) is a highly conserved mitotic regulator that prevents chromosome segregation errors during cell division, thereby maintaining genome stability. Variation in the strength of the SAC (i.e. the ability to delay mitotic progression in the presence of spindle assembly defects) is a widespread, but poorly understood feature of checkpoint regulation. Using in situ live imaging, we have shown that C. elegans germline stem cells have a stronger SAC than somatic embryonic blastomeres, providing an excellent opportunity to examine variability in SAC activity in vivo and to uncover cell fate-specific adaptations. Here we show that the embryonic progenitors of the adult germline, the P lineage blastomeres, also display a stronger SAC, relative to their somatic peers. Using both Nocodazole treatment of permeabilized embryos and a novel, inducible monopolar spindle assay, we find that cells in the germline lineage exhibit longer mitotic delays than cells with a somatic fate. These differences are entirely dependent on an intact checkpoint and only partially attributable to differences in cell size, which has recently been reported to impact SAC strength in C. elegans embryos. In the 2-cell embryo, we find that roughly half of the difference in checkpoint strength, between the larger somatic AB and the smaller germline P1 blastomere, is due to cell size, with the remainder requiring the asymmetric partitioning of cytoplasmic cell fate determinants downstream of the PAR polarity proteins. To further dissect lineage-specific adaptation of SAC strength, we are using live imaging and a semi-automated tracking and fluorescence quantification approach to monitor the levels and dynamics of key kinetochore and SAC proteins in different cell lineages during embryogenesis. Altogether our results suggest that germline cell fate correlates with enhanced SAC activity, raising the interesting possibility that variation in SAC strength is adaptive, and reveal a novel relationship between asymmetric cell division and SAC regulation.
P2944
Board Number: B225
Spatiotemporal delay of chromosome alignment causes chromosomal instability.
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The number of chromosomes is kept 46 in normal human cells. In contrast, most solid tumors have abnormal numbers of chromosomes (aneuploidy), usually resulting from high rates of chromosome missegregation (chromosomal instability: CIN). As defects in the essential processes for mitotic progression lead to cell death, it is plausible that CIN in tumors is caused by the defects in the non-essential processes for mitotic progression. For equal chromosome segregation, kinetochores on sister chromatids have to attach to the ends of microtubules (end-on attachment) from opposite spindle poles (bi-orientation). After nuclear envelope breakdown, kinetochores bind to the microtubule lateral surface, referred to as lateral attachment, and quickly move toward the spindle equator. Lateral attachment is then converted to end-on attachment before establishing bi-orientation. Forming end-on attachment at the spindle equator would be advantageous to establish bi-orientation, because there are comparable numbers of microtubules from both spindle poles. Therefore, we hypothesized that efficient chromosome alignment at the spindle equator contributes to bi-orientation establishment and high-fidelity chromosome segregation. Here we verified the hypothesis in several experimental settings. Recently, we reported that a chromokinesin Kid is involved in efficient chromosome alignment (Nat Commun, 2015). Kid-depleted cells progressed through mitosis without mitotic arrest, but took longer time to align chromosomes and showed increased chromosome missegregation. We found that cells depleted of other motor proteins non-essential for mitotic progression also showed increased time to align chromosomes as well as elevated levels of chromosome missegregation. Increased chromosome missegregation was also seen when these motor proteins were overexpressed, a condition often found in cancer cells, suggesting that expression levels need to be within a proper range. We also generated tetraploid cells that have twice as many chromosomes as normal cells as another situation having difficulty in chromosome alignment. Tetraploid cells also showed increased time to align chromosomes and increased levels of chromosome missegregation. Furthermore, abnormal kinetochore-microtubule attachment was preferentially seen in late-aligning chromosomes. These data suggest that spatiotemporal delay of chromosome alignment causes chromosomal instability, which might be related to CIN in cancer cells.

P2945
Board Number: B226
Mechanisms of quantitative transmission of CENP-A nucleosomes through the germline.
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Accurate inheritance of genetic information is achieved by replicating DNA and partitioning it equally into daughter cells to quantitatively transmit correct copy numbers of genes. Centromeres direct partitioning of chromosomes, but their position on chromosomes is not specified by a particular DNA sequence. They are instead epigenetically defined by the presence of a histone H3 variant, CENP-A, and the location is inherited by maintaining CENP-A nucleosomes. This poses a unique challenge in the germline as CENP-A nucleosomes are maintained quantitatively through both a prolonged prophase I
arrest in mammalian oogenesis and widespread histone replacement in spermatogenesis. Maintaining CENP-A chromatin through gametogenesis preserves centromere structure and ensures reproductive success, but the underlying mechanisms remain unclear. We considered two possible mechanisms for determining the number of CENP-A nucleosomes inherited. First in a template-dependent model, each nucleosome directs local stoichiometric loading of a new CENP-A nucleosome, analogous to semi-conservative DNA replication. Second in a template-independent model, a fixed number of CENP-A nucleosomes is assembled regardless of the abundance of pre-existing CENP-A nucleosomes. To distinguish between these models in the male and female mouse germline, we perform crosses between male and female cenpa +/- mice, which each have reduced CENP-A levels at centromeres, to produce cenpa +/- progeny with wild type expression of CENP-A protein. If epigenetic centromere inheritance is template-dependent, the progeny should inherit a reduced number of CENP-A nucleosomes centromeres despite having normal expression of CENP-A protein. If it is template-independent, CENP-A levels at centromeres will be restored to wild type. In the male germline, CENP-A levels at centromeres are only partially restored after a single generation, indicating epigenetic memory in support of the template-dependent model. We conclude that to maintain centromere identity through the germline, both the position and the quantity of CENP-A nucleosomes is inherited epigenetically.

P2946

Board Number: B227

The kinetochore-dependent and -independent formation of the CDC20-MAD2 complex and its functions in HeLa cells.

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The mitotic checkpoint complex (MCC) is formed from two sub-complexes of CDC20-MAD2 and BUBR1-BUB3, and current models suggest that it is generated exclusively by the kinetochores after nuclear envelope breakdown (NEBD). However, neither sub-complex has been visualised in vivo, and when and where they are formed during the cell cycle and their response to different SAC conditions remains elusive. Using single cell analysis in HeLa cells, we show that the CDC20-MAD2 complex is cell cycle regulated with a “Bell” shaped profile and peaks at prometaphase. Its formation begins in early prophase before NEBD when the SAC has not been activated. The complex prevents the premature degradation of cyclin B1. Tpr, a component of the NPCs (nuclear pore complexes), facilitates the formation of this prophase form of the CDC20-MAD2 complex but is inactive later in mitosis. Thus, we demonstrate that the CDC20-MAD2 complex could also be formed independently of the SAC. Moreover, in prolonged arrest caused by nocodazole treatment, the overall levels of the CDC20-MAD2 complex are gradually, but significantly, reduced and this is associated with lower levels of cyclin B1, which brings a new insight into the mechanism of mitotic “slippage” of the arrested cells.
P2947
Board Number: B228
Spindle assembly checkpoint function in the mouse preimplantation embryo.
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Chromosome segregation errors during early embryonic divisions result in embryos comprising a mixture of euploid and aneuploid cells. While such mosaicism is commonly observed in human embryos, how and why it arises remains elusive. Here, we examine the presence, strength and sensitivity of the spindle assembly checkpoint (SAC) in mouse preimplantation embryos. We combine immunofluorescence and live cell imaging approaches to examine chromosome segregation dynamics and SAC function in cleaving embryos. Fast-acquisition live imaging of H2B:RFP-expressing embryos under standard conditions revealed pre-anaphase misaligned chromosomes in ~5% of divisions, suggesting that SAC function is weak in early mouse embryos. To ascertain whether a SAC operates in preimplantation development, embryos were treated from the two-cell to blastocyst stage with the specific SAC inhibitor AZ3146. Immunofluorescence analysis revealed a higher incidence of micronuclei after SAC inhibition (p=0.038). Consistent with this, live imaging experiments show that SAC inhibition results in premature anaphase with increased chromosome misalignment and chromosome segregation errors such as lagging chromosomes (p=0.002). In addition, SAC inhibition is capable of overcoming the mitotic arrest induced by high concentrations of the spindle poison Nocodazole. Thus, a SAC operates in embryos. We next probed SAC strength performing live imaging of embryos in Nocodazole at different developmental stages. Surprisingly, only at morula stage, we observe no anaphase delay despite an increased rate of chromosome mis-segregation, suggesting that a failure to activate SAC signaling in response to spindle damage results in chromosome segregation errors. Interestingly, unlike in mouse oocytes and C. elegans embryos, experimentally reducing cell volume by cytoplasmic removal does not strengthen the SAC in mouse morula stage embryos. Taken together, our results demonstrate that embryos possess a SAC that does limit chromosome mis-segregation, but it is not sufficiently effective to prevent all errors. SAC insufficiency provides a potential mechanistic explanation for mosaicism.

P2948
Board Number: B229
Quantitative analysis of the biochemical cascade that generates the Mitotic Checkpoint Complex.
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Accurate chromosome segregation during cell division requires that kinetochores of sister chromatids attach to microtubules emanating from opposite spindle poles. In the absence of microtubule attachment, kinetochores generate a diffusible signal that inhibits the Anaphase Promoting Complex/Cyclosome (APC/C) throughout the cell to delay anaphase onset. This inhibitory signal, known as the Mitotic Checkpoint Complex (MCC), is a complex of four signaling proteins: Bub3, BubR1, Cdc20, and Mad2. To prevent anaphase onset, an unattached kinetochore must recruit all four signaling proteins rapidly, and then efficiently assemble them into the MCC. The mechanisms that realize these
objectives are poorly understood, primarily because of two reasons. First, the biochemical cascade that recruits the signaling proteins is extremely complex and incorporates parallel recruitment pathways. Second, the biochemical reactions that lead to MCC formation are spatially localized in a nanoscale volume. To circumvent these difficulties and dissect the mechanisms of MCC assembly, we have engineered a system to ectopically drive the biochemical cascade that assembles the MCC in human cell lines using the knowledge that the phosphorylation of signaling proteins by the Mps1 licenses downstream reactions. Our system uses chemically induced dimerization of the kinase domain of Mps1 with various functional regions of either KNL1, Bub1, or Mad1, three signaling proteins that act as scaffolds in the biochemical cascade that generates the MCC. Upon activation (dimerization in vivo), this system delays the onset of anaphase in a dose-dependent manner. Using this ectopic activation system, we conducted quantitative analyses for various functional regions of all three proteins to study how the duration of mitosis scales with the concentration of the activator complex. Such ‘dose-response’ analysis revealed a novel, prozone-like effect involving BubR1 in MCC generation, wherein the limited abundance of BubR1 produced non-monotonic dose-response characteristics. We will continue employing the ectopic activation system to understand the molecular mechanisms that enable rapid and highly efficient MCC assembly in unattached kinetochores.

Spindle Assembly 3

P2949

Board Number: B230

KIF18B is regulated by distinct interactions with EB1 and importin α through its tail domain.

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The mitotic apparatus of a dividing cell comprises functionally distinct subpopulations of microtubules whose dynamics are intricately regulated by diverse proteins to ensure proper spindle formation and accurate DNA segregation. Previous studies showed that KIF18B, a member of the Kinesin-8 family that destabilizes microtubules, localizes to the plus tips of astral microtubules and attenuates astral microtubule length and number during prometaphase. Plus-tip localization of KIF18B is mediated by EB1 binding to the Kif18B tail, but how KIF18B and EB1 coordinately regulate microtubule dynamics at plus-tips is still not fully understood. Additionally, KIF18B also contains putative nuclear localization signals (NLS) in the tail, which may serve as regulatory sites for the Ran pathway. To dissect how EB1 and the Ran pathway modulate KIF18B function, we developed in vitro FRET biosensors to examine the interactions of Kif18B with EB1 and importin α and to map their binding sites. Using these biosensors, we show that the tail of Kif18B interacts independently with either EB1 or importin α/β and that binding of EB1 to Kif18B can be competitively inhibited by importin α/β. Furthermore, addition of RanQ69L, a GTP analog of Ran, disrupts the interaction between importin α/β and the tail of KIF18B. The tail of Kif18B also binds to microtubules, and its binding to microtubules is inhibited by importin α/β but not by EB1. Using site-directed mutagenesis, we identified two NLS and two EB1 binding sites within the tail domain of Kif18B. The tail of KIF18B contains one non-classical bipartite NLS and one monopartite NLS. Mutation of both NLS sites is required to abolish intermolecular FRET between the tail of KIF18B and importin α/β. Mutation of the NLS sites also reduces the affinity of the tail for microtubules, suggesting that importin α/β binding is used to modulate the interaction of Kif18B with microtubules. The EB1 binding sites do not contain the classical SxIP motif described for EB1 binding, but are rather variations of this site, SFLP and ARLP. Taken together, these data suggest that the presence of redundant binding sites may ensure robust binding of Kif18B with EB1 and importin α/β in vivo. Alternatively, the additional
sites could serve to fine tune Kif18B interaction with its binding partners in a temporal and context-dependent manner in vivo. Testing the importance of these interactions in vivo will give further insights into how KIF18B regulates the dynamics of astral microtubules during mitosis.

P2950
Board Number: B231
Chromosome velocities in the absence of microtubules in *Mesostoma ehrenbergii* spermatocytes are affected by drugs that alter myosin.
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Throughout cell division in *Mesostoma* spermatocytes the three bivalent chromosomes oscillate towards and away from either pole, at average speeds of 5-6 µm/min, without stopping until anaphase. In experiments reported at previous ASCB meetings, after nocodazole (NOC) was added to cells to depolymerize the microtubules all chromosomes selectively detached from only one pole, curled to one side, and quickly moved towards the opposite pole with initial velocities of up to 200 µm/min or more. Chromosomes then slowed down as they neared the opposite pole. Confocal microscopy confirmed there are no remaining kinetochore-microtubule connections at the detached pole, indicating that this rapid movement does not appear to be due to microtubules.

The current work addresses the following question: if not microtubules, what is responsible for the very rapid chromosome movements to opposite poles? We tested whether myosin might be involved by adding ML7 or BDM, which inhibit MLCK and myosin ATPase respectively, to prometaphase spermatocytes for 5-10 minutes, after which we added 10 µM NOC in conjunction with either ML7 or BDM. If myosin plays a role in chromosome movement then inhibiting myosin should decrease the speed of chromosome movement or stop the movement altogether. In all cells treated with NOC plus ML-7 chromosomes moved more slowly to the opposite pole as compared to NOC alone (average 16 µm/min vs. 35 µm/min). In approximately half of the cells treated with NOC plus BDM chromosomes did not move and in the other half of cells chromosomes moved more slowly at an average 13 µm/min. These results seem to indicate that myosin is involved in the high speed chromosome movements after NOC treatment. We will further test this by seeing if chromosome movement is sped up with Calyculin A, which hyper-activates myosin.

P2951
Board Number: B232
Elastic tethers extend between the telomeres of separating anaphase chromosomes in a broad range of animal cells.
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“Tethers”, originally described in crane-fly spermatocytes, are elastic components that extend between separating telomeres in anaphase. They exert force on chromosome arms opposite to the direction the anaphase chromosomes move. Our experiments using laser microbeam irradiation of anaphase cells to cut chromosome arms and to cut the tethers themselves show that tethers exist in a broad range of animal cells from Turbellarian flatworms to humans. Thus they are previously unrecognised components of general mitotic mechanisms. They extend between separating telomeres until late anaphase and
cause the arms of the chromosomes to be stretched by about 10%, as indicated by shortening of arms when their tethers are cut. Tethers need to be accounted for in general models of mitosis in terms of forces on chromosomes and in terms of what their roles might be.

P2952
Board Number: B233
Dual spindle formation around zygotic pro-nuclei explains parental genome separation.
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Intriguingly, in the early mammalian embryo, chromosomes are compartmentalised in a parent-of-origin specific manner. In contrast to many other animal species, karyogamy in mammals does not occur through fusion of the pronuclei after fertilisation but instead parental chromosomes are brought together after replication on the metaphase plate of the first mitosis. Surprisingly, maternal and paternal chromatin occupies distinct hemispheres in the nuclei of the two-cell embryo, and this separation only gradually decreases during subsequent stages of development. Both the mechanism underlying parental genome separation and its functional importance for embryonic development are currently unclear. Utilising the high spatio-temporal resolution of our recently developed inverted light-sheet microscope, we reveal that the formation of two separate bipolar spindles around each parental pronucleus keeps maternal and paternal genomes apart during the first cleavage of the zygote. Based on this mechanistic understanding we were able to experimentally mix parental chromosomes during the first embryonic division in order to understand if separate nuclear compartments are required for normal development.

P2953
Board Number: B234
GTSE1 REGULATES SPINDLE MICROTUBULE DYNAMICS TO CONTROL AURORA B KINASE AND KIF4A CHROMOKINESIN ON CHROMOSOME ARMS.
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In mitosis, the dynamic assembly and disassembly of microtubules are critical for normal chromosome movement and segregation. Microtubule turnover varies among different mitotic spindle microtubules, dictated by their spatial distribution within the spindle and by their interactions with each other and with other organelles such as kinetochores, chromosome arms, and the cell cortex. How turnover among the various classes of spindle microtubules are differentially regulated and the resulting significance of differential turnover for chromosome movement remains a mystery. As a new tactic, we used GAMMA, a bioinformatic method, to identify novel regulators of mitosis. GTSE1 (G2 and S phase expressed protein 1) was selected for further analysis based on previous findings demonstrating GTSE1 association with microtubules. GTSE1 is expressed exclusively in late G2 and M phase. Additionally, GTSE1 has been shown to function as an EB1-dependent plus-end tracking protein. However, following nuclear envelope breakdown in mitosis, GTSE1 microtubule plus-end tip tracking is inhibited until anaphase onset and instead the protein decorates the microtubule lattice. During this time GTSE1 binds
preferentially to the most stable populations of mitotic spindle microtubules and promotes their turnover. Cells depleted of GTSE1 show defects in chromosome alignment at the metaphase plate coupled with an increase in the proportion of stable mitotic spindle microtubules. Photoactivation data show that GTSE1 fosters turnover of the most stable population of microtubules within the mitotic spindle from prometaphase to anaphase onset. At anaphase onset, GTSE1 returns to tip tracking and redistributes to the ends of astral microtubules. We speculate that this redistribution prevents GTSE1 from destabilizing midzone microtubules during anaphase and telophase. Cells depleted of GTSE1 display hyper-stabilized spindle microtubules, which in turn affects the activity of the mitotic kinase, Aurora B, on chromosome arms with negligible effects on Aurora B at centromeres. The loss of Aurora B activity on chromosome arms diminishes accumulation of the chromokinesin Kif4A. In sum, we have identified a novel pathway in which GTSE1 is an upstream regulator of microtubule stability, chromosome alignment, spindle pole integrity, and timely mitotic progression.

P2954
Board Number: B235
Interplay between microtubule bundling and sorting factors ensures acentriolar spindle stability during C. elegans oocyte meiosis.
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During mitosis, duplicated centrosomes nucleate microtubules and form the spindle poles. However, oocytes of most species lack centrioles, so spindles assemble using a different pathway. Recently, we reported that acentriolar spindle assembly in C. elegans oocytes proceeds by: 1) formation of a cage-like microtubule array inside the disassembling nuclear envelope, 2) sorting of microtubule minus ends to the periphery of the array, and 3) focusing of these ends into nascent poles that coalescence until bipolarity is achieved. Moreover, we demonstrated that the kinesin-12 family motor KLP-18 acts during the sorting step to force the minus-ends outwards where they can be organized into the spindle poles (1). Now, we have uncovered additional insights into the principles underlying this specialized form of cell division, through studies of KLP-15 and KLP-16, two highly homologous members of the minus-end-directed kinesin-14 family. Fixed and live imaging of KLP-15/16-depleted oocytes revealed that following nuclear envelope breakdown, microtubules form a transient cage-like structure, but then microtubules collapse into a disorganized ball-shaped structure surrounding the chromosomes. These results suggest that KLP-15/16 bundle and organize microtubules during acentriolar spindle assembly, and consistent with this proposed function, these proteins localize to spindle microtubules during the cage stage and remain microtubule-associated throughout the meiotic divisions. However, despite the severe spindle assembly defects observed following KLP-15/16 depletion, we were surprised to find that these disorganized microtubules were then able to reorganize into a spindle capable of segregating chromosomes during anaphase, revealing the existence of additional mechanisms that can act to bundle and organize spindle microtubules. This phenotype therefore enabled us to identify factors promoting microtubule organization and assembly during anaphase, whose contributions are normally imperceptible in wild-type worms. First, we discovered that SPD-1 (PRC1), which loads onto microtubules in early anaphase, is required for the formation of anaphase microtubule bundles in the absence of KLP-15/16. Moreover, we found that KLP-18, which sorts microtubules during spindle assembly, can then function during anaphase to sort these bundles into a functional orientation capable of mediating chromosome segregation. Therefore, our studies have revealed an interplay between distinct mechanisms that together promote spindle formation and chromosome segregation in the absence of structural cues such as centrioles. (1) Wolff, et.al., (2016) MBoC
P2955
Board Number: B236
Chromokinesin Kif4 is required for faithful chromosome segregation in mammalian oocytes.
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Spindles in female meiotic cells (oocytes) lack centrosomes, which usually serve to nucleate microtubules and organize spindle poles. Despite the importance of oocyte meiosis to successful reproduction, how acentrosomal spindles interact with chromosomes and mediate accurate segregation is poorly understood. We are investigating the contribution of a kinesin, Kif4, to the meiotic divisions in mouse oocytes. Kif4 is a plus-end directed microtubule motor in the kinesin-4 family that contributes to chromosome organization and midzone formation in mitotically-dividing cells. However, its roles in oocyte meiosis are less well understood. Using immunofluorescence microscopy, we found that, similar to mitosis, Kif4 localizes to chromosomes from prometaphase to metaphase. However, in oocytes Kif4 displayed a dynamic localization pattern during these stages, as it was excluded from kinetochores and only on chromosomes arms during prometaphase but then became kinetochore-enriched at metaphase, the stage at which kinetochore-microtubule attachments are formed in mouse oocytes. During anaphase, Kif4 relocalized to the spindle midzone; it first appeared in stretches along microtubules and then concentrated into distinct ring-like structures that appeared to organize bundles of microtubules. Other midzone proteins, MgcRacGAP (part of the centralspindlin complex) and PRC1 (protein regulator of cytokinesis 1), also localized to these structures. During mitosis, PRC1 bundles microtubules and binds to Kif4, and our localization data suggest that this relationship is maintained in oocytes. Together, the observed Kif4 localization pattern raised the possibility that this motor could be involved in chromosome dynamics and spindle midzone formation in oocytes. Consistent with this idea, depletion of Kif4 using morpholinos resulted in severe anaphase defects, including chromosome bridges and defective midzone formation, indicating that Kif4 has important functions in oocytes. Building on these results, we also found that treatment of oocytes with AZD1152, an Aurora B/C kinase inhibitor, inhibited localization of Kif4 to metaphase chromosomes and to the anaphase spindle midzone, suggesting that Aurora B/C regulate Kif4 localization. In addition, this treatment caused severe chromosome architecture and segregation defects, some of which resemble Kif4 depletion defects. Moreover, the anaphase spindles appeared disorganized and unstable, indicating that Aurora B/C plays an important role in anaphase spindle organization and thus proper chromosome segregation. Taken together, our work has revealed essential roles for Kif4 during the meiotic divisions and forms the basis for further understanding mechanisms promoting accurate chromosome segregation in acentrosomal oocytes.

P2956
Board Number: B237
Proper rotation of the mitotic spindle requires an equatorial spindle centering mechanism in human cells.
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Successful rotation requires a well-defined rotational center. The mitotic spindle is precisely rotated to a pre-determined position, which determines the plane of cell division and thus, the position of
daughter cells within tissues. While it is known that Dynein powers spindle rotation in human cells, it is not clear how the center of rotation is controlled. To address this we analyzed spindle movements in 100s of single-cells exposed to protein depletion or drug treatment regimens and uncovered a role for MARK2 in maintaining the spindle at the geometric center of human epithelial cells. In MARK2 depleted cells, spindles fail to rotate and instead glide along the cell cortex; this failure to center and rotate normally leads to a failure in establishing the correct plane of cell division. Surprisingly, spindle off-centering in MARK2 depleted cells is not due to excessive pull by cortical Dynein. We show that MARK2 modulates mitotic microtubule growth and length, and increasing microtubule stability is sufficient to rescue the off-centering of spindles in MARK2-depleted cells. Thus, we report a novel MARK2-dependent spindle centering mechanism and provide the first insight into a spindle centering mechanism that ensures the proper rotation of spindles and plane of division in human cells.

**P2957**

**Board Number: B238**

Rapid degradation of gamma-TuRC component GCP2 causes spindle collapse in mitosis.

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The gamma-tubulin ring complex (gamma-TuRC) acts as a structural scaffold for microtubule nucleation. The complex is composed of GCP2, GCP3, GCP4, GCP5 and GCP6, and localizes to centrosomes, microtubules, kinetochores and the nuclear envelope. GCP2 and 3 bind gamma-tubulin to form the highly conserved gamma-tubulin small complex (gamma-TuSC), and upon association with GCP4, 5 and 6 comprise gamma-TuRC. Studies aimed at elucidating the role of gamma-TuRC components in microtubule nucleation have traditionally relied on siRNA-based approaches which require long incubation to achieve complete protein depletion. With the aim of depleting the protein in minutes, we biaxially inserted an Auxin-Inducible Degron (AID) tag onto the endogenous locus of GCP2 using CRISPR/Cas9 in DLD-1 cells. In the resultant cells, the bulk of GCP2 was degraded within 15 minutes of auxin addition, becoming undetectable within 45 minutes. Under GCP2-depleted conditions, we observed that GCP3 and gamma-tubulin are displaced from the centrosome, whereas other gamma-TuRC members remain unaffected. The majority of GCP2-depleted cells arrested in metaphase with a monopolar-like spindle. Some of the arrested cells eventually appeared to exit mitosis and decondense their chromosomes, possibly indicating spindle assembly checkpoint failure after extended arrest. Notably, live imaging showed that even established bipolar spindles underwent spindle collapse after rapid degradation of GCP2. Our results indicate that mitotic DLD-1 cells can maintain extensive microtubule arrays in the absence of gamma-TuRC, but that the normal spindle morphology and function are dramatically impaired. We will discuss our efforts to understand these defects at the level of microtubule dynamics and spindle organization.
**P2958**  
**Board Number:** B239  
**Cortical Pulling Drives Pronuclear Migration and Rotation, and Spindle Positioning and Oscillation.**  
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In the *C. elegans* early embryo, the pronuclei complex migrates to the cell center and rotates such that the mitotic spindle forms along the anterior-posterior axis. Subsequently, the spindle undergoes transverse oscillations as it moves toward the posterior. This asymmetric spindle positioning is crucial for asymmetric cell division and proper development. The contributions of pushing, cortical pulling, and cytoplasmic pulling forces to those dynamic centrosome positioning events are not fully understood. To study these processes, we constructed a novel laser ablation system capable of creating nearly arbitrary 3D cuts of astral microtubules at any desirable timing. We used this system to dissect the relative contribution of pushing and pulling forces throughout pronuclei and spindle motions. Our results suggest that all of these motions are dominated by net pulling. We used microinjected fluorescent nanodiamonds to track cytoplasmic fluid flow, which indicates that cortical pulling forces dominate over cytoplasmic pulling at all stages. We used computer simulations and mathematical modeling to interpret our experimental data. Taken together, our results strongly argue that cortical pulling drives pronuclear migration and rotation, metaphase spindle positioning, asymmetric spindle positioning and all aspects of spindle oscillations.

**P2959**  
**Board Number:** B240  
**Colorectal cancer cells require glycogen synthase kinase-3 for sustaining mitosis via translocated promotor region (Tpr)-dynein interaction.**  
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Glycogen synthase kinase (GSK)-3 mediates fundamental cellular signaling pathways and emerges as a potential therapeutic target in many cancer types including colorectal cancer (CRC). During mitosis, GSK3 localizes in mitotic spindles and centrosomes, however its function is largely unknown. We previously demonstrated that translocated promotor region (Tpr: a nuclear pore component) and dynein (a molecular motor) cooperatively contribute to mitotic spindle formation. Such knowledge encouraged us to investigate putative functional interaction among GSK3, Tpr and dynein in mitotic machinery in CRC cells. Here we show that, inhibition of GSK3 attenuated proliferation of CRC cells and induced cell cycle arrest at the G2/M phase and their apoptosis. Morphologically, GSK3 inhibition disturbed chromosome segregation, mitotic spindle assembly and centrosome maturation during
mitosis and ultimately resulting in mitotic cell death. These changes in CRC cells were associated with decreased expression of Tpr and dynein as well as disruption of their functional colocalization with GSK3 in mitotic spindles and centrosomes. Clinically, we showed that Tpr expression was increased in CRC databases and primary tumors of CRC patients respectively. Furthermore, Tpr expression in SW480 cells xenografted in mice is reduced following treatment with GSK3β inhibitors. Together, these results indicate that GSK3 sustains steady mitotic process for proliferation of CRC cells via interaction with Tpr and dynein, thereby suggesting that the therapeutic effect of GSK3 inhibition depends on the induction of mitotic catastrophe in CRC cells.

P2960
Board Number: B241
Ectopic JNK activity during aging increases symmetric divisions by altering spindle pole orientation.
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Proliferation of stem cells (SC) is carefully regulated to maintain tissue regeneration without becoming cancerous. As animals age, SC function becomes aberrant and leads to failure in tissue maintenance, contributing towards many age-associated diseases and ultimately, limiting lifespan. Signaling pathways that maintain SC activity, including JNK, become overactive in the aging condition and cause overproliferation and cell fate defects. The mechanisms in which these pathways cause these stem cell defects, however, are not well understood. Using the Drosophila intestine as a genetically accessible model to explore stem cell function, we find that spindle pole orientation in stressed or aging intestinal stem cells (ISCs) is dramatically shifted parallel to the basement membrane, generating more symmetric divisions. Overactivating JNK phenocopies the age-associated spindle pole orientation defect while disrupting JNK in stressed or aging conditions is sufficient to restore proper spindle pole orientation. We find evidence supporting that JNK regulates spindle pole orientation both directly and transcriptionally. Restoring normal spindle pole orientation in aging and age-associated conditions not only restores cell fate, but also preserves the barrier function of the intestine and increases overall longevity, providing a mechanism to the aging process. These results deepen our understanding of the cell fate regulation in the aging barrier epithelia and provide insight into mechanisms preserving long-term tissue homeostasis.

P2961
Board Number: B242
Activated Ezrin controls cortical MISP levels to ensure correct NuMA localization and spindle orientation.
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Correct spindle orientation is achieved through signaling pathways that provide a molecular link between the cell cortex and astral microtubules in an F-actin dependent manner. Activation of ERM proteins (Ezrin/Radixin/Moesin) controls localization of LGN and NuMA force-generator complexes at the cell cortex. Here we show that the actin-binding protein MISP that has been implicated in spindle orientation directly interacts with Ezrin. Moreover, activated Ezrin ensures appropriate cortical MISP
levels in mitosis by preventing its excessive accumulation. Enrichment of MISP in turn abolishes cortical NuMA polarization and leads to hyper-stabilization of astral microtubules. On the contrary, loss of MISP results in enhanced cortical NuMA localization. Cdk1-dependent phosphorylation of MISP is required for polarized NuMA localization. Our data implicate that MISP functions as a molecular linker between Ezrin and NuMA. We propose that enrichment of MISP at the cortex promoted by SLK/LOK-activated Ezrin is required for proper NuMA localization and spindle orientation.

P2962
Board Number: B243
Importin alpha/beta regulates XCTK2 localization within the spindle and promotes parallel microtubule cross-linking and sliding.
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The mitotic spindle is critical for the accurate segregation of chromosomes into daughter cells. A major regulator of spindle assembly is the small GTPase Ran, which forms a gradient of RanGTP around the chromatin that extends to the spindle poles. RanGTP activates spindle assembly factors (SAFs) by releasing the inhibitory action of importin alpha/beta. Our previous work demonstrated that XCTK2, a Kinesin-14 that cross-links and slides microtubules (MTs), was regulated by the Ran system; and we proposed that the Ran gradient around the chromosomes would release the importins from XCTK2, allowing it to cross-link and slide MTs in that vicinity. To test this model, we generated a series of FRET biosensors of XCTK2 and importin alpha to look at their interactions in the spindle and in vitro. CyPet-XCTK2 interacts directly with importin alpha-YPet and importin beta through its tail domain, resulting in a high FRET ratio that can be reduced by the addition of the RanGTP analog RanQ69L. Surprisingly, our initial fluorescence lifetime imaging microscopy (FLIM) studies suggest there is a bias of importin alpha interaction with XCTK2 around the chromatin compared to the poles. Consistent with these results, we see an enrichment of importin alpha on the chromatin relative to the spindle and cytoplasm. Addition of a nuclear localization signal mutant of XCTK2 in our FLIM assay resulted in reduced association around chromatin and enrichment of the mutant XCTK2 near the poles, consistent with its reduced affinity to importin alpha/beta. Together these results suggest that importin alpha association may facilitate the loading of XCTK2 to the plus ends of MTs near chromatin where it could walk toward the poles. To understand how importin alpha/beta affect XCTK2 MT cross-linking and sliding, we reconstituted XCTK2 MT cross-linking and sliding in vitro and found that importin alpha/beta reduced the overall MT sliding events, consistent with the idea that the importins reduce MT association. Surprisingly, the anti-parallel MT sliding events were specifically reduced relative to the parallel MT sliding events in the presence of the importins, suggesting a bias toward parallel MT sliding that could facilitate pole formation. These results suggest that Ran regulation of spindle assembly may not be simply through the release of the importins along a gradient, but may also involve directed localization within the spindle.
P2963

Board Number: B244

Xenopus borealis egg extracts: a new system to investigate spindle variation.
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Egg extracts of the African clawed frog, Xenopus laevis, have provided an instrumental in vitro cell-free system to elucidate fundamental aspects of the cell cycle, including mechanisms of spindle assembly. To study mechanisms of spindle and organelle size control, we have previously utilized a smaller, related frog, Xenopus tropicalis. Here, we investigate the Marsabit clawed frog, Xenopus borealis. While the allotetraploid X. laevis and diploid X. tropicalis are 48 million years apart, X. borealis is more closely related to X. laevis, having diverged 17 million years ago. Both X. laevis and X. borealis possess allotetraploid genomes of 36 chromosomes, although X. laevis frogs (~10 cm adult body length) are larger than X. borealis (~7 cm). In contrast, X. tropicalis frogs possess a diploid genome with 20 chromosomes and are significantly smaller (~4 cm). Scaling at the organismal level between X. laevis and X. tropicalis is accompanied by differences in the size of the egg as well as nuclei and spindles formed in egg extracts. We developed an X. borealis egg extract to provide a new system to further investigate interspecies spindle scaling and morphometric variation. As with X. laevis and X. tropicalis, spindles formed in extracts prepared from metaphase cytostatic factor (CSF) arrested X. borealis eggs appeared very similar to meiosis II spindles in vivo. CSF-arrested X. borealis formed spindles around various sources of DNA, including sperm and embryo nuclei and chromatin-coated beads, and could be cycled through interphase. Interestingly, X. borealis cycled spindles showed similar length but decreased width compared to X. laevis, and the microtubule distribution across the length of the spindle differed noticeably from both X. laevis and X. tropicalis. Mixing experiments revealed that titration of X. borealis extract with X. laevis extract did not alter spindle size, whereas addition of X. tropicalis extract caused X. borealis spindles to shrink, potentially indicating common scaling phenomena across the three species. Ongoing experiments aim to evaluate the role of known key players in spindle assembly and microtubule organization, such as the RanGTP gradient, in establishing spindle size and morphology in X. borealis extract.

P2964

Board Number: B245

CENP-E-PRC1 interaction provides a temporal cue for central spindle assembly.
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Mitotic kinesin CENP-E plays key roles in chromosome congression and spindle checkpoint satisfaction. We have recently identified and characterized syntelin, a novel allosteric inhibitor selective for CENP-E (Ding et al., 2010. Cell Res. 20, 1386-1389; Mo et al., 2016. Nat. Chem. Biol. 12,226-232; Liu et al., 2016. J Mol Cell Biol. 8,144-156). Cells treated with syntelin progress through interphase, enter mitosis normally with a bipolar spindle and lagging chromosomes around the poles. Our optical trap analyses show that syntelin is an allosteric inhibitor which tightens CENP-E-microtubule interaction by slowing inorganic phosphate release. To delineate the role of CENP-E in reorganization of interpolar microtubules into an organized central spindle, metaphase synchronized cells were exposed to syntelin and other mitotic motor inhibitors. Syntelin does not perturb inter-polar microtubule assembly but abrogates the anti-parallel microtubule bundle formation. Real-time image shows that CENP-E inhibited

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cells undergo central spindle splitting which is confirmed by transmission electron microscopic analyses. Using a syntelin affinity matrix, we identified a novel CENP-E complex containing PRC1. Interestingly, inhibition of CENP-E did not alter the interaction between CENP-E and PRC1 but perturbed temporal assembly of PRC1 to the midzone. These findings reveal a previously uncharacterized role of CENP-E motor in temporal control of central spindle assembly. Currently, we are reconstituting the central spindle sliding assay in vitro and delineate how CENP-E-PRC1 interactions establish the central spindle and promote spindle elongation using super-resolution imaging analyses.

P2965
Board Number: B246
She1 Preferentially Crosslinks Parallel Microtubules to Ensure Spindle Stability for Spindle Positioning.
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Dynein mediates spindle positioning in budding yeast by pulling on astral microtubules from the cell cortex. The microtubule-associated protein She1 regulates dynein activity along astral microtubules and directs spindle movements toward the bud cell. In addition to localizing to astral microtubules, She1 also targets to the spindle, but its role on the spindle remains unknown. Using function-separating alleles, live-cell spindle assays, and in vitro biochemical analyses, we show that She1 is required for the maintenance of metaphase spindle stability. She1 binds microtubules via a C-terminal microtubule-binding site and preferentially crosslinks microtubules in a parallel configuration. EM and sedimentation analyses reveal that She1 self-assembles into ring-shaped oligomers, formation of which appear to be disrupted by Ipl1/Aurora phosphorylation. In cells, She1 stabilizes interpolar microtubules, preventing spindle deformations during movement, and we show that this function is regulated by Ipl1/Aurora B phosphorylation during cell cycle progression. Our findings reveal how She1 ensures spindle integrity during spindle movement across the bud neck and suggest a potential link between regulation of spindle integrity and dynein pathway activity.

Centrosome Assembly and Functions 2

P2966
Board Number: B247
Vesicular trafficking plays a role in centriole disengagement and duplication.
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Centrosomes, as the major microtubule-organizing centers of cells, play a crucial role in microtubule anchoring, organelle positioning, primary ciliogenesis, and cell cycle progression. At the centrosome core lie a tightly-associated or ‘engaged’ mother-daughter centriole pair. During mitotic exit, centrosomes are licensed to duplicate by the process of ‘disengagement’ when the inter-centriolar linkers that hold mother-daughter centrioles together are degraded. The degradation of such linkers usually renders the removal of pericentriolar matrix components, including Cep215/Cdk5RAP2 and
pericentrin/PCNT. This process ensures that centrosomes duplicate only once each cell cycle, which is critical for proper centrosome function. Despite the importance of Cep215 and pericentrin removal on disengagement and duplication, little is known about the mode by which they are eliminated. Herein, we explore a new mechanism involving vesicular trafficking for the removal of Cep215 from the centrosome. We show that EHD1, an ATPase and endocytic regulatory protein, is required for the transport of Cep215 from centrosomes to the spindle midbody, thus facilitating disengagement and duplication. Cep215 is transported on transferrin-containing vesicles away from centrosomes in an EHD1-dependent manner. We also show that EHD1 and Cep215 are in a complex with the distal appendage protein CP110, which is functionally involved in EHD1-mediated removal of Cep215. Our data supports a previously unknown role for vesicular trafficking in the regulation of the centrosome cycle.

P2967
Board Number: B248
The small ovary (sov) gene is essential for centrosome function and cell cycle progression during embryogenesis.
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In the germline stem cell niche, cues for either stem cell renewal or differentiation arise both cell autonomously and from neighboring somatic niche cells. Proper orchestration of these cues is critical for germline development. Using clonal knockdown approaches, we demonstrate that the Drosophila small ovary (sov) gene, or CG14438, is differentially required in somatic and germline lineages during female germline development. Loss of sov in the niche results in massive tissue degradation, failed oogenesis, and ovarian tumors. Conversely, while sov knockdown in the germline permits oogenesis, the resulting embryos exhibit numerous defects. Consistent with a role in germline development, embryos deficient in sov produce fewer primordial germ cells. Moreover, sov mutant embryos display increased nuclear fallout, dysmorphic nuclei during metaphase/anaphase, and aneuploidy, ultimately resulting in embryonic lethality within the first few hours of embryogenesis. In the syncytial embryo, centrosomes are microtubule-organizing centers essential for error-free nuclear divisions. Further analysis confirmed that loss of sov results in centrosome disorganization, detachment, and mispositioning. Taken together, these data suggest Sov regulates centrosome function and is essential for proper cell cycle progression and embryonic viability.

P2968
Board Number: B249
Proximity labeling to define the nucleoporin-interactome at centrioles.
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Nuclear pore complexes (NPCs), which control the exchange of molecules between the nucleus and cytoplasm, are massive assemblies of proteins called nucleoporins (nups). Nups also moonlight in other subcellular locations with several established connections to the mitotic apparatus (1). For example, in previous work, we showed that the inner ring nucleoporin Nup188 localizes to the bases of cilia, surrounding the mother and daughter centrioles (2). A key challenge, however, is that it remains difficult to determine a specific pericentriolar function for Nup188 outside of its established role at NPCs.
Therefore, we have undertaken a proximity-dependent biotin identification (BioID) strategy to interrogate the pericentriolar-specific interactome for Nup188. We generated stable cell lines expressing the promiscuous biotin ligase BirA or BirA-Nup188 fusion protein and validated the BirA-Nup188 specific biotin labeling of both NPCs and centrosomes by fluorescence imaging. Mass spectrometry analysis of biotinylated proteins derived from total cell extracts identified all previously reported NPC-specific interactions for Nup188, supporting the functionality of the BirA-Nup188 construct. To specifically assess pericentriolar interactions, we purified centrosomes by density-gradient centrifugation and affinity purified biotinylated proteins from these isolated fractions. We identified several pericentriolar proteins including Cep152, Cep192 and PCM1, in addition to several, potentially centriolar-specific nups. Interestingly, depletion of either Cep152 or Cep192 leads to the loss of Nup188 localization specifically at centrioles, suggesting a model in which key centriolar components recruit Nup188 to the pericentriolar material (PCM). These interactions provide a genetic framework to be able to directly probe the function of Nup188 outside of NPCs.

References:

P2969
Board Number: B250
Activation of the centrosome’s microtubule-assembly activity by the Zika virus (ZIKV).
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Recent outbreaks of the Zika virus (ZIKV) in the Americas have resulted in a public health crisis due to its links to microcephaly and additional birth defects collectively known as congenital Zika syndrome. Our lab investigates the inherited forms of microcephaly, which are due to mutations in centrosome protein-encoding genes. The centrosome, found in most animal cells, is the major microtubule-organizing center (MTOC). However, the functional link between centrosomes and the development of microcephaly remains unclear. We are investigating how ZIKV infection affects centrosome function. We found that the centrosome’s MTOC activity is elevated upon ZIKV infection. We also found a close association between the centrosome and the assembly of the viroplasm, a ER-derived compartment that is responsible for virus replication and assembly. These viroplasms have a toroid shape with the centrosome positioned in its center with a core of dense microtubules composed of both tyrosinated and acetylated tubulin. When treated with centrinone, a drug that results in centrosome elimination, viroplasm assembly is impaired. The activated centrosome appears to organize the toroid shape of the viroplasm as this structural element is lost in the infected drug-treated cells. One particular centrosomal protein is recruited at higher levels to centrosomes in ZIKV infected cells compared to uninfected cells. This protein is recruited to the centrosome in response to ZIKV, and we surmise that it is required for centrosomal activation during viral infection. We further propose that ZIKV-mediated centrosome activation is required for ZIKV viroplasm organization.
P2970
Board Number: B251
Identification of novel regulators of centriole duplication in Caenorhabditis elegans.
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Centrosomes are organelles comprised of two centrioles (one mother and one daughter) and a surrounding pericentriolar material (PCM). The centrosome is the major microtubule-organizing center of the cell and aids in building the mitotic spindle. In quiescent cells, the mother centriole serves as a basal body for cilia assembly. Centrioles are duplicated only once per cell cycle and this involves the formation of a single daughter centriole next to each mother. Dysregulation of this process can result in aneuploidy, a hallmark of cancer cells. Therefore, it is critical that centriole duplication be tightly regulated. All the core components of the centriole duplication pathway that have been identified in C. elegans are conserved in humans. We are interested in understanding the molecular mechanisms by which proper centriole number is maintained in the cell. The kinase ZYG-1 (Plk4 in humans) is a master regulator of centriole duplication. Depleting ZYG-1 prevents centriole duplication while increasing its levels or activity causes centriole over-duplication. We are currently performing genetic suppressor screens for a hypomorphic loss-of-function zyg-1 allele (zyg-1(it25)) as well as using biochemical assays and mass spectrometry to identify novel proteins that regulate centriole duplication. Using our genetic suppressor approach, we have identified the proteins PP1, I-2, SDS-22 and CHD-1 as negative regulators of centriole duplication. We recently showed that PP1 acts as a molecular brake for centriole duplication and its activity is required to ensure that one and only one daughter centriole forms next to each mother during centriole duplication. We have also identified the chromatin remodeling protein CHD-1 as an additional negative regulator of centriole duplication. At the restrictive temperature, the loss of CHD-1 function partially rescues the embryonic lethality and centriole duplication defects of the zyg-1(it25) mutant. To complement our genetic approach, we have screened for proteins that physically interact with ZYG-1. Preliminary experiments indicate that one of the candidate proteins positively regulates centriole duplication. We also identified LIN-23, a known negative regulator of centriole duplication as a ZYG-1-interacting protein in our assay. Thus, using this approach, we can expect to identify both positive as well as negative regulators of centriole duplication. In the future, we will assay whether any of the other ZYG-1-interacting proteins regulate centriole duplication and will also determine the underlying mechanism.

P2971
Board Number: B252
Using Genome Wide CRISPR/Cas9 Screens to Elucidate How Cells Arrest Following Centrosome Amplification.
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Centrosomes are the main microtubule organizing centers of mammalian cells and play an important role in guiding the formation of the bipolar spindle apparatus upon which chromosome are segregated. Centrosome copy number is normally precisely controlled so that cells possess exactly two copies of the centrosome during cell division. Alterations in centrosome number reduce the fidelity of cell division by disrupting the structure of the mitotic spindle, leading to chromosome segregation errors. Extra copies of centrosomes are common in human cancer cells and are sufficient to promote genome instability and tumorigenesis in mice. Surprisingly, while centrosome amplification is frequently observed in cancer
cells, it is poorly tolerated in non-transformed cells. Our lab has performed genome wide loss-of-function screens to identify key proteins that are required to arrest the cell cycle in response to centrosome amplification. We have identified several proteins that, when knocked out, allow the continued growth of cells with extra centrosomes. We are currently examining how these proteins function to prevent the proliferation of cells with extra centrosomes. Understanding how the genes involved in this pathway ‘sense’ abnormal centrosome numbers and transmit this signal to arrest cell growth may lead to novel targets for cancer therapeutics.

P2972
Board Number: B253
Structural and functional analyses of the C. elegans Spindle-Defective Protein 2 ASH domain.
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Centrosomes are organelles that nucleate and organize microtubules in the cell. They have central roles in the formation of the bipolar spindle during the division of the spermatocytes and early embryo, and they are essential for the formation of the primary cilia and flagellum. Aberrant centrosome structure and/or function is a hallmark of cancer, and results in developmental abnormalities and ciliopathies including, polycystic kidney disease, nephronophthisis, Senior-Loken syndrome, orofaciiodigital syndrome and Bardet-Biedl syndrome. Our overall goal is to understand mechanisms of accurate centrosome inheritance during meiosis and mitosis. At the G2/prophase checkpoint of the cell cycle, centrosome maturation becomes apparent as the centrosome increases in size and pericentriolar material. Upon entry into the mitotic/meiotic division, activated centrosomes form the microtubule-organizing center (MTOC) of the bipolar spindle. Our focus here is to identify and test in vivo the role of the domains required for the normal localization and function of the centrosomal SPD- 2/CEP192 protein during mitosis/meiosis in C. elegans. The Spindle-defective protein 2, SPD-2, is a highly conserved protein with key roles in centrosome maturation and duplication. In addition, SPD-2 serves to recruit different proteins to the centrioles and to the pericentriolar material. Its numerous roles in centrosome structure and function make it an excellent target to study mechanisms of centrosome inheritance. Here we present the modeled three-dimensional structure of the ASH domain of C. elegans SPD-2 using template based modeling and its biophysical characterization. We also show localization of the SPD-2 ASH domain to the centrosome utilizing a GFP-tagged ASH construct. Using several domain mutation constructs, we present the sufficiency of specific residues for localization and function of the ASH domain. These studies not only expand our knowledge of centrosome function, but also provides a new tool for targeting proteins to the centrosome in C. elegans.

P2973
Board Number: B254
Pericentrin reduction is essential for efficient spermatogenesis.
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During spermiogenesis, centrioles are significantly remodeled in a process termed centriole reduction. This process includes the removal of many core centriole proteins. Previous studies in Drosophila have shown that a failure to remove the centriole protein Asterless (Asl) does not result in a defect in fertilization, but rather results in defects in embryogenesis caused by the unreduced paternal centriole (Khire et al. 2016). In this study, we find that pericentrin-like protein (PLP) is reduced prior to Asl
reduction, suggesting its removal might be a critical first step in the reduction process or be occurring for a distinct reason. To test the importance of PLP-reduction, we ectopically expressed PLP throughout spermatogenesis, which resulted in its sustained presence on the centriole. Furthermore, we found ectopic expression of PLP causes complete male infertility as a result of catastrophic failure to produce mature sperm. By investigating all stages of spermatogenesis, we determined that meiosis is unaffected by ectopic PLP expression in spermatocytes. However, significant defects appear in later stages of spermatid development, including in the attachment of the centriole to the nucleus and in the proper individualization of spermatids. Together, these data suggest that sperm centrioles must reduce PLP for reasons distinct from those for Asl. Interestingly, we found that ectopic PLP expression can lead to ectopic PCM and microtubule (MT) recruitment to basal bodies. We propose that PLP reduction is a highly regulated mechanism required to strip basal bodies of PCM and MTs for proper nuclear docking and are currently using Drosophila genetics in combination with high-resolution microscopy to test this model.

P2974

Board Number: B255

Unravelling the structural aspects of core Centriole proteins using various biophysical techniques.

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Centrioles are cylindrical microtubule-based organelles that aid in bipolar spindle formation and serve as basal bodies for nucleating cilia. They are characterized by a nine-fold symmetrical arrangement of microtubules at the periphery of the cylinder. Centriole duplication is tightly controlled in dividing cells. Misregulation of this process has been linked to a growing number of diseases such as cancer and microcephaly. Studies in C. elegans have identified core centriole proteins: SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4. Orthologs of these proteins have been found across genera including humans and the general theme of assembly is evolutionarily conserved with only slight variations. Although the molecular machinery has been worked out, structural details of centriole biogenesis are still obscure. The insolubility of centriolar proteins when expressed heterologously in bacteria, has hampered structural studies. In this study, we have devised improved methods for in vitro refolding of full-length ZYG-1, SAS-5, SAS-6 and SAS-4 expressed as inclusion bodies in bacteria. We subjected the full-length proteins to a variety of biophysical methods to elucidate their structural features. Circular Dichroism spectra of these proteins revealed the presence of defined secondary structural elements - SAS-6 and SAS-5 were predominantly alpha-helical, while ZYG-1 may have a mixture of both beta-sheets and alpha-helices. The in vitro refolded ZYG-1 is active, confirming that the polypeptides have attained their native conformation. The oligomeric status of these proteins was determined by size exclusion chromatography. ZYG-1 eluted as a single peak corresponding to a mean molecular mass of 994 kDa, indicating that ZYG-1 is approximately a 13mer. SAS-6 and SAS-5 eluted at the exclusion limit showing either the presence of large oligomers or that they form assemblies with an elongated morphology. SAS-4 exhibited a broad elution profile within the separation range, a feature of polydisperse ensembles. Thus, our study demonstrates that the full-length core centriolar proteins could be refolded in vitro and that they are amenable to biophysical analysis. The ability to refold these proteins ensures unhindered supply for structural investigations.
P2975
Board Number: B256
A centrosome-localized PLK1 scaffold ensures mitotic fidelity.
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PLK1 mis-regulation drives chromosome segregation errors. We are testing a PLK1 scaffold, Gravin, 
during this process. In both 3-D primary prostate cancer cell cultures that are prone to Gravin-depletion 
and control prostate epithelial cells treated with a Gravin shRNA an increase in cells containing 
micronuclei was noted when compared to control cells that express Gravin. To examine whether loss of 
Gravin affected PLK1 distribution and activity we utilized Fluorescence Recovery After Photobleaching 
(FRAP) and a PLK1 activity-biosensor. Gravin-depletion resulted in an increased PLK1 mobile fraction and 
activity. Gravin-loss, and subsequent increase in PLK1 activity, resulted in the spindle pole component, 
CEP215, having increased S/T phosphorylation and disorganization. Gravin-loss led to defects in 
microtubule re-nucleation from spindle poles, decreased K-Fiber formation, chromosome misalignment, 
and subsequent micronuclei formation following mitosis completion. Murine Gravin rescued 
chromosome misalignment and micronuclei formation, but a mutant Gravin (Gravin-T766A) that cannot 
bind PLK1 could not. These findings suggest that disruption of a Gravin–PLK1 interface likely leads to 
inappropriate PLK1 activity contributing to chromosome segregation errors, micronuclei formation, and 
subsequent DNA-damage.

P2976
Board Number: B257
Genetic Analysis of a sas-6 Mutant Suggests an Instructive Role for the Mother Centriole in 
Centriole Assembly.
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Centrioles are nine-fold symmetric barrel-shaped structures that exist as mother-daughter pairs, and 
participate in the assembly of bipolar spindles and the formation of cilia and flagella. During S phase of 
each cell cycle, centrioles are precisely duplicated through a process that involves the formation of a 
new daughter next to each preexisting mother centriole. While the mother centriole clearly dictates 
where a new centriole will form, its role in daughter centriole formation, beyond providing spatial 
control, has been controversial. SAS-6 is a protein that forms the central scaffold of the centriole and 
recent work has shown that SAS-6 can assemble into nine-fold symmetric rings in vitro suggesting that 
daughter centrioles can self-assemble. Indeed in vivo, centrioles can form de novo without a mother 
centriole, however these centrioles are often structurally abnormal. Here we describe a missense (D9V) 
mutation in C. elegans SAS-6 that compromises the ability of centrioles to act as mothers. The SAS-
6(D9V) protein can be assembled into centrioles (D9V centrioles) which organize normal-sized 
centrosomes and spindle poles. Further, D9V centrioles possess SAS-7, a marker of mature centrioles 
and are able to form daughter centrioles from cytoplasmic pools of wild-type SAS-6 protein. Thus, in 
many respects D9V centrioles appear normal. However, in the presence of a cytoplasmic pool of SAS-
6(D9V) protein, D9V centrioles almost always fail to form daughters. In contrast, wild-type centrioles 
can readily assemble cytoplasmic SAS-6(D9V) into daughter centrioles, demonstrating that SAS-6(D9V) is
assembly competent. Together these data indicate that centriole duplication failure associated with SAS-6(D9V) arises in part from a mother centriole defect and suggest some type of molecular communication between the mother centriole and the assembling daughter. Experiments are underway to identify which step in daughter centriole assembly is defective in D9V mothers.

P2977
Board Number: B258
Genetic screen for centrosomin synthetic lethality reveals novel proteins required for acentrosomal cell division.
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At least sixteen centrosomal proteins have been shown to be involved in microcephaly and primordial dwarfism syndromes; however, the underlying cellular and molecular mechanisms remain poorly understood. For this study, we focused on a single disease gene in Drosophila melanogaster known as centrosomin (cnn). Cnn facilitates proper assembly of the matrix of proteins known as pericentriolar material (PCM) and is responsible for coordinating the microtubule organizing center (MTOC) activity of mitotic centrosomes. Additionally, Cnn plays a critical role in neural stem cells where it is recruited asymmetrically to the younger “daughter” centrosome and is required for correct spindle pole polarity. Cells lacking cnn, however, are capable of assembling bipolar, anastral spindles at mitosis despite the fact that the cells lack functional centrosomes. Centrioles themselves are not necessary for proper division; furthermore, certain cell types can successfully complete multiple rounds of division without the centrosome. Cell division in plants and many unicellular eukaryotes, as well as oocyte meiosis in the majority of animal species, routinely occurs without the help of centrosomes.

We hypothesize the existence of a “backup” pathway that allows cnn mutant somatic cells to faithfully undergo mitosis despite their lack of functional centrosomes. To investigate this phenomenon, we conducted a screen in Drosophila to isolate mutations on the X chromosome that are synthetically lethal with cnn. We reasoned that mutants that were viable, but whose mutations were lethal in combination with a cnn null mutation, would reveal key components of the “backup” pathway. Isolated mutants reveal a variety of functions such as kinetochore microtubule stability, spindle pole focusing, spindle microtubule size control, and other spindle microtubule integrity effects.

Signaling Pathways and Target Screens

P2978
Board Number: B260
Characterization of aPKC-Related Signalling Pathways in Normal and Oncogene-Transformed Cells.
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Loss of epithelial homeostasis is considered a prerequisite for tumour formation, progression and developmental abnormalities. Atypical Protein Kinase C (aPKC) is required for cell polarity, proliferation and migration, and is commonly up-regulated in multiple cancer types including lymphoma, breast, prostate, pancreas and colon cancer. Partitioning defective 6 (Par6) is a regulatory element for aPKC that
regulates its localization and activity. Recent studies indicate that aPKC is required for Ras-induced transformation and carcinogenesis in many cancer types. However, our understanding of aPKC-mediated signaling pathways in normal cells and during carcinogenesis is incompletely understood particularly in 3D systems. To examine aPKC-related signalling pathways in normal and oncogene-transformed 3D cultured cells, Caco-2 cells were stably modified to express constitutively active KRAS-G12V. Non-transformed Caco2 cells form regular hollow central lumen and polarized spheroids in 3D culture. However, KRAS-transformed cells produce solid, irregular structures with disrupted cell polarity that exhibit a 3-fold increase in cyst size, which was blocked by aPKC knockdown. To examine aPKC targets in control and KRAS-transformed cells we used BioID. In addition to confirming known aPKC interacting proteins (including Pard3, Lgl2 and Pard6b) we identified 138 novel aPKC-interacting proteins. One of these is Aminopeptidase N (ANPEP), which is a widely-expressed type II membrane-bound metalloprotease. ANPEP is frequently overexpressed in diverse tumour cells, including breast, ovarian, and prostate cancer. We confirmed that ANPEP is in a complex with aPKC and Par6b, and that the PB1 domain of Par6 is necessary for association with ANPEP. We found that ANPEP co-localizes with aPKC and Par6 at the apical domain in 3D cysts. Interestingly, knockdown ANPEP in KRAS-transformed cells, cells presented significantly smaller than KRAS-transformed cells, this indicates that ANPEP associates with the apical Par complex and is required for KRAS-induced overgrowth and proliferation. Further characterization of the function and the pathway mechanisms of polarity-interacting proteins in 3D systems will give more insight to potential targets in epithelial transformation.

P2979
Board Number: B261
The lateral mobility of membrane-tethered KRAS4b revealed spatiotemporal complexity of signaling.
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RAS proteins function as GTP/GDP-dependent control switches to regulate signaling networks in cells and play a major role in human cancer. The major difference across the isoforms of RAS proteins (KRAS4a, KRAS4b, NRAS, and HRAS) lies within the C-terminus hypervariable region (HVR, aa 167-189) and its post-translational lipid modifications. One hypothesis is that the differential signaling behavior from RAS isoforms is controlled by the recruitment and organization of RAS into distinct membrane nano-domains. Association with these nano-domains is determined by RAS lipid-tethers and key residues in the HVR. However, very little is known about the molecular dynamics of membrane-tethered RAS molecules and how these dynamics influence the downstream signaling cascade within a living cell. Herein, the molecular mobility of membrane-tethered Ras variants was characterized by using single molecule tracking method that further estimated underlying mobility states. Detailed analysis of tracks revealed that KRAS4b exhibit confined mobility with three diffusive states in the active plasma membrane of living cells. This diffusion characteristic was unique to KRAS4b and influenced by both the hypervariable region and globular domain of the protein, compared to all the other RAS isoforms. Importantly, the occupancy of each diffusive states was altered for the oncogenic mutant of KRAS4b, implicating enhanced signaling activity. Our study begins to decipher the underlying principle of KRAS functionality in real time on the membrane of living cancer cells that will augment our ability to develop novel therapeutic strategies for targeting oncogenic KRAS4b.
Contract HHSN261200800001E - Funded by the National Cancer Institute
The Ras family of small GTP-binding proteins is frequently activated by mutations, including NRAS (20%), KRAS (2%), and HRAS (1%), in melanoma. In addition to mutations, Ras isoforms can also be activated by the inactivation of Ras GTPase activating proteins (RasGAPs), such as \( NF1 \), \( RASA1 \), and \( RASA2 \). In our recent study, we observed that the inactivation of \( RASA1 \) (RAS p21 protein activator 1, also called p120RasGAP) suppressed melanoma via its RasGAP activity toward the R-Ras (related RAS viral \( r-ras \) oncogene homolog) isoform. We hypothesized that, although not mutated, R-Ras is activated in melanoma through the inactivation of RasGAPs and that RasGAP/R-Ras pathway activation cooperates with BRAF activation in melanoma tumorigenesis.

In this study, we addressed the importance of R-Ras, a previously less appreciated member of the Ras family, in melanoma tumorigenesis and investigated the molecular mechanisms underlying R-Ras signaling in BRAF mutant melanoma. We observed frequent activation of R-Ras in BRAF mutant human melanoma cell lines. In addition, RNAi-mediated reduced expression of R-Ras suppressed anchorage-independent colony growth and tumor growth. Moreover, among the 3 major RAS effector pathways, reduced R-Ras expression suppressed Ral-A activation, which may explain the mechanism of Ral-A activation in BRAF mutant melanoma. Interestingly, anchorage-independent growth driven by \( RASA1 \) inactivation and subsequent R-Ras activation was suppressed by both genetic (siRNA targeting Ral-A) and pharmacological (Ral inhibitor BQU57) inhibition of Ral-A. To further investigate the impact of \( RASA1 \) loss, and thus R-Ras activation, on BRAF mutant melanoma development \textit{in vivo} we generated a \( RASA1^{+/\footnote{1}} \); BRAF \( ^{CA/CA}_L \); Tyr-CreERT2 mouse model in which treatment with 4OHT results in the expression of constitutively activated mutant BRAF and the deletion of \( RASA1 \) in melanocytic lineage cells. Preliminary analysis shows hyperpigmentation of the ear, tail, and foot pad in \( RASA1^{1/\footnote{1}} \) BRAF \( ^{CA/CA}_L \) mice compared to \( RASA1^{1/\footnote{1}} \) BRAF \( ^{CA/CA}_L \) littermates and the development of melanoma in \( RASA1 \) mutant mice. This study demonstrates the importance of the \( RASA1/R-Ras/Ral-A \) signaling pathway in BRAF mutant melanoma and supports the possible combinatorial treatment strategy targeting both the BRAF/MAPK and Ral signaling pathways.

**P2981**

**Board Number: B263**

\textbf{To identify the subcellular EGFR interactome in NSCLC cells by quantitative proteomics.}

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The alteration of the epidermal growth factor receptor (EGFR)-driven signaling network is a characteristic feature of non-small cell lung cancer (NSCLC), and inhibition of EGFR represents a treatment strategy. However, EGFR-targeted interventions have been largely ineffective. Aberrant
subcellular translocation and distribution of EGFR represent a major yet underappreciated NSCLC development mechanism. These include the translocation of EGFR to non-canonical sub-cellular locations including the nucleus and mitochondria. Mapping the relationship between the subcellular interactome of EGFR and NSCLC can yield a fundamental understanding of the mechanisms governing tumor progression and therapeutic resistance, leading to alternative treatment strategies. In this study, we employed isobaric mass tags (iTRAQ)-based quantitative proteomics approach to identify subcellular proteins that interact with EGFR in the highly invasive CL1–5 NSCLC cells. The resulting MS/MS spectra were analyzed using the UniProtKB/Swiss-Prot sequence database with the MASCOT algorithm. The search results were further evaluated using the TPP software (version 4.1) with stringent criteria regarding protein probability (>0.90) and at least two peptide hits for one protein identification. Using such an analysis, we identified 58, 79, and 67 EGFR-interacting proteins in the cytosol, mitochondria, and nucleus, respectively. To deduce the potential biological processes involving subcellular EGFR interactome, the EGFR-interacting proteins identified in the iTRAQ experiment were uploaded into DAVID v6.7 and analyzed for enrichment of categories belonging to biological processes. The proteins of direct interactions with mitochondrial EGFR are correlated with biological process of metabolism and mitochondrion organization. The EGFR-interacting proteins in nucleus are exclusively related to process of RNA processing/splicing. We have confirmed a number of these interactions. These data indicated that EGFR at the different subcellular locations elicits distinctly different signaling. Variations in the subcellular translocation and distribution of EGFR within NSCLC cells could also serve as an additional molecular signature for therapeutic choice, as those variations could have a dramatic impact on therapeutic efficacy.

P2982
Board Number: B264
Quantitative analysis of calcium signaling to identify specific pathways that promote drug resistance in neuroblastoma.
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Neuroblastoma (NB) is the most common extra-cranial solid tumor and the second most common pediatric tumor in children. The range of clinical outcomes in NB is broad, from spontaneous regression to aggressive tumors, and poor outcome is often due to disseminated disease and the development of multi-drug resistance (DR). The mechanisms whereby NB cells switch to a DR phenotype remain relatively unknown. Calcium is a well-known second messenger with a crucial role in cell viability, cell differentiation, and cell proliferation. Intracellular calcium levels are tightly controlled through calcium signaling at the plasma membrane, as well as the endoplasmic reticulum (ER) and mitochondria, which serve as calcium reservoirs. However, many of these calcium signaling pathways are deregulated in cancerous cells, resulting in uncontrolled growth, increased metastatic potential, increased survival signaling, resistance to cell death and DR. We hypothesize that altered calcium-related pathways, including calcium signaling between subcellular compartments, can exacerbate cell survival or reduce cell apoptosis leading to the DR phenotype in NB cells. MYCN-amplified, drug-sensitive (SKNBe1) or drug-resistant (SKNBe2(c)) cell lines were tested using Western blot, cell survival assays, ratio-metric Fura-2-AM calcium measurements, and confocal microscopy when exposed to anti-cancer drugs. Several anti-cancer drugs (doxorubicin, etoposide, vincristine and cisplatin) alter cell survival and expression levels of calcium-signaling-related proteins. Intracellular calcium levels of drug-sensitive (DS) or DR cells were measured in a confocal microscope using fluorescent dyes or genetically encoded calcium indicators specifically targeted to the ER or mitochondria. To distinguish the sources of intracellular
calcium, we have developed analytical protocols of signal co-localization to obtain Pearson’s correlation coefficients (PCC) to discriminate between compartments. Using a microperfusion system, transient applications of anti-cancer drugs induce rapid changes in intracellular calcium levels. These new findings suggest that this methodology can clearly differentiate specific calcium-related signaling that may be involved in the switch to a more aggressive DR phenotype, and reveal novel targets for the development of more effective treatments for high-risk NB patients.

P2983
Board Number: B265
HBx-induced Ca²⁺ aberrancy in hepatocellular carcinoma.
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Chronic infection of hepatitis B virus (HBV) is the leading cause of hepatocellular carcinoma (HCC), but how HBV viral proteins induce cancer initiation and progression remains unknown. Previous researches revealed that one of the viral proteins, HBx, induced liver cancer in mouse models. Further investigations also indicated that HBx regulated intracellular Ca²⁺ to promote the replication of HBV and the death of hepatocytes. However, how such HBx-related acute events are linked to the development of HCC remains elusive. We therefore studied how HBx regulated intracellular Ca²⁺ signaling and its functional significance in HCC. We firstly detected [Ca²⁺] in cytosol, endoplasmic reticulum (ER) and mitochondria of HBx-expressing hepatocyte or HCC cell lines, using live-cell fluorescence Ca²⁺ imaging. We noticed that cells expressing HBx had universally decreased [Ca²⁺] in cytosol, ER and mitochondria in different cell lines, indicating that HBx might decrease the internal Ca²⁺ storage and subsequently reduce intracellular Ca²⁺ signals in affected hepatocytes or HCC cells. We further studied the mechanism how HBx decreased intracellular Ca²⁺ in HCC cells, through examining whether or not HBx changed Ca²⁺ flows between intracellular compartments. By manipulating Ca²⁺ pump activities and extracellular Ca²⁺ levels, we noticed that HBx increased Ca²⁺ excretion from cytosol to the extracellular space. Interestingly, neither the Ca²⁺ release from ER to cytosol nor the store-operated Ca²⁺ entry (SOCE) was directly affected by HBx. Therefore, the plasma membrane Ca²⁺-ATPase (PMCA), Na⁺-Ca²⁺ exchanger (NCX) or other Ca²⁺ extrusion systems may be potential HBx targets. We are currently working on (1) how HBx mechanistically suppresses Ca²⁺ excretion, and (2) whether or not HBx-induced Ca²⁺ alterations are linked to the initiation and progression of HCC. These approaches will probably resolve the mystery of HBx, leading to the improvement of current HBV treatment and HCC prevention.

P2984
Board Number: B266
Long noncoding RNA HOTAIR and miR-203 regulate tumorigenesis in renal cell carcinoma by targeting the epithelial-to-mesenchymal transition pathway.
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Objective: Long non-coding RNAs (lncRNAs) and miRNAs are known to be involved in gene regulation in several cancers, but their regulatory mechanisms are far from clear. Several studies show that HOTAIR, an lncRNA, is highly expressed, whereas miR-203 has low levels of expression in various types of cancer, however the role of HOTAIR in renal cell carcinoma (RCC) remains poorly understood. We hypothesized
that lncRNA HOTAIR and miR-203 interaction may be crucial for renal cell carcinoma (RCC) proliferation, invasion, and tumorigenesis. Thus, the focus of the present study was to investigate the role of oncogenic lncRNA HOTAIR and its regulatory miRNA miR-203 in renal cancer using in vitro and in vivo models.

Experimental Design: Human renal cancer cell lines (ACHN and Caki-1), normal renal epithelial cells HK-2 and tumor tissues were used for this study. Expression levels of HOTAIR and miR203 were evaluated by quantitative real-time PCR and their interaction was confirmed by luciferase assay. Cells were transiently transfected with HOTAIR siRNA (25nM) and miR-203 mimic (10nM) for 72 hours and used for functional studies. Statistical analysis was performed to determine the clinical significance of HOTAIR and miR-203 in kidney cancer patients. The biological role of miR-203 and its interaction with HOTAIR was investigated using cell lines and nude mouse models.

Results: HOTAIR expression in RCC cell-lines and clinical specimens was observed to be higher and that of miR-203 lower when compared to normal cells and tissues. Overexpression of miR-203 altered the cell cycle, inhibited epithelial-to-mesenchymal transition (EMT) and decreased proliferation with induction of epithelial marker proteins, PTEN, p21, p27 and decreased pAKT. Luciferase assay confirmed direct binding of miR-203 to HOTAIR. Knockdown of HOTAIR reduced cell migration, invasion, and induced apoptosis, mimicking the effects of miR-203 overexpression. Administration of miR-203 mimic to established tumors in nude mice significantly suppressed tumor growth compared to controls. Statistical analysis using patient samples showed that both HOTAIR and miR-203 may serve as diagnostic and prognostic biomarkers for renal cell carcinoma.

Conclusion: Our results demonstrate that miR-203 overexpression inhibits RCC progression through HOTAIR knockdown and reversal of epithelial-to-mesenchymal transition. These studies show that HOTAIR and miR-203 may be useful in RCC diagnostics and therapeutics.

P2985

Board Number: B267

Influence of breast cancer drivers on mammary gland architecture.

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Though many putative drivers of breast cancer have been identified, there is limited understanding of the mechanisms by which they promote cancer progression at the level of tissue structure. Specifically, their impact on the transition from in situ to invasive cancers is largely unknown. In the normal breast, the milk-secreting luminal cells (LEPs) are surrounded by the contractile myoepithelial cells (MEPs). This bilayered architecture constitutes a tumor suppressive microenvironment for transformed luminal cells, and its systemic breakdown is the hallmark of progression to invasive cancers.

We recently demonstrated that the tissue architecture is maintained by a balance of interfacial tensions between LEPs, MEPs, and their extracellular matrix (ECM). Specifically, the unique ability of MEPs to strongly interact with the ECM is central to their capacity to maintain tissue architecture through self-organization. Consequently, we hypothesize that physical changes to the interfacial properties of LEPs and MEPs upon oncogenic activation can cause architectural disruption, thereby promoting the progression to invasive cancers. While changes in the physical properties of both tumor cells and surrounding matrix during cancer progression have been reported, a direct mechanistic link between oncogenes, altered interfacial mechanics, and a breakdown in tissue architecture has not been made.

To this end, we are characterizing the influence of putative breast cancer driver genes on the self-organizing capacity of the mammary gland. Starting with primary human mammary epithelial cells from
healthy human donors, we are systematically analyzing the self-organization, biophysical properties, and transcriptional dynamics of these cells expressing defined combinations of driver genes for fixed periods of time. For example, we found that LEPs expressing a mutant form of PIK3CA fail to self-organize with MEPs, likely due to PIK3CA activation-induced changes to cellular mechanics. In contrast, the expression of cyclin D1, another common oncogene which does not have a major role in cytoskeletal regulation, does not have a significant impact the tissue self-organization.

Collectively, by quantitatively linking cancer-driver expression, biophysical changes in cell and tissue mechanics, and the associated transcriptional changes, we aim to determine the molecular underpinnings of tissue architectural disruption in breast cancer.

P2986
Board Number: B268
MiR-133a function in the pathogenesis of dedifferentiated liposarcoma.
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Objective: Sarcomas are malignant heterogeneous tumors of mesenchymal derivation. Dedifferentiated liposarcoma (DDLPS) exhibits aggressive biological behavior with an 80% local recurrence rate and metastasis in approximately 20% of patients. Our laboratory characterized a unique miRNA expression signature associated with human DDLPS tissue. The objective of this study is to characterize the biological and molecular consequences of miR-133a in DDLPS.

Methods: Taqman real-time PCR was used to evaluate expression levels of myomiRs in human DDLPS tissue, normal fat tissue, and available human DDLPS cell lines. To evaluate the effects of miR-133a expression on DDLPS cell line behavior in vitro, cells were stably transduced with miR-133a vector or empty lentiviral vector and the effects on cell cycle, proliferation, apoptosis, and migration were assessed. DDLPS cells were injected subcutaneously in mice to form xenograft tumors. Agilent Seahorse Bioanalyzer system was used to assess metabolism in vitro and ex vivo from sectioned xenografts. We performed an in silico search for predicted targets of miR-133a using target prediction databases and compared these target genes to known genes that are highly expressed in human DDLPS tissue.

Results: The expression levels of myomiRs were significantly decreased in human DDLPS tissue compared to normal human fat as well as in human DDLPS cell lines compared to a human preadipocyte cell lines. Overexpression of miR-133a decreased cell proliferation, decreased glycolysis, and increased spare respiratory capacity. There was no significant effect on cell cycle, apoptosis, migration, or in vivo tumor growth. Oxidative respiration was increased in xenograft tissue formed from cells overexpressing miR-133a. Using in silico target gene analysis, we identified 18 potential targets of miR-133a.

Conclusions: We have demonstrated that enforced expression of miR-133a decreased proliferation and metabolism in human DDLPS cells. We have identified putative gene targets of miR-133a and are currently dissecting the molecular mechanism by which miR-133a may mediate proliferation and metabolism in DDLPS cell lines. Taken together, these results suggest that miR-133a regulates metabolism and proliferation, and its dysregulation might contribute to the oncogenic phenotype of DDLPS.
P2987

Board Number: B269

BRCA1 regulation of miRNA expression in breast cancer.
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Hereditary mutations in BRCA1 are a well-known susceptibility factor in breast cancer. Women with one defective copy of this protein end up with increased chances to acquire breast cancer. Partial disruption of the function of this protein is enough to cause cancer susceptibility. There are several functions attributed to BRCA1 that explain its role in carcinogenesis. Some studies have found that BRCA1 can be a transcription factor and others suggest it is a protein involved in DNA double strand repair. Additional work suggests BRCA1 acts through regulating DNA methylation by biding to DNA methyltransferase-1. Other effects of BRCA1 epigenetic regulation have been less explored. Here we proposed to study the effect of partial depletion of BRCA1 in the breast cancer cell line MCF7. We treated the cells with limited amounts of siRNA (5nM) targeting BRCA1 and determine the levels of expression by qRT-PCR. Our results showed that the mRNA levels of BRCA1 decreased by 40% (BRCA1 mRNA expression = 58.62% ± 3.16%). In the cells where BRCA1 was partially expressed, levels of DNMT1 and DNMT3B were slightly decreased by 20% and 10%, respectively (DNMT1 mRNA expression = 82.30% ± 6.10% and DNMT3B mRNA expression = 90.91% ± 9.01%). We will determine how the partial loss of BRCA1 affects the levels of miRNAs in the cell, by examining miRNA expression using the Human miRNA CodeSet from NanoString. We will later confirm the positive hits by qRT-PCR. These findings will greatly increase our understanding of BRCA1 function and the role it plays in susceptibility to breast cancer.

P2988

Board Number: B270

A Genome-Wide miRNA Screen to Identify Regulators of Tetraploid Proliferation.
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Tetraploid cells, commonly generated by errors in cell division, are genomically unstable and well known to promote tumorigenesis. Recent studies indicate that ~40% of solid tumors undergo a whole-genome doubling event during tumor development. This suggests that tetraploidy, which promotes aggressive and invasive phenotypes, plays a significant role in shaping the evolution of tumors. To combat the pro-oncogenic effects of tetraploidy, it was recently discovered that tetraploid cells activate the Hippo tumor suppressor pathway and stabilize p53 to restrict their growth. To successfully proliferate and become oncogenic, tetraploid cells must therefore overcome this G1 cell cycle arrest; however, how tetraploid cells circumvent this arrest remains poorly understood. We hypothesized that overexpression of specific miRNAs, which are small non-coding RNAs that are commonly deregulated in various human malignancies, may enable the proliferation of tetraploid cells. We performed a comprehensive, gain-of-function genome-wide screen to identify miRNAs that promote the proliferation of arrested tetraploid cells. Our screen identified ~20 miRNAs whose expression significantly promoted tetraploid proliferation. The vast majority of these miRNAs promote tetraploid growth by enhancing growth factor signaling (e.g. miR-191-3p); however, we also identified several miRNAs that disable the p53-p21 pathway (e.g. miR-523-3p), and a single miRNA (miR-24-3p) that inactivates the Hippo pathway, in part
via downregulation of the tumor suppressor gene NF2. Collectively, our data reveal several new avenues through which tetraploid cells may regain the proliferative capacity necessary to drive tumorigenesis.

P2989
Board Number: B271
Evolution of genetic instability through single-hit mutations.
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Genetic instability, defined as an increase in the mutation rate, accelerates evolutionary adaptation. Repeatedly selecting for antibiotic resistance selects for genetically unstable mutants in E. coli. Genetic instability is universal in cancer and is thought to arise primarily from the inactivation or removal of both copies of genes whose products function in DNA metabolism and cell cycle regulation.

We evolved and characterized genetic instability in diploid strains of the budding yeast, Saccharomyces cerevisiae, by selecting for the sequential inactivation of three independent growth suppressor genes, yeast analogs of tumor suppressor genes. Our selection produced clones with increased point mutation, mitotic recombination, and chromosome loss rates. Whole genome sequencing and genetic segregation analysis identified candidate, heterozygous mutations and when these were engineered, individually, into the ancestral, genetically stable diploid strain, they induced genetic instability. Conversely, restoring the wild-type allele in the evolved clone reduced genetic instability. 20% of the genes we found have homologs implicated in human cancer, they include essential genes and genes not previously implicated in genetic instability, they are enriched for genes which function in transport, protein quality control, and DNA metabolism, and they differ from genes found in previous screens for genetic instability.

We conclude that single genetic events can cause genetic instability in diploid yeast cells, and propose that similar, single-hit, mutations may initiate genetic instability in cancer.

P2990
Board Number: B272
Stereotyped p53 binding tuned by chromatin accessibility.
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Differential regulation of transcription factors give cell types their identity. Many transcription factors are ubiquitously expressed yet nonetheless regulate specific targets in different cellular contexts. The degree to which the differential activities represent different transcription factor binding or more downstream regulation of their activity at the promoter or system wide level is unclear. We focused on the tumor suppressing transcription factor p53 and compared in vitro binding to in vivo ChIPseq data. We observe that while a position weight matrix/motif predicts p53 binding in vitro well, in vivo the p53 motif is only weakly associated with binding suggesting other cellular factors modulate binding. To explore if in vivo regulators of p53 binding could vary across cell lines leading to different p53 dependent phenotypes we used ChIPseq to compare p53 binding across 13 cell lines. Interestingly, we observe quite uniform p53 binding, with only ~5% of p53 binding sites varying substantially across cell lines. The binding of p53 to these ‘variable’ binding sites was lineage specific with, for example, p53 associating with several novel ER regulated target genes in an ER+ breast cancer line. Indeed, in this cell line ER binding was a significant predictor of adjacent p53 binding. We wondered to what extent these interactions represented direct regulation or indirect control through chromatin accessibility. Using
ATACseq we found that chromatin accessibility was a strong predictor of p53 binding and that when controlling for accessibility all other factors tested (e.g. ER or Myc binding) were not significant. These results show the limited diversity of p53 binding across cell lines and identify the key role of chromatin regulation in regulating cell type specific p53 dna binding.

P2991
Board Number: B273
E6 Proteins from Diverse High-Risk HPV Types Differ in their Target Specificities.
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At least twelve HPV types have been defined as definitely carcinogenic in humans, and a further thirteen types as probably or possibly carcinogenic. All of these have PDZ-binding motifs (PBM)s on the extreme C-terminus of the E6 oncoprotein.

Using an unbiased proteomic approach we have previously shown that each of these E6 PBM}s has a particular set of preferred cellular targets, and that increased oncogenic potential of the virus appears to correlate with increased functional flexibility of the PBM, and preferred selection of a subset of PDZ domain-containing target proteins. We also identified the ZO2 protein as a novel E6 target that is stabilised, rather than degraded, in the presence of HPV E6. We have now identified another cellular protein, bound through a different motif, that also appears to be stabilised by binding the E6 proteins from certain HPV types. It is becoming clear that the outcome of any infection is the result of interplay between the lineage of the infected cell and the precise type of HPV infecting it. Accordingly we have now extended our studies to look at how the target protein repertoire of these PBM}s might change under different growth conditions, and in cells from different origins. In addition we have found that certain non-PDZ-containing proteins are also targeted by this region of E6, apparently in a phosphorylation-dependent manner.

P2992
Board Number: B274
IDENTIFICATION OF CANDIDATE GENES ASSOCIATED WITH TRIPLE NEGATIVE BREAST CANCER.
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Triple negative breast cancer (TNBC) is a heterogeneous disease. Although the disease is defined as a single subtype, patients (or cell lines) within the group can be very different. Microarray analyses of TNBC samples show that at least five different subclasses exist within the subtype. One of the subclasses has been defined as enriched for immune-related genes. For the current project we identified TNBC cell lines and clinical patient microarray datasets from Gene Expression Omnibus (GEO). The TNBC cell line datasets were separated into two groups based on low or high expression of immune-related genes. Unsupervised bioinformatics-analysis of the microarray datasets was performed and a larger list of differentially expressed genes were selected. Following further comparison to clinical patient samples and experimental validation, six candidate genes were ultimately selected. The six candidate genes included IL32, GATA3, MYBL1, ETS1, TMEM158 and PTX3. The candidate genes demonstrated differential gene expression in approximately 60% of TNBC patient samples compared to other TNBC, normal, luminal A, luminal B and Her2 patient samples. This study outlines the approach utilized to select the final list of candidate genes. At this point, it is unclear as to the clinical utility of the six genes
either individually or jointly. The first approach is to confirm the use of the genes to further characterize TNBC heterogeneity.

Tumor Invasion and Metastasis 3

P2993
Board Number: B275
TGF-beta determines the pro-migratory potential of bFGF signaling in medulloblastoma.
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The mechanistic understanding of how tumor cell dissemination is controlled by microenvironment-derived cues is still incomplete. In this study, we investigated the integration of microenvironment-derived growth factor signaling and how this affects cancer cell migration and invasion. We developed a phenotype-based screening approach1 to identify mechanisms and regulators of cell migration/invasion in the pediatric brain tumor medulloblastoma (MB), the most common malignant brain tumor in children. The combination of this approach with validations in organotypic cerebellum slice culture2 and an orthotopic PDX model allowed us to determine at the molecular, cellular and tissue levels how tumor cells integrate parallel signals from different, microenvironment-induced pathways.

We identified tumor growth factor beta (TGF-b) function as a key determinant of pro-migratory basic fibroblast growth factor (bFGF) signaling in MB. We demonstrate that bFGF promotes migration/invasion through FGF receptor substrate 2 (FRS2), and that at low bFGF concentrations, TGF-b antagonizes FRS2 activation through Rho kinase (ROCK) and Extracellular Regulated Kinase (ERK1/2). Latter prevents activating FRS2 tyrosine phosphorylation only in the context of non-canonical TGF-b signaling via contractility-dependent relocalization of ERK1/2. Conversely, at high bFGF concentrations where FRS2 activation is blocked by negative feedback through ERK1/2, TGF-b restores FRS2 activation and enables migration/invasion. Thus, by controlling FRS2 function through coinciding ERK1/2 and ROCK activation, TGF-b determines the pro-migratory response of the tumor cells to the variable abundance of bFGF in the microenvironment.

This study identified FRS2 as a coincidence detector that integrates variably abundant microenvironmental cues to control pro-migratory cell functions, and it exemplifies a novel paradigm of rheostat regulation of growth factor signaling.

**P2994**  
**Board Number: B276**  
**Illuminating the role of neurogenic regulator REST in Medulloblastoma dissemination.**  
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Perturbed developmental signaling is characteristic of the pediatric brain tumor Medulloblastoma (MB). In fact, MB is subgrouped by activation of various molecular pathways including sonic hedgehog (SHH) and wingless (WNT) signaling, with two other less defined subgroups named group 3 and group 4. Across all subgroups, patients with metastasis display increased levels of the protein, RE-1 silencing transcription factor (REST). Consistent with this, an increased incidence of leptomeningeal dissemination is observed in a novel transgenic mouse model expressing REST in the cells of origin of SHH MB, cerebellar progenitor cells. We demonstrate here that elevated REST results in an abnormal cytoarchitecture in the postnatal cerebellum. Further analysis of these defects revealed REST-dependent dysregulation of the CXCR4 signaling pathway, a cascade known to play a role in proper cerebellar lamination. The activation of CXCR4 is an established marker of negative prognosis in MB patients. Consistent with this, when we elevated REST in the tumor environment, there was a concomitant increase of CXCR4 signaling and tumor cell migration. Our findings suggest that REST-dependent activation of CXCR4 contributes to the aggressive tumor behavior displayed by subgroups of MB patients and open the discussion to treatment options targeting the REST complex in REST-high tumors.

**P2995**  
**Board Number: B277**  
**Optimized Isolation of Plasma Extracellular Vesicles for Use as Potential Biomarkers in Patients with Glioblastoma.**  
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Glioblastoma (GBM) is the most common brain tumor and is nearly universally fatal within five years despite aggressive treatment. Tissue histopathology is required for definitive diagnosis. Imaging alone is inadequate to distinguish tumor progression from treatment-related inflammatory pseudoprogression, particularly following immunotherapy. Therefore, there is a need for non-invasive diagnostic alternatives. GBM’s release extracellular vesicles (EV’s) including exosomes (<100nm) and microvesicles (100nm–1000nm). GBM EV’s in plasma may have biological effects including mediating immunosuppression and could serve as liquid biopsies as they contain signature tumor-associated antigen immunosuppressive protein profiles. However, plasma EV isolation techniques have not been optimized. Here, we present a simple technique to isolate plasma EV’s from grade 2, 3 and 4 glioma patients (n=21) with new or recurrent tumors that are isocitrate dehydrogenase (IDH) mutant or wild type (WT), and normal donors (n=19) using a two-step ultracentrifugation protocol. After ultracentrifugation using a sucrose gradient (90 minutes at 100,000 x g), nanotracker (NanoSight) analysis revealed that glioma patients with tumors that are IDH WT (n=7) have smaller particle size (13.04 ± 5.660) (P<0.05), but increase plasma EV concentration (-1.993X1011 ± 9.207X1010) (P<0.05) compared to normal donors. Further ultracentrifugation (16 hours at 100,000 x g) concentrated EV’s efficiently for additional analysis, but resulted in EV aggregation that skewed nanotracker results. Western blot analysis of isolated plasma EVs confirmed exosome markers CD63 and HSP70 in both
glioma patients and healthy donors. Interestingly, a higher expression of the immunosuppressive molecule programmed death-ligand 1 (PD-L1) was observed in glioma patients in comparison to healthy donors. Cytokine array analysis showed higher concentration of the Th1 cytokine IFN-γ in plasma EV's from normal donors in comparison to plasma EV's from glioma patients (34.50 ± 9.856) (Ps0.05). These findings suggest that a simple two-step ultracentrifugation protocol efficiently isolates plasma EVs with distinct differences between glioma patients and normal donors that could be used as a non-invasive biomarker.

P2996
Board Number: B278
Emerging role of a sulfhydryl oxidase in glioma cell behavior.
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Quiescin Sulfhydryl Oxidase 1 (QSOX1) is a flavo-enzyme that catalyzes the oxidation of free thiols to generate disulfide bonds with the reduction of molecular oxygen to hydrogen peroxide. QSOX1 has been reported to be upregulated in a number of cancers including breast, prostate, pancreatic and certain brain cancers. Overexpression of QSOX1 has been correlated with aggressive cancers and poor patient prognosis. Gliomas are primary brain tumors that occur mostly in adults. Glioblastoma Multiforme (GBM) is a stage IV glioma that is very aggressive and has been practically impossible to treat successfully. GBM cells invade normal brain tissue very quickly and, thus, escape surgery to lead to an often more aggressive recurrence. There is a continuing need to understand the mechanism of GBM cell migration and invasion. Here we investigate the function of QSOX1 in human gliomas. We lentivirally introduced shRNA to knockdown QSOX1 in a human GBM cell line, T98G. These QSOX1 knockdown cells showed decreased cell migration in vitro as demonstrated by a quantitative Super Scratch assay. We also used the embryonic chick brain xenograft model to demonstrate glioma cell invasion in vivo. Therefore, reduced QSOX1 expression leads to decreased glioma cell migration and invasion both in vitro and in vivo, respectively.

P2997
Board Number: B279
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Biological self-organization is when a process or a biological multicellular structure forms at the macroscopic level as a result of interactions among lower-level components, i.e., individual cells. Interactions among the individual components only utilize local information. Cancer growth is mostly understood as random. The role that stereotypical structures may have in tumor growth has not been carefully considered. During the study of both rodent and human brain tumors we determined the existence of multicellular structures which we named oncostreams. Oncostreams are elongated cells 5-20 cells wide and of variable length. Oncostreams express proteins that are typical of brain tumors, i.e., GFAP, nestin, twist, MMP2, retinoblastoma, olig2, amongst others. The density of immunocytochemically distinct proteins varies between oncostream and non-oncostream glioma cells.
The function of oncostreams was explored utilizing cell biological, molecular, and mathematical modeling experiments. Oncostreams mediate glioma invasion, as they form tight fingers of parallel elongated cells that push into surrounding normal brain. Oncostreams can also serve as preferential highways for the movement of glioma cells throughout the brain. Injection of slowly moving human brain glioma stem cells into rapidly streaming rodent glioma cells results in the alignment of the human glioma cells to the oncostreams, and their fast distribution throughout the tumor along these structures. Oncostreams may also limit immune cells’ entry into gliomas as they appear to be excluded from these structures. To test the hypothesis that oncostreams are formed solely from interactions between individual glioma cells, we built a series of agent-based mathematical models. These allowed us to discover that only elongated cells form structures resembling oncostreams. Circular cells never do so. The length:width ratio of the elongated oncostream-forming cells is not significantly different from the ratio measured in vivo. Finally, to understand whether oncostreams differ molecularly from surrounding glioma cells, we dissected oncostreams and surrounding glioma tissue using laser scanning microdissection, RNA-Seq and bioinformatics. The set of gene expression patterns differs significantly between the two areas, thus enabling us to identify the molecular individuality of oncostreams. Network analysis has identified fyn and STAT-1 as highly connected nodes. Deletion studies are now allowing us to demonstrate that fyn appears to play a role in oncostream formation and malignant tumors’ phenotype. In conclusion, we demonstrate that oncostreams are a novel cancer structure with individual molecular makeup and function, and have an important role in determining the phenotype of malignant brain gliomas.

P2998
Board Number: B280
Mechanisms Involved in Microglia Stimulation of Glioblastoma Invasion.
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Glioblastoma multiforme (GBM) is one of the deadliest human cancers with a median survival rate of 12 months post diagnosis. One of the major hallmarks of GBM is its highly infiltrative nature which makes advanced tumors nearly impossible to fully resect. Therefore increasing our knowledge of the mechanism GBM cells use to invade normal brain is of critical importance in designing novel therapies. We recently showed that tumor associated microglia stimulate GBM cell invasion and this process is mediated by CSF-1R signaling. This finding is consistent with the great body of literature that supports a role for myeloid cells in invasion and metastasis of a variety of cancers regardless of cellular origin. In this study, we seek to identify factors that are upregulated in GBM-stimulated microglia as well as uncover potential signaling mechanisms that promote GBM invasion. We assayed cDNA and protein from microglia treated with conditioned media from the murine GBM cell line GL261, and discovered that the EGFR ligand amphiregulin (AREG) and the chemokine CCL3 (MIP1a) are significantly induced. Using RNA interference, we show that microglia depleted of AREG cannot promote invasion of GL261 cells into matrigel-coated invasion chambers as effectively as controls. In addition, an AREG blocking antibody strongly attenuates the ability of THP1 macrophages to activate human GBM cell line U87 invasion. We have also demonstrated that novel small molecule CCR1 specific antagonists also inhibit the ability of microglia to stimulate microglia-stimulated glioblastoma. Interestingly, the ability of microglia to fully stimulate GBM cell invasion requires cell-cell contact. Three dimensional spheroid invasion assays confirm the extensive cell-cell contacts that occur between invasive GL261 and U87 cells and microglia. We have identified the tyrosine kinase receptor EphB2 as a key mediator of microglia activation of invasion. Microglia and other macrophage cell lines were found to express abundant levels
of Ephrin-B1, the principal ligand for EphB2. Consistent with the observation that juxtacrine interactions are critical, microglia are unable to stimulate invasion of EphB2-depleted U87 GBM cells. Taken together, we observe in two models of GBM that CSF-1 secreted by tumor cells reprogram microglia and macrophages to stimulate GBM invasion via several mechanisms including AREG/EGFR, CCL3/CCR1 and Ephrin-B/EphB2.

**P2999**  
**Board Number: B281**  
**Downregulation of ASCT2 (alanine-serine/cysteine transporter 2 / SLC1A5) in human cancer.**  
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ASCT2 (alanine-serine/cysteine transporter 2 / SLC1A5) is highly expressed in cancer cells to support their continuous growth and proliferation and is a promising pathological marker for predicting a worse outcome in certain types of cancer (Semin Cancer Biol 15:254-266,2005). The present study examined the effect of ASCT2/SLC1A5 gene down regulation by RNA interference on the expression and function of the transporter in human cancer cells. Target sites within ASCT2 gene (NM_001145144) were selected from the respective human mRNA sequences. Nucleotide homology searching was performed against nonredundant and dbEST using BLAST via online connection to NCBI. The commercial siRNA SI00079730 that targets the human ASCT2 mRNA sequence and negative control siRNA commercial sequences NC-SI03650318 and NC-SI03650325 (Qiagen) were tested. Cells (human liver adenocarcinoma SK-HEP-1, human fibrosarcoma HT-1080 and human bladder carcinoma T24) were cultured for 48 h (in RPMI and DMEM-hg, respectively). ASCT2 mRNA and protein expression, inward transport of [14C]-L-alanine, and effects of siRNA anti-ASCT2 upon [14C]-L-alanine uptake were analyzed. Antibodies raised against ASCT2 and GAPDH were used, and images were obtained by scanning at both 700 nm and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences). For RT-PCR, total RNA was converted to cDNA and qPCR analysis was made in the StepOnePlus instrument (Applied Biosystems). Primers for ASCT2 and for the endogenous control gene GAPDH were used. All cell types expressed ASCT2 mRNA and protein. The abundance of ASCT2 mRNA differed among the tumor cell lines, being more intense in HT-1080, followed by SK-HEP-1 and T24 cells. The abundance of ASCT2 protein followed the same trend. The sodium-dependent inward transport of non-saturating concentrations (0.25 μM) of [14C]-L-alanine, the preferred substrate of ASCT2, was linear with time for up to 10 min of incubation. In order to determine the kinetics of the transporter, cells were incubated for 1 min with [14C]-L-alanine (0.25 μM) in the absence or in the presence of increasing concentrations of unlabelled substrate L-alanine (1 to 3 mM). The kinetic parameters of [14C]-L-alanine uptake (Km in mM; Vmax in nmol/mg protein/min) were determined by non-linear analysis of the specific analysis of inhibition curve for L-alanine and are as follows: SK-HEP-1, Km=0.221±0.023, Vmax=6.746±0.570; T24, Km=0.225±0.015, Vmax=8.799±0.103. Transfection with siRNA against ASCT2 gene, but not the negative control siRNAs, reduced the [14C]-L-alanine accumulation in all cancer cell lines. In conclusion, anti-ASCT2 siRNA decreased [14C]-L-alanine uptake, possibly by downregulation of ASCT2 expression and function.
P3000

Board Number: B282

Over-Expression of the βII Isotype of Tubulin and Especially Its Localization in Cell Nuclei Correlates with Poorer Outcomes in Colorectal Cancer.

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Tubulin, the subunit protein of microtubules, is a heterodimer of α and β subunits, each of which exists as isotypes differing in amino acid sequence and encoded by different genes, with the differences being highly conserved in evolution. Specific isotypes of tubulin have associations with cancer that are still not well understood. Previous studies noted that the βII-tubulin isotype is expressed in a number of transformed cells where the normal tissues do not express βII (Yeh, & Ludueña, Cell Motil. Cytoskeleton 57: 96, 2004). Even more interesting, in many of these tumors, βII is found in cell nuclei in non-microtubule form (Walss et al. Cell Motil. Cytoskeleton 42: 274, 1999). The association of βII expression and its nuclear localization with cancer progression has been noted to be more frequent in cancers but its potential clinical significance has not been addressed. Here we report a study with patients with colorectal cancer. We have previously reported that, while normal colon expresses little or no βII or βIII, inflamed colon expresses βIII while cancerous colon expresses both βII and βIII (Portyanko et al. Virchows Arch 454:541, 2009; Portyanko et al. J. Crohn's & Colitis 10, Suppl. 1: S102, 2016). We have found that patients whose tumors over-express βII-tubulin have a greatly decreased life expectancy, compared to patients whose tumors do not express βII-tubulin. We have also found life expectancy is even shorter in those patients with nuclear βII. These results are consistent with a model whereby, as a cancer progresses, it begins to over-express βII and then to localize it in the cell nuclei and therefore that βII expression and localization could be a useful prognostic marker. We have also observed that otherwise normal colon epithelial cells adjacent to the tumor contain nuclear βII. This observation could decrease the probability of a false negative biopsy. Our results suggest that expression of βII and its localization to the nucleus in non-microtubule form may be important for cancer cell growth and proliferation, and may be part of an as yet unknown signaling pathway. Our results raise the following mechanistic and pharmacological questions: 1) How does transformation lead to expression and nuclear localization of βII? 2) What does nuclear βII do for the cancer cell? 3) How does a cancer cell stimulate nearby cells to express βII? 4) Is the currently accepted paradigm that anti-tubulin drugs work by freezing microtubule dynamics sufficient or do we need a new set of drugs to target tubulin in non-microtubule form?
P3001
Board Number: B283
Association of cell stiffness and migratory potential of head and neck and prostate cancer cells exposed to cytostatics.
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It has been shown that cell stiffness could be used as a marker for cancer progression and metastatic potential. Furthermore, changes in cytoskeletal architecture induced by stress (anti-cancer drugs or fluid shear stress in the circulatory system during metastatic processes) were shown to significantly influence biomechanical features of cancer cells. In this study, we were focused on cell stiffness changes in separated CD90-positive cells from tumour tissue of head and neck squamous cell cancer patients and prostatic cell lines PNT1A. 22Rv1, LNCaP, and PC-3 after exposure to antineoplastic drugs docetaxel and cisplatin.

Primary cell lines were prepared from tumour tissue obtained at surgery. For separation of CD90-positive subpopulations derived from primary cell line magnetic particles were used. Atomic-force microscopy (AFM), wound healing assay, and gene expression followed by cytostatic treatment was used.

AFM was performed on in sum 68 LNCaP, 42 PC-3, 20 22Rv1, and 64 PNT1A non-treated cells. Young’s modulus was 1153±52 kPa for PNT1A, 671±52 kPa for 22Rv1, 997±52 kPa for LNCaP and 1210±52 kPa for PC-3. The Young’s modulus of primary tumor’ 22Rv1 was significantly different from those obtained for other cell lines (both non-tumor and metastatic, p<0.005 in all cases). On the other hand, exposure of cells to cytostatic treatment increased the cell stiffness of all studied cell lines significantly. Regarding CD90+ head and neck cancer, cell stiffness of tumour cells derived from patients with stage of lymph node metastasis N0-1 was significantly higher than cell stiffness of CD90-positive tumour cells derived from patients with stage N2-3. Results indicate that changes of cell stiffness could be promising predictor of metastatic and/or aggressive behaviour of various cancers.

This work was supported by the Czech Science Foundation GA16-12454S and by Ministry of Health of the CR 16-29835A

P3002
Board Number: B284
Oncosomes as a Biomarker for Quantifying Metastatic Cancer Dynamics in Real-Time.
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INTRODUCTION AND OBJECTIVES: Tumor cells acquire qualities that enable them to succeed at key steps of the metastatic cascade, but very little is known about how individual cells accomplish these feats in a challenging hemodynamically active environment. Using intravital imaging, we observe that oncosome release is a key event during cancer cell extravasation in various prostate cancer cell lines. Oncosomes are large cell fragments released by cancer cells at various stages of cancer progression. Having observed their release in vivo during cancer cell extravasation, we sought to determine at what other
Stages of metastasis oncosomes were released. METHODS: Using PC-3, LnCAP, Du145 cells, intravenous injection into the chorioallantoic membrane (CAM) of chick embryos, a gold standard of visualizing cancer cell extravasation, was employed and confocal resonance scanning microscopy was used to visualize the release of oncosomes and other smaller extracellular vesicles in vivo. Blood at various timepoints was also collected to enumerate the number of CD9+ve and STEAP1+ve oncosomes released by extravasating cells. Primary tumors were also formed and blood collected in the same manner to ascertain the extent of oncosome release in vivo. RESULTS: At the key step of extravasation, arrested cancer cells release oncosomes into the microcirculation which are observed to exhibit a diameter >900 nm and expressing surface antigens found on the surrogate prostate cancer cell such as CD9 and STEAP1. We explored the abundance and biophysical characteristics (size diameter range) of extracellular vesicles (EVs) released during the metastatic cascade and found that oncosomes are not consistently released by primary tumors or metastases and that these large cancer cell fragments are specifically released by actively extravasating cancer cells. CONCLUSIONS: Circulating oncosome levels in patient plasma are a novel biomarker or “liquid biopsy” for actively metastasizing cells in the body, representing a powerful tool for monitoring metastatic cancer dynamics. We show that oncosome biogenesis is a specific byproduct of extravasating cells and not by primary tumors or metastatic deposits even in the presence of pro-apoptotic or pro-necroptotic stimuli. Our findings in plasma samples from patients on first-line treatment for metastatic prostate cancer support the concept of oncosomes as a promising biomarker for monitoring cancer metastasis dynamics in realtime, a novel "liquid biopsy" for metastatic prostate cancer treatment response.

P3003
Board Number: B285
Single CTC isolation and Analysis of PCa CTCs Using Celsee™ PREP SingleCell system.
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Prostate cancer (PCa) is the second most common cancer in American men accounting for an estimated 180,890 new cases and about 26,120 deaths in 2016 (Siegel, Miller, & Jemal, 2016). PCa metastasis is responsible for the vast majority of PCa-related deaths (Kelly & Yin, 2008). Circulating tumor cells (CTCs) are considered the focal points of the metastatic cascade. Recently, our understanding of cancer heterogeneity indicates that metastasis is a complex problem consisting of heterogeneous populations residing within the tumor. Due to lack of a sensitive technology, the majority of studies on PCa metastasis have focused on CTC enumeration and cell signaling of bulk cells. With the advent of the microfluidic technology it has now become possible to capture rare cell populations from peripheral circulation. However, isolating rare cells in a single cell format with high efficiency has been a long-standing challenge. In this study, we will present evidence that PCa CTCs can be isolated from peripheral blood of mice that have C4-B PCa cells xenografts, using the Celsee PREP CTC enrichment method. Following this, cells are captured onto a Celsee SingleCell chip and single CTCs are isolated using Celsee single cell retriever for downstream analysis such as single cell expression and morphology analysis. The Celsee SingleCell chip is fabricated with an optically compatible polymer and consists of 256,000 nano wells which captures cells with high efficiency in less than an hour. The Celsee single cell retriever allows precision movements over nano-meter distance and isolates single cell from the chip while maintaining cell morphology and viability. The overall objective of this study is to demonstrate the capability of the Celsee PREP technology to isolate single CTCs most likely undergo transformation to metastasize in the peripheral blood from the site of injection. The isolated CTCs may further be used for any high resolution molecular analysis.
P3004
Board Number: B286
Overexpression of VRK1 promotes mammary epithelial cell proliferation in three-dimensional culture, slows cell migration and induces a partial mesenchymal to epithelial transition (MET) phenotype.
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Vaccinia-related kinase 1 (VRK1) is a nuclear serine/threonine kinase that shows elevated expression in highly proliferative tissues, tumors and several cancer cell lines. Mice engrafted with VRK1-depleted MDA-MB-231 cells develop smaller primary tumors and fewer, if any, distal metastases to the lung and brain than seen with control cells (Molitor & Traktman, 2013). In contrast, VRK1 overexpression has been associated with poor clinical outcome in ER+ breast cancer patients. Here, we describe the impact of overexpressing VRK1 (or, as a control, 3XF-kinase dead-VRK1) on MCF10A cells using stable cell lines generated by lentiviral transduction. RNA microarray analysis of control vs 3XF-VRK1 monolayers revealed altered expression of several genes associated with cell migration, cell-cell and cell-substratum adhesion. In a series of follow-up studies, we determined that VRK1 overexpression significantly delays cell spreading, sheet migration, filopodia formation and transwell invasion. 3XF-VRK1 cells show reduced accumulation of the mesenchymal marker vimentin and increased accumulation of the epithelial cell-cell adhesion markers E-cadherin, and claudin-1. Strikingly, 3XF-VRK1 overexpression is also associated with reduced levels of the transcriptional repressors snail, slug, and twist, which regulate E-cadherin and claudin-1 expression. Because these findings suggest that VRK1 overexpression augments the epithelial properties of MCF10A cells, we also studied 3D matrigel cultures, in which cells proliferate and form spherical epithelial sheets surrounding hollow lumens (acinus). When we examined VRK1-overexpressing cells in 3D culture, we observed that initial acinus growth was accelerated relative to control cells. When acini were pulsed with BrdU, we found that constitutive VRK1 overexpression led to an increase in the S-phase population at days 4 and 5. By days 7 and 12, however, the vast majority of cells were in G0/G1 in all cell lines. VRK1 overexpression clearly promotes initial acinus growth, but does not block the later stages of acinar maturation, which are characterized by a cessation of cell proliferation and the apoptosis of luminal cells that are not participating in cell:cell or cell:matrix interactions. In sum, we have shown that VRK1 overexpression promotes MCF10A proliferation in 3D culture, and retards cell spreading, migration and invasion in 2D culture. By doing so, VRK1 may play a role in promoting the mesenchymal-to-epithelial transition involved in cancer cell colonization and metastatic spread, perhaps by regulating the transcription repressors snail, slug, and twist1.

P3005
Board Number: B287
The effects of bisphenol (BPA) and BPA structural analogs on metastasis of the MCF-7 breast cancer cell line.
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Bisphenols are plasticizers that share a similar structure to estrogen and are, therefore, classified as estrogen disrupting compounds. When bisphenols are present, they can bind to estrogen receptors and signal both classical and non-classical pathways to elicit a cellular response. The natural hormone
estrogen is known to modify enzyme function and elicit cell proliferation of certain cell types, including breast cancer cells. Similar effects, including an increase in metastasis, are seen in breast cancer cells exposed to bisphenol A. This is partly achieved through the hyper-production and excretion of lysosomal proteases known as cathepsins. Although many plastic-producing companies have removed bisphenol A from the manufacturing process, it is often being replaced with similar structural analogs. Through the use of cathepsin-specific fluorescence activity assays and transwell migration assays, we have found that BPA structural analogs (BPF and BPS) also modify cathepsin activity and cell metastasis of the MCF-7 line of human breast cancer cells.

P3006
Board Number: B288
Role of mDia2 at Adherens Junctions in Epithelial Ovarian Cancer.
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Epithelial ovarian cancer (EOC) cells disseminate within the peritoneal cavity, in part, via the peritoneal fluid as single cells, clusters, or spheroids. Initial single cell egress from a tumor mass involves the disruption of cell-cell adhesions in a process commonly known as epithelial-mesenchymal transition (EMT). In epithelial cells, adherens junctions (AJs) are characterized by homotypic linkage of E-cadherins on the plasma membranes of adjacent cells. AJs are anchored to the intracellular actin cytoskeletal network through a complex involving E-cadherin, p120 catenin, β-catenin, and αE-catenin. However the specific players involved in the interaction between the junctional E-cadherin complex and the underlying junctional actin is complex and remains unclear. Recent evidence indicates that the actin-nucleating family of mammalian Diaphanous-related (mDia) formins plays a key role in epithelial cell AJ formation and maintenance. Binding of αE-catenin to linear F-actin inhibits association of the branched-actin nucleator Arp2/3, while favoring linear F-actin bundling. Our previous published work specifically implicated mDia2 and not mDia1 as a downstream effector of RhoA in maintaining EOC spheroids. Loss of mDia2 was associated with spheroid invasive egress. Our current work indicates that mDia2 has a role at adherens junctions in EOC cells and human embryonic kidney (HEK) 293 cells through its association with αE-catenin and β-catenin. Both inhibition of mDia2 activity and depletion of mDia2 in EOC cells leads to disruption of cell-cell junctions, supporting the necessity of mDia2 for the stability of the junctional E-cadherin/catenin complex. Further experiments will be performed to determine the impact of mDia2 depletion compared to mDia2 agonism on AJ formation and stability.

P3007
Board Number: B289
Melanoma cell malignancy does not correlate with migratory rates in three different highly metastatic cell lines.
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Malignant melanoma is characterized by its ability to metastasize to different organs, and is responsible for 90% of skin cancer mortality. Metastasis requires cancer cell migration from the primary tumor to blood and lymph vessels, where the cells become disseminated to and populate distant organs. Cell migration is a complex process that requires dynamic remodeling of the cytoskeletal and integrin-based focal adhesions (FAs). However, it is unclear whether metastatic potential correlates directly with cell migration behavior or cytoskeletal and FA phenotype. To test this, we examined cell migration and the
cytoskeleton and FAs in 3 different human melanoma cell lines that differ in their metastatic potential: the highly metastatic Lu 1205, A375 cells with an intermediate metastatic potential, and less metastatic SK-mel-28 cells. Cell motility was evaluated by time-lapse phase contrast microscopy in wound healing assays over 48 hours, and cell velocities were measured using kymograph analysis. We found that SK-mel-28 cells migrated 2 times faster than Lu1205 and A375 cells. To see if cytoskeleton and FA organization related to the migratory behaviors, we performed immunofluorescence analysis using antibodies to myosin IIA and paxillin as an FA marker and stained the actin filaments with Alexa 565 phalliodin. Fluorescence images showed that SK-mel-28 cells presented small focal adhesions and dense, well-organized myosin IIA ribbons on a cortical actin meshwork, while A375 and Lu 1205 cells had larger focal adhesions and myosin IIA filaments organized into peripheral arcs and thick actin stress fibers. We then used polyacrylamide gel electrophoresis and western blot to analyze the FAK signaling pathway across the 3 cell lines. This showed that Lu 1205 cells presented highest phosphorylation of FAK on tyrosine 397 (p-FAK), while SK-mel-28 had the least p-FAK in comparison. Since higher p-FAK correlates with strong integrin binding to the ECM, it is likely Lu 1205 cells exert higher integrin activation in their large FAs, which could correlate with slower FA turnover and slower migration. In contrast, in SK-mel-28 cells, lower p-FAK, small focal adhesions and well organized myosin likely result in the observed fast motility. However, because the slow-migrating Lu 1205 cell line has a higher metastatic potential than the fast-migrating SK-mel-28, our results suggest that there is no correlation between metastatic potential and in vitro motility rate and corresponding cytoskeletal and adhesion phenotype.

P3008
Board Number: B290
Role of AMPK isoforms during breast cancer cell migration.
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AMP-dependent kinase (AMPK) has made its way to the forefront of cancer biology following a 2005 retrospective study showing that diabetic patients have a reduced incidence of cancer when following a treatment regimen consisting of metformin therapy, a biguanide pharmaceutical that is a known AMPK activator. As a master metabolic regulator, AMPK serves to suppress energy expenditures under nutritional stress and instead tilt signaling pathways towards catabolic processes to restore energy levels. In a cancer cell setting, it has been shown that AMPK activation will reduce cell growth, halt cell cycle progression, and even promote apoptosis under appropriate conditions. This has resulted in a widespread acceptance that AMPK functions to suppress tumor growth and should be targeted for therapeutic opportunities. Upon closer examination of AMPK signaling pathways, however, a paradox surfaces showing that AMPK’s efforts to restore energy levels may also enhance survival mechanisms – in both normal and cancer cells - through angiogenesis, autophagy, and migration/invasion. This study investigated the potential role for AMPKα1 and -α2 isoforms in regulating MDA-MB-231 breast cancer cell migration. A wound healing assay was performed by plating cells to confluence, establishing a “wound” using a pipet tip, and imaging the rate of wound closure periodically over the course of 24 hours. Results indicate that MDA-MB-231 cells exhibited a baseline wound closure rate of 41.4 ± 9.9% under standard culture conditions. When the AMPKα isoforms were activated with metformin, there was a significant dose-dependent increase in wound closure with values of 76.2 ± 3.2% (p=3.2x10^6) with 2.5mM metformin and 85.5 ± 4.6% (p=1.4x10^-7) with 5mM metformin. This migratory effect was ablated when AMPK was inhibited with compound c and wound closure values were reduced to 18.7 ± 3.2% (p=1.2x10^4) and 15.8 ± 4.2% (p=3.7x10^-5), respectively. The experiment was also repeated using siRNA oligos targeting AMPKα1 or AMPKα2 to determine whether this phenotype was isoform specific. Results showed partial knockdown of each AMPKα isoform (ranging from 50-75% ablation at the protein
level) but did not demonstrate any influence on migratory behaviors. Lastly, the localization of phosphorylated AMPKα protein was evaluated by performing immunofluorescence on cells fixed during the wound healing process. Staining reveals that activated AMPK localizes to the leading edge of the migratory cells, suggesting a possible linkage between cytoskeletal rearrangement and AMPK signaling. Overall, it is seen that AMPK activation promotes migration in MDA-MB-231 breast cancer cells and should be further explored to better understand this adverse survival phenotype prior to clinical use.

**P3009**
**Board Number: B291**
**Expression and roles of lectin galactoside-binding soluble 3 binding protein (LGALS3BP) in cholangiocarcinoma cell lines.**
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Cholangiocarcinoma (CCA), a biliary duct cancer, is highly prevalent in northeastern area of Thailand. The prognosis of CCA is poor with short survival time, therefore new therapeutic targets for CCA treatment is required. The secreted glycoprotein galectin-3-binding protein (LGALS3BP) is found to be overexpressed in serum and tissue of cancer patients, and it has been reported to have both positive and negative influences on cancer prognosis. However, the expression and roles of LGALS3BP on CCA are still unknown. We found that the expression levels of LGALS3BP protein were upregulated in CCA cell lines compare to human cholangiocyte cells. Role of LGALS3BP in two CCA cell lines, KKU-M213 and HuCCA-1 was examined using lentiviral shRNA. Knockdown LGALS3BP decreased CCA cell proliferation and survival. Surprisingly, we found that knockdown LGALS3BP increased migration and invasion abilities of both of CCA cell lines. The expression levels of LGALS3BP partners (CD82, CD9, β1 integrin and galectin-3) were not altered in knockdown LGALS3BP cells. Moreover, knockdown LGALS3BP decreased Akt (Ser473) and however increased p70S6K (Thr389) phosphorylation. These results indicate that the decreasing of cell proliferation and survival in knockdown LGALS3BP CCA cells might be mediated through inhibition of Akt activation. In contrast, increasing of cell migration and invasion after LGALS3BP suppression might be partly mediated by activation of p70S6K. Herein, we demonstrated for the first time about the dual effects of LGALS3BP on CCA phenotypes. Our finding highlights the precaution of using LGALS3BP as therapeutic target or prognostic biomarker for CCA.

**P3010**
**Board Number: B292**
**Thy-1/CD90 induces metastatic breast cancer cell migration.**
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Cell migration is essential for the development and homeostasis of multicellular organisms, tissue repair and regeneration, and contributes significantly to disease states, such as tumor cell metastasis. Previous studies have shown that Thymus cell antigen 1 (Thy-1/CD90), a cell adhesion molecule of activated endothelial cells, mediates the adhesion of melanoma cells to the endothelium through interactions...
with integrins in cancer cells. However, a role for Thy-1 in the migration of breast cancer cells remains undefined. Here, we studied whether Thy-1 may participate in the migration of metastatic human breast cancer MDA-MB-231 cells. We determined the migration of cells in a wounded monolayer stimulated by Thy-1. We observed that Thy-1 induced a rapid increase in ATP release, focal adhesion formation and cell migration in MDA-MB-231 cells. In the presence of Apyrase, Thy-1-induced cell migration was decreased, suggesting that ATP is required for migration of breast cancer cells. Moreover, blockers of the P2X7 purinergic receptor also decreased cell migration. Thus, our results reveal that ATP signaling via P2X7 receptors is required for Thy-1 induced migration of breast cancer cells. These findings suggest that purinergic receptors may represent an attractive therapeutic target to prevent breast cancer metastasis.

Acknowledgements: FONDECYT #3160349 (MB); #1170925 (AFGQ); #1150744 (LL). CONICYT-FONDAP #15130011 (AFGQ)

P3011
Board Number: B293
CX3CR1 chemokine receptor antagonism inhibits migration and contact-independent growth in pancreatic cancer epithelial cells.
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Pancreatic ductal adenocarcinoma (PDAC) has become the third-leading cause of cancer death in the United States. Most patients present with advanced metastatic disease that is resistant to conventional therapies, which is in part due to a unique tumor microenvironment (TME). The PDAC TME includes a dense desmoplastic stroma comprised of heterogeneous cellular and fibrotic components that exert both pro- and anti-tumorigenic effects. These phenotypes are mediated through intercellular crosstalk and interaction with the extracellular matrix. CX3CL1 is a chemokine known to play an important role in extravasation, homing and distal establishment of breast and prostate cancer cells through its receptor CX3CR1. Furthermore, previous results show that PDAC perineural locoregional invasion within the pancreas is driven by CX3CR1:CX3CL1 traction. However, it remains unknown if this signaling axis contributes to other elements of PDAC tumor primary development and maintenance or its metastasis to distal sites. We hypothesize that CX3CR1 signaling is a driving force of growth, survival, dissemination and metastatic progression through TME interaction. Here we demonstrate CX3CL1:CX3CR1 signaling regulating cancer cell mobility and proliferation. Our data suggest that CX3CL1 contributes to cancer cell motility, and inhibition of the CX3CR1 receptor by selective small molecule antagonists diminishes cell migration and viability in contact-independent cultures, in part through loss of phosphorylation of AKT. Interestingly, log phase 2D cell viability remained unaffected by CX3CR1 inhibition, suggesting a particular role for CX3CL1 in the three-dimensional organization and progression of PDAC tumors through TME cell-cell interaction and/or signaling. Our findings suggest that CX3CR1 inhibition may serve as a viable therapeutic intervention for PDAC.
P3012

Board Number: B294

Differential Expression of miRNAs and EMT-Related Targets in Cutaneous Squamous Cell Carcinoma Tissues.

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Cutaneous Squamous Cell Carcinoma (cSCC) is a common form of skin cancer predominantly induced by exposure to UV radiation (sunlight), with most cases remaining localized at the site of initiation. As cSCC is commonly treated by surgical excision, the amount of research investigating how cSCC can progress from a relatively benign state to becoming invasive, and potentially metastatic, is sparse. Metastasis of tumor cells is mediated by a process known as epithelial-to-mesenchymal transition (EMT). During EMT, epithelial cells undergo a dedifferentiation process whereby they transition to a highly mobile and invasive mesenchymal cell type with metastatic capacities. Although numerous factors have been identified as influencing the EMT process in a variety of cancers, we chose to examine the role of miRNAs. miRNAs are small, non-coding RNA species that are known to epigenetically regulate a host of genes within a given cell type. We hypothesized that miRNAs are differentially expressed between normal vs. cSCC tissue and that these differences in expression contribute to the progression of cSCC. To test this hypothesis, we compared the expression pattern of ten miRNAs (miR-21, -31, -34a, -125b, -181, -199a, -200b, -200c, -204, and -1246) between fresh primary tumor samples and fresh adjacent normal tissue (ANT) collected from patients diagnosed with either cSCC, SCC in situ, or invasive SCC. Total RNA was isolated from each of the samples and qPCR was performed to determine the expression level of each miRNA relative to control. Of the ten miRNAs tested, two were significantly upregulated (miR-21 and miR-31) in the patient cSCC samples compared to ANT. We then examined the expression of miR-21 and miR-31 targeted EMT markers via qPCR and/or Western blot from RNA and protein isolated from both cSCC and ANT tissues. TGF-β1 and TGF-βR2 mRNA were significantly increased relative to normal tissue, suggesting EMT may be being initiated in the cancerous tissue. mRNA expression of E-cadherin was significantly increased in cSCC as well, and protein expression of phosphorylated E-cadherin was also significantly higher, potentially indicating increased E-cadherin-mediated signaling in the cancerous cells, although this remains to be verified experimentally. Further work investigating the function of these differentially-expressed miRNAs will elucidate the role miRNAs are playing in the progression of cSCC.

P3013

Board Number: B295

DDR1 regulates tumor dormancy by balancing p38/ERK signaling.

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Background: Metastasis is the primary cause of death in cancer patients. However, metastatic manifestation can takes years after primary tumor removal. This delay in metastatic growth is a consequence of tumor dormancy, a reversible growth arrest that can be regulated by the interaction of disseminated tumor cells (DTCs) with the environments they encounter, through balancing p38/ERK (quiescence/proliferative) signals. Tumor cells interact with collagen through integrins, DDRs, GPVI and LAIR-1 receptors. The collagen receptor Discoidin Domain Receptor 1 (DDR1) is part of a dormancy
signature established in ER+ breast tumors; however its role in tumor dormancy is not well understood. Here, we investigate the role of DDR1 in tumor dormancy.

Methods: We used breast cancer cell lines and a well-described dormancy model of head and neck squamous cell carcinoma (HNSCC) to study the role of DDR1 in tumor dormancy. To analyze the status of p38/ERK signaling pathways at the single cell level we used recently developed p38 and ERK biosensors to study the activation of these signaling nodes in vivo by using intravital multiphoton microscopy.

Results: We have determined that DDR1 is overexpressed at both RNA and protein levels in breast cancer and HNSCC dormant cells when compared with their proliferative counterparts. We demonstrated that the downregulation of DDR1 induces proliferation of dormant cells in vivo: in chicken embryo CAMs and in nude mice, by balancing p38/ERK signaling. Finally, we utilized p38 and ERK biosensors to identify where proliferative and dormant cells reside in vivo.

Conclusion. We demonstrated that DDR1 expression maintains quiescence of tumor cells and prevents metastasis formation through the regulation of p38/ERK signaling.

P3014
Board Number: B296
The role of srGAP1 in regulating cancer cell motility and invasion.
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The Rho family of small GTPases, which regulates actin cytoskeleton organization, contributes to many key cellular functions, including cell motility, polarity, and vesicle trafficking. Activation and deactivation of Rho GTPases is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). In pathological processes, such as cancer, regulators of Rho GTPases are often mutated and overexpressed; however, their contribution to disease progression is relatively unknown.

The Slit-Robo GAP (srGAP) family was initially identified as Robo-interacting proteins that regulate neuronal cell migration. We find that srGAP1, an F-BAR domain containing protein, is expressed in mesenchymal, triple-negative breast cancer cells, but not in ER+ non-migratory breast cancer cells. srGAP1 localizes to both lamellipodia and invasive protrusions (invadopodia and linear invadosomes), which are F-actin rich structures that degrade the extracellular matrix. siRNA-mediated depletion of srGAP1 expression results in an increase in 2D and 3D cell motility on collagen I fibrils and in collagen I plugs, respectively, as well as an increase in matrix degradation on collagen I fibrils.

To analyze the effects of srGAP1 on tumor cell dissemination in vivo, we have developed an intravital imaging platform that allows us to analyze disseminated tumor cell (DTC) behavior and measure single-cell motility parameters, such as protrusion formation, in sites of dissemination (e.g. the lymph nodes). We aim to utilize intravital multiphoton imaging to further understand srGAP1 effects on DTC seeding, motility, and metastasis formation.
P3015

Board Number: B297
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Background: The SW13 cancer cell line has two naturally occurring, well-defined and epigenetically distinct cell subtypes. The SW13(Vim-) has a phenotype that resembles typical epithelial tumors and displays increased growth and proliferation with a low level of invasiveness. The SW13(Vim+) subtype has mesenchymal-like phenotype that is commonly associated with invasive and metastatic epithelial cancers. The phenotype of the SW13(Vim-) cell subtype can be driven toward the SW13(Vim+) phenotype when treated with histone deacetylase inhibitors. This SW13 cell line model is used to study the epigenetic phenotypic differences in primary tumor versus metastatic cells. Gene chip data obtained from the SW13 subtypes documents differential expression of genes involved in cholesterol, sphingolipid, and GPI-anchored protein metabolism, all of which are essential components of lipid rafts.

Hypothesis: As genes affecting the synthesis of all components of lipid rafts are differentially expressed in the SW13 subtypes, we hypothesize that 1) lipid raft protein composition is distinctly different between the two SW13 cell subtypes and that 2) differences in the composition of lipid rafts may play a role in the unique oncogenic phenotypes of the two subtypes.

Methods: Utilizing low-temperature detergent-resistant lipid raft isolation techniques, western blotting, and mass spectrometry, we investigated protein composition differences between the two SW13 cell subtypes. Cholesterol assays were used to assess the distribution and total cholesterol content of each SW13 subtype to evaluate the impact of the differentially expressed cholesterol genes detected in gene chip data obtained from the subtypes. Cholesterol depletion techniques utilizing cyclodextrin for acute disruption and simvastatin for chronic disruption have been used to perturb lipid rafts and observe their role in the unique oncogenic phenotypes of the SW13 cell subtypes.

Results: Preliminary results in cholesterol assays confirm differential cholesterol levels in the two SW13 cell subtypes. Western blot analysis suggests differential protein composition of lipid rafts, several of which are confirmed cancer biomarkers, isolated from each SW13 subtype. Ongoing experiments are measuring the metastatic capacity in the SW13(Vim+) after lipid raft and cholesterol perturbations.

Conclusions: Results may detail a mechanism in which cholesterol perturbations are capable of decreasing metastasis, improving the outcome of patients with epithelial cancers. Specifically, this data may provide a mechanism by which treatment with statin drugs has led t

Cancer Therapy: Targeting the Tumor Microenvironment

P3016

Board Number: B298
SIRPA-inhibited, marrow-derived macrophages engorge, accumulate, and differentiate in antibody-targeted regression of solid tumors.
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Marrow-derived macrophages are highly phagocytic, but whether they can also traffic into solid tumors and engulf tumor cells is questionable, given the well-known limitations of tumor-associated macrophages (TAMs). Here, SIRPα on macrophages from mouse and human marrow was inhibited to
block recognition of its CD47 ligand, a ‘marker of self’ on all other cells. These macrophages were then systemically injected into mice with fluorescent human tumors that had been antibody targeted. Within days, the tumors regressed, and fluorescence analyses showed that the more the SIRPα-inhibited macrophages engulfed, the more they accumulated within regressing tumors. Human marrow-derived macrophages engorged on the human tumors, while TAMs were minimally phagocytic, even toward CD47-knockdown tumors. Past studies have opsonized tumors in situ with antibody and/or relied on mouse TAMs but have not injected SIRPα-inhibited cells; also, unlike past injections of anti-CD47, blood parameters remained normal here. Consistent with tumor-selective engorge-and-accumulate processes in vivo, phagocytosis in vitro inhibited macrophage migration through micropores that mimic features of dense 3D tissue. Accumulation of SIRPα-inhibited macrophages in tumors favored tumor regression for 1-2 weeks, but donor macrophages quickly differentiated toward non-phagocytic, high-SIRPα TAMs. Analyses of macrophages on soft (like marrow) or stiff (like solid tumors) collagenous gels demonstrated a stiffness-driven, retinoic acid-modulated upregulation of SIRPα and a mechanosensitive nuclear marker, lamin-A. Mechanosensitive differentiation was also evident in vivo and likely limited the anti-tumor effects, as confirmed by re-initiation of tumor regression by fresh injections of SIRPα-inhibited macrophages. Macrophage motility, phagocytosis, and differentiation in vivo are thus coupled.

P3017
Board Number: B299
Extracellular matrix-binding peptide conjugation to immune checkpoint blockades enhances anti-tumor efficacy and reduces adverse events.
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Therapy using anti-CTLA4 and anti-PD-1/PD-L1 antibodies (Abs) has exhibited considerable anti-tumor effects, but instances of severe side-effects have been previously reported. We hypothesized that conjugation of an extracellular matrix (ECM)-binding peptide derived from placenta growth factor-2 (PIGF-2123-144) to anti-CTLA4 and anti-PD-L1 Abs would accomplish localized immune checkpoint blockade therapy, minimizing the Abs’ systemic exposure. Here, we show that enhanced tissue retention and lower Ab concentrations in the blood plasma resulted from PIGF-2123-144 conjugation when the Abs were administered peri-tumorally compared to wild-type (wt) Abs administered by the same route at the same dose. This resulted in decreases in TNFa and IFNg concentrations in the serum and a decrease in liver tissue damage, as was assessed both biochemically and histologically. These results indicate lower systemic side-effects. Particularly, we show that PIGF-2123-144 conjugation reduced the risks of checkpoint blockade-induced auto-immune diabetes in the male non-obese diabetic (NOD) mouse. Remarkably, wt anti-PD-L1 induced type 1 diabetes in 100% of male NOD mice, while our matrix-binding variant did not induce type 1 diabetes in any mice. Because diabetes induced by PD-1/PD-L1-inhibition has been reported in the clinic, this strongly supports the idea that PIGF-2123-144-conjugation to Abs could reduce treatment-related adverse events. This observation with localized PIGF-2123-144-Abs could be an important breakthrough in treating patients who have discontinued checkpoint blockade therapy due to the associated side-effects, as well as those who are not amenable to systemic chemotherapy. In addition, we show that PIGF-2123-144 anti-CTLA4 and -anti-PD-L1 Abs treatment exhibited significantly higher anti-tumor activity compared to wild-type forms in three cancer models: B16F10 melanoma, Tyr:Cre-ER+/LSL-BrafV600E/Ptenfl/fl inducible autochthonous melanoma, and MMTV-PyMT breast.
cancer. Importantly, local injections of Plgf-2123-144-Combination Abs increased the number of activated CD8+ and CD4+ T cells within the tumor, resulting in growth suppression of both the distant tumor and re-challenged tumor. This data suggests the feasibility of treating patients with oligometastatic tumors by administration of Plgf-2123-144-Combination Abs into one available metastasis. Our data suggest that local injection of Plgf-2123-144-Combination Abs efficiently activates tumor-antigen-specific T cells while maintaining systemic immune homeostasis by avoiding influence on non-tumor antigen specific T cells. This simple approach of using engineered ECM-binding Abs may hold potential for clinical translation of locally-delivered immune checkpoint blockade as a cancer therapeutic.

P3018
Board Number: B300
Extracellular matrix-binding form of anti-Cd40 agonistic antibody reduces systemic damages and increases anti-tumor efficacy.
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Anti-Cd40 agonistic antibody maturates antigen-presenting cells, showing considerable anti-tumor effects, but previous studies have reported severe side-effects. We hypothesized that peri-tumoral injections of extracellular matrix (ECM)-super affinity peptide conjugated anti-Cd40 antibody would accomplish local immune therapy via antigen presenting cell maturation around the tumor. Here, we conjugated an ECM binding peptide: placenta growth factor-2 (Plgf-2123-144), to anti-Cd40 antibody and observed a decrease in concentrations in blood plasma. This reduces cytokine concentration in the serum and liver tissue damages, indicating lower systemic side-effects. Plgf-2123-144-Cd40 antibody administration exhibit significantly higher anti-tumor efficacy compared to its wild-type form in B16F10 mouse melanoma model. Importantly, Plgf-2123-144-anti-Cd40 treatment slowed growth of B16F10, at low dosages where wild-type anti-Cd40 antibody had no effect. Plgf-2123-144-Cd40 antibody administration activates CD8+ T cells (defined as PD-1+ and CD62L-Cd44+) in both tumor and tumor-draining lymph nodes as much as its wild-type form. Plgf-2123-144-Cd40 antibody, but not wild-type form, increased maturated dendritic cells (defined as % CD80+CD70+ of CD11c+) within tumor. Plgf-2123-144-Cd40 antibody but not wt Cd40 antibody increased mature B cells (defined as % MHCIIhigh CD80+ of B220+ cells) in tumor and tumor-draining lymph node. Plgf-2123-144-Cd40 antibody but not wt Cd40 antibody increased plasmablasts (defined as % Blimp I of B220+ cells) in tumor-draining lymph node. Also, Plgf-2123-144-Cd40 antibody but not wt Cd40 antibody increased mature CD11b cells (defined as % MHCIIhigh CD80+ of B220+ cells) in tumor-draining lymph node. Finally, the activation of immune cells efficiently suppresses the growth of a distant tumor when Plgf-2123-144-Cd40 antibody was injected peri-tumorally to one tumor. This ECM-binding Cd40 antibody may be useful to achieve effective cancer local immunotherapy avoiding its systemic side-effect.
P3019
Board Number: B301

NMK-057, a novel triazole derived γ-secretase inhibitor attenuates Notch mediated-EMT and stemness in triple negative breast cancer cells by inducing autophagic cell death.

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Autophagy plays a dual role in cancer depending on its role in the survival or death of cancer cells. The relationship between autophagy and Notch signaling is not clear and only a few reports have come across in highly reputed journals in last one year. Notch signaling is reported to be deregulated in several malignancies including breast and the enzyme γ-secretase plays important role in the activation and nuclear translocation of Notch intracellular domain (NICD). Hence pharmacological inhibition of γ-secretase might lead to the subsequent inhibition of Notch signaling in cancer cells. But whether inhibition of γ-secretase triggers autophagy is an interesting topic of investigation and not reported in detail. In search of novel γ-secretase inhibitors (GSIs), we screened a series of triazole-based compounds, for their potential to bind γ-secretase and we observed that 3-(3′,5′-Trimehtoxyphenyl)-5-(N-methyl-3′-indolyl)-1,2,4-triazole compound (also known as NMK-T-057) can bind to γ-secretase complex. Very interestingly, NMK-T-057 was found to inhibit proleiferation, colony forming ability, and motility in various triple negative breast cancer cells such as MDA-MB-231, MDA-MB-468, 4T1 and also MCF-7, with negligible cytotoxicity towards non-cancerous MCF-10A, and PBMC. NMK-T-057 also showed limited toxicity in Swiss albino mice, as determined by measuring the hematological and clinical parameters. Moreover NMK-T-057 treatment resulted in the reprogramming of the EMT process in TNBCs, accompanied by the drastic reduction of CD44high/CD24low population. NMK-T-057 also inhibited the spheroid forming ability of TNBCs and reduced the stem cell enriched side population. It was also observed that in NMK-T-057 treated cells, Notch-1 and its downstream targets such as Hes-1 and pAkt were significantly down regulated. The in silico study revealing the affinity of NMK-T-057 towards γ-secretase, was further validated by fluorescence based γ-secretase activity assay, which confirmed the inhibition of γ-secretase activity in NMK-T-057 treated TNBC cells. Very interestingly, we observed that NMK-T-057 induces significant autophagic responses in TNBCs and administration of the autophagy inhibitor 3-MA, attenuates NMK-T-057 induced cell death. Similar results were observed on the treatment of TNBC cells with commercially available γ-secretase inhibitor DAPT. Thus we might conclude that NMK-T-057 could be potential drug candidate against breast cancer, which can trigger autophagy mediated cell death in breast cancer cells by inhibiting the γ-secretase –mediated activation of Notch-signaling.
P3020

**Board Number: B302**

**Hematopoietic cell kinase (HCK) inhibitor as a potent antiproliferative compound for the development of novel acute myeloid leukemia treatment.**

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Src kinase family (SFK) is a family of non-receptor tyrosine kinases that includes nine members, which are aberrantly expressed and/or activated in diverse bone marrow malignancies, as acute myeloid leukemia (AML), a heterogeneous neoplasm with high incidence in older person. The better understanding of molecular targets that participate in the disease pathogenesis has led to new drug development and novel therapeutic strategies. In this concern, the aim of this study was to investigate the role of hematopoietic cell kinase (HCK), the unique SFK member restricted expressed in hematopoietic cells. We found that HCK is overexpressed in hematopoietic stem cell (HSC) isolated from total bone marrow of AML patients. Therefore, we did an effort to develop a selective inhibitor for HCK (iHCK). Using the iHCK, we evaluated its effects on cell growth and death as well as on regulation of the oncogenic pathways MAPK and PI3K, which usually is superexpressed in hematological neoplasm. The iHCK treatment significantly decreased cell growth and induced apoptosis in myeloid leukemic cell lines and in primary AML patient’s cells without changing in healthy donors HSC survival or death. Similar results were found in a 3D culture system (bioscaffold) that works as an optimal microenvironment: after iHCK treatment and culture into a bioscaffold for 7 days, primary AML patient’s cells showed an decreased proliferation when compared to healthy donors HSC. We also induced leukemia in mice using human leukemic cells. After the confirmation of the leukemia development, the mice were treated with iHCK. As occurred in vitro assays, the in vivo treatment with iHCK led to a decrease phosphorylation of ERK, AKT and P70S6K proteins in leukemic mice compared to untreated mice. These results demonstrated a reduced activation of MAPK/ERK and PI3K/AKT pathways in leukemic cells after HCK pharmacological inhibition. We also analyzed the combinatory effects of iHCK with Cytarabine or 5-Azacitidine, both drugs that are actually used in chemotherapy. The combinatory treatment of iHCK and Cytarabine or iHCK and 5-Azacitidine demonstrated additive effects relative to either drug alone on the reduction of cell growth and death, with increased BAX (pro-apoptotic) and reduced BCL-XL (anti-apoptotic) protein levels. In conclusion, the selective HCK inhibitor has an antiproliferative activity in leukemic cells without alter cell death and survival of normal HSC. Besides, iHCK treatment was able to abolish the activation of MAPK/ERK and PI3K/AKT pathways. Our results indicate that this compound only acts onto cells with higher HCK expression and activity, as occurs in leukemia, and could be useful for a novel AML treatment, mainly in combination with other chemotherapy drug.
P3021
Board Number: B303
Programmed death ligand 1 (PD-L1), Immune-Checkpoint Blockade with Combination Therapy in Syngeneic Colon Carcinoma Model.
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Cancer cells sometimes find ways to use these checkpoints to avoid being attacked by the immune system. But drugs that target these checkpoints hold a lot of promise as cancer treatments. Combination therapy is becoming more and more general in the treatment of oncological diseases. Now that safety and clinical activity has been demonstrated in the monotherapy setting, the field is moving in the direction of testing novel combinations. The aim of the study was to explore the role of programmed death ligand 1 (PD-L1) and the antitumor effect of anti- cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4), Lymphocyte-activation gene-3 (LAG-3) and mucin-domain containing-3 (TIM-3) combination therapy in Colon Carcinoma. In this in vivo trial combination the standard immunotherapeutic treatment; the PD-L1 regulatory antibody and a CTLA-4, LAG-3 and TIM-3 antibody will be tested in Syngeneic Colon Carcinoma model. Monoclonal antibodies that target either PD-L1 or combination can block this binding and boost the immune response against cancer cells. These drugs have shown a great deal of promise in treating certain cancers.

P3022
Board Number: B304
HDAC6/DHPS signaling drives hypusination and nuclear export of eIF5A to promote TGFbeta-induced EMT and associates with a novel SOX2 signature to predict decreased breast cancer patient survival.
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Progression of solid tumors to a metastatic stage accounts for over 90% of cancer mortality. Thus, it is critical to identify therapeutic strategies that target both primary and metastatic tumors. Epithelial-mesenchymal transition (EMT) negatively correlates with therapy response, contributing to intratumoral heterogeneity and systemic dissemination in breast cancer. We previously reported that pseudopodium-enriched atypical kinase one (PEAK1) promotes breast cancer cell EMT and metastasis by potentiating fibronectin - transforming growth factor beta (TGFβ) signaling cross-talk. Since eukaryotic initiation factor five A (eIF5A), a unique translation factor that is activated by post-translational hypusination, is required for PEAK1 expression, we hypothesized that TGFβ may directly regulate eIF5A activity as a novel means of promoting EMT, and that targeting this pathway may inhibit or reverse metastatic progression. In this regard, we provide evidence of an active eIF5A-EMT program in undifferentiated breast cancer tissue. Notably, blockade of eIF5A hypusination (via deoxyhypusine synthase, DHPS, inhibition) reduces PEAK1 translation, cell viability and TGFβ-induced EMT. Conversely, we demonstrate that TGFβ induces post-translational hypusination of eIF5A in metastatic breast cancer cells. TGFβ is known to activate histone deacetylase six (HDAC6) and HDAC6 was independently reported to promote eIF5A deacetylation and nuclear export, supporting its translation activity. When delivered in combination, HDAC6 and DHPS inhibitors synergize to sequester eIF5A to the nucleus, suppress eIF5A-dependent translation and potently kill metastatic breast cancer cells. To identify candidate pathways downstream of the eIF5A/PEAK1 axis during EMT, we generated a Cytoscape interactome using eIF5A signaling and PEAK1-induced EMT genes as search terms. All interactome component genes were then
analyzed across two breast cancer patient studies available on the Cancer Bio Portal. Interestingly, SOX2, PIK3CA and EIF4A2 were the interactome nodes that exhibited copy number amplifications among patients harboring genomic alterations in the initial interactome search genes, and SOX2 amplification significantly and independently associated with decreased patient survival (p = 0.0476). Taken together, our results establish a novel signaling pathway by which TGFβ stimulates HDAC6 and DHPS function to activate cytoplasmic elf5A and promote EMT and survival in breast cancer cells. This work also identifies new therapeutic reagents and strategies that may improve cancer patient survival.

P3023
Board Number: B305
Cordycepin induces human lung cancer cell apoptosis by inhibiting nitric oxide mediated ERK/Slug signaling pathway.
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Nitric oxide (NO) is an important signaling molecule and a component of the inflammatory cascade. Besides, it is also involved in tumorigenesis. Aberrant upregulation and activation of the ERK cascade by NO often leads to tumor cell development. However, the role of ERK inactivation induced by the negative regulation of NO during apoptosis is not completely understood. In this study, treatment of A549 and PC9 human lung adenocarcinoma cell lines with cordycepin led to a reduction in their viability. Analysis of the effect of cordycepin treatment on ERK/Slug signaling activity in the A549 cell line revealed that LPS-induced inflammatory microenvironments could stimulate the expression of TNF-α, CCL5, IL-1β, IL-6, IL-8 and upregulate NO, phospho-ERK (p-ERK), and Slug expression. In addition, constitutive expression of NO was observed. Cordycepin inhibited LPS-induced stimulation of iNOS, NO, p-ERK, and Slug expression. L-NAME, an inhibitor of NOS, inhibited p-ERK and Slug expression. It was also found that cordycepin-mediated inhibition of ERK downregulated Slug, whereas overexpression of ERK led to an upregulation of Slug levels in the cordycepin-treated A549 cells. Inhibition of Slug by siRNA induced Bax and caspase-3, leading to cordycepin-induced apoptosis. Cordycepin-mediated inhibition of ERK led to a reduction in phospho-GSK3β (p-GSK3β) and Slug levels, whereas LiCl, an inhibitor of GSK3β, upregulated p-GSK3β and Slug. Overall, the results obtained indicate that cordycepin inhibits the ERK/Slug signaling pathway through the activation of GSK3β which, in turn, upregulates Bax, leading to apoptosis of the lung cancer cells.

KEYWORDS: Cordycepin; ERK; apoptosis; lung cancer; nitric oxide; slug
P3024

Board Number: B306

Expressional assessment of mouse embryonic stem cell, lung cancer and somatic fibroblast cell lines on the basis of EMT, MAPK and Inflammation.

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Life is a continuous cycle of the cells, composed of beginning, surviving and death. Cells keep dividing, differentiate and form somatic cells; and soon organism is formed. These somatic cells at some point can be turn into cancer cells and share characteristics of stem cells. The processes during these periods are important for the identifying the tendency to abnormality in somatic cells and transformation to the cancer cells. Cancer progenitor cells are the challenging concept not only arise, development and recurrence of the cancer but also sharing the characteristics of embryonic developmental stages. To control emergence and progress of the cancer, mechanisms take part in the surviving become significant, especially EMT and inflammation. EMT occurs in both embryonic developmental stages and cancer progression and molecular basis of this process can be the therapeutic target for cure. For the promising agents in cellular and molecular targeted therapy, this study was conducted in cancer, somatic and embryonic stem cells; compares similarities and differences in central signalling pathways EMT, MAPK and inflammation via gene profiling and protein expression. For cancer cell mouse squamous lung cancer cells (SqLCs), for somatic cells mouse skin fibroblast cells (MSF) and for embryonic stem cell mouse embryonic stem cells (mESCs) were used. Three signalling pathways were compared at the gene expression level with EMT, MAPK and inflammation related genes in mESC, SqLC and MSF. With immunofluorescence staining protein level of ERK 1/2, vimentin and twist. We selected four to five most significant fold changes of RT-PCR results in each panel. ERK ½ protein expression in SqLCs and MSFs were similar while mESCs were less intensity. In addition, while ERK 1/2 protein expression was rich in nuclear region of MSFs, SqLCs had leaky in nucleus. Likely the gene expression, Vimentin protein expression was the highest in MSF and the least in SqLCs. Differently, MSF had significant vimentin fibrils. Twist expression, on the other hand, has similar intensity in SqLCs and mESCs while MSF had higher. Twist was expressed more in dividing cells into MSF cells. Mostly SqLC had expression into cytoplasm (no protein in nucleus), whereas mESC into the nucleus. These results show that the molecular basis of the EMT and inflammation supported by the MAPK signalling pathway were similar in SqLCs and mESCs while MSF relatively altered, therefore the molecules can be the potential driving force for therapeutic target.
**P3025**  
**Board Number: B307**  
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Mechanical properties of extracellular matrices surrounding cancer cells is critical for the cancer progression and survivability. Increase of the matrix stiffness make the cancer cells much more sensitive to drug-resistance. However, the underlying molecular relationship between matrix stiffening and anti-cancer drug resistance remains largely unknown. In this study, we report that the level of tubulin acetylation depending on the matrix stiffness is important for regulation of drug resistance during chemotherapy. Aggressive breast cancer cell lines cultured in a stiff substrate became more sensitive to anti-cancer drugs such as cisplatin or eribulin than on soft one, whereas expression of stem cell marker genes profoundly increased in cells cultured on the soft substrate. We also found that the cancer cells cultured in the stiff matrix rapidly upregulated the level of tubulin acetylation in prior to the onset of cell death upon the treatment of anti-cancer agents. However, in the case of cells resistance to anti-cancer drugs, treatment with anti-cancer drugs did not increase the tubulin acetylation. Interestingly, disruption of a-tubulin acetyltransferase gene, ATAT1 by CRISPR/Cas9 system reduced the death of breast cancer cells upon treatment of anti-cancer drugs. Moreover, recovery of tubulin acetylation by overexpression of ATAT1 or treatment of HDAC6 inhibitor, tubacin in drug resistance cell reduced the expression of stem cell marker genes and increased the cell death. Taken together, our results indicate that level of tubulin acetylation under variety of mechanical cancer microenvironment is critical for the regulation of drug sensitivity in chemotherapy.

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**P3026**  
**Board Number: B308**  
Therapeutic Effect of FCH domain only 1 shRNA in K-ras¹⁰¹¹ Mice.  
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The FCH domain only 1(FCHO1) protein is associated with early working proteins at the surface clathrin assembly as key coat nucleators in a previous study. However, the possible involvement of FCHO1 protein in cancer is not clear. In this study, we provide evidence that FCHO1 regulates mitotic proteins by global proteomics. In addition, we aimed to demonstrate the therapeutic effect of FCHO1 protein down-regulation, especially lung cancer. Lenti-viral shRNA is a biocompatible carrier used for aerosol gene delivery. Repeated aerosol delivery of lenti-viral FCHO1 shRNA was performed to 8-week-old female K-ras¹⁰¹¹ mice twice a week for 4 weeks in a nose-only exposure chamber. Aerosol delivery of lenti-viral FCHO1 shRNA inhibits lung cancer in K-ras¹⁰¹¹ mice compared to control and scramble control groups. In addition, FCHO1 siRNA led to the cell death caused by cell division failure in A549 cell. Therefore, Down-regulation of FCHO1 significantly suppresses lung cancer in vitro and in vivo.

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Tuesday-194
P3027

Board Number: B309

A New Targeting Agent that Specifically Binds to Breast Cancer Cells?


Targeted therapy is one of the major improvements for treating cancer. Targeted molecular imaging agents (TMIAs) can be applied for more effective diagnosis of primary and metastatic tumors by locating and illuminating cell types of interest bearing the molecular marker that allows targeting. To be an effective TMIA, a molecule must exhibit binding affinity and specificity for the desired cell type. Purpose: To evaluate a new molecular imaging agent that targets specifically to breast cancer cells. Methods: A newly developed TMIA which we have named M1 consists of a fluorescent dye (Cy5.5) conjugated to a peptide found to bind to keratin 1 of breast cancer (BrCa) (Soudy et al, 2017). Results: M1 binding was studied via in vitro models using the BrCa cell line MDA-MB-231. MDA-MB_231 cells were observed to stain well with the M1 stain exhibiting internalized localization within vesicles. Visualization of M1 staining was achieved via confocal fluorescent microscopy. The M1 staining of MDA-MB-231 was more significant than control cancer lines such as lung and prostate cancer lines. M1 was reported to display specificity toward tumors over healthy breast tissue and greater binding specifically to BrCa. Conclusion: The M1 TMIA holds great potential as a cancer imaging agent. Characterizing the binding and processing of M1 in BrCa cells should aid in our understanding of the interaction of TMIAs like M1 with cancer cells and hopefully lead to better treatment of cancer patients and improved clinical outcomes.

P3028

Board Number: B310

GLE regulates stemness properties in Inflammatory Breast Cancer cells via STAT3 regulation.

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Inflammatory Breast Cancer (IBC) is the most lethal and aggressive type of breast cancer where patients have a 43% higher risk of death when compared to non-IBC. It accounts for 5% of all breast cancer cases in the U.S., with a five year survival rate of 20-50% when treated with chemotherapy. IBC is associated with a deprived outcome and an elevated risk of recurrence and metastasis compared with non-inflammatory locally advanced breast cancer. IBC cells contain a high cancer stem cell (CSC) composition, which display the CD44+/CD24− surface marker phenotype, and contribute to metastasis, recurrence and drug resistance. It has been demonstrated that the signal transducer and activator of transcription 3 (STAT3) is involved in oncogenesis and takes part in mediating the effects of maintaining a stem cell phenotype. A recent study reported that CD44+ tumor-initiating breast cancer cells had preferential activation of STAT3, suggesting that STAT3 may be a potential therapeutic target in breast cancer. Our data indicates that Ganoderma lucidum extract (GLE) decreases STAT3 activation in the IBC cell model SUM-149. A constitutively active mutant of STAT3 (cSTAT) was developed in order to determine the role of STAT3 in SUM-149 cells. Therefore, we tested the hypothesis that GLE decreases stem cell properties in IBC cells via STAT3 regulation. Our preliminary flow cytometry results show that cSTAT cells have a larger population of CD44+/CD24− cells compared to wild type (wt) SUM-149 cells. Importantly, our results demonstrate that GLE treatment significantly decreases the CD44+/CD24− cell population in cSTAT cells. To further analyze if GLE is decreasing stem cell properties, we performed
western blot analysis of stem cell-related transcription factors (Nanog, Oct4, and Sox2), which have a strong correlation with the CSCs phenotype. Our data demonstrates an increased expression of these proteins in cSTAT cells compared to wt SUM-149 cells. Interestingly, our results show that GLE decreases the expression of these transcription factors in both cSTAT and wt SUM-149 cells. Our results suggest that GLE is decreasing stemness properties in SUM-149 cells regulated by STAT3. We highlight the effectiveness of GLE in decreasing stem cell properties and identified STAT3 as a potential IBC target for therapy. This project was sponsored by Title V PPOHA and Title-V-Cooperative #P031S130068 US Department of Education #P031M105050 to UCC, NIH/RCMI MD007583 to UCC/MMM, NIH/INBRE GM103475 to UPR/UCC/MMM, NIH/SC3 #GM111171 to UCC/MMM, and PRSTRT SGRP 2017-00143 (MMM) and NIH/NIGMS R25GM110513 to UCC/TJR. The content is solely the responsibility of the authors and does not necessarily represent the official views of the supporting agencies.

P3029
Board Number: B311
CTLA-4 in cancer cells transmits high forces via the bond to CD80.
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Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is abundantly expressed on regulatory T cells and responsible for immune suppression. We examined two breast cancer cell lines, MDA-MB-231 (human) and EO771 (mouse), and found that the expression of CTLA-4 is significantly higher than their respective normal counterparts, MCF10A and EPH4-EV cells. The observation suggests that CTLA-4 by cancer cells might play a role in immune suppression. Immune suppression might be achieved by transendocytosis of CD80/CD86, or triggering signaling pathways that inhibit antigen presenting cells (APCs), or T cell activation, or both. We hypothesize that mechanical forces transmitted through CTLA-4/CD80 bond are involved in mediating either transendocytosis or immune suppression signaling. To test our hypothesis, we measured the forces between CTLA-4 and CD80 using DNA-based tension gauge tether (TGT) and the micropillar method. The TGT measurement results showed that EO771 and MDA-MB-231 cells exert higher pulling forces via the CTLA-4/CD80 bond, in the range between 12 pN and 54 pN, compared to the control EPH4-EV and MCF10A cells. Interestingly, micropillar measurement showed MDA-MB231 cells could transmit significantly higher (more than 2-fold) traction forces via CTLA4/CD80 bond, compared the bond between integrin and the RGD motif in ECM proteins. This finding implies mechanical forces might be significant in immunosuppression of breast cancer cells. Furthermore, it was observed that CTLA-4 co-localizes with phosphorylated myosin in cancer cells. In summary, our study provides a possible force-dependent mechanistic model responsible for immune suppression in breast cancer.
P3030

Board Number: B312

Therapeutic strategies for osteosarcoma stem cells by regulating adipocyte differentiation based on actin dynamics.

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Cancer stem cells (CSCs) are a subset of tumor cells that are responsible for initiating and maintaining the disease. In the clinical point of view, the most important characteristics of CSCs include their resistance to various therapeutic interventions. However, the underlying mechanisms of the resistance remain unclear. To address this question, we established a mouse osteosarcoma (OS) model by overexpressing c‐MYC in bone marrow stromal cells derived from Ink4a/Arf (−/−) mice. These OS cells were composed of two distinctly different clones: highly tumorigenic cells (termed AX cells), similar to bipotent‐committed osteochondral progenitor cells, and low tumorigenic tripotent cells (termed AO cells), similar to mesenchymal stem cells. We found that the loss of adipogenic potential is an essential event for AO cells to become AX‐like tumorigenic cells. Although AO cells are not found tumorigenic as far as they maintain the adipogenic differentiation potential, AO cells are found to be highly resistant to chemotherapeutic agents such as Adriamycin. Then, AO cells survived after the chemotherapy eventually change to AX‐like cells by losing their adipogenic differentiation potential to become tumorigenic. Therefore, our understanding of regulatory mechanisms of adipocyte differentiation would contribute to control OS tumorigenesis. We have recently found a novel regulatory mechanism of adipocyte differentiation. Regulation of the transcriptional coactivator MKL1 (megakaryoblastic leukemia 1) by actin cytoskeleton dynamics drives adipocyte differentiation mediated by peroxisome proliferator‐activated receptor (PPARγ), a master transcriptional regulator of adipogenesis. We found that disruption of actin stress fibers through the inactivation of RhoA‐ROCK signaling induces the rapid increase in monomeric G‐actin, leading to the interaction of G‐actin with MKL1, which prevents nuclear translocation of MKL1 and allows expression of PPARγ followed by adipogenic differentiation. Our findings thus provide new mechanistic insight into the relation between the actin dynamics and transcriptional regulation during cellular differentiation. Based on this discovery, we attempted to induce adipocyte differentiation in OS stem cells by treatment with ROCK inhibitors. ROCK inhibitors induced adipocyte differentiation of OS stem cells and significantly suppressed their in vitro growth and in vivo tumorigenesis. Our findings suggest that induction of trans‐differentiation of cancer stem cells by regulating actin cytoskeleton dynamics is a potential approach for some tumor types.

P3031

Board Number: B313

Inhibition of IL‐8 uptake by breast cancer cells suppresses vasculogenic mimicry.

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Highly aggressive cancer cells have been found to act as endothelial cells by forming capillary‐like networks for blood supply to the developing tumor in a process known as vasculogenic mimicry (VM). This implies that interventions targeting angiogenesis‐endothelial cell based vasculature – may not be

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completely effective at disrupting the growth of the tumor. The aim of the study was to investigate the effect of transgelin knockdown on VM in vitro, and on the expression of angiogenesis-related markers during VM. The formation of VM was accompanied by the expression of CD34 as revealed by flow cytometry and immunocytochemistry. Silencing of transgelin by siRNA suppressed VM but did not affect the expression of angiogenesis-related markers such as VEGFR2, CD34, VE-cadherin and IL-8 as shown by flow cytometry, immunocytochemistry analyses and RT-PCR. Suppression of vasculogenic mimicry by transgelin downregulation however, led to increased levels of IL-8 in the culture supernatant. Blocking the IL8 receptor, CXCR2, also inhibited VM and led to increased levels of IL-8, confirming that IL-8 uptake is associated with VM. Findings from these studies highlight the need for more work to elucidate the molecular mechanisms involved in vasculogenic mimicry that will eventually help in the development of improved interventions that do not only target angiogenesis, but the entire vasculature, for a more effective therapy.

P3032
Board Number: B314
Investigating mitotic kinesins as therapeutic targets for triple negative breast cancer.
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Patients with triple negative breast cancer (TNBC) display a particularly high risk for metastatic spread and disease recurrence, and 5-year overall survival rates associated with regional (85%) and distant (25%) metastases are lower than those for locally controlled tumors (99%). TNBC, molecularly defined by the absence of estrogen receptor, progesterone receptor and overexpressed human epidermal growth factor receptor 2 (Her2), accounts for ~15% of all breast cancers and poses significant treatment challenges. There are currently no targeted therapies available for TNBC, leaving cytotoxic agents as the primary systemic option. TNBC cells exhibit two common features not found in normal cells that we predict can be exploited for novel treatments: (1) they display high levels of chromosome instability, and (2) they exhibit significant changes in the expression of genes that regulate mitotic spindle function. Mitotic spindle integrity is dependent on a balance of mechanical forces generated by molecular motor proteins and microtubule dynamics. We hypothesize that changes in the expression of mitotic spindle proteins within TNBC cells reduces spindle integrity by disrupting this normal force balance, making the tumor cells more susceptible than normal cells to inhibition of spindle force regulators, such as kinesins. We are testing this “fragile spindle” model by assessing the effects of inhibiting five different kinesin motor proteins in cell models for three TNBC subtypes (basal-like 1, basal-like 2, and mesenchymal), as well as normal breast epithelial cells. Our results indicate that inhibition of Kif18A significantly slows the growth of TNBC cells but not normal cells. This slow growth is correlated with a mitotic delay and the formation of abnormal spindle structures. Taken together, our results support Kif18A as a promising target to specifically limit the growth of TNBC cells with low toxicity to other cell types.

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P3033
Board Number: B315
Telomerase inhibitors TMPyP4 and Thymoquinone decreased cell proliferation and induced cell death in NSCLC cell line LC-HK2, modifying the pattern of focal adhesion.
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The telomere is a repetitive double-strand sequence of DNA that protects the chromosomes ends. Its length is maintained by telomerase, whose expression is low or absent in somatic cells but is present in most cancer cells. As the cell continues to divide, telomeres shorten and undergo a process called telomeres dysfunction. This DNA breaks activate p53, a tumor suppressor that induces cell senescence, or other checkpoint responses depending on the cell type. G-quadruplexes are structures formed at the ends of the telomere, this structure is rich in guanines and are stabilized by molecules that bind to specific sites by Hoogsten-base paring. TMPyP4 and Thymoquinone are small that bind to the G-quadruplexes helping to form and to stabilize them. They have drawn attention for their role as telomerase inhibitors. The aim of this study was to evaluate the effects of telomerase inhibitors on cellular proliferation, senescence and death. Two cell lines LC-HK2 (Non-Small Cell Lung Cancer) and RPE-1 (hTERT) were treated with TMPyP4 (5 μM) and Thymoquinone (10 and 40 μM) (telomerase inhibitors) for 72 and 120 h. Both inhibitors were effective in decreased telomerase activity (TelO TAGGG Telomerase PCR ELISA assay). TMPyP4 increased the percentage of cells with membrane damage associated with cell death induction as evidenced by the zombie green assay and decreased the frequency of cells in S-phase (evaluated by EdU incorporation). Alterations in cell morphology and in the pattern of microtubules and microfilaments organization were more evident when cells were treated over longer periods (10 days). TMPyP4 changed the cell adhesion ability decreasing the number of attached cells and modified the pattern of focal adhesion, confirmed by slowdown of vimentin and vinculin mRNA expression in the qRT-PCR assay. Thymoquinone acted in a dose-dependent manner, and differently from TMPyP4, increased the frequency of senescent cells in the β-Galactosidase assay. It induced to cell cycle arrest in the G1 phase observed in the analysis by flow cytometry. In conclusion, the effects of both telomerase inhibitors on tumor cell line and non-tumoral cells were similar, despite the differences between them.

P3034
Board Number: B316
A "Trojan Horse" strategy to target lysosomes in cancer mediated by acidotropic nanomedicine. Z. Yang1, X. Zhang1, M. Feng1, C. Wang1;
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Lysosomes are acidic catabolic organelles in which intracellular wastes and foreign materials are degraded and recycled by various lysosomal enzymes. As a destination of several cellular pathways including endocytosis and autophagy, lysosomal dysfunction could perturb cell homeostasis, resulting in apoptotic or necrotic cell death. Therefore, it seems a promising strategy to destabilize the lysosomes in cancer cells using nanomedicines which are readily taken up into lysosomal compartments via endocytosis. However, it remains a challenge to specifically deliver lysosomal-targeting nanomedicine to the tumors while ignoring the normal tissues, since lysosomes are present in almost all the mammalian cells. In our work, we developed one acidotropic nanomedicine (PAC/HA) which preferentially accumulated in the acidic tumor than in normal tissues. Subsequently, PAC/HA overloaded in tumor cells induced lysosomal alkalinization and autophagy blockage accompanying with lysosomal membrane

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permeabilization (LMP) and oxidative stress, and thereby triggered the cell death. All the in vitro and in vivo data demonstrated the tumor-acidity-selective cytotoxicity of PAC/HA. Hence, this facile strategy offers an opportunity to target lysosomes via our well-defined acidotropic nanomedicine in the treatment of cancer.

P3035
Board Number: B317
Pharmacological effect of three tryptophan analogues with HDAC inhibitory activity in MDA-MB-231 triple negative breast cancer cells.
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The mechanisms leading to epigenetic modifications such as acetylation and deacetylation of histones by the enzymatic activity of HAT and HDAC, play an important role in regulating gene transcription. It has been shown that many diseases, particularly cancer is caused when epigenetic regulation mechanisms is aberrant. Breast cancer is unhealthy therefore epigenetic mechanisms of deregulation emerging pathology associated with aging and lifestyles. The subtype of breast cancer triple negative (TNBC), named for the absence of receptors for estrogen, progesterone and HER2 / neu is characterized as the most aggressive, and not respond to trastuzumab and endocrine therapies. As TNBC cells overexpressing HDAC8 in our working group have been designed and synthesized three analogues of tryptophan with inhibitory activity HDAC8. So the objective of this work was to determine the degree of inhibition of HDAC8 inducing analogs FTrpa tryptophan, and Trp1 FTrpc; the antiproliferative effect and morphological changes that induce each of these compounds on the MDA-MB-231 cells TNBC.

Methodology: The degree of inhibition of HDAC8 was performed using a commercial kit from SIGMA-ALDRICH. To indirectly determine the number of surviving cells to treatment with each of the analogues of tryptophan the proliferation of TNBC cells was quantified by the colorimetric MTT assay in MDA-MB-231 seeded in 96 wells, incubated at 37 ° C and 5% CO2 DMEM F12 medium, 10% FBS (100uL / well) to a 90% confluence, were added 100uL / well of each compound at 8 different concentrations were 0310-10 mM, DMEM F12 medium was used as a positive control and 10 mM TSA HDACi, as negative control. And the evaluation of cellular morphological changes were made by Giemsa staining. Our results show that FTrpa, and Trp1 FTrpc compounds had the ability to inhibit the activity of HDAC8 being Trp1 the most effective. All three compounds had the ability to inhibit proliferation of MDA-MB-231 being the most TNBC powerful Trp1. They also had the ability to induce the death of MDA-MB-231 cells TNBC. In conclusion Trp1 compound has a greater chance of success as an antitumor agent in TNBC.

P3036
Board Number: B318
Experimental therapeutics of orthotopic colon cancer models.
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To the potential impact on colon cancer growth of different low-dose chemotherapy regimens, including combinations with the angiogenesis inhibitor bevacizumab, we generated orthotopic models of the human HT29 cancer cell line in immunodeficient mice. HT29 cells were transfected to express GFP (Green Fluorescent Protein), luciferase or human chorionic gonadotropin (hCG), and subsequently injected intracaecally or intrasplenically into female mice. Tumors were subsequently monitored via
Prostate cancer (PCa) is one of the most common cancers in American men. While initial resection and radiation therapy is usually successful, recurrence is common. Upon recurrence, PCa patients are often treated with androgen deprivation therapy (ADT), which impairs proliferative signaling through the androgen receptor (AR). Despite ADT treatment, many cancers eventually recur as hormone treatment refractory cancers. These castration resistant prostate cancers are uniformly lethal. In some cases, such ADT insensitive tumors may display neuroendocrine characteristics. These cancers are termed neuroendocrine prostate cancer (NEPC) and are particularly aggressive. Clinical evidence suggests that incidence of the NEPC phenotype is increasing concurrently with increasing use of potent new ADT medications, such as abiraterone and enzalutamide.

We aim to understand the effects of prenylation on ADT-related neuroendocrine differentiation of PCa. Prenylation is a post translational modification in which proteins are modified by the addition of a farnesyl or geranylgeranyl group derived from mevalonate via the isoprenoid pathway. Prenylated proteins have roles in cancer development, metastasis, cytoskeletal regulation, and differentiation. Here, we investigated digeranyl bisphosphonate (DGBP) as a potential modulator of neuroendocrine differentiation. This compound inhibits the synthesis of geranylgeranyl pyrophosphate and thus reduces the geranylgeranylation of regulatory proteins, including members of the Rho family. We found that DGBP reduces PCa progression and metastasis in both in vitro and xenograft models. Reporter assays and Western blotting suggest that short term DGBP treatment of PCa cells reduces AR activity, but not protein levels. Fluorescence microscopy revealed this is due to reduced nuclear localization of the AR protein. In the context of long term treatment, however, it appears that DGBP can restrict unfavorable changes toward the NEPC phenotype in both steroid-reduced and ADT induced cell culture models of prostate cancer, while also reducing AR expression. We conclude that geranylgeranylation of downstream proteins is required for both PCa progression and AR nuclear translocation, and inhibition of this process may enhance patient responses.
P3038

Board Number: B320

In vitro antitumor activity of new quaternary hydroxypyridine-based phosphonium salts.
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Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 5.4 million deaths in 2017, and, therefore, novel approaches are required for improvement of cancer therapies. Phosphonium salts are one of promising classes of compounds with antitumor activity, which show great promise for the diagnosis and treatment of neoplasms. Some phosphonium salts have been reported to show anti-proliferative activity in several cancer cell lines and a xenograft model of ovarian cancer based on their ability to disrupt mitochondrial ultrastructure and alter cellular lipid content. Studies of phosphonium salts as contrast agents for diagnostic imaging have elucidated two key points concerning the selectivity of this class of compounds: 1) these agents are capable of preferentially accumulating within tumor cells, 2) phosphonium cation itself does not impart cytotoxicity. In this study, we assessed the effects of new quaternary phosphonium salts based on 3-hydroxypiridine and 4-deoxyxypyrdoxine on OVCAR-4 ovarian cancer cells. In particular, the following phosphonium salts were studied: 3-hydroxy-2-[tris(p-tolyl)phosphoniomethyl] pyridinium dichloride, 3-hydroxy-2,6-bis-[tris(p-tolyl)phosphoniomethyl] pyridinium dichloride, 3-acetoxy-2,4-dimethyl-5-[tris(p-tolyl)phosphoniomethyl] pyridine chloride. Anti-cancer activity and cytotoxicity of these compounds were evaluated in vitro. Cell proliferation and viability after treatment with the tested compounds was assessed by colony formation and MTT assays. The effects of compounds on apoptosis and cell cycle were determined by flow cytometry using Annexin V FITC/PI and PI staining, respectively. Effects of compounds on mitochondrial membrane potential and intracellular reactive oxygen species were determined using TMRE and DCFHA staining. Western blot analysis was used to assess changes in the expression of Bcl-xL, Bax and caspase-3 apoptotic proteins. Treatment of ovarian adenocarcinoma cells OVCAR-4 with the studied compounds led to expressed inhibition of growth and induced cell cycle arrest in G1-phase. 3-Hydroxypyridine derivatives induced apoptosis via hyperexpression of BAX and caspase-3, while 4-hydroxypyridine derivative induced cell death partly via ROS generation and caspase-3 hyperexpression. These results indicate that the studied hydroxypyridine-based phosphonium salts may be promising therapeutic agents for the treatment of ovarian cancer.

Tumor Microenvironment 2

P3039

Board Number: B321

Tunneling Nanotubes, a Novel Mode of Tumor Cell-Macrophage Communication in Tumor Cell Invasion.
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The interaction between tumor cells and macrophages is crucial in promoting tumor invasion and metastasis. These two cell types are engaged in a mutual interaction in which tumor-associated macrophages produce epidermal growth factor (EGF) to activate tumor cells. In turn, tumor cells produce colony-stimulating factor-1 (CSF-1) that stimulates macrophages. The tumor cell - macrophage
pairs formed in response to this paracrine signaling are then attracted toward blood vessels under an endothelial cell produced HGF gradient. This mutual signaling interaction leads to the co-migration and invasion of both cell types as imaged both in vitro and in vivo. Recent studies have revealed an additional novel mechanism of intercellular communication between macrophages that can transmit signals over long distances through membranous actin-based tunneling nanotubes (TNTs). Our data demonstrates that heterotypic TNTs form between macrophages and tumor cells in co-culture. This novel interaction induced changes in tumor cell morphology consistent with a more invasive phenotype which was dependent on EGF-EGFR signaling. Moreover, the presence of these heterotypic TNTs was important for tumor cell invasion in an in vitro 3D invasion assay. Furthermore, reduction of M-Sec (TNFAIP2) in macrophages, a protein involved in TNT formation, inhibited tumor cell elongation and blocked the ability of tumor cells to invade. Using a modified 1D assay that mimics macrophage-dependent tumor cell streaming observed in vivo, we show a significant increase in long distance directional migration of tumor cells towards an endothelial-coated bead in a TNT dependent manner. We also employed an in vivo zebrafish model that recreates macrophage mediated tumor cell invasion in a more physiological fashion. The presence of macrophages increased tumor spread from the injection site, number of metastatic foci, and the distance of metastatic spread in a macrophage TNT-dependent manner. Overall, our studies support a role for TNTs as a novel means of interaction between tumor cells and macrophages that may lead to tumor progression and metastasis.

P3040

Board Number: B322

Substratum stiffness and tumor dormancy.
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About 90% of cancer-related deaths are due to the spread of cancer cells to secondary sites in a process called metastasis. Current treatments can effectively remove or shrink the primary tumor and slow down the growth of metastatic recurrences, however, they are only temporary. Breast cancer has a high risk of recurrence that can develop over the course of decades due to “dormant” tumor cells that, at one time, disseminated from the primary tumor to secondary sites. Although it is known that breast cancer cells preferentially metastasize to the lung, brain, bone marrow and liver, the nature of physical and biochemical interactions between disseminated cancer cells and their local microenvironment is not known. Here we investigated whether the physical microenvironment, specifically host tissue stiffness, affects the propensity of disseminated tumor cells to become dormant at secondary sites. Given that substratum stiffness affects cell phenotype, motility, adhesive properties, and proliferative behavior, it is likely to also regulate some of the mechanisms that trigger dormancy. Breast cancer cells were cultured on “soft” (E~130 Pa) or “stiff” (E~4020 Pa) polyacrylamide gels that mimic the stiffness of the normal and tumorigenic mouse mammary gland respectively. We found that tamoxifen and 5-fluorouracil induce cell dormancy in estrogen receptor-positive breast cancer cells that were cultured on soft or stiff substrata as determined by the immunofluorescence of Ki67. Quantifying the transcript levels of CD44, Nanog, and ILK in tamoxifen-treated breast cancer cells revealed upregulation in the expression of cancer stem cell markers. Our data suggest that tamoxifen and 5-fluorouracil might contribute to chemoresistance by inducing dormancy and stemness. Elucidating the respective roles of dormancy and stemness in chemoresistance and defining the characteristics of a microenvironment that harbors chemoresistant metastases could pave the way for more effective cancer treatments.
P3041
Board Number: B323
Interplay between mechanotransduction and force generation underlies mitosis in three-dimensional microenvironments.
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Cell division underlies the development of humans from embryo to full-grown adult, regenerative processes such as wound healing, and diseases such as cancer. The process of cell division requires extensive morphological changes, including elongation along the mitotic axis, critical for proper segregation of chromosomes and other intracellular materials and guiding orientation of daughter cells. In physiological tissues such as packed epithelial monolayers or growing tumor spheroids, cells are surrounded by adjacent cells and extracellular matrix (ECM), which mechanically constrain the growth and morphological changes required for mitosis. While much is known about the intracellular aspects of mammalian cell division, how the mechanical aspects of the microenvironment regulate cell cycle progression, and how cells overcome mechanical constraints to undergo mitosis, remain unknown. Here, we investigated cell division in viscoelastic hydrogels, which served as three-dimensionally confining microenvironments. We found that hydrogel stress relaxation, related to hydrogel viscosity, regulates cell cycle progression of tumor cells. In fast relaxing, or highly viscous, matrices, cells proliferated extensively to form large spheroids, while in slow relaxing, or more elastic, matrices, cells were less proliferative and mostly arrested in the G1/G0 phase of the cell cycle. Mechanistically, mechanical stress imposed by the surrounding microenvironment against cell growth regulated PI3K and p27 activation to control cell cycle progression. Next, we examined the mechanics of how cells undergoing cell cycle progression are able to divide in the mechanically confining viscoelastic microenvironments. We found that dividing cells generate substantial protrusive forces that deform the microenvironment along the mitotic axis, clearing space for mitotic elongation to proceed. These forces result from interpolar spindle elongation and lateral contraction of the actomyosin ring, which drives expansion along the mitotic axis through the Poisson effect. Together, these results reveal the mechanical interactions that occur between cells and their microenvironments, which sense mechanical properties of the microenvironment to control cell cycle progression and then enable cell division to occur in mechanically confining microenvironments.

P3042
Board Number: B324
Early stage breast cancer spheroids mechanically remodel the microenvironment more significantly compared to their normal and metastatic counterparts.
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It is known that cancer cells remodel the tumor microenvironment in terms of the extracellular matrix (ECM) fiber density, organization, and stiffness by facilitating activation of mechanosensitive pathways in favor of tumor progression. However, how cancer cells remodel the microenvironment dynamically has yet to be characterized. We establish a 3D model system where spheroids of breast epithelial cells of increased malignancy [Eph4-EV (normal), 67NR (early-stage), and 4T1 (metastatic)] are embedded and cultured in thick collagen gel at 5\(\times\)10\(^4\) cells per spheroid, in mimicry of the physiological conditions in
vivo. The dynamic interaction between the spheroids and their microenvironment was recorded using time-lapse microscopy. The remodeling of the tumor microenvironment was then quantified in terms of changes in collagen density, fiber alignment, and porosity around the spheroid over time. We also used traction force microscopy to measure the mechanical forces exerted by the spheroids in 3D. 67NR cells remodeled the ECM such that they become more porous (~2.1% increase), more aligned (~8-fold increase), and denser (~17% increase) compared to EPH4-EV. It was also observed that 67NR cells generated higher forces compared to EPH4-EV. We also noticed that cancer spheroids incapable of generating relatively high mechanical forces were not capable of remodeling the ECM. Quite intriguingly, we observed that 4T1 cells invaded the collagen within 24hrs of getting embedded in collagen, while 67NR cells showed bulk spheroid behavior and hence dramatically modulated the collagen. Our findings provide a direct connection between force generation, dynamic ECM remodeling, and tumor progression.

P3043

Board Number: B325

Meaningful connections: Homotypic cell-cell interactions in pancreatic ductal adenocarcinoma’s desmoplastic stroma.

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Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a 7% 5 year survival rate. PDAC is mediated via complex interactions between tumor cells and the surrounding stroma, known as desmoplasia, that mainly includes cancer-associated fibroblasts (CAFs) and the dense extracellular matrix (ECM) they produce, as well as infiltrating nerves and immune cells. Desmoplasia is important to PDAC development as it 1) comprises over 70% of the cancer mass, 2) facilitates tumor-stromal interactions, and 3) imparts both pro- and anti-tumorigenic effects. Thus, elucidating how cell-cell interactions contribute to the adverse effects of desmoplasia is imperative. Comparing normal fibroblast vs. CAF gene expressions revealed increased mRNA and protein levels of the neural extracellular laminin-related glycoprotein netrin G1 (NG1) in CAFs, while its receptor, NG1 ligand 1 (NGL1) was detected in both tumor cells and CAFs. Interaction of these proteins was sufficient to establish a synapse between homotypic and heterotypic cell types. Strikingly, CRISPR-Cas9 knock out of either NG1 or NGL1 in CAFs significantly altered the CAF secretion profile, thereby suggesting that NG1/NGL1 expression in CAFs, and their engagement, may have important roles in stromal maintenance that can contribute to the pro- and anti-tumorigenic effects. While heterotypic CAF-PDAC cell interactions were found to be NG1-dependent and essential for PDAC cell survival under starvation, the role of homotypic CAF-CAF interactions in this process remains unknown. Therefore, the goal of this project is to 1) elucidate the NG1/NGL1 signaling transduction cascade within CAFs and 2) determine how this signaling contributes to cell-cell interactions effects on the tumor microenvironment. Taken together, the work done in this project will help elucidate how phenotypic changes to the stroma affect heterotypic cell-cell interactions that are necessary for PDAC survival.
P3044
Board Number: B326
Age-related gene expression and cancer susceptibility in luminal epithelial cells is driven by a microenvironment made from myoepithelial cells.
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Luminal epithelial cells in breast gradually alter expression of proteins and genes with age, appearing to lose lineage-specificity by acquiring myoepithelial-like characteristics. We hypothesize that the luminal lineage is particularly sensitive to microenvironment changes, and age-related microenvironment changes cause altered luminal cell phenotypes. To evaluate the effects of different microenvironments on the fidelity of luminal and myoepithelial gene expression via epigenetic regulation, we generated a set of lineage-specific probes for genes that are controlled through DNA methylation. Primary luminal cells cultured in conditions that favor myoepithelial survival caused reprogramming at the level of gene methylation and expression towards a more myoepithelial-like state. Primary myoepithelial and luminal cells were used to recreate bilayers, providing cues necessary to maintain luminal cells in culture without losing lineage-specific gene expression. Isogenic stromal fibroblast co-cultures were unable to maintain the luminal phenotype. Mixed-age luminal-myoeipithelial bilayers revealed that luminal cells adopt transcription and methylation patterns consistent with the chronological age of the myoepithelial cells. We provide evidence that the luminal epithelial phenotype is exquisitely sensitive to microenvironment conditions, and that states of aging are cell non-autonomously communicated through microenvironment cues over at least one cell diameter in human breast tissue.

P3045
Board Number: B327
Targeting the alliance between tumor cell sub-populations.
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Tumor heterogeneity is a major obstacle in the development of effective cancer treatment. Resistant clones, which may only make up a small fraction of the total cell mass, persist and expand to lethal tumors following treatment. Suitable models and technologies are lacking that allow eavesdropping on tumor cell-cell communication and facilitate quantification of the emergent behavior resulting from this cooperation.

We have developed a rigorous and reproducible model of tumor cell cooperation that demonstrates active maintenance of tumor heterogeneity and coordinated regulation of cell migration. Two subclones from the U87MG glioblastoma cell line were each created by expansion from a single, randomly picked cell. Both clones displayed a unique pattern of cytogenetic abnormalities as well as differential activation of key signal phosphoproteins. Clone A had a significantly slower growth rate (1.5 fold) compared to clone B. This prompted us to question why the slower growing clone was not outcompeted by the more aggressively growing clone.

Combining clones A and B in a 1:1 mixed-clone culture resulted in the temporary synchronization of growth rates, increasing the growth rate for clone A and restraining growth for clone B. In long-term cell culture (>100 days), clone A was maintained at a constant fraction of around 5% of the total cell number. The rate of cell migration out of 3D spheroids was comparable between both clones when
spheroids were monoclonal. However, in spheroids composed of 1:1 clonal mixes of clone A and B the migration of clone B cells was abolished by the presence of clone A. Thus, our model shows multiple layers of cooperation: (a) persistence of a slow growing clone, (b) slowed growth of a fast growing clone, and (c) inhibition of migration of the fast growing clone. Paradoxically, this indicates the slow growing clone as the driver of this interclonal cooperation.

We found high phosphorylation of Stat3 Ser727 in clone A, but not clone B or the original U87MG cell line. In a wound healing assay, inhibition of Stat3 phosphorylation using Napabucasin had no effect on cell migration in monoclonal cultures. However, the constraining effect of clone A on the migration speed of clone B was significantly exacerbated when Stat3 was inhibited in 1:1 mixed-clone cultures. This straightforward and highly reproducible in-vitro model of tumor cell-cell cooperation provides a unique opportunity to study the mechanisms underpinning interclonal communication. Our model is easily scalable to allow future screening of compounds that disrupt clonal cooperation and may thus help provide a new approach to treat cancer by eliminating driver clones that actively maintain tumor homeostasis and cause driver sub-populations to persist.

P3046
Board Number: B328

Desmoplasia meets nerves in pancreatic cancer progression.
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Pancreatic ductal adenocarcinoma (PDAC) is recognized as one of the most deadly types of cancer, with a 5-year survival of less than 8%. One of the major features of PDAC is the rich fibrous stroma, known as desmoplasia, characterized by the expansion of cancer associated fibroblasts (CAFs) and their extracellular matrix (ECM). In this hypoxic and immunosuppressive environment, an uncommon phenomenon takes place: neoneurogenesis, or de novo innervation, which is associated with PDAC "perineural invasion" and accentuated neuropathy. The expansion of nerves, together with desmoplasia, is related to poor prognosis in PDAC. Although there is an established neurotropic role for CAF and PDAC, the relationship between these cells and neural cells within PDAC microenvironment is complex and poorly understood. Recently our lab identified the ectopic expression of the neural pre-synaptic protein NetrinG1 (NG1) in CAFs and of its binding partner NetrinG1 ligand (NGL1) in PDAC cells in samples from PDAC patients, which led us to ask if NG1-NGL1 were involved in this complex network between CAFs, PDAC and neural cells. To better understand this connection, we generated CRISPR/CAS9 mediated knockout (KO) of NG1 or NGL1 in CAFs and co-cultured them with neurons from E16 embryonic hippocampus, in a 3D culturing system, using CAFs-derived extracellular matrix as the substrate. Preliminary data indicates that neurons had increased survival when co-cultured with wild type CAFs under stressful conditions, compared with neurons cultured alone or in the presence of NG1 KO CAFs, suggesting that CAFs supports neuron survival in a NG1-NGL1 dependent way. Moreover, we observed that neurons and CAFs establish heterotypic interactions and that there is a material flow between these cells. Interestingly, the flow from neurons to CAFs was disrupted either when NG1 in the neurons and NGL1 in the CAFs were inhibited, suggesting a “pre-synaptic role” for NG1 and a “post-synaptic role” for NGL1. Additional studies will confirm these observations. Finally, we correlated the levels of NG1-NGL1, neoneurogenesis and desmoplasia at different time points in samples from a murine spontaneous PDAC model and in samples from PDAC patients, in order to establish the relationship between NG1-NGL1 expression and PDAC progression, and perhaps uncovering two new putative targets for clinical interventions or diagnostics.
P3047
Board Number: B329
Desmoplastic expression of NetrinG1 fuels pancreatic cancer growth and promotes metastasis through nutritional supply and immunosuppression.
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Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease, with a dismal 5-year survival rate of <8%. A specific PDAC characteristic is desmoplasia; which consists of the expansion of activated stellate cells, known as cancer associated fibroblasts (CAFs), and the active deposition and remodeling of their extracellular matrix. PDAC’s desmoplasia results in a microenvironment that contains collapsed blood vessels and is immunosuppressive to both cytotoxic T and natural killer (NK) cells. Cell-cell interactions between PDAC, CAFs and immune cells, within the desmoplastic milieu, are not fully understood. In this study, we uncovered the ectopic expressions of NetrinG1 (NG1), a neural pre-synaptic protein, in CAFs, and of NG1’s post-synaptic binding partner, NetrinG1 ligand (NGL1), in PDAC cells. Using a multi-cellular 3D culturing system, we observed that heterotypic CAF-to-PDAC cell-cell interactions, via NG1/NGL1 engagement, are critical for providing survival advantages to nutrient deprived PDAC cells and for CAFs to protect PDAC from NK cell-driven elimination. Mechanistically, we uncovered that PDAC starvation is overcome by NG1 expressing CAFs that provide nutrition via material transfer, based on an intact glutamine/glutamate cycle. Further, CRISPR/CAS9 mediated knockout of NG1, in CAFs, significantly reduced the production of both immunosuppressive cytokines, such as IL-6, IL-8, and TGF-β and pro-tumorigenic factors, such as GM-CSF and CCL20. Moreover, loss of NG1 from CAFs resulted in anti-tumor NK cell activation. Excitingly, results were confirmed in an orthotopic PDAC mouse model in which ablation of NGL1, in syngeneic mouse PDAC cells, significantly halted tumor growth and metastasis. Translationally, in a cohort of surgically resected samples from PDAC patients, we questioned if stromal NG1 and tumoral NGL1 expressions correlated with our recently established outcome predictive stromal signature. This study proposes a possible novel desmoplastic immune cell checkpoint and added means to influence tumor-stromal interactions.

P3048
Board Number: B330
Secretory IgM exacerbates tumor progression by inducing accumulations of MDSCs in mice.
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We generated and characterized an antigen-specific MD4/εμ-TCL1 CLL mouse model. B cells from young MD4/εμ-TCL1 mice harbor a monoclonal B cell receptor (BCR) against hen egg lysozyme (HEL) and secrete IgM against HEL. Surprisingly, all CLL cells developed in older MD4/εμ-TCL1 mice fail to recognize HEL, but they still respond to goat F(ab’2) anti-mouse IgM by activating robust BCR signaling, suggesting that MD4/εμ-TCL1 CLL cells reactivate a parental Ig gene allele to produce non-HEL-specific BCR and
secretory IgM (slgM). Compared with age-matched Eμ-TCL1 mice, MD4/Eμ-TCL1 mice survive significantly shorter, produce high levels of slgM, and generate significantly increased granulocytic and monocytic myeloid cells in the peripheral blood, spleens and bone marrow. We demonstrated that these granulocytic and monocytic myeloid cells purified from the spleens of MD4/Eμ-TCL1 mice can suppress the proliferation of CD8+ T cells in an antigen-dependent manner, qualifying these cells as myeloid-derived suppressor cells (MDSCs). To test the hypothesis that slgM may account for the accumulation of MDSCs, we crossed μS-/− mice, which can only produce membrane-bound BCR but not slgM, with Eμ-TCL1 mice. The μS-/−/Eμ-TCL1 mice develop significantly lower numbers of MDSCs, and survive significantly longer than Eμ-TCL1 mice. In XBP-1-deficient B or CLL cells, the synthesis of slgM is significantly downregulated. To test whether targeting XBP-1 in CLL cells can reduce the production of slgM and lead to decreased MDSCs, we crossed B cell-specific XBP-1KO mice with MD4/Eμ-TCL1 mice. The resultant XBP-1KO/MD4/Eμ-TCL1 mice indeed produce significantly reduced amounts of slgM and decreased numbers of MDSCs. In addition, pharmacological inhibition of XBP-1s also leads to compromised immunosuppressive functions of MDSCs, suggesting the therapeutic potential of targeting XBP-1s in inhibiting immunosuppressive functions of MDSCs. To test whether slgM also plays a role in regulating MDSCs in solid tumors, we implanted μS-/− mice with Lewis lung carcinoma, and showed that the function of MDSCs in suppressing anti-tumor T cells is abrogated by the absence of slgM, resulting in the slower growth of the lung cancer. These results clearly demonstrate that slgM produced by B cells can upregulate the functions of MDSCs in tumor-bearing mice to aggravate cancer progression.

P3049
Board Number: B331
A Systems Model of Human Metastatic Melanoma from Invasion to Colonization Identifies Tenascin-C as a Driver of Resistance and Emergence.
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The lethal progression of metastatic melanoma is driven by refractory micrometastases. These small tumors are generally chemoresistant, and under growth arrest. Dormancy confers drug resistance and secures eventual ectopic emergence. Limited information is known in regard to drivers of metastatic emergence. Human tissue biopsies indicate that metastatic melanoma harnesses the tumor microenvironment (TME) to develop a drug resistant niche, at which point durable therapeutic responses are rare. While genetically engineered mouse models have been instrumental in identifying the impacts of melanoma driver mutations, the study of cell programs governing early ectopic seeding, survival, and emergence have been constrained by inherent complexity. To address this we have developed the first all-human microphysiological systems (MPS) model of metastatic melanoma to study key events from dissemination to colonization. Using the MPS, an all-human organotypic skin organ has been connected via microfluidic circulation to a functional 3D human liver organoid to model critical changes in the primary and ectopic TMEs. Pathophysiologic changes in the TME occur early in primary melanoma which is marked by increased expression of the ECM protein, Tenascin-C (TNC). The presence of TNC in melanoma tumors is correlated to increased malignancy and poor patient prognosis. Invasive outgrowth of melanoma tumor spheroids is increased by melanoma derived TNC, and by stimulatory epidermal growth factor (EGF) and inflammatory ligand lipopolysaccharide. However, invasive outgrowth appears to be more readily influenced by cancer associated fibroblasts (CAFs). CAFs are the primary source of cancer ECM within primary and ectopic TMEs, and are hypothesized to create a tumor

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permissive ECM for outgrowth and promote tumor survival via secretion of TNC which contains intrinsic low affinity, anti-adhesive, EGF-like repeats. Preliminary studies in the MPS using high TNC expressing metastatic melanomas indicate that TNC is key to driving vertical invasion into collagen matrices. Strategies will be aimed at targeting stromal and cancer cells producing TNC to limit outgrowth and invasion within the TMEs.

P3050
Board Number: B332
Collagen Increases Proliferation and Drug Resistance of Papillary Thyroid Cancer Cells Harboring BRAFV600E Mutations.
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Thyroid cancer is the most common endocrine cancer, and incidence is increasing worldwide. Thyroid cancer can be classified as either well-differentiated or poorly differentiated. Of well-differentiated thyroid cancers, papillary thyroid cancer is most common, and is associated with activating BRAF mutations. While our understanding of the genetic basis for thyroid cancer is fairly extensive, less is known about how the tumor microenvironment alters tumorigenic characteristics of thyroid tumor cells. Recently, Jolly et al. reported that papillary thyroid tumors derived from cells harboring activating BRAF<sup>V600E</sup> mutations and PTEN deletions are enriched with fibrillar collagen which is associated with decreased survival. In this study, we investigated whether growth on collagen enhanced tumorigenic characteristics of papillary thyroid cancer cell lines with BRAF<sup>V600E</sup> mutations. Three distinct cell lines derived from mouse papillary thyroid cancer tumors were grown in the presence and absence of collagen and assessed for increased proliferation, increased sensitivity to apoptosis, morphological changes indicative of epithelial to mesenchymal transition, and increased drug resistance. Interestingly, our results suggest that growth on collagen contributes to a more mesenchymal morphology, increased proliferation, and decreased sensitivity to chemotherapy drugs. Additionally, our results suggest that collagen increases resistance to apoptosis in an AKT-dependent manner. These and other results implicate an important role for collagen in the progression of thyroid cancer.

P3051
Board Number: B333
Evaluating the overexpression of mitotic checkpoint silencing protein TRIP13 in human breast cancer cell lines in 2D and 3D cell culture models.
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Breast cancer is the most prevalent cancer afflicting women worldwide, and is responsible for hundreds of thousands of deaths each year. Several studies have linked overexpression of the TRIP13 gene with breast cancers with poor prognosis, and a variety of other cancer types. TRIP13 is one of many members of the AAA-ATPase family, which collectively have diverse roles in cells that include signal transduction, regulation of gene expression, and protein degradation. Our lab recently established that TRIP13 plays a major role in silencing the mitotic checkpoint, a regulatory mechanism that ensures proper attachment of chromosomes to the mitotic spindle during cell division. Premature silencing of the checkpoint can lead to chromosomal instability (CIN) and aneuploidy, hallmark facilitators of tumorigenesis. Although TRIP13 overexpression has also been correlated with CIN in clinical samples, there is not yet any direct evidence of a causative influence of TRIP13 overexpression on either CIN or tumor development. In the
current study, we sought to investigate the effects of TRIP13 overexpression in human mammary epithelial cells (MCF-10A) as well as in established breast cancer cell lines representing major subtypes of breast cancer: MCF-7 (Luminal A), ZR75-30 (Luminal B), MDA-MB-231 (Basal). All cell lines were engineered with inducible (doxycycline) control over TRIP13 expression. Although TRIP13 overexpression attenuated cell proliferation in all three of the breast cancer cell lines, it almost completely arrested cell proliferation in the non-tumorigenic MCF-10A cells. Flow cytometric analyses of the MCF-10A cells further revealed that TRIP13 overexpression increased G0/G1 cells while reduced cells progressing into S-phase. This was accompanied by a slight increase in CIN, as measured by FISH probing of chromosomes 8 and 12. In addition, when both MCF-7 and MCF-10A cells were plated in a 3D spheroid culture, TRIP13 overexpression noticeably reduced the circularity and compactness of the spheroids in both groups, but only the MCF-10A spheroids exhibited a decrease in total cell viability. These findings suggested that TRIP13 overexpression itself is insufficient to trigger CIN and tumorigenesis, however it is likely to synergize with other genetic or epigenetic alterations in driving the survival and uncontrolled proliferation of precancerous cells. Future work will determine how TRIP13 overexpression might specifically be advantageous for cancer cells, and whether such activities are required for the transition to metastasis.

P3052
Board Number: B334
Mechanical control of nuclear blebbing and micronuclei in hepatocellular carcinoma cells.
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The mechanical environment of cells changes during cancer development, especially in the liver, where stiffening from fibrosis is often a precursor to cancer initiation. In many cancer cell types, there is correlation between deregulated nuclear morphology or protein expression and cancer grade, which is poorly understood. In this work we report the effect of substrate stiffness on nuclear shape and defects in the human hepatocellular carcinoma cell line Huh7, which exhibits distorted nuclear morphology compared to normal human hepatocytes. The percentage of blebbing nuclei and the number of micronuclei depend on the stiffness of the substrate. Both blebbing and micronuclei are rare on substrates mimicking the stiffness of healthy liver (<1 kPa) and increase as substrate stiffness increases to levels found in cirrhotic livers and liver tumors (10 kPa). Nuclear membrane tension is assessed by dynamic nuclear envelope (NE) fluctuation through live cell imaging, showing larger fluctuations on softer substrates. To investigate the underlying mechanism of nucleo-mechanosensing, we perturbed actin and microtubule systems. Inhibiting ROCK to decrease actomyosin contractility slows down nuclear fluctuation. These in vitro results provide a background with which to compare the impact of stiffness differences on micronuclei formation in normal and HCC tissues from patient samples.
Extracellular matrix (ECM) stiffness has emerged as an important regulator of cellular processes and a significant parameter of the tumor microenvironment. In general, solid tumors are known to be much stiffer than normal tissue, with increased fibrosis being indicative of a worse prognosis for many cancers. The present study focuses on pancreatic ductal adenocarcinoma (PDAC), in which ECM desmoplasia is exceptionally pronounced. Such stromal amassment has been proposed to be a driver of malignancy and a key barrier to efficient drug delivery. With a five year survival rate of 5%, PDAC is in urgent need of research into more effective and personalized therapies. Here we investigate the interplay of varying stiffness with pancreatic stellate cells (PSCs), and their influence on tumor cell behavior. As an integral element of the stromal compartment, PSCs have simultaneously been suggested to be a driver of malignancy and a restraint on cancer progression. Our studies aim to bridge missing links in our understanding of PSC regulation and activity, particularly in regards to ECM stiffness and heterogeneity and their influence on cancer progression. We assess stiffness effects (0.2, 4, and 12 kPa; 3 GPa) on PSC secretomes across patients, and examine tumor-stroma crosstalk with and without treatment. Interestingly, cytokine secretion levels vary in response to stiffness, and differ between tumor-derived PSCs and normal immortalized pancreatic fibroblasts. Intermediate stiffness levels upregulate growth factors that promote fibrosis (TSP-1), increase migratory potential (PTX3) and are predictive of worse prognosis (IGFBP-3), among others. Importantly, we observed expression differences between 3 GPa (plastic) and 4 and 12 kPa (biologically relevant range), indicating that the mechanical environment of typical cell culture methods introduce considerable secretome deviations. We also observe inter-patient variation in PSC morphologies in response to stiffness, as well as differing molecular signatures. These studies highlight the significance of the stromal compartment in PDAC, both with respect to patient heterogeneity and ECM stiffness.

Hypoxia is an important phenomenon in many physiological processes and involved in many human diseases including cancer, cardiovascular and neurodegenerative diseases. The study of hypoxia has been complicated with the lack of sensitive dyes to measure hypoxia at greater than 1% O2. Here, we describe a live cell-based method to conveniently measure hypoxia using a new Image-iT™ Hypoxia Green Probe. The Image-iT™ Hypoxia Green Probe is a hypoxia sensing fluorescent probe, and has excitation and emission peaks of 488 and 520 nm respectively. The probe is sensitive to varying concentrations of oxygen and can detect as low as 5% O2 concentrations in cells. Using this probe, we measured hypoxia in several cell lines including A549, HeLa and U-2 OS using fluorescence microscopy, high content imaging and fluorescence plate reader. The Image-iT™ Hypoxia Green Probe is multiplex
able with other important physiological parameters like mitochondrial membrane potential, apoptosis and oxidative stress. Image-iT™ Hypoxia Green Probe works well in detecting hypoxia in 3D tumor spheroids. The Image-iT™ Hypoxia Green Probe is formaldehyde-fixable that allows end-point measurement of hypoxia in cells. The Image-iT™ Hypoxia Green Probe facilitates sensitive, robust and reproducible measurements of hypoxia in cells.

**Gene Transcriptional Networks**

**P3055**  
**Board Number: B338**  
**Binding Dynamics of nFGFR1 in Chromatin Architecture and Promoter Machinery.**  
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Fibroblast growth factor receptor 1 in its nuclear form (nFGFR1) binds to genomic sites on every chromosome in both the mouse and human cell models. Upon mouse embryonic stem cell (mESC) neuronal differentiation with Retinoic Acid (RA), there is an increased influx of nFGFR1 into the nucleus, increased nFGFR1 proximal promoter binding, and a shift in GO associated nFGFR1 binding locations from stem cell maintenance associated loci bound in ESCs, to binding at mRNA processing, protein folding, and differentiation associated loci post RA treatment. nFGFR1 has widespread binding across the genome and ChIP-seq data from our lab has shown that several binding sites can be present across entire developmentally important gene clusters, one of which being the Hoxa gene cluster. We hypothesize that the widespread binding of nFGFR1 may reflect its potential involvement in chromatin structure organization. Using the Chromatin Conformation Capture (3C) assay and ChIP-qPCR we are investigating the involvement of nFGFR1 chromatin binding dynamics with how it relates to structural changes in topological domains in the Hoxa gene cluster. The current results indicate that nFGFR1 binding is increased at Hoxa topological structures which are maintained between ESCs and RA induced neuronal cells and reduced at sites where topological domains are lost as a result of RA treatment. Human Neuronal Progenitor Cells (NPCs), like mESCs, result in FGFR1 nuclear influx and neuronal differentiation as result of RA treatment. PD173074 (PD) is a specific FGFR1 kinase inhibitor which is able to inhibit nFGFR1 from binding to and affecting gene transcription. RNA-seq was performed on NPCs treated under control, RA, PD, and RA + PD conditions. Comparative analyses showed treatment versus control NPCs having altered expression in 896 genes. Three of the top dysregulated genes (WNT4, GHR, TUBBIII) were chosen to quantify nFGFR1 promoter binding using ChIP-qPCR. The current data shows increased promoter binding resulting from RA treatment and unexpectedly also an increase to promoter binding following PD treatment. Further work is being completed to continue 3C analyses with nFGFR1 binding in human NPCs and following PD treatment. The data together indicates that FGFR1 binding activity may be involved in regulating chromatin architecture through modulating nFGFR1 levels, and that PD inhibition of FGFR1 results in increased FGFR1 binding, possibly due to upregulation of nFGFR1 activity to offset its activity reduction by PD. Supported by NYSTEM (C026415, C026714), NSF (CBET-1555720, CBET-1706050) and Patrick P. Lee Foundation.
Midbrain Dopamine neurons (mDA) can arise from the floor plate of the midbrain and are responsible for the symptoms of Parkinson’s disease when they cease to function. mDA neurogenesis and maturation are regulated by multiple genes such as Shh, Foxa2, Lmx1a/b, Wnt1, among others. Genes that promote formation of mDA have potential to be used for clinical therapy development in Parkinson’s disease. One gene involved in dopamine neurogenesis is the basic helix-loop-helix transcription factor Nato3. However, its mechanism of action is not well known. We hypothesized that Nato3 had the ability to upregulate the LMX1 markers in vivo and in vitro. Previous unpublished data produced by our lab using qPCR and immunostaining showed that overexpression of Nato3 upregulates LMX1b gene in vivo. These current data show upregulation of the LMX1a gene expression by Nato3 in the immortalized mouse midbrain SN4741 cell line, shown through qPCR data. Nato3 upregulation of Lmx1 genes indicate that Nato3 can influence the expression of genes known to drive dopamine neurogenesis, raising it as a potential target for therapeutic development.

Construction of a complex functional system, such as a living organism, requires not only raw building materials (genes encoding structural and other functional proteins), but also an assembly program, organized into flexible feedback and feed-forward subroutines that can function within, and readily adapt to a nonstable environment. Our studies utilizing pluripotent stem cells and organoids revealed a new pan-ontogenic mechanism, Integrative Nuclear FGFR1 signaling (INFS), which underwrites gene programming during development. Genetic experiments have indicated that the fgfr1 gene sits on top of the gene hierarchy that governs gastrulation, as well as the subsequent development of the major body axes, nervous system, muscles, and bones, by affecting downstream genes that control the cell cycle, pluripotency and differentiation, as well as microRNAs. The regulatory control exerted by INFS is due to a single protein, the nuclear isoform of FGFR1 (nFGFR1), which integrates signals from development-initiating factors and operates at the interface of genomic and epigenomic information. nFGFR1 cooperates with a panoply of transcription factors, and targets thousands of genes encoding mRNAs, as well as miRNAs in critical ontogenic networks. nFGFR1 binds to promoters of ancient proto-oncogenes and tumor suppressor genes which serve as switches in cell proliferation, binds and regulates the pluripotency core genes as well as metazoan morphogens that delineate body axes, construct the nervous system and the mesodermal and endodermal tissues. The panontogenic gene programming by INFS feed-forward and feedback loops expands our understanding of ontogeny, the roots of cancer, schizophrenia and other developmental diseases, and holds new promise for reconstructive medicine and cancer therapy. This work was supported by grants from New York State Department of Health (NYSTEM C026415, C026714), National Science Foundation (CBET-1555720) and Patrick P. Lee Foundation.
P3058
Board Number: B341
Dissecting the cell-type specific regulatory landscape of a Nuclear Hormone Receptor in C. elegans.
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Metazoan Transcriptional Regulatory Factors (TFs) bind to genomic response elements, controlling gene expression precisely to establish and maintain tissue-specific cellular identity. Importantly, TFs also display remarkable plasticity, governing distinct gene networks in different tissues and developmental stages. For example, the conserved C. elegans TF, NHR-25, regulates two different developmental programs in larvae: vulva patterning and molting. To characterize NHR-25 activity and its role in defining these distinct cell types, we used CRISPR/Cas9 to introduce GFP, 3xFLAG, and an auxin inducible tag (AID) into the endogenous NHR-25 gene. The multiple tags simultaneously enabled live imaging of NHR-25 in L1 larvae, ensuring NHR-25 expression in both the developing vulva and seam cells, and ChIP-seq. We found that 75% of our NHR-25 enrichment sites overlapped with those in the modENCODE ChIP-seq NHR-25 dataset at a similar developmental time. However, we identified about one-third fewer enrichment peaks for NHR-25 than modENCODE, suggesting that even modest overexpression of TFs results in substantial promiscuous binding. Sequence analysis of the enriched regions revealed an NHR-25 consensus motif highly similar to its mammalian orthologs as well as an additional consensus motif for another C. elegans’ TF, EOR-1. Interestingly, RNA-seq analysis of worms where NHR-25 was transiently depleted for four hours through addition of auxin, revealed that genes directly adjacent to an NHR-25 enriched site were not necessarily responsive to loss of NHR-25. Thus, NHR-25 may act at a distance. To definitively correlate NHR-25 binding to its target gene, we have deleted NHR-25 occupied regions utilizing CRISPR/Cas9. Phenotypic and transcriptional characterization of these mutants leads us poised to define functional NHR-25 response elements responsible for establishing cell-type specific gene regulatory networks.

P3059
Board Number: B342
Repression of CDON expression by Nuclear Factor One.
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Nuclear Factor One (NFI) family of transcription factors regulate gene expression by associating with and maintaining open chromatin sites during embryonic development as well as in certain types of cancer. NFI family comprises four members: NFIA, NFIB, NFIC, and NFIX. NFIB, can both act as and oncogene and tumor suppressor, depending on the context. For example, in glioblastoma, low NFIB expression is associated with classical and mesenchymal subtypes, where if expressed NFIB induces genes associated with differentiation; while in neural and proneural subtypes, it is upregulated and promotes proliferation and invasiveness. In the developing mouse brain, cell adhesion associated, oncogene regulated (CDON) is upregulated when NFIIs are silenced. Interestingly, analysis of data from The Cancer Genome Atlas (TCGA) found that expression of CDON is negatively correlated with NFIB expression in these glioblastoma subtypes. CDON, an Ig superfamily glycoprotein, takes part in a signaling complex to
promote differentiation of neural and muscle stem cells. Bioinformatic analysis of the 5 kb of sequence upstream of the human CDON gene transcription start site identified five NFI binding sites which cluster in the first 1 kb region, or in the 3.5 kb upstream region. Electrophoretic mobility shift and supershift assays showed that NFIIB binds to all five sites. Furthermore, using chromatin immunoprecipitation, we found that NFIIB occupies two sites in the first 1 kb region in vivo. In luciferase reporter assays carried out in HEK293T cells, overexpression of all NFI members represses the CDON promoter by up to 85% and this repression can be reversed by mutation of one NFI motif. As CDON’s regulatory role in development is also emulated in cancer where it has a pro-apoptotic effect in neuroblastoma cells, we are now investigating if NFIIB indeed regulates CDON expression in brain cancer cell lines. We gratefully acknowledge funding from TUBITAK (KBAG 212T017 and 115Z524) and Istanbul Technical University Research Fund.

P3060
Board Number: B343
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Introduction: Atherosclerosis is characterized by an accumulation of cholesterol-loaded macrophages in the vascular wall. Several factors regulate macrophage cholesterol content. Lipoprotein lipase (LPL) hydrolyzes triglycerides from circulating lipoproteins thereby promoting cellular lipid uptake. ATP-Binding Cassette A1 (ABCA1) facilitates cholesterol efflux from cells toward high-density lipoprotein (HDL) formation. Peroxisome Proliferator-Activated Receptors (PPAR) are nuclear receptor transcription factors which bind to PPAR response elements (PPRE) on target genes to drive their expression. LPL contains a PPRE in its promoter region, indicating that transcription of LPL is PPAR-γ-dependent. ABCA1 is also regulated by PPAR-γ, by way of another nuclear receptor transcription factor, Liver X Receptor alpha (LXR-α). In previous studies, we demonstrated an upregulation of ABCA1 in LPL-knock-down (KD) THP-1-derived macrophages. Hypothesis and Objective: We hypothesized that fatty acid products of LPL’s activity may act as agonists/antagonists for PPARs, thus the objective of this study was to determine if the upregulation of ABCA1 by LPL-silencing may be via a PPAR-dependent pathway. Methods: Our objective was to examine the relationship between LPL and PPAR-γ in differentiated THP-1 macrophages. Short hairpin RNA was used to specifically silence LPL or PPAR-γ gene, and lentivirus was used to over-express PPAR-γ in THP-1 macrophages. We performed end-point reverse transcriptase polymerase chain reaction (RT-PCR) to compare gene-specific transcript levels in wild-type (WT) cells and either LPL-KD or PPAR-γ-KD macrophages. RT-PCR was performed on PPAR-γ-expressing cells to observe differences in LPL expression. A PPAR-luciferase reporter gene assay was performed to investigate the PPAR-activating potential of lipid extracts or lysates prepared from WT or LPL-KD macrophages. Results: RT-PCR showed that the level of PPAR-γ transcripts in LPL-KD cells was 93% of that in WT cells. Specific repression of PPAR-γ resulted in down-regulation of LPL to 27% of WT levels, while over-expression of PPAR-γ upregulated transcription of LPL two-fold. Luciferase assay data showed that lipid extracts from LPL-KD macrophages exhibited more PPAR activation than WT lipid extracts, while lysates from WT macrophages induced greater PPAR activation than LPL-KD lysates. Conclusion: Our data show that PPAR-γ expression levels directly affect LPL transcription. It appears that while LPL expression levels may not influence PPAR-γ transcription, lipolysis products of LPL may repress the transcription of PPAR-γ-responsive genes. Thus, LPL silencing may upregulate ABCA1 transcription because of the absence of lipolysis products capable of inhibiting PPAR-γ activation.
P3061
Board Number: B344
The lower expression of ABCA1 in LPL-expressing THP-1 macrophages may be mediated by fatty acid products of lipolysis.
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Introduction: Atherosclerosis is characterized by the thickening and hardening of vascular walls, a process initiated by infiltration of cholesterol-loaded macrophages. Cellular cholesterol accumulation is the net result of receptor-mediated uptake and transporter-mediated efflux. Scavenger receptors (CD36, SR-AI, SR-BI) mediate cellular cholesterol uptake whereas the ATP-binding cassette transporter A1 (ABCA1) mediates the unidirectional efflux of excess cholesterol. Lipoprotein lipase (LPL) is secreted by macrophages and catalyzes the hydrolysis of circulating triglycerides, facilitating cellular lipid uptake. We recently demonstrated that the expression of ABCA1 as well as ABCA1-mediated cholesterol efflux is upregulated in LPL-knock-down (LPL_KD) THP-1 cells

Hypothesis and Objectives: We hypothesized that in addition to regulating cholesterol efflux, LPL may also regulate the expression of macrophage scavenger receptors responsible for cellular cholesterol uptake. Further, we hypothesized that regulatory effects of LPL may be mediated by fatty acid products of LPL’s lipolytic activity. Thus, the objective of this study was (i) to determine if LPL expression levels correlate with the transcription of scavenger receptors and (ii) to directly examine the effect of fatty acids on the expression of ABCA1.

Methods: The LPL gene was silenced in THP-1 macrophages (LPL-KD) by siRNA interference and its expression was compared against wild type (WT) macrophages. Transcript levels of scavenger receptors were compared in WT and LPL-KD macrophages by RT-PCR. The mass of total cholesterol in cell lysates was compared using a cholesterol oxidase-based colorimetric quantification assay. To mimic the presence of lipolysis products, WT cells were incubated in the presence of various saturated and unsaturated fatty acids, followed by measurement of ABCA1-mediated cholesterol efflux.

Results: LPL-KD cells showed an increase in ABCA1 expression (161% of WT). Cholesterol content in LPL-KD cells was 75% compared to WT macrophages. The expression of scavenger receptors SR-BI and CD36 was lower in LPL-KD THP-1 cells compared to WT macrophages (55% and 60%, respectively). Treatment of cells with Oleic acid reduced ABCA1 expression and efflux to 55% of control. Stearic acid and Linoleic acid also significantly reduced ABCA1 transcription compared to a cholesterol loaded control group (67 and 65% respectively).

Conclusion: LPL may function as a signaling molecule that modulates cellular cholesterol content by regulating the expression of scavenger receptors as well as the cholesterol transporter. The presence of fatty acid products of lipolysis may contribute to repress the expression of ABCA1 in WT cells resulting in lower transcript levels compared to LPL-KD cells.

P3062
Board Number: B345
Regulation of anterior lineage genes in C. elegans embryogenesis.
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Embryonic patterning requires many signaling pathways to control cell fates. Anterior-posterior axis patterning depends on the Wnt pathway, which acts through the transcription factor TCF and its
coactivator β-catenin. Classic Wnt targets are activated when Wnt signaling is on by the TCF:β-catenin complex binding to cis regulatory regions and are repressed when Wnt signaling is off by TCF binding without β-catenin. In contrast, emerging evidence suggests the existence of direct “opposite” targets, activated by TCF without β-catenin and repressed by TCF:β-catenin. In the C. elegans embryo, almost all cell divisions, including anterior-posterior divisions, are patterned by the Wnt/β-catenin asymmetry pathway, in which nuclear POP-1/TCF is high and SYS-1/β-catenin is low in the unsignaled anterior sister cell and the reverse is true in the signaled posterior sister cell. Although many genes expressed in posterior sister lineages are classic Wnt targets, the regulation of anterior lineage genes is not well understood. These genes may be direct opposite Wnt targets. I am working to determine which anterior genes are opposite targets and how they are regulated. I am testing if POP-1 and SYS-1 are necessary for regulation of anterior genes and identifying sites bound by POP-1 in their cis regulatory regions. I am identifying anterior gene enhancers, and determining if their POP-1 bound sites are necessary for anterior expression. I will also determine if POP-1 binds to opposite Wnt target sites directly, as in mice and Drosophila, or indirectly by interacting with a binding partner, as for a target in C. elegans. If the Wnt pathway does not directly regulate anterior genes, I will determine what other mechanisms regulate their expression. Regardless of the mode of regulation, this work will clarify mechanisms regulating expression of anterior genes in the C. elegans embryo, and may have implications for Wnt regulation of genes expressed in unsignaled cells in other species. So far, I have identified four anterior lineage genes that depend on POP-1 and/or SYS-1 for their wild type expression patterns.

P3063
Board Number: B346
Age-dependent regulation of the FOXO transcription factor DAF-16 by SMK-1 in the roundworm Caenorhabditis elegans.
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In the roundworm Caenorhabditis elegans the insulin/IGF-1(IIS) DAF-2 signaling pathway confers stress-resistance and determines lifespan by modulating the activity of DAF-16, an evolutionarily conserved Forkhead box (FOXO) transcription factor. Transcriptional targets of DAF-16 encode proteins that primarily function in immunity, detoxification, and DNA repair. In the presence of ligands of the DAF-2 insulin receptor, an inhibitory phosphate group added by the AKT-1 kinase prevents DAF-16 from translocating to the nucleus. However, in younger animals DAF-16 is activated in response to acute environmental stresses such as heat shock and ultraviolet irradiation. Several studies suggest that through an association with one or more cofactors in the nucleus, the transcriptional output of DAF-16 is tailored to a particular stressful stimulus. Through functional analysis of DAF-16 in reproductively mature and post-reproductive animals, we found that aging alone triggers DAF-16 transcriptional activity. In non-stressed, well-fed, wild type adults, the expression of many DAF-16 targets increases in an age-dependent manner. Accordingly, DAF-16 plays a role in the innate immunity of adult but not larval stage worms. To understand the mechanistic basis of the dynamic activity of DAF-16 during aging, we took a candidate gene approach to ask about the role of specific nuclear proteins and found that SMK-1 is necessary for the age-dependent increase in DAF-16 function. SMK-1 is the C. elegans orthologue of mammalian SMEK1 (Suppressor of MEK1) and is a regulatory subunit of the Protein Phosphatase 4 (PP4) complex. Functional analysis of genes encoding other putative subunits of this complex in C. elegans, including PPFR-1, suggest a role for PP4 in modulating the function of DAF-16 during adulthood. Phylogenetic analysis suggests that PP4 is closely related to the PP2 and PP6 phosphatases, and some subunits appear to associate with more than one of these phosphatase
complexes. Using an RNAi-based reverse genetic approach to probe the role of individual complex members, we are investigating the possibility that all three phosphatases may coregulate DAF-16 later in life, and our preliminary data suggest that this is the case. We anticipate that our studies will have implications regarding regulatory modules that govern gene expression during aging in evolutionarily diverse species, including humans.

**P3064**
**Board Number: B347**
cAMP Reduces Non-Specific DNA-Protein Interactions During Transcription Regulation in Mycobacterium Tuberculosis.
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The cAMP Receptor Protein in Mycobacterium tuberculosis (CRPMTB) is a transcription factor responsible for regulating genes involved in virulence, carbon metabolism and energy conservation. CRPMTB is a homodimer capable of binding to DNA promoter sequences and up to two cAMP molecules. Here, we investigate the linkage mechanisms between cAMP binding cooperativity and DNA interactions. By using isothermal titration calorimetry and fluorescence spectroscopy, we find that negative cooperativity exists between the binding of the first and the second cAMP molecule to CRPMTB. Interestingly, protein-DNA interactions monitored by fluorescence anisotropy show that the affinity between CRPMTB and its promoter sequence is similar in the presence and in the absence of cAMP, indicating that the cyclic nucleotide does not regulate transcription at the level of binding affinities to specific DNA promoter sequences. Instead, we find that cAMP stabilizes a one-to-one stoichiometric CRPMTB-DNA complex, prevents the formation of high-order CRPMTB-DNA oligomers, and reduces the affinity of CRPMTB for non-specific DNA sequences. Moreover, DNA binding assays taken as function of cAMP concentration show that only one cAMP molecule bound per homodimer is required to fully reverse and dissociate high-order CRPMTB-DNA oligomers into stable one-to-one CRPMTB-DNA complexes. In combination, these results provide an archetype of cAMP-mediated regulation that is significantly different from those described previously for other structurally similar CRPs, like the Escherichia coli homolog.

**P3065**
**Board Number: B348**
Glandular cell-specific DNA demethylation in a carnivorous plant Drosera adelae.
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Using carnivorous plants, we are studying the relationship between the environmental responses of plants and gene evolution (Nishimura et al., Planta 2013, 2014; Arai et al., Biochem. Biophys. Res. Commun. 2015). Carnivorous plants have adapted themselves to the nutrient-deficient habitats. They can obtain nitrogen and phosphate, which are necessary for amino acid and nucleotide syntheses, from insects. We showed that the carnivorous plant Drosera adelae (sundew) contains an abundance of an S-like ribonuclease (RNase) in the digestive liquid (Okabe et al., FEBS Lett. 2005). Besides the S-like RNase, recently, we also identified 8-1, 3-glucanase, a class I chitinase, a thaumatin-like protein, a cysteine protease, a lipid-transfer protein and several proteins belonging to the PR4 and PR17 families in the digestive liquid of D. adelae. Interestingly, they all are self-defense-related proteins. More recently, we clarified that most of the genes encoding these proteins are expressed in a glandular cell-specific expression manner. Here, we report on the mechanism underlying the glandular cell-specific expression of these genes.
genes. The analyses of DNA methylation showed that the promoters of the genes encoding the S-like RNase (da-1), the β-1, 3-glucanase (DaGlu) and the protein belonging to the PR4 (DaPR4-L) are unmethylated only in the glandular cells, while the promoters of the other genes are unmethylated in all organs examined (Nishimura et al., *Planta* 2013, 2014; in preparation). All of these genes are the self-defense-related genes. Furthermore, we revealed that the gene encoding the DNA demethylase DEMETER is also expressed highly in the glandular cells. Many studies have shown that plants, in response to biotic stress, regulate the expression of the self-defense-related genes by DNA methylation. Accordingly, it seems that *D. adelae* makes use of the self-defense-related genes for carnivory by making these genes be constitutively unmethylated in the glandular cells.

**P3066**  
**Board Number: B349**  
**Yeast Hsf1 drives a dynamic gene restructuring program in response to heat shock.**  
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The evolutionarily conserved heat shock response is an essential mechanism that allows eukaryotic organisms to survive thermally stressful conditions. The master regulator of this response is Heat Shock Factor 1 (Hsf1). In the yeast *Saccharomyces cerevisiae*, several domains of Hsf1 are well-characterized. These allow Hsf1 to respond to proteotoxic conditions triggered by thermal stress: two transactivation domains (N-terminal, C-terminal), a DNA binding domain, a trimerization domain, and a regulatory domain (CE2). Recent work from our lab has revealed that robust activation of Hsf1-regulated *Heat Shock Protein* (*HSP*) genes is accompanied by a dynamic restructuring of the chromatin landscape. Using a sensitive version of chromosome conformation capture (3C), we have discovered that intra- as well as inter-chromosome interactions (5′-3′ end gene looping, intragenic ‘crumpling’, and intergenic coalescence) take place amongst *HSP* genes during heat shock. Strikingly, the coalescence that we observe with Hsf1-regulated genes is not seen with other transcriptionally active genes, even those activated by heat shock. Indeed, DNA-bound Hsf1 is absolutely required for *HSP* gene coalescence. We hypothesize that a domain within Hsf1 is responsible for driving *HSP* genes into coalesced foci within the yeast nucleus during heat shock.

In order to test this hypothesis, we are using *S. cerevisiae* strains bearing different Hsf1 domain deletions. Using ChIP, we have obtained preliminary evidence that Hsf1 bearing a deletion of its N-terminal activation (NTA) domain leads to its increased occupancy of *HSP* genes under basal conditions. This enriched binding is further increased during acute heat shock (39°C). An explanation for this is the unmasking of the potent C-terminal activation domain (CTA) in the Hsf1ΔNTA mutant. It will be interesting to determine whether enhanced binding of Hsf1ΔNTA is accompanied by an increase in looping, crumpling and coalescence of Hsf1-target genes. This will be addressed in our future studies. Identification of the domain within Hsf1 responsible for coalescence would give us a tool to understand the mechanism and biological significance of this fascinating phenomenon. Moreover, it will allow the engineering of heterologous transcription factors capable of driving the coalescence of their own regulons.

Support by a National Science Foundation grant (MCB-1518345) awarded to D.S.G. and Ike Muslow predoctoral fellowships awarded to S.C. and A.S.K.
Chromatin and DNA Repair

P3067
Board Number: B350

Chromosome analysis of Glyptotendipes glaucus and Glyptotendipes paripes (Chironomidae, Diptera) from reservoirs of the Kaliningrad city, Russia.
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G.glaucus and G.paripes are species of Chironomidae family, the larvae of which are sensitive indicators of water contamination by toxic substances. The polytene chromosomes in salivary glands of larvae allow to observe the level of chromosomal alterations in their populations and make conclusions about the ecological conditions of water reservoir. Widespread species G.glaucus and G.paripes have high level of chromosomal polymorphism that allows use them as biological indicators of a reservoirs condition. Here we investigate karyotypes of G.glaucus and G.paripes populations living in the same reservoirs of Kaliningrad and analyze key indicators of their chromosomal polymorphism. The materials for these studies were samples of 4-th instar larvae collected in 2008-2010 during the winter and summer periods from three reservoirs in Kaliningrad: Shkolnoe lake, Isakovskiy (Chistiy) pond and Karasevka ponds system. Collected larvae were fixed in 96% ethanol and concentrated acetic acid (3:1). The polytene chromosome preparations have been made by a standard aceto-orcein technique. From 156 totally studied samples of G.glaucus only 16 (10,25%) samples had a standard karyotype owing to prevalence of the inverted sequence of gla B2 in population. In G.paripes populations the standard karyotype had only 25% of individuals: chromosomal arms A and B were the most polymorphic, frequency of inversions in these arms relating to standard arms averaged 56.5% and 66.7%, respectively. These data correspond to the findings of other authors about G.paripes populations of the Saratov region where heterozygous inversions in A and B shoulders also most often met. The number of heterozygous inversions on individuals in population of G.glaucus varies in low limits from 0.91 to 1.0, average 0.95. This value doesn't exceed the level of natural polymorphism in the previously studied populations of G.glaucus in Saratov region where the number of heterozygous inversions on an individual had average 0.97. In G.paripes population this indicator varies from 0.77 to 1.0 and average 0.85, that exceed the data of populations of this species in Saratov region (0.667). This indicator also higher, than in European G.paripes populations (Bulgarian and Hungarian). In the G. paripes population studied, genomic polymorphism (the presence of B-chromosome in a karyotype), which is characteristic of a number of other populations of species, was also found. In Kaliningrad region G.glaucus and G.paripes are mass and can be recommended as convenient indicators of reservoirs condition from the point of view of the karyotype indicators.

This work was supported by Contract №14.575.21.0074, unique identifier of applied scientific research and experimental development RFMEFI57514X0074.
P3068

Board Number: B351

Analysis of irradiation resistance mechanism in S. cerevisiae.

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Epigenetic control is critical for regulation of gene expression. Our purpose is that reveal how the epigenetic alteration effect on organisms. There is silencing mechanism as one of epigenetic control. In Saccharomyces cerevisiae, Sir complex consisting of Sir2, Sir3 and Sir4 is a key regulator of silencing mechanism. Sir2 is a histone deacetyltransferase that deacetylates histone H4 lysine 16. Deacetylation of histones promotes chromatin compaction, leading to gene repression. Sir2 is need for telomere, HM and rDNA silencing. Especially, rDNA silencing requires Sir2 but not Sir3 or Sir4. Irradiation causes dramatic changes of epigenetic profile in organisms. We determined whether Sir complex genes play critical role in epigenetic changes by measuring survival rate after irradiation. We found that sir3 or sir4 deletion strains increased radiation resistance. Interestingly, sir4 deletion strain exhibited various survival rates. In order to reveal the resistance mechanism, we performed microarray analysis of sir4 deletion strain and wild type. Compared with wild type and sir4 deleted low survival strain, iron and copper related genes were decreased in sir4 deleted high survival strain. Iron can mediate generation of reactive oxygen species (ROS) in response to irradiation. Therefore, we hypothesized that low iron concentration in cell was a key factor for resistance to irradiation. In addition to iron genes, anaerobic genes were also decreased in sir4 deleted high survival strain. These genes are thought to be inversely correlated with mitochondrial activity. Furthermore, it was reported that telomere dysfunction activate mitochondrial activity and that Sir complex maintain telomere length. Thus we presumed below model. First, sir4 deletion activates mitochondrial activity. Then, cellular iron concentration is decreased. Finally, decreased ROS generation contributes to high survival after irradiation. We would like to report and discuss our data at this meeting.

P3069

Board Number: B352

Determining the role of chromatin context on repair of DNA double-breaks formed by a Cas9-linked camptothecin mimic.

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DNA double-strand breaks (DSBs) can cause genome instability or genetic diversity in cells, depending on their context and the repair outcome. The response to DSBs is called the DNA damage response (DDR), which includes repair by either non-homologous end joining (NHEJ) or homologous recombination (HR). Previous research indicates that chromatin is a major contributor in the DDR signaling pathways which can include cell death, cell cycle arrest, or activation of one of the two DSB repair processes. The long-term goal of this project is to understand the role of chromatin landscape in DSB vulnerability and repair outcome in Saccharomyces cerevisiae. To begin to examine this, the formation and repair frequency of a DSB in a genome region with an active chromatin signature and one with a repressed signature will be compared. In order to induce DNA DSBs at specific regions, we use inducible CRISPR-Cas9 systems under a galactose promoter to target DSBs to active and inactive chromatin regions. We have found in pilot studies that the break frequency can be accurately quantified using the reduction in the real-time PCR signal across the targeted region upon breakage. Using this method, we will test the hypothesis that
active chromatin regions are more vulnerable to DSBs, however they are repaired more rapidly by the NHEJ pathway.

The CRISPR-Cas9 systems that will be used include wild-type Cas9 nuclease but also a novel system composed of a topoisomerase I mutant (Top1-T722A) fused to nuclease-dead Cas9 enzyme. This system will induce DSBs that have a protein-bound DNA end, which requires further processing, and mimics the mechanism of topoisomerase poisons that are used for cancer therapy like camptothecin. These results help us understand the mechanism by which DSBs are processed and repaired, which has implications for understanding both how cells maintain their genome and how it can be manipulated for driving genetic change. Also, these differences can provide insight on how the chromatin environment may restrict or enhance the cytotoxic effects of chemotherapy drugs.

**P3070**

**Board Number: B353**

The Optimization of CRISPR Systems for Double Strand Break Induction in *S. cerevisiae*.

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Double strand breaks (DSBs) are a form of DNA damage that can result from an array of chemicals as well as ionizing radiation. Unresolved DSBs are detrimental to cell health, necessitating efficient repair; however, DSBs can result in mutations that knock out gene function. While DSB repair mechanisms are heavily studied, the relationship of repair kinetics to chromatin environment is weakly understood. To study repair kinetics in the context of chromatin modifications, DSBs can be induced in a controlled manner and their repair can subsequently be studied; however, we must first optimize the induction of DSBs before beginning to study repair.

CRISPR-Cas9 is a robust new system for DSB induction (and genome editing) that can be directed to almost any site in a genome simply by changing its guide RNA sequence. While Cas9 is a useful tool for DSB induction, modifications of this system have also been engineered to increase specificity. fCas9 is comprised of catalytically inactive Cas9 fused to the FokI endonuclease, which must dimerize to induce a DSB. This increases specificity by doubling the required guide RNA hybridization. Cas9 nickases also double the required gRNA binding by only inducing single stranded “nicks.” The goal of our study is to compare the cutting efficiency and specificity of fCas9, dual Cas9 nickase complexes, wild-type Cas9, I-SceI, and the HO endonuclease in the yeast genome. The hypothesis is that the Cas9 enzyme will have the highest frequency of cutting at the target site, but with the lowest specificity, while Cas9 nickase and FokI-dCas9 will have lower cutting efficiencies, but higher specificity. In order to test this hypothesis, qPCR will be performed with amplification across break sites, causing breaks to knock down initial template quantities. Preliminary testing of the HO and I-SceI assays have yielded calculated cutting frequencies of 25 and 90 percent, respectively. While further optimization is required, these results demonstrate that qPCR assays are an effective approach to a measurement of DSB induction.
P3071
Board Number: B354
Characterizing the interaction between Rad54 and PCNA.
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Rad54 is a eukaryotic protein known to be involved in homologous recombination (HR), a process used by cells to repair DNA double-strand breaks (DSB). Malfunctions in HR drive human diseases such as breast and ovarian cancer, and Fanconi anemia. The goal of this study is to investigate the function of the interaction between two proteins that are critical in HR, Rad54 and the replication clamp PCNA (proliferating cell nuclear antigen). Using a candidate approach we identified two N-terminal point mutations, K102A and R103A, in Saccharomyces cerevisiae that we believed would exclusively disable the Rad54-PCNA interaction. It was important that this mutation, rad54-KR/AA, only affected the interaction with PCNA so we could study it in isolation. Microscale thermophoresis assays revealed that rad54-KR/AA creates a fivefold decrease in interaction between Rad54 and PCNA. To verify that rad54-KR/AA is a separation-of-function mutation, we tested its ability to form displacement loops (D-loops). Rad54 normally aids in the formation of D-loops to facilitate strand invasion during HR; if D-loops cannot form, HR cannot happen. Results showed that rad54-KR/AA can in fact form D-loops in levels comparable to wild type. We then examined the rad54-KR/AA phenotype for defects seen in rad54\textsuperscript{Δ} cells, particularly, sensitivity to DNA damage and genetic interactions. First, we tested if KR/AA interacted with Rad27, a flap endonuclease whose deletion causes synthetic lethality with rad54\textsuperscript{Δ} due to an accumulation of replication intermediates that depend on HR for resolution. Double mutants had a synthetic sick phenotype and is extremely sensitive to DNA damage, indicating a defect in repair. In a follow-up spot assay, we investigated if non-homologous end joining (NHEJ) played a role in the synthetic interaction between rad54-KR/AA and rad27\textsuperscript{Δ}, by testing the effect of mutating a gene required for the initiation of the NHEJ, Ku70. Interestingly, the triple mutant with ku70\textsuperscript{Δ} did not exhibit a synthetic sick phenotype but instead grew nearly as well as wild type. The growth rescue in its absence could indicate that either NHEJ processes are creating toxic intermediates, or the Ku70 protein itself is inhibiting repair of replication damage in the KR/AA mutant. Future experiments will examine other NHEJ proteins, such as Lif1 to determine which is the correct hypothesis. We will also use a primer extension assay to gauge the cells ability to perform repair synthesis during recombination events, and a two hybrid assay to see if rad54-KR/AA can interact with other HR proteins like Rad51. Understanding the mechanisms of HR is essential for advancing research in other fields that pertain directly to human health, including genomic stability, hereditary diseases, and cancer.

P3072
Board Number: B355
Signaling via DNA breaks and RUVB proteins activates the germline genome.
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In \textit{C. elegans}, the primordial germ cells Z2 and Z3 are born during early embryogenesis and then held in a transcriptionally quiescent state until hatched larvae begin to feed. During this period of transcriptional quiescence Z2/Z3 are blocked at G2 of the cell cycle. Upon feeding, RNAPII is abruptly and globally activated and the ensuing gene expression breaks the cell cycle arrest and drives Z2/Z3
division. A previously documented (1) yet unexplained feature of germline genome activation in the worm is the appearance of numerous DNA breaks coincident with RNAPII activation. Here, we describe a novel pathway whereby nutrients trigger topoisomerase II-dependent DNA breaks that, in turn, recruit RUVB proteins to decompact the chromatin. Chromatin decompaction then allows for global activation of RNAPII and ensuing gene expression. Our results suggest that DNA break-induced chromatin decompaction may be a general mechanism for the rapid induction of signal-mediated gene expression. Our results also provide just the third-ever example of programmed DNA breaks in eukaryotic biology, after meiotic and V(DJ) recombination.


P3073
Board Number: B356
The co-repressor complex mSin3A/HDAC1 is involved in the down-regulation of CRTC2 target genes during B cell differentiation.
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Introduction: Germinal center is the principal source of memory B cells and plasmatic cells. During class switch recombination (CSR), the cytidine deaminase induced by activation (AID) produces double-strand breaks in the DNA that requires to be repaired by the DNA damage response (DDR). This response is attenuated in B cells allowing to the cells of the germinal center replicate without entering to apoptosis or senescence. Recently, it was shown that B cell differentiation is associated with genotoxic stress produced by DNA damage. During this process, the co-transcriptional activator CRTC2 is phosphorylated, inactivated and translocated to the cytoplasm, which results in the down-regulation of several genes. It was shown that CRTC2 is part of an alternative DDR that couples genotoxic stress and cellular differentiation, however, it’s not the well understood the molecular mechanism of this process. Results obtained by our laboratory points to the co-repressor complex mSin3A/HDAC1 as a potential complex involved in the down-regulation of CRTC2 target genes. Therefore, our main goal was to discover if this complex is involved in the transcriptional regulation of CRTC2 target genes. Material and Methods: We used Ramos cell line to study B cell differentiation and etoposide as a double-strand breaks inductor. Through immunoprecipitation, western blot and chromatin immunoprecipitation assays we evaluated the participation of this complex during this process. Results: Our results showed that mSin3A and HDAC1 interacted with CRTC2 in the nucleus and this interaction is decreased under genotoxic stress. Also, we showed an enrichment of mSin3A and HDAC1 in the promotor region of the target genes of CRTC2 during genotoxic stress. The presence of this repressor complex is accompanied by an increase of the epigenetic mark of repression H3K9me3 and a decreased of the epigenetics marks of activation, H3K9Ac, H3K27Ac and H3K4me3 in the promoter regions of the CRTC2 target genes. Discussion: Our results showed that co-repressor mSin3A/HDAC1 complex could be involved in the transcriptional regulation of CRTC2 target genes during B cell differentiation. Regardless this process is necessary to the formation of plasma cells that produce high-affinity antibodies, it is associated with cancer, since most B-cell lymphomas originate from B cell differentiation process, which why is important to study the molecular biology associated with this event.
P3074
Board Number: B357
Histone dynamics during oocyte meiosis in \textit{C. elegans}.
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During meiosis, the process through which gametes are formed, homologous chromosomes must pair, undergo recombination, and segregate from each other. Chromatin dynamics, which have been studied extensively in somatic cells, have been less explored in the context of meiosis. To gain new insights into this process, we are using live imaging in \textit{C. elegans} to examine chromatin dynamics during various stages of meiotic prophase. Unexpectedly, we have found widespread histone H2B exchange in the late stages (diplotene and diakinesis) of oocyte meiotic prophase. We have confirmed this observation in three different ways: 1) by the complete recovery of mCherry::H2B fluorescence after photobleaching in a strain carrying a mCherry::H2B transgene (indicating new unbleached histones are loaded onto chromatin), 2) by the disappearance of photoconverted Dendra2::H2B in a strain overexpressing the Dendra2::H2B transgene in the germline (indicating that converted histones are unloaded, and new unconverted histones are loaded onto chromatin), and finally, 3) by the redistribution of photoconverted H2B::Dendra2 from a sub-region of chromatin to the entire chromatin in a strain where the Dendra2 tag was inserted at an endogenous H2B locus by CRISPR. This suggests that H2B histones are widely unloaded from chromatin at the diplotene and diakinesis stages in meiosis. Furthermore, our data suggests that when the nucleoplasmic pool of the tagged H2B is limited (as in the endogenously tagged strain), the converted histones are reloaded, and when the nucleoplasmic pool is large (as in the over-expressing transgenic strain), they are degraded. This widespread exchange is specific to meiosis, as we have not observed this phenomenon in terminally differentiated somatic cells in the timeframe assayed. The histone exchange at this stage of meiosis is surprising, as no replication is occurring and transcription is thought to be shut down by diakinesis. The mechanism and role of histone exchange at this stage are unknown, but possible roles include enabling changes in chromosome configuration important for the meiotic divisions, and/or resetting of chromatin marks. In future work, we will screen candidate histone chaperones and chromatin remodelers to find factors involved in this meiotic histone exchange. We will also examine the consequence of blocking histone exchange, which may lead to defects in meiosis or embryo development.

P3075
Board Number: B358
Oligo-conjugated antibodies and massively parallel single-cell sequencing reveal the high-parameter correlation of protein and mRNA expression in individual immune cells.
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Recent developments in high-throughput single-cell mRNA sequencing have enhanced our knowledge of the cell populations found in individual tissues or disease states. However, prior studies have shown a low correlation between mRNA expression levels and protein expression levels, suggesting that the addition of high-parameter single-cell protein expression data would significantly improve our understanding of cellular functions and states. Traditional single-cell protein assays (flow cytometry and microscopy) are limited in the number of parameters that can be achieved within one experiment, and can be difficult to combine with mRNA expression analysis in a single workflow.
Here we use oligo-conjugated antibodies (BD Ab-seq) in combination with massively parallel single cell mRNA sequencing on the BD Rhapsody™ platform to simultaneously measure the protein and mRNA content of individual human peripheral blood mononuclear cells (PBMCs). To accomplish this, a 30+ parameter oligo-conjugated antibody panel against immune relevant cell-surface markers was created and paired with the BD Rhapsody Immune Response Panel (a targeted gene expression panel consisting of 399 targets). The combined sequencing data and subsequent analysis generated from this single workflow experiment provides a measurement of both gene expression and protein expression. These digital measurements have significantly reduced PCR bias through the use of the unique molecular indices (UMIs). Our results showed that protein molecule expression detected with BD Ab-seq was highly sensitive and specific. Protein expression patterns correlate well with results from flow cytometry data on the same samples. BD Ab-seq allowed the robust detection of protein markers like CD4 and CD8, even when their cognate mRNA transcripts were found in low abundance. Certain markers cannot be easily distinguished at the mRNA level, like T-cell memory marker isoforms CD45RA and CD45RO, but are easily distinguished at the protein level. Finally, we are able to correlate the mRNA and protein expression on 30+ genes in thousands of individual cells. Our study shows the successful combination of high-parameter protein and mRNA expression data within a single workflow, and should enable the further elucidation single cells within different tissues, developmental time points, and disease states.

P3076
Board Number: B359
The Role of Mps3 and Htz1 in Telomere Cohesion and Telomere Position Effect.
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The yeast nuclear envelope protein Mps3 functions in several components of chromosome metabolism such as sister chromatid cohesion, telomere clustering, and DNA damage repair. Mps3 also physically and functionally interacts with the histone variant Htz1 in DNA damage repair. Htz1 is found near the telomeres and is involved in preventing DNA from forming silent heterochromatin as a way to regulate transcription and provide chromosome stability. Because Mps3 functions in cohesion and has been also shown to have a defect in cohesion at the telomeres, our lab is interested in determining if Htz1 also functions in cohesion near the telomeres. In addition because both Mps3 and Htz1 function near/at telomeres, our lab is interested in determining if either function in telomere position effect (TPE). TPE is a phenomenon where genes located next to telomeres are transcriptionally repressed. It has been extensively studied in Saccharomyces cerevisiae and recent studies in mammalian cells show TPE is implicated in several human syndromes where genes are relocated near the telomeres and then lose their expression. To better understand TPE our lab used a yeast strain that contains URA3 adjacent to the VII-L telomere. Mutations of Mps3 or a deletion of Htz1 in this strain was then observed for defects in TPE.
RNA Localization and Transport

P3077
Board Number: B360
Muscleblind-like RNA binding proteins form RNA transport granules associated with Kif1b.
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Myotonic Dystrophy, Type 1 (DM1) is an autosomal dominant disease that affects multiple tissues of the body, including muscle and neurons. DM1 is caused by expanded CTG repeats in the 3' UTR of DMPK, which form toxic intranuclear RNA foci that sequester Muscleblind-like RNA binding proteins (MBNL). While regulation of alternative splicing by MBNL proteins in the context of muscle development and DM1 have been extensively studied, MBNL proteins also regulate mRNA localization in myoblasts and young neurons by binding to cis-elements in 3' UTRs. However, mechanisms of MBNL-mediated RNA trafficking is not well understood. We have investigated the role of cytoplasmic isoforms of MBNL in mRNA localization in differentiated cortical neurons in culture. MBNL proteins were localized in a punctate distribution in axons and dendrites. Live cell imaging of GFP tagged cytoplasmic MBNL isoforms revealed particles that exhibited bidirectional transport. A split kinesin assay revealed selective interactions of MBNL proteins with the tail domain of Kif1bα that was required for transport. MBNL did not interact with the splice variant, Kif1bβ, or other kinesins. Interaction of Kif1bα with RNA transport granules has not been previously reported. Using a biochemical approach, overexpression of Kif1bα but not Kif1bβ increased the association of cytoplasmic MBNL with microtubules. Experiments in progress are to identify MBNL target mRNAs that are transported by Kif1bα. These results suggest a new function for Kif1bα-MBNL interactions in mRNA localization that may be impaired in DM. Cytoplasmic MBNL isoforms may play a key role in coupling cytoskeleton-dependent mRNA transport that is altered in DM.

P3078
Board Number: B361
Localizing mRNAs in skeletal muscle cells.
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Skeletal muscle is formed by multinucleated myofibers, the biggest cells in the human body. The multiple nuclei in these cells are regularly positioned so that the distance between them is maximized. We previously found nuclear positioning to be important for skeletal muscle function. We hypothesize that each nucleus influences the nearby cytoplasm by differential mRNA transport along myofibers. Using highly matured mouse myofibers differentiated in vitro, we found that overall mRNA distribution depends on nuclear position. smFISH demonstrated mRNA clustering around the nucleus along with myofiber maturation in a dynein dependent manner. We have also confirmed that the levels of protein translation can depend on nuclear location. Interestingly, a peculiar subset of mRNAs localizes regardless of where the nucleus is placed. We are now dissecting the intrinsic properties of these mRNAs that determine their distribution in order to question the reason for this distinct localization pattern. Understanding the mechanisms of mRNA transport and anchoring that govern its subcellular destinations in myofibers may be the key to understand how nuclear positioning impacts muscle activity.
P3079  
**Board Number: B362**  
Certain types of inverted repeat sequences can organize local chromatin infrastructure in *Saccharomyces cerevisiae*.  
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DNA segments that read the same from 5' to 3' in either strand are called inverted repeat sequences or simply IRs. Although IRs are widely found in both prokaryotic and eukaryotic genomes, their *in vivo* functions, if any, are still enigma. We developed a computer program that can identify the IRs with cruciform-forming potential in a given genome. Using the *S. cerevisiae* genome and this computer program, we performed genome-wide analyses for the distribution, occurrence frequency, position, sequence characteristics and relevance to chromatin structure for the IRs. We clarified the following points. Firstly, nucleosome-excluding IRs are rare in open reading frames. Secondly, the ApT- or TpA-rich IRs function in the 3'-UTRs, and the A-tract (or T-tract)-rich IRs function in the core promoter regions as the nucleosome depletion centers. The mechanism underlying the nucleosome depletion, however, seems to be different between the two types of IRs. Thirdly, there is a strong structural correlation between the IRs and the poly(A) signals in the mRNA precursor. We will discuss possible roles of the IRs in transcription and the formation of mRNA 3'-end in the yeast.

P3080  
**Board Number: B363**  
Spatial and temporal control of the *Neurospora crassa* molecular clock.  
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*Neurospora crassa* has long been an important model for circadian biology. However, the spatio-temporal dynamics of the *N. crassa* clock components at the single cell level have remained elusive. The core oscillator in the Neurospora clock is a negative feedback loop in which WC-1 and WC-2 acting together as a transcription factor drive expression of frequency (frq) whose product, the Intrinsically Disordered Protein FRQ, dimerizes, complexes with the DEAD-Box Helicase FRH and with CK1, and depresses the activity of WC-1/WC-2. We set out to investigate whether frq mRNA is non-randomly positioned in the cytoplasm. We hypothesized that frq mRNA translation may be spatially controlled through the association with an RNA-binding protein. To examine if frq transcript is spatially patterned, we employed single molecule RNA FISH coupled with quantitative image processing. We also used immunofluorescence to understand the relationship between FRQ protein and mRNA localization. The image analysis suggested that there is non-random clustering of frq, indicating that there is spatial regulation of mRNA position. We are currently investigating the role of known frq binding proteins in this phenomenon to better understand the spatio-temporal dynamics of FRQ throughout the circadian day.
P3081
Board Number: B364
Transcription factor-mediated targeting of genes to the nuclear pore complex is the major pathway controlling peripheral localization of genes in budding yeast.
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Hundreds of genes physically interact with the nuclear pore complex (NPC) and many inducible genes reposition to the nuclear periphery when they are activated. In budding yeast, interaction with the NPC is controlled by cis-acting targeting elements (or “DNA zip codes”) and requires both NPC proteins and transcription factors (TFs). Here, we describe the mechanistic dissection of the function of TFs and transcriptional regulators/mRNA export factors in mediating targeting to the nuclear periphery. Further, we have performed a global screen of all ~200 yeast transcription factors to test the generality of this phenomenon. Each TF was tagged with the DNA binding domain from LexA and crossed against a strain having the LexA binding site integrated at a locus that normally localizes to the nucleoplasm. The position of this locus with respect to the nuclear envelope was scored using confocal microscopy. Approximately 50% of the tested transcription factors were sufficient to cause localization to the nuclear periphery. The major pathway by which this targeting was mediated required the NPC; CRISPR-meditated mutations in the NPC protein Nup2 blocked peripheral targeting by >95% of these TFs. This suggests that controlling gene positioning within the nucleus is an important and unappreciated function of TFs and that interaction with the NPC is the major pathway by which genes localize at the nuclear periphery. The TFs identified in the screen regulate over 1000 genes, including housekeeping genes as well environmentally or developmentally regulated genes. The TFs are found in all transcription factor families in budding yeast and a large subset (50%) of the transcription factors identified have human homologs. A human TF was able to mediate targeting to the nuclear periphery in yeast, suggesting that the molecular mechanism of gene recruitment to the NPC has been deeply conserved and likely plays a fundamental role in genome organization.

P3082
Board Number: B365
Asymmetric Distribution of Hexose Transporter mRNA Provides a Growth Advantage.
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Asymmetric localization of mRNA is important for cell fate decisions in eukaryotes and provides the means of localized protein synthesis in a variety of cell types. Here we show that hexose transporter mRNAs are retained in the mother cell until metaphase-anaphase transition (MAT) and then released in the bud in S. cerevisiae. The retained mRNA was translationally inactive but was already ribosome-bound before MAT. Treatment with a translation initiation inhibitor caused degradation of HXT mRNA, while blocking elongation, prevented release of the mRNA into the bud, indicating translational control of mRNA localization. Consistently, unlike in large added cell, in small and medium budded cells, HXT proteins were not found at the plasma membrane in the bud. Importantly, when cells were released from starvation into rich, glucose containing medium, HXT2 mRNA, but none of the other HXT mRNAs tested, was enriched in the bud after MAT. This enrichment was dependent on the cAMP/Ras2/PKA pathway, microtubules and correct nuclear segregation into the bud. Using strains that only expressed one hexose transporter allowed us to demonstrate that Hxt2 only strains grow faster than their counterparts. Therefore, asymmetric distribution of HXT2 mRNA provides a growth advantage for young
daughters who are better prepared for nutritional changes in the environment. Our data provides strong evidence that asymmetric mRNA localization is an important factor in determining cellular fitness and may influence lifespan as well as aging.

**P3083**
**Board Number: B366**

Functional map of the DEAD-box ATPase Dbp5 at single amino acid resolution.
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Dbp5p (DDX19) is an essential DEAD-box protein that interacts with multiple nuclear pore complex (NPC) proteins to drive the directional export of mRNAs through RNA-protein complex remodeling. However, Dbp5p also shuttles between the nuclear and cytoplasmic compartments and has been functionally linked to transcription, export of pre-ribosomal subunits, DNA damage responses, and translation. The mechanism(s) by which Dbp5p contributes to these other cellular processes, or is shuttled through the nucleus, remains largely unknown. Towards understanding the specific molecular interactions mediating these functions of Dbp5p, we have generated a yeast mutant collection containing strains with single amino acid substitutions at residues 2-482 of Dbp5p. Phenotypic screening of this collection to identify separation-of-function mutants has included assays to define sensitivity to transcriptional and translational inhibitors, sensitivity to DNA damage, and defects in mRNA or rRNA export. Through this approach, we have identified key residues mediating functions of Dbp5p outside of mRNA export, thus providing a means to detail the molecular mechanisms by which Dbp5p may be contributing to these processes. The mutant collection was further screened for changes in Dbp5p subcellular localization, which led to the identification of a nuclear export signal (NES) within Dbp5p. Mutation of the NES results in the localization of Dbp5p mainly to the nucleus and is not lethal, suggesting that the normally observed enrichment at NPCs and large cytoplasmic pool of Dbp5p are not required for the essential functions of this protein. Synthetic genetic array (SGA) analysis with a strain carrying the mutated Dbp5p NES identified genes encoding the nuclear exosome components Rrp6p and Lrp1p, providing a link between Dbp5p shuttling and exosome mediated RNA processing and surveillance. We expect that continued characterization of these separation-of-function mutants will provide detailed mechanistic insight into the multiple functions of Dbp5p in gene expression and beyond.

**P3084**
**Board Number: B367**

Schawman-Diamond Syndrome: inside the structure of EFL1, SBDS proteins and their complex.
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Ribosome biogenesis is closely linked to the cell growth and proliferation. Dysregulation of this process causes several diseases collectively known as ribosomopathies. For the final step of the maturation of the ribosome, the nascent 40S and 60S subunits are exported from the nucleus to the cell cytoplasm. To
prevent premature association of these ribosomal subunits, eukaryotic initiation factor 6 (elf6) binds the 60S subunit within the nucleus. Its release in the cytoplasm requires the interaction of EFL1 and SBDS proteins. In Shwachman-Diamond syndrome (SDS), a defective SBDS protein prevents elf6 eviction, inhibiting its recycle to the nucleus and subsequent formation of the active 80S ribosome. We have shown that the interaction of EFL1 with SBDS resulted in a decrease of the Michaelis-Menten constant (KM) for GTP and thus SBDS acts as a GEF for EFL1 [1]. Subsequent studies demonstrated that SBDS debilitates the interaction of EFL1 with GDP without altering that for GTP [2]. The interaction of EFL1 alone or in complex with SBDS to guanine nucleotides is followed by a conformational rearrangement. Understanding the molecular strategy used by SBDS to disrupt the binding of EFL1 for GDP and the associated conformational changes will be key to understand their mode of action and alterations occurring in the disease. In this study, we aim to show, using BIO-SAXS and ab-initio modelling techniques, the conformational changes in EFL1 during its mutation [3] and the results from the interactions between EFL1 and its binding partners, the SBDS protein and the guanine nucleotides.

The authors acknowledge financial support PGR2015-2017 "Con il contributo del Ministero degli Affari Esteri e dalla Cooperazione Internazionale, Direzione Generale per la Promozione del Sistema Paese".


The Nuclear Envelope and Nuclear Pore Complexes 2

P3085

Board Number: B369

The SUMO-Specific Isopeptidase SENP2 is Targeted to Intracellular Membranes via a Predicted N-Terminal Amphipathic α-Helix.

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Sumoylation regulates a wide range of essential cellular functions, many of which are associated with activities in the nucleus. Although there is also emerging evidence for the involvement of SUMO at intracellular membranes, the mechanisms by which sumoylation is regulated at membranes is largely unexplored. In this study, we report that the SUMO-specific isopeptidase, SENP2, uniquely associates with intracellular membranes. Using in vivo analyses and in vitro binding assays, we show that SENP2 is targeted to intracellular membranes via a predicted N-terminal amphipathic α-helix that promotes direct membrane binding. Furthermore, we demonstrate that SENP2 binding to intracellular membranes is regulated by interactions with the nuclear import receptor karyopherin-α (Kap-α). Consistent with membrane association, BioID-mass spectrometry (BioID-MS) revealed interactions between SENP2 and ER, Golgi and inner nuclear membrane-associated proteins. Collectively, our findings indicate that SENP2 binds to intracellular membranes where it interacts with membrane-associated proteins and has the potential to regulate their sumoylation and membrane-associated functions.
P3086
Board Number: B370
The VAP family member Scs2 functions to organize chromatin at the yeast inner nuclear membrane through recruitment of the SUMO E3 ligase Siz2 and telomeres.
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The nuclear envelope (NE) provides an environment for the proper regulation and organization of interacting chromatin. In budding yeast, NE-associated chromatin includes telomeres and silenced subtelomeric regions. Multiple mechanisms are employed to tether these chromatin regions to the NE and silence resident genes. Among the factors required for telomere association with the NE is Siz2, a SUMO E3 ligase (Ferreira, et al., 2011. Nature Cell Biology, 13(7), 867–74). We further investigated the role of Siz2 and sumoylation in telomere association with the NE. We found that Siz2 is predominantly distributed throughout the nucleoplasm during interphase, but is recruited to the NE during the early stages of mitosis, when newly replicated telomeres re-associate with the NE. We show that enrichment of Siz2 at the NE is dependent on its mitotic phosphorylation, a SUMO interacting motif within Siz2, and a nuclear pore complex protein, Nup170, which is known to interact with subtelomeric chromatin (Van De Vosse et al., 2013. Cell, 152(5), 969–983). In all cases, mutant cells which fail to recruit Siz2 to the NE results in telomere tethering defects. We hypothesize that NE recruitment of Siz2, during mitosis, functions to target specific proteins for sumoylation that are required for proper chromatin organization at the NE. Consistent with this model, we show that NE recruitment of Siz2 correlates with the mitotic specific sumoylation of several proteins. This includes Scs2, an integral membrane protein and member of the VAP protein family. Scs2 resides in the endoplasmic reticulum (ER) and the NE where it appears to function in phospholipid metabolism, endoplasmic reticulum/plasma membrane interactions, and telomeric silencing. We show that Scs2 is required for telomere association with the NE and our data suggests that Scs2 functions as a receptor for Siz2 on the inner nuclear membrane. Data on the role of Scs2 in subtelomeric gene silencing and its functional links to nuclear pore complex proteins to will also be discussed. Together these data suggests that Scs2 plays an essential role in the spatial and temporal control of sumoylation at the inner nuclear membrane, an important post-translational modification involved in chromatin organization.

P3087
Board Number: B371
Metazoan Nuclear Pores provide a scaffold for poised genes and stabilized induced Enhancer-Promoter contacts.
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Understanding how patterns of gene expression are established, maintained, and transmitted during cell division is crucial to decipher how cells initiate and preserve their developmental identity. A growing number of components of gene expression machinery are known to be non-randomly arranged in the nucleoplasm but located in distinct structural and functional nuclear compartments and scaffolds. Concomitant with this non-random organization, individual chromosome regions can be repositioned in nuclear space, establishing contacts between distant genomic loci as well as protein components of nuclear scaffolds. One of the most prominent nuclear scaffolds is the Nuclear Pore Complex (NPC) which is embedded in the Nuclear Envelope, and consists of approximately 30 different subunits, called
Nucleoporins (Nups). This large protein complex mediates nucleo-cytoplasmic transport of macromolecules, but it has also been implicated in additional cellular processes such as regulation of gene expression via direct binding to the genome. In yeast, gene recruitment to the NPC has been linked to transcriptional activation and to epigenetic memory of the activated state. Yet in animal cells, it is still unclear what function NPC-genome association brings to gene regulation. In Drosophila melanogaster culture cells and isolated tissues, we have identified genome-wide presence of multiple Nups at regulatory DNA elements, including promoters, enhancers, and insulators. Strikingly, we discovered that the NPC component Nup98 facilitates the looping contacts between promoters and enhancers, revealing Nups as a new class of architectural proteins that organize the 3D architecture of the genome and therefore, influence gene regulation. Consistent with this idea, we characterized the role of different Nups in a new mechanism of developmental transcriptional memory, in which Ecdysone hormone-induced genes are more rapidly or more strongly expressed in cells that have previously experienced the presence of the hormone. Interestingly, loss of Nup98 did not affect transcription during initial induction, but resulted in slow activation during re-induction, demonstrating a complete loss of transcriptional memory. To further characterize this phenomenon, we used single-molecule mRNA quantification to measure the magnitude of fluctuations in the expression of the Ecdysone-induced gene, E74. Together, our findings introduce a previously unrecognized role of metazoan nuclear pores in functioning as scaffolds for topological genome organization and suggest that in animal cells, nuclear pores stabilize enhancer-promoter loops and facilitate transcriptional memory.

P3088
Board Number: B372
Chromatin-bound nuclear pore proteins recruit chromatin remodeling complexes to induce DNA decondensation in Metazoan cells.
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Nuclear pore complexes (NPCs) span the eukaryotic nuclear envelope and regulate nucleo-cytoplasmic traffic of large molecules, RNA and protein. NPCs are comprised of about 30 distinct nuclear pore proteins (nucleoporins, Nups), each present in many copies to form an 8-fold rotationally symmetric pore structure. In recent years, it has become clear that NPCs interact with the genome, and this interaction has a functional impact on gene expression. In yeast, recently activated metabolic genes re-localize to NPCs, and this interaction is required for the phenomenon of transcriptional memory, whereby subsequent gene induction results in dramatically faster and higher levels of gene transcription. Transcriptional memory of inducible genes has also been found in Drosophila and human cells, and in both organisms, NPCs, or separate “off-pore” nucleoporins, respectively, have been found to be critical for the increased second round of transcriptional up-regulation. Genome-wide binding data in Drosophila and human cells show specific nuclear pore proteins at genetic targets throughout the genome, both as components of the peripheral pore complex and as intranuclear off-pore entities. These genomic targets tend to be open chromatin associated with active, developmental or cell cycle genes, suggesting a possible role for nucleoporins in regulating expression of developmental programs. One specific nucleoporin, Nup98, commonly binds enhancers and promoters of target genes, and is required for E-P looping, as well as transcriptional memory, upon target gene transcriptional induction. The mechanism by which Nups are involved in these processes however, and at what stage of gene activation, is not known. To probe this, we have developed a tethering system in Drosophila melanogaster by which to study the function of Nup-genome interactions. Using transgenic Drosophila containing integrated genomic lacO arrays and LacI-Nup fusion proteins, we have determined that
tethering nucleoporins Nup98, Nup62, and Sec13 to ectopic lacO loci is sufficient to induce chromatin decondensation of larval salivary gland polytene chromosomes in a chromatin context dependent manner. Furthermore, by probing the functions of Sec13 specifically, we are able to show that at an endogenously condensed lacO locus, tethering Sec13 is sufficient to recruit a Brahma chromatin remodeling complex, as well as GAGA Factor, another Drosophila protein associated with chromatin decondensation, and subsequently decondense the target chromatin. These experiments show a role for components of NPCs in recruiting chromatin remodelers to target loci to facilitate chromatin decondensation, regulating a crucial step at an early stage in gene activation.

P3089
Board Number: B373
Uncovering a role for nucleoporin Megator in a novel nuclear scaffold structure.
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The Nuclear Pore Complex consists of ~30 Nucleoporin proteins or Nups. Beyond their structural transport role, Nups have recently been shown to play a role in genome organization and transcriptional regulation. These gene regulatory events occur during Drosophila melanogaster development. The details and the mechanisms by which Nups regulate gene expression is not well understood. Our lab has identified a novel nuclear structure formed by specific Nups. We refer to this structure as the nuclear scaffold or nuclear cables since this accurately describes the localization of the Nups – binding in large interspersed domains along chromatin, as observed by immunofluorescence staining of polytene chromosomes of Drosophila larval salivary glands. One of the functional players is a nuclear basket Nup, Megator (Mtor), which has been implicated in both transcriptional regulation and transport functions of the nuclear pore. The goal of my research is to define the biological role of the Mtor formed nuclear scaffold as well as its effect on gene regulation. To determine if Mtor plays a role in defining the rate of transcription, we have performed single molecule RNA FISH experiments in whole mounted larval salivary glands targeting a developmentally important transcription factor that responds to ecdysone hormone, Eip74. Results from confocal imaging indicate that upon Mtor knock down: 1) Eip74 transcription seems to be upregulated, as indicated by a bigger, brighter site of nascent transcription and 2) there is an accumulation of single mRNAs in the nucleus. We are also interested in a possible implication of Mtor in the targeting of non-coding RNAs (ncRNA) Rox1 to chromatin since Mtor has been shown to play a role in dosage compensation. Preliminary results from single molecule RNA FISH experiments against Rox1 ncRNA in larval salivary glands indicate that upon Mtor knock down, targeting of ncRNA to the X chromosome may not be as efficient as in wild type conditions. This work will help to establish a potential role for the Mtor nuclear scaffold in transcriptional regulation as well as potentially in ncRNA transport and targeting for the epigenetic process of dosage compensation.

P3090
Board Number: B374
Mapping Nucleoporins in the D. Melanogaster Genome.
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Regarded as the gateway to the genome, nuclear pore complexes (NPCs) consist of approximately 30 proteins termed Nucleoporins (Nups). While the canonical function of the 30-120 MDa NPC is to act as a
selective barrier and regulate intranuclear traffic, recent studies have shown that Nups are also involved in chromatin-related processes such as the regulation of genes and chromatin architecture. However, the organization of individual Nups on chromatin and how Nups are recruited to chromatin and remains poorly understood. To decipher how Nups are organized on chromatin we conducted ChIP-seq of three Nups in two different D. melanogaster cell lines. One of the Nups, Elys, is the only Nup that exhibits DNA-binding ability. The other two Nups, Nup93 and Nup107, are members of the inner and outer ring structural subunits of the NPC, respectively. Due to the roles of Nup93 and Nup107 as structural Nups we hypothesized that these two Nups would share the majority of their binding sites and demarcate NPC binding sites. Preliminary analysis shows that contrary to expectation, these two Nups share only a small number of their binding sites. Moreover, Nup107 and Elys share a large fraction of their sites independent of Nup93 suggesting that either we have captured immature pores or that Elys is recruiting Nup107 for another function. While a majority of Nup binding sites are conserved between cell types, all three Nups exhibit cell type-specific binding patterns. Through this study we have begun to map Nups on chromatin and by expanding upon this study we will be able to further understand what roles Nups have in regulating chromatin architecture and gene regulation. Additionally, by cross referencing these data sets with all available data sets of chromatin/DNA binding proteins we will be able to predict candidates which recruit Nups to chromatin.

P3091
Board Number: B375
Epigenetic modifications and DNA replication in striated muscles rely on LINC-mediated mechanotransduction coupling.
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Nuclear mechano-transduction has been implicated in epigenetic control of gene expression, however its in vivo contribution to mature contractile striated myofibers, has yet to be elucidated. We find that perturbation of nuclear constraints mediated by the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex induced differential nuclear size caused by a varying degree of DNA endocycle progression within myonuclei of single myofibers of Drosophila larvae. Unlike control myonuclei, temporal regulation of DNA replication and its arrest were abolished in LINC mutant myonuclei resulting with variable DNA content in the myonuclei of single myofibers. Furthermore, altered levels of epigenetic chromatin modifications were observed in the LINC mutant myonuclei, which varied between myonuclei of individual myofibers, implicating variability in the transcription activity of the mutant myonuclei. Genomic analysis, using muscle-specific expression of Dam-RNA Pol II indicated that the altered epigenetic modifications correlated with modified RNA-Pol II occupancy of essential genes. Specifically, expression of the chromatin regulator Barrier-to-autointegration Factor (BAF), and the Ca++-dependent myosin regulator Troponin-C were downregulated. We propose that in mature myofibers nuclear constraints determine cell cycle progression, and epigenetic chromatin modifications required for muscle function.
P3092
Board Number: B376
Macronuclear positioning in the giant ciliate, *Stentor coeruleus*.
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The giant ciliate *Stentor coeruleus* takes nuclear positioning to the extreme: this unicellular organism can reach 1 mm in length, and it possesses one of the largest known nuclei. This nodulated macronucleus occupies a specific location and orientation within *Stentor*, and extends down its anterior-posterior axis. *Stentor* has a complex cortical structure that is highly asymmetric. The anterior end features an oral apparatus, while the posterior end tapers into a holdfast. *Stentor* also has the ability to regenerate its oral apparatus after sucrose shocking. During the process of regeneration, the macronucleus dramatically changes shape, condensing into a rod and then renodulating at defined stages of regeneration. How does *Stentor* achieve precise nuclear positioning over large length scales? How is the shape of the macronucleus regulated? Our first molecular foothold into these questions comes from an RNAi screen of genes differentially expressed during oral regeneration in *Stentor*. Knockdown of the exportin gene CSE1 results in *Stentor* with rounded posterior ends and macronuclei that are mispositioned to the anterior end of the cell. Another differently expressed gene contains a SUN domain. SUN proteins are components of the LINC complex, which connect the nuclear envelope to the cytoskeleton and play a role in nuclear positioning. RNAi knockdown of one of *Stentor*’s SUN-domain genes results in cells with irregularly sized macronuclear nodes. Further work will identify more key proteins used to position the macronucleus and begin to parse the mechanisms *Stentor* uses to achieve this positioning.

P3093
Board Number: B377
Characterizing LINC complex assembly in budding yeast meiosis.
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The conserved linker of nucleoskeleton and cytoskeleton (LINC) complex is composed of a SUN-domain integral membrane protein of the inner nuclear membrane and a KASH-domain integral membrane protein of the outer nuclear membrane. In most organisms investigated to date, a direct interaction between the SUN and KASH domains, which takes place in the lumen of the nuclear envelope, mediates LINC complex assembly.

In budding yeast, Mps3 is the SUN-domain protein, whereas two KASH-like proteins, Csm4 and Mps2, have been proposed. Unlike Mps2, which is present in both mitosis and meiosis, Csm4 is meiosis-specific and is required for organizing actin microfilaments that engage telomere motility during meiotic prophase I. The predicted transmembrane domain of Csm4 is located very close to its C-terminus, leaving about three amino acids available for its interaction with the SUN-domain of Mps3 in the nuclear lumen.

In this study, we seek to understand how Csm4 interacts with Mps3 and mediates LINC complex assembly in budding yeast. We have confirmed the genetic interaction between Csm4 and Mps3 by ectopically expressing Csm4 in vegetative yeast cells. We propose two models for how Csm4 interacts with Mps3. First, the very C-terminal end of Csm4 is sufficient for direct binding to the SUN-domain of Mps3. Alternatively, another factor is present to mediate the interaction between Csm4 and Mps3. To distinguish between these two models, we use a combined genetic and biochemical approach. Our
preliminary findings support the idea that a third component is required for bridging Csm4 and the SUN domain of Mps3, thus indicating the formation of a trimeric LINC protein complex in budding yeast meiosis.

**P3094**

**Board Number: B378**

**Molecular insights into the mechanisms of SUN1 oligomerization in the nuclear envelope.**

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SUN proteins are inner nuclear membrane proteins, which interact with the outer nuclear membrane KASH proteins to form conserved nuclear envelope-spanning molecular bridges that mechanically integrate the nucleus with the cytoskeleton. These bridges, or linker of nucleoskeleton and cytoskeleton (LINC) complexes, are important for several cellular functions including DNA damage repair, mechanotransduction, meiotic chromosome pairing, and nuclear positioning. Underscoring their significance is a growing list of human diseases associated with genetic mutations in LINC complex proteins including ataxia, cancer, and muscular dystrophy.

In this study, we combined sophisticated multifunctional computational modeling approaches with in vivo biochemical experiments to understand the functional specification of the two major mammalian SUN proteins: SUN1 and SUN2. While SUN1 and SUN2 play functionally redundant roles in several cellular functions, more recent studies have revealed diverse and distinct functions for SUN1. There is also evidence in the literature to suggest that SUN1 may assemble a wider range of homo-oligomers than SUN2. To understanding the molecular mechanisms underlying SUN1 oligomerization in the nuclear envelope we first made structural models of SUN1 based on the structure of SUN2. Our models predict that both SUN1 and SUN2 favor trimeric SUN domains, however, our results imply that in monomeric (inactive) SUN1, the alpha helical regions preceding the SUN domain adopt a conformation that is distinct from that of SUN2 which may result in differential activation mechanisms for SUN1. Having established the likelihood that SUN1 can homo-trimerize, we next sought to investigate how SUN1 homo-trimers might be assembled into higher-order oligomers as observed experimentally. We show that SUN1 but not SUN2 trimer can associate through their SUN domains to form lateral complexes. In agreement with our models, our in vivo Z-scan Fluorescence Fluctuation Spectroscopy (FFS) results show that exchanging the SUN domains of SUN1 and SUN2 can alter their oligomer states. Finally, by predicting the large-scale functional motions of SUN proteins, we propose a model in which bending of the luminal coiled coil regions may mediate clustering and higher order assembly formation of SUN domain proteins.

**P3095**

**Board Number: B379**

**Regulating interactions between SUN and KASH proteins to mediate nuclear migration and anchorage.**

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The LINC complex, consisting of KASH proteins in the outer nuclear membrane and SUN proteins in the inner nuclear membrane mediates nuclear positioning to specific locations in a cell. Defects in LINC
components result in failure to position nuclei during fertilization, cell migration, cell polarization, neuronal development, and muscle development. The cores of LINC complexes are the interfaces between KASH and SUN domains in the perinuclear space. However, the regulation of the SUN/KASH interaction is poorly understood. Crystal structures predicted that KASH peptides form three interfaces with SUN proteins. Our goal here is to test the functional consequences of disrupting these three interfaces. Significantly, we show that a conserved disulfide bond between SUN and KASH proteins plays an important role in the developmental switch between nuclear migration and anchorage. We use a three-pronged approach combining in vivo C. elegans developmental genetics, a functional assay for nuclear migration in polarizing tissue culture cells, and molecular dynamic simulations to better understand the molecular mechanisms for how SUN and KASH domains interact. The C-termini of KASH proteins are pointed into a SUN protomer forming the first SUN/KASH interface. We showed that extension of the KASH domain by a single alanine residue completely blocked the nuclear migration function of C. elegans UNC-83. At the second interface, KASH peptides then interact in a cleft between two SUN protomers. We showed that mutation of tyrosine at -7 to an alanine blocks UNC-83 function. Molecular modeling showed that the alanine is too short to reach a conserved region in of the SUN domain necessary for hydrogen-bond formation. Finally, before leaving for the outer nuclear membrane, KASH peptides stretch along the surface of a second SUN protomer and form a di-sulfide bond. Interestingly, the C. elegans nuclear migration KASH protein UNC-83 does not contain this third interaction domain, while the anchorage KASH ANC-1 and its mammalian ortholog Nesprin-2G does. Mutations of the conserved cysteines in SUN or KASH disrupted ANC-1 dependent nuclear anchorage in C. elegans and Nesprin-2G/SUN2 dependent nuclear movements in polarizing NIH3T3 fibroblasts. However, the SUN cysteine mutation did not disrupt UNC-83 function during nuclear migration. Finally, molecular dynamic simulations showed that the disulfide bond is necessary to transfer maximal forces from the cytoskeleton to the nucleoskeleton. Thus, the intermolecular disulfide bond is critically important for LINC complex-mediated mechanotransmission and the developmental switch from nuclear migration to nuclear anchorage.

P3096
Board Number: B380
The LINC complex contributes to epithelial cell homeostasis.
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Mechanical forces, both at cell-cell junctions and cell-matrix adhesions, have been shown to be important for regulating homeostatic processes of the epithelium, such as proliferation, collective cell migration, and 3D organization. More recently the nuclear LINC (linker of the nucleoskeleton and cytoskeleton) complex has emerged as another critical structural and mechanosensitive region of the cell. However, the majority of studies of the LINC complex have occurred in fibroblasts. We hypothesized that the LINC complex, both structurally and mechanically, was necessary for epithelial function. Using a previously developed nesprin-2G force biosensor, we confirmed that the LINC complex is subject to mechanical tension in 2D MDCK cell monolayers. We also observed that biaxial stretch increased the mechanical force applied across the LINC complex, indicating that externally applied forces affect LINC complex force. Additionally, we measured higher nesprin-2G force in 3D epithelial acini (grown in Matrigel) as compared to 2D monolayers. To disrupt the LINC complex, we developed an MDCK II cell line expressing inducible dominant negative (DN) KASH, a peptide which disrupts the endogenous nesprin-SUN interactions and blocks nuclear-cytoskeleton interactions. Cells expressing DN KASH exhibited slower migration speeds in response in a scratch wound cell migration assay, consistent
with prior reports in fibroblasts. Expression of DN KASH in MDCK 3D acini cultures resulted in a rapid filling of the central lumen with cells, suggesting that the LINC complex is necessary for acini equilibrium. This filling of the lumen occurs without measurable increases in cellular proliferation, suggesting either a defect in apoptosis, migration, or cellular polarity. Taken together our results indicate that the LINC complex is a critical structure in the epithelium.

**P3097**
**Board Number: B381**
Identification and characterization of *Medicago truncatula* LINC complex components with potential functions in root symbioses.
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Nuclear movement is vital for cellular and developmental processes across eukaryotic life. Nuclear movement often requires LINC complexes, which link the nucleoskeleton and cytoskeleton and bridge the nuclear envelope (NE) through the interaction of outer nuclear membrane (ONM) KASH proteins and inner nuclear membrane SUN proteins. These proteins interact in the NE lumen through the C-terminal SUN-interacting tail (SIT) of KASH proteins and the KASH-binding lid of SUN proteins. LINC complexes are conserved throughout eukaryotes, and recently our lab has shown that they are involved in nuclear movement and positioning in the model plant *Arabidopsis thaliana*. Our interest in plant LINC complexes stems from the many elements of the plant life cycle that involve nuclear movement and positioning, from cell division to fertilization. Among these, one of the most important is symbiosis between plants and soil-dwelling microbes. It has long been known that nuclei move during symbiosis initiation, but it has never been established whether these movements are functional or incidental. *Arabidopsis* does not form symbiotic interactions, thus we have adopted the model legume *Medicago truncatula* - an alfalfa relative widely used as a model for root symbiosis - to determine if plant LINC complexes are involved in symbiosis initiation. Using bioinformatic tools, we identified *Medicago* genes that encode 12 putative proteins: nine KASH proteins, two ONM KASH interacting partners (WIT), and one SUN protein. Several of them are limited to non-Brassica Rosids, and thus may have functionally diversified. Ten of the 12 candidate proteins are properly targeted to the plant NE. NE-associated KASH candidates bind plant SUN, and the interaction depends both on the SIT of the KASH protein and the KASH-binding lid of the SUN protein. Furthermore, NE targeting of all SUN-binding KASH proteins depends on their ability to interact with SUN. Together, these data indicate that *Medicago* expresses *bona fide* LINC complexes that function *in planta*. To broadly inhibit LINC complex function, a dominant-negative version of *Arabidopsis* SUN2 was created that resides in the ER lumen, disrupts LINC-complex function at the NE in *Arabidopsis*, and can bind to *Medicago* KASH proteins. Data showing its effect on both nuclear movement in root hairs and symbiosis initiation and establishment will be presented.
P3098
Board Number: B382
Nesprin-2G, a key player in regulating nuclear mechanics.
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Nucleo-cytoskeletal coupling directly affects nuclear mechanics, which is critical in various cell functions such as migration and differentiation. Linkers of the nucleoskeleton and cytoskeleton, known as LINC complexes, physically anchor the nucleus to the cytoskeleton. Nesprin-2G is a giant component of the LINC complex directly connecting the actin cytoskeleton to SUN proteins, another core component of the LINC complex in the perinuclear space. Nesprin-2G was shown to undergo tension in various nuclear planes and transmit forces to the inner nuclear space. The present study employs integrated experimental and computational techniques across different scales with the aim to investigate the relation between tension along nesprins and nuclear mechanics. Specifically, we explored molecular response of nesprin-2G to cytoskeletal forces using a structural model of nesprin-2G in molecular dynamics simulations. Then, we performed in vitro pulling experiments on nesprin-2G with magnetic tweezers in order to study the effect of mutations identified in silico on force-extension curves. At the cellular level, we observed the effect of these mutations in rescuing the rearward motion of NIH 3T3 fibroblasts. Past efforts have been focused on fully disrupting the connection between the nucleus and the cytoskeleton, which results in completely impaired nuclear anchorage. Here, we designed a protocol for fine-tuning mechanoresponse at the molecular level by introducing site-specific mutations, which can result in major advances in controlling the emergence of nuclear phenotypes to physiological cues.

P3099
Board Number: B383
Nesprin-1alpha 2 mediates MTOC and motor protein recruitment to the nuclear envelope during myogenesis to control myonuclear positioning.
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At the onset of myogenesis, the microtubule (MT) network is reorganised to facilitate the changes in cell morphology and myonuclear positioning that occur upon myotube formation. MT nucleating capacity transfers from the centrosome to the NE through recruitment of pericentriolar material (PCM) components to the NE, including PCM1, pericentrin and γ-tubulin. Following myoblast fusion, MTs then reorganise to form a longitudinal array and their associated motor proteins, dynein and kinesin-1, are recruited to the NE to drive regular myonuclear spreading along the length of the myotube. The LINC complex, comprised of SUN and nesprin proteins, resides in the NE and connects the nucleus to the cytoskeleton. Here, we provide evidence supporting a role for the LINC complex in nuclear-MT connection to control myonuclear positioning. We found that nesprin-1 expression at the NE is induced early in myogenesis, whereas nesprin-2 is largely cytoplasmic. Furthermore, nesprin-1 is required for recruitment of PCM1, pericentrin and kinesin light chains 1/2 to the NE, whilst SUN1/SUN2 act redundantly. Expression of the muscle-specific nesprin-1α2 isoform is induced at the onset of myogenesis, concomitant with pericentrin recruitment to the NE. In nesprin-1 null myotubes, exogenous expression of nesprin-1α2 was sufficient to rescue defects in PCM protein recruitment to the NE. Our data support a model in which nesprin-1α2, anchored at the NE through redundant SUN1/SUN2 interactions, acts as a muscle-specific receptor for recruitment of the MTOC and motor proteins to the
NE. In line with this, muscular dystrophy-associated mutations in LINC complex proteins impaired pericentrin recruitment and MT nuclear nucleation at the NE and led to defects in myonuclear spreading. These defects are likely to contribute to muscle disease pathophysiology.

**P3100**  
**Board Number: B384**  
**MULTIPLE ISOFORMS OF NESPRIN1 ARE INTEGRAL COMPONENTS OF CILIARY ROOTLETS.**  
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Multiple isoforms of Nesprin1 associate homogenously with the nuclear envelope (NE) where they interact with Sun proteins to form Linkers of the Nucleoskeleton and the Cytoskeleton (LINC complexes) that span the whole NE. In a stark departure from this biological paradigm for Nesprin1 at the NE, we show that Nesprin1α colocalizes with the ciliary rootlets of mouse photoreceptors and also forms asymmetric NE aggregates that dock rootletin filaments at the nuclear surface of photoreceptors. Strikingly, recombinant filaments of rootletin expressed in NIH3t3 cells induce the aggregation of specific Sun2/Nesprin1 LINC complexes that are required for the docking of rootletin filaments at the nuclear surface. In agreement with these data, we show that the N-terminal region of rootletin interacts with the cytoplasmic domain of Nesprin1α and that larger isoforms of Nesprin1 are integral components of ciliary rootlets of multiciliated ependymal and tracheal cells. From a biological standpoint, these data provide a novel paradigm for multiple isoforms of Nesprin1 at ciliary rootlets and suggest a unique role for Nesprin1 in anchoring rootletin filaments at the nuclear surface in vivo. From a clinical standpoint, our data suggest that the wide spectrum of human pathologies linked to nonsense mutations of SYNE1, which encodes isoforms of Nesprin1, may in part originate from ciliary defects due to a loss of function of ciliary Nesprin1.

**P3101**  
**Board Number: B385**  
**Dissecting the role of LINC complex in meiotic chromosome pairing and synapsis.**  
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Meiosis is the specialized cell division that enables sexual reproduction by producing haploid gametes from diploid cells. Following one round of DNA replication, the two parental copies of each chromosome, known as ‘homologs’, are segregated such that the resulting gametes only inherit one copy of each. To achieve this accurate segregation, each chromosome must first pair with its homologous partner and establish a physical connection between them called the synaptonemal complex (SC). Despite the identification of many of the molecular components essential for this process, how homology is recognized and how synapsis is prevented between nonhomologous chromosomes remain important unsolved questions. In *C. elegans*, homologous recognition requires the pairing centers, specialized cis-regulatory regions on each chromosome, connected to the microtubule motor dynein through the LINC complex. Without dynein, synapsis fails to initiate even when homologous pairing centers eventually pair; without SUN-1 function, promiscuous synapsis occurs between nonhomologous chromosomes. Loss of SUN-1 function allows extensive synapsis formation even in the absence of dynein, although SC forms between nonhomologous chromosomes (Sato et al., 2009). This
epistasis suggests that SUN-1 is part of a barrier that inhibits promiscuous synapsis while dynein acts to promote synapsis between homologs by overcoming this barrier. To test and extend this model, we are investigating the roles of additional LINC complex components. The LINC complex spans inner and outer nuclear envelopes, and the component of LINC complex connecting dynein and SUN-1 is the KASH-protein ZYG-12. ZYG-12 is essential for development and germline proliferation, but can be depleted specifically during meiosis using the auxin-inducible degradation (AID) system. Acute depletion of ZYG-12 results in distinct defects in paring and synapsis, suggesting a boundary of the putative inhibitory barrier necessary to prevent synapsis between nonhomologous chromosomes. Our data also suggest that different components in the LINC complex connected to the dynein motor could have different functions in controlling pairing and synapsis, during the mechano-regulation of meiotic homology recognition.


P3102
Board Number: B386
Novel human mutation in KASH5 transmembrane domain causes protein mislocalization and male infertility.
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Azoospernia is a type of male infertility characterized by a complete absence of sperm production. The recent identification of a human mutation in the KASH5 transmembrane domain (TMD) has been correlated with an azoospernia phenotype. KASH5 is the most recently identified member of the KASH domain protein family. The KASH domain proteins localize to the outer nuclear membrane through an interaction with inner nuclear membrane SUN domain proteins. KASH and SUN protein interactions form a physical bridge spanning the nuclear envelope, which is known as the LINC complex (linker of Nuclear Envelope and Cytoskeleton). LINC complexes play a key role in the mechanical force transmission required for various cellular processes such as migration and differentiation. During prophase of meiosis I, the SUN1/KASH5 LINC complex connects telomere ends of meiotic chromosomes to the molecular motor protein dynein, which is necessary for chromosome movement and homologue pairing.

The recently identified KASH5 TMD mutation has been found in a family where two affected brothers are carrying homozygous mutations. The mutation consists of a single base pair substitution resulting in an amino acid change from leucine to glutamine at the position 535 (L535Q). In this study, we aim to characterize the KASH5 L535Q mutation and its involvement in the cellular and molecular mechanism leading to azoospernia.

To assess potential changes in secondary structure caused by this mutation, we utilized an in-silico structure prediction program (I-TASSER, University of Michigan) and compared wild-type KASH5 to L535Q mutant. The simulation showed no alteration of the secondary structure and a preserved alpha-helical TMD in the L535Q mutant, similar to what is predicted for wild-type KASH5. However, when we analyzed the TMD using the GES hydrophobicity scale, we found that KASH5 L535Q is less hydrophobic than the wild-type KASH5, with a TMD hydrophobicity of 31.7 and 38.6 kcal/mol, respectively. Changes in the hydrophobicity of TMDs have been shown to affect the targeting of proteins to various organelles. Indeed, when we expressed a GFP-tagged versions of the wild-type and L535Q KASH5 proteins in HeLa
cells, we found a mislocalization of the mutant KASH5 to the mitochondria. We predict that this mislocalization is a key factor underlying the azoospermia phenotype in patients.

**P3103**
**Board Number: B387**
*SUN2 regulates mitotic duration in response to extracellular matrix rigidity.*
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How cells adjust their growth to the mechanical properties of their surrounding environment is central to many aspects of biology. During interphase, changes in extracellular matrix (ECM) rigidity affect gene expression and cell cycle progression, which can compromise tissue homeostasis. Here we examined the effect of extracellular matrix rigidity on cell division. We found that cells divide more rapidly when cultured on rigid substrate. Surprisingly, we observed no effect of extracellular matrix rigidity on rounding or post-mitotic spreading duration, but instead we found that changes in matrix stiffness impact metaphase to anaphase transition. We nest compared the proteomic content of cells dividing on soft and stiff substrates and we found that ECM rigidity regulates the stability of the LINC complex component SUN2, whose level of expression affects mitotic progression. On rigid substrates high level of SUN2 promotes cortical tension and mitotic progression, while low level of SUN2 delays the onset of anaphase when cells are grown on soft extracellular matrix.

**Nuclear Bodies and Dynamics**

**P3104**
**Board Number: B388**
*DNA Damage Causes Rapid Accumulation of Nuclear Phosphoinositides for ATR Signaling.*
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Phosphoinositide lipids (PPIs) are enriched in the nucleus and the role of nuclear PPI signaling has remained enigmatic. A hint from earlier studies suggests involvement of nuclear PPIs in DNA damage response and is evident from the transient increase of nuclear PIP2 level upon DNA damage. To investigate the role and visualize the distribution of the PPIs in response to DNA damage, we followed NLS-tagged GFP-PH fusions that localized to specific PPIs. Expression of nuclear-targeted PH domains that sequester specific PPIs dramatically inhibit recruitment of Ataxia telangiectasia and Rad3-related protein (ATR) plus ATR interacting protein (ATRIP) to the damage site and consequently reduces activation of Chk1, as confirmed by both laser microirradiation and western blots. PPI-binding domains rapidly (<1s) accumulate at damage sites with local enrichment of PPIs, as observed from immunostaining against anti-PIP2 antibody. The magnitude of accumulation is altered by wortmannin and decreased with ATP depletion or lower temperatures. Accumulation of PIP2 in complex with the nuclear receptor protein, SF1, at damage sites requires phosphorylation by inositol polyphosphate multikinase (IPMK) and promotes actin assembly at damage sites that is required for the recruitment of endogenous ATR. Suppressed ATR recruitment/activation is confirmed with Latrunculin A and
wortmannin treatment as well as IPMK or SF1 depletion. Other DNA repair pathways involving ATM and DNA-PKcs are unaffected by PPI sequestration. Together, these findings revealed that nuclear PPI metabolism mediates an early DNA damage response through the IPMK-dependent pathway to specifically recruit ATR/ATRIP complex for Chk1 activation.

P3105
Board Number: B389
Direct visualization and quantitative classification of interphase hetero- and euchromatin using two-color, 3D super-resolution microscopy.
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Limitations impeding the direct visualization of both histones and DNA of interphase chromatin is a substantial barrier to understanding the dynamic organization of the genome within the eukaryotic nucleus. The building block of chromatin is the nucleosome, which is ~11nm in diameter and comprised of DNA wrapped around a cylindrical octamer of the core histone proteins. Higher levels of chromatin organization are broadly categorized into either transcriptionally active euchromatin or repressed heterochromatin. Though these two general organizations can be visualized via conventional light or electron microscopy, the nucleosome organization that underlie them have not been resolved due to a lack of either spatial resolution or molecular specificity, respectively. Single-Molecule Localization Microscopy methods, such as STORM and PAINT, overcome these shortcomings and have spatial resolutions approaching the length scales necessary for determining nucleosome organization (20nm laterally, 50nm axially). Previous studies have utilized STORM to visualize interphase DNA structure [Zessin 2012] and histone H2B structure [Ricci 2015], but no super-resolved overlay of the two structures exists due to the difficulty in capturing both structures in the same nuclear plane at the nanoscale level. Indeed, we have found that many STORM-compatible dyes failed to reconstruct nucleosome organization at high density. Here, we combine STORM with DNA-PAINT [Jungmann 2014] to resolve this deficiency and simultaneously visualize DNA and histone H2B in 3D. Our approach enables the first direct visualization of both DNA and histones of interphase chromatin in intact nuclei. By chemically inducing the euchromatin conformation via hyperacetylation, we demonstrate the capacity to quantify chromatin compaction between hetero- and euchromatin, and correlate it with nucleosome organization.

P3106
Board Number: B390
Canonical, Alternative and Multistranded DNA, and Cell Death.
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Cell death [e.g., apoptosis, necrosis and terminal differentiation (i.e., denucleation)] plays an important role in normal and pathological conditions. Nucleic acids have many different structures that regulate gene expression in normal and diseased tissues. Our goal was to see the effect of two different types of
cell death on nucleic acid structure and function. Therefore, we immunohistochemically [i.e., on frozen and fresh tissue samples (FFPE)] examined the effect of cell death on the integrity of canonical [e.g., right-handed double-stranded (ds) B-DNA], alternative (e.g., left-handed ds-Z-DNA), and multistranded (e.g., quadruplex DNA) nucleic acids, using a variety of different normal and diseased adult and embryological tissues (e.g., adult epidermis, adult crystalline lens and embryologically developing crystalline lenses). Different fixatives (e.g., formalin and non-formalin-based fixatives) were used in order to obtain information on how different nucleic acid structures are affected by diverse types of cell death; apoptosis versus terminal differentiation (i.e., denucleation). Some of the fixatives used were: 10% NBF, Formalin-Alcohol-Acetic Acid, Clarke’s, Carnoy’s, Methacarn, HOPE, Bouin’s, Zenker’s and acetone-methylbenzoate-xylene. Both fluorescent and colorimetric techniques were employed with positive and negative controls, along with Hematoxylin & Eosin staining and the Feulgen Reaction (i.e., DNA content). We used anti-B-DNA, anti-Z-DNA, anti-single-stranded DNA, and G4 anti-quadruplex DNA monoclonal and polyclonal antibodies to investigate the effects of different types of cell death on the presence and amounts of canonical, alternative and multistranded nucleic acids. Data was quantified using a computerized image analysis system, i.e., mean optical density (MOD) units. Our data reveals that each type of nucleic acid structure manifests a different pattern of destruction during cell death, and that apoptosis and terminal differentiation (i.e., denucleation) have certain factors in common, and many differences. Understanding the relationship between cell death and the degradation of nucleic acids will shed light on the pathways that can be targeted by nucleic acid therapeutics. The goal of this project is to provide new knowledge on the processes of cell death including morphology, cell biology, biochemistry and molecular biology. This research is supported by a NYIT-ISRC grant and a TriBeta Biological Honor Society Research Foundation Grant.

P3107
Board Number: B391
Dengue virus NS5 protein targets PML nuclear bodies involved in intrinsic immunity.
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Promyelocytic leukemia (PML) protein is the key organizer of sub nuclear structures called nuclear bodies (PML-NBs). PML-NBs are known to be involved in many basic cellular processes including apoptosis, proliferation, DNA repair and antiviral response. Accumulating reports have revealed that PML-NBs can restrict the replication of many DNA viruses (that must replicate in the nucleus), but also RNA viruses (that replicate in the cytoplasm), such as dengue virus (DENV). A total of 390 million DENV infections occur each year around the world, turning DENV into a major public health problem. DENV can cause dengue fever, which is often a self-limited febrile illness. However, more severe forms of the disease lead to 500,000 hospitalizations and 25,000 deaths worldwide annually. Here, we shed light on the antiviral role of PML in the in-vitro replication of DENV as well as the mechanism underlying this effect.

In order to determine if PML has an inhibitory effect on DENV replication, the expression of PML in A549 cells was silenced by using siRNAs. Then, cells were infected with four DENV serotypes (DENV 1-4) and the amount of virus particles produced was quantified. A549 cells silenced for PML produced 3-4 times more DENV particles than control cells. Next, to determine if DENV infection has an impact on PML-NBs distribution, confocal microscopy of DENV-infected cells was performed. Image analysis revealed that the number of PML-NBs was significantly lower in infected cells.

Even though flaviviruses, such as DENV, replicate in the cytoplasm of infected cells, DENV proteins C, NS3 and NS5 can localize to the nucleus. This nuclear localization is still considered enigmatic in DENV
biology. To determine if the disruption of PML-NBs could be a consequence of the interaction between PML and viral proteins, cells were transfected with vectors encoding for C or NS5. Confocal images showed that quantitative expression of NS5, but not C, was sufficient to reduce the number of PML-NBs. Furthermore, immunoprecipitation studies confirmed the interaction between NS5 and PML. Finally, we observed that overexpression of PML isoforms III and IV, but not the other nuclear PML isoforms induced an accumulation of NS5 at PML-NBs. Overall, we show for the first time a phenotypic as well as functional interplay between PML and DENV. DENV-2 NS5 is found in a complex with PML and its expression alone is sufficient to disrupt PML-NBs. We conclude that NS5 nuclear localization may be important for inhibiting PML-mediated antiviral response.

P3108
Board Number: B392
Optogenetic control of nuclear body assembly.
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Nuclear bodies are membrane-free sub-organelles found in the nucleus of mammalian cells. Recent studies show that nuclear bodies such as nucleoli assemble by phase transition through liquid-liquid demixing, based on evidence from in vitro reconstitution and in vivo observation. However, our understanding of this process in vivo and its functional consequences is limited due to the lack of tools to experimentally manipulate phase transition in live cells. We developed an optogenetic approach to induce phase transition in live cells by controlling protein-protein interactions using photocaged chemical inducers of protein dimerization. We applied these tools to probe nuclear body assembly and its biological functions in vivo, focusing on a subset of promyelocytic leukemia (PML) nuclear bodies that associates with telomeres in telomerase-negative cancer cells. Those cells employ an alternative lengthening of telomere (ALT) pathway using homologous recombination to maintain telomere length. ALT-associated PML nuclear bodies (APBs) are a hallmark of ALT cancers and are used for diagnosis, but the assembly mechanism and functional role of APBs are not clear. We induced de novo APB assembly with light and provide evidence that APBs assemble by liquid-liquid demixing, which promotes telomere clustering in ALT cells. Our results demonstrate the use of our optogenetic tools for manipulating phase transition to probe biological function in vivo.

P3109
Board Number: B393
RICC-seq: Variable chromatin structure revealed by in situ spatially correlated DNA cleavage mapping.
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Chromatin structure at the length scale encompassing local nucleosome–nucleosome interactions is thought to play a crucial role in regulating transcription and access to DNA. However, the local three-dimensional structure of chromatin remains poorly understood compared with the structure of single nucleosomes or long-range looping interactions. We report a genome-wide map of chromatin conformation in human cells at the 1–3 nucleosome (50–500 bp) scale, obtained using ionizing
radiation-induced spatially correlated cleavage of DNA with sequencing (RICC-seq). RICC-seq can serve as a nuclease-independent orthogonal method for mapping nucleosome positions, complementing methods that sometimes yield contradictory results about nuclease-labile fragile nucleosomes. Importantly, RICC-seq data from low radiation doses that cause sparse DNA damage also reveal DNA–DNA contacts that are spatially proximal via spatially correlated DNA strand breaks. Analysis of RICC-seq signal spanning tri-nucleosome units reveals regional enrichment of DNA fragments characteristic of DNA-DNA contacts between alternating nucleosomes. These contacts are particularly enriched in H3K9me3-marked heterochromatin and depleted in open or transcriptionally active chromatin. Our data support a model of chromatin architecture consisting of fibers with local zig-zag order and variable longitudinal compaction that correlates with changes in histone modifications.

P3110
Board Number: B394
Phase transitions in the nucleolus: modulating material properties and protein dynamics using optogenetics.
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The nucleolus is a liquid-like membrane-less nuclear body which plays an important role in cell growth and size control. By modulating nucleolar component concentration through RNAi conditions that change C. elegans cell size, it was previously found that nucleoli only assemble above a threshold concentration; moreover, the ripening dynamics of nucleated droplets are consistent with the hypothesis that the assembly of the nucleolus represents an intracellular liquid-liquid phase transition. A key question following this work is how the material properties of the nucleolus are tuned and linked to the primary function of the nucleolus, in transcribing and processing ribosomal RNA. To address this, we perturb protein-protein interactions in the nucleolus using CRY2olig, an optogenetic clustering protein. Our results suggest that using 488nm light, we can tune the concentration and mobility of nucleolar proteins as well as nucleate and recruit nucleolar proteins from the nucleoplasm. Finally, we present results showing changes in timescale of nucleolar fusion in the X. laevis nucleus. These results lay the foundation for a quantitative understanding of the regulation of nucleolar material properties and their effect on biomedically-critical RNA-processing steps.

P3111
Board Number: B395
Nuclear speckle biology as revealed by HIV-1 infection.
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HIV-1 integration into the host cell chromatin is an essential part of virus replication. HIV-1 selectively integrates into gene dense regions of chromosomes as a result of CPSF6 interaction with capsid (CA). CPSF6 is present broadly in the nucleoplasm of cells, but accumulates in nuclear speckles soon after HIV-1 infection. CPSF6 enrichment in speckles depends on interaction with CA and occurs within hours of infection. Speckles are nuclear bodies present at 15-30 copies per cell that are enriched in transcription and splicing factors. We considered the possibility that HIV-1 trafficked to speckles to facilitate
integration in gene dense regions of chromatin. We obtained fluorescent in situ hybridization (FISH) probes to known hotspots for CPSF6-dependent HIV-1 integration and FISH probes to CPSF6-independent HIV-1 integration hotspots. Indeed, genetic hotspots for HIV-1 integration on 8 different chromosomes co-localized with nuclear speckles, whereas hotspots for HIV-1 integration in the absence of CPSF6-interaction were rarely associated with nuclear speckles. FISH probes were also designed to detect HIV-1 DNA. In the absence of CPSF6 interaction, HIV-1 failed to access speckles and integrated outside of gene-rich regions. Finally, we examined the physical location of speckle-associated regions of chromatin. Speckle-associated regions were in fixed, regular proximity to the nuclear membrane in comparison to other regions that were otherwise accessible to HIV-1 integration. Collectively, our data indicate that the speckles serve as functional organizing centers for HIV-1 after nuclear entry, providing a critical waypoint that HIV-1 uses to access factors that shuttle to gene-rich regions of chromatin. We hypothesize that speckles are anchored to the nuclear lamin in mitotic cells and may hold decondensed, gene-rich regions of chromatin in a fixed position in proximity to the nuclear membrane.

P3112
Board Number: B396
Loss of capsid protein at the nuclear membrane is a pre-requisite for translocation of HIV-1 pre-integration complexes into the nucleus.
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HIV-1 cores released into the cytoplasm as a result of virus-cell fusion undergo reverse transcription and shed a major portion of the capsid protein (CA) as they traffic to the nucleus. The extent, the sites and the dynamics of CA loss (referred to as uncoating) are poorly understood. We visualized single HIV-1 uncoating in living cells, using a novel fluorescent CA marker, cyclophilin-A DsRed (CypA-DsRed), which binds tightly to the viral core without considerably affecting infectivity. Imaging single cores co-labeled with integrase-GFP (IN-GFP) and CypA-DsRed revealed distinct uncoating phenotypes in the cytoplasm: an abrupt loss of CypA-DsRed from cores at early times post-infection vs. gradual release of this marker over several hours. To assess the relevance of these uncoating phenotypes to productive infection, we tracked single IN-GFP labeled complexes that entered the nucleus and thus had reasonable probability of integrating in the host genome. We examined temporal and spatial characteristics of CA release from IN-GFP complexes entering the nucleus by 3-color live cell imaging of HeLa-derived cells expressing a nuclear membrane marker, EBFP2-lamin. Nearly all IN-GFP complexes entering the nucleus (n=105) contained detectable amounts of CypA-DsRed upon arrival at the nuclear envelope (NE). On average, these complexes docked at the NE for 36 min prior to nuclear import. Importantly, all docked HIV-1 cores lost CypA-DsRed before entering the nucleus. By contrast, double-labeled docked particles that did not lose a major portion of the CypA-DsRed at the NE never entered the nucleus, suggesting that loss of CA at the nuclear pore is a necessary step for nuclear import. Of note, a fraction of nuclear complexes contained small amounts of CypA-DsRed and stained for p24/CA, indicating that CA shedding at the nuclear pore does not always reach completion. The requirement for uncoating at the NE is further supported by the lack of nuclear entry of the hyper-stable E45A CA mutant that retains CypA-DsRed and accumulates in the perinuclear area. We also found evidence that uncoating in the cytoplasm destabilized IN complexes, as evidenced by the loss of the IN-GFP signal. Collectively, our results suggest that loosing CA in the cytoplasm disfavors the nuclear entry of IN complexes, whereas partial or full loss of CA at the NE is a prerequisite for productive infection. This work was supported by the NIH R01 GM054787 and AI129862 grants to GBM.
P3113
Board Number: B397
Defining the Genetic Context of the D. Melanogaster Histone Locus Body.
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Many proteins bind locations all over the genome, yet promote different functions at different loci. One example is the Chromatin Linked Adaptor for MSL Proteins (CLAMP), a D. melanogaster transcription factor that recognizes GA-repeats throughout the genome. CLAMP binding to GA-repeats on the male X-chromosome recruits Male Specific Lethal (MSL) complex, which increases transcription of male X-linked genes during dosage compensation. However, CLAMP also binds at the locus of the arrayed, repetitive histone genes, where GA-repeats are contained within the histone3/histone4 promoter (H3-H4p). In contrast to the male X-chromosome, at the histone locus CLAMP recruits Histone Locus Body (HLB) factors that promote histone biosynthesis. It is not understood why CLAMP does not recruit HLB factors to the male X-chromosome or MSL complex to the histone locus. Interestingly, another GA-repeat binding factor, GAGA-Factor (GAF) is specifically excluded from both the male X-chromosome and the HLB, suggesting that one function of CLAMP at both loci is to exclude GAF. However, GAF recruits to the HLB when CLAMP is depleted, suggesting it may partially compensate for CLAMP function.

Our experiments aim to systematically separate the effects of CLAMP binding to specific sequences from the genomic context in which they reside. To do this we created a series of transgenes that introduce non-endogenous DNA sequences into the H3-H4p region of a single copy of the histone gene array. A transgene containing a single copy of the intact array attracts HLB factors when inserted ectopically into the genome. We engineered transgenes in which the GA-repeats in the H3-H4p are replaced with X-chromosome CLAMP binding sites (which normally attract MSL complex), GA-rich GAF binding sites (which do not normally attract CLAMP, MSL complex, or HLB factors), and the H3-H4p region of the Drosophila virilis histone gene array (which contains shortened GA-repeats but still attracts CLAMP and HLB factors). We created stable, homozygous transgenic lines carrying each transgene and immunostained third instar larval salivary gland polytene chromosomes to determine which proteins (CLAMP, MSL complex members, HLB factors, GAF) are recruited to each transgene. CLAMP and HLB factors are not recruited to the transgene if the GA-repeats are deleted from the H3-H4p region. By determining which factors bind to each modified transgenic array, we will learn about how CLAMP promotes distinct functions in different genomic locations and thus better understand how one protein can have multiple context-specific functions.

P3114
Board Number: B398
Molecular Dissection of Zc3h8 Localization to Nuclear Bodies.
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We have identified the zinc-finger protein Zc3h8 as a promoter of cancer and aggressive cellular phenotypes including cell proliferation, migration, invasion, and 3D growth. Overexpression of Zc3h8 also promotes tumor growth in mice while knockdown of Zc3h8 reduces tumor incidence and growth. Very little is known about Zc3h8, but sequence analysis indicates a putative casein kinase 2 (CK2) phosphorylation site at the n-terminus and three C3H zinc fingers at the c-terminus that are suspected to bind RNA. Furthermore, we have localized Zc3h8 to nuclear bodies that contain PML (promyelocytic...
leukemia protein). This localization can be disrupted and changed by altering the CK2 phosphorylation site on Zc3h8 (T32), or the use of specific kinase inhibitors that block CK2 activity. A Zc3h8 T32A mutation results in larger nuclear bodies, while a T32E mutation results in a large increase in the number of, but a decrease in the size of, nuclear bodies. Similarly, the CK2 inhibitor TBB causes nuclear bodies to become larger and fewer in number. Disruption of Zc3h8 also seems to affect correct localization of other nuclear body proteins including PML. At these nuclear bodies, Zc3h8 may interact with nascent RNA (or DNA) and have a critical role in the modification of RNA including alternative poly-adenylation. The zinc finger motifs are important for nucleic acid binding and, therefore, Zc3h8’s function in cells. We have generated stable cell lines expressing Zc3h8 with mutations in one or more zinc finger and have found some domains are more important for generating aggressive cellular characteristics. Here we present a molecular dissection of Zc3h8 to describe amino acid motifs that are important for localization to nuclear bodies, maintenance of nuclear bodies, and function. Ascertaining the implications of the amino acid motifs of Zc3h8 is critical for understanding the molecular mechanisms that result in altered cellular behavior and oncogenic phenotypes.

P3115
Board Number: B399
Nuclei spin prior to mitosis in epithelial tissues.
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The force-generating machinery within cells is critical for sculpting tissue, powering cellular locomotion, and maintaining a properly functioning organism. On a smaller length scale, cells also generate forces to move organelles, change their shape, and partition cellular material during cell division. Here, we found that nuclei in mammary epithelial cells often spin during the time immediately preceding cell division. We combined live-cell imaging and quantitative image analysis to provide insight into the biophysics underlying nuclear spin. In particular, we developed and implemented a four-dimensional (4D) imaging strategy to quantify nuclear rotation from nucleolar movements. Using this strategy, we found that nuclear spin requires a dynamic microtubule cytoskeleton and myosin-mediated cytoskeletal contraction. Furthermore, analyzing E-cadherin-knockout cells and isolated epithelial cells revealed a non-intuitive instructional role for the local microenvironment. Taken together, our results characterize a hitherto unexplored epithelial cell behavior and highlight how dynamic subcellular motion arises within a multicellular cohort.

P3116
Board Number: B400
Genetically Encoded Multimeric nanoparticles (GEMs) to visualize the biophysical properties of the nucleus.
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The mechanisms that determine the physical properties of the nucleus remain largely unknown. We recently developed Genetically Encoded Multimeric (GEM) nanoparticles to study the physical properties of the cell. GEMs are homomultimeric proteins that self-assemble into bright, stable fluorescent particles of defined size and shape. By adding a nuclear localization signal (NLS), we were
able to direct GEMs to assemble within the nucleus. Once assembled, the NLS was buried within the interior of the icosahedral GEM compartment, thus the NLS did not cause significant interactions with the nuclear transport machinery. Tracking the diffusion of nuclear GEMs allowed us to infer the biophysical nature of the nucleoplasm. Using this approach we discovered that mTORC1 inhibition doubles the effective diffusion coefficient of 40 nm particles in the nucleus. This effect on nuclear rheology is similar to the effect of mTORC1 on cytoplasmic crowding through ribosome biogenesis. rRNA can account for 80% of nucleic acid in the nucleus, therefore we hypothesise that ribosomal assembly intermediates have a significant impact on the physical properties of the nucleus. We will now screen mutants to identify the dominant mechanisms that set the rheology of the nucleoplasm.

P3117
Board Number: B401
Nuclear envelope rupture under confinement triggers TREX1-dependent DNA damage and cell senescence.
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During in vivo invasion, intracellular forces are transmitted across the cytoskeleton to the nucleus. Nuclear deformability limits the cell ability to pass through tight spaces. It was recently shown that cells migrating in confined spaces display nuclear envelope (NE) rupture events (assessed by a cytosolic DNA sensor) and DNA damage. Of note, only cells displaying entry of the DNA sensor into the nucleus showed an increase in DNA damage (assessed by 53BP1 foci). This finding suggested that 1) DNA damage associated to confinement might stem from NE rupture, 2) that diffusing cytoplasmic molecules/organelles are entering the nucleus after NE rupture and attacking the DNA. In this study, we used confining slides containing a layer of micro-pillars of different heights to apply a controlled deformation to cells and their nuclei. In order to test if DNA damage is associated to NE rupture, we first determined the confinement heights that induce either NE deformation without NE rupture or NE deformation with NE rupture. We found that at 2μm confinement, 70% of the cells displayed NE rupture as opposed to only 25% and 10% of the cells, at 3 and 4μm respectively. Moreover, cells confined at 2μm displayed higher number of 53BP1 foci compared to cells confined at 3 and 4μm. Careful analysis showed that DNA damage was pronounced only in cells displaying NE rupture, thus demonstrating that DNA damage is linked to NE rupture. Critically, compromised DNA integrity may trigger non-lethal changes, such as premature aging. Therefore we explored whether DNA damage associated to NE rupture caused cell senescence. Cells confined at 2 but not 4μm displayed senescence phenotypes such as increased percentage of β-Gal positive cells and number of HP1y heterochromatin foci. Next, we aimed at identifying the factor responsible for DNA damage following NE rupture. TREX1 is the most abundant cytosolic exonuclease and it was shown to attack chromatin bridges that persisted in cytokinesis, following rupture of the NE that reformed around them. Strikingly, DNA damage was greatly reduced in TREX1-depleted cells (rescued to the same levels of cells not displaying NE rupture). Importantly, TREX1 knockdown rescued cell senescence in cells confined at 2μm, suggesting that TREX1-associated DNA damage following NE rupture is promoting cell senescence. Here we described for the first time a causal relationship between NE rupture, DNA damage and cell senescence in cells under confinement and identified TREX1 as a key player in this pathway. Importantly, cancer cells often migrate through narrow environments and if cell senescence is not activated upon DNA damage they might survive confined migration to colonize distant tissues with an increased genomic instability.
P3118
Board Number: B402
A potential link between DNA damage-induced nuclear actin filaments and the cell cycle.
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Nuclear actin filaments form upon DNA double strand breaks, which are in the most detrimental class of DNA damage. Previous work from the lab has shown an increase in 53BP1 foci, a canonical DNA damage marker, when the cell’s ability to form nuclear actin filaments is inhibited. This result indicates that nuclear actin filaments play a role in the repair process, and that DNA damage persists in the absence of nuclear actin filaments. The role of nuclear actin filaments in DNA repair has been the subject of ongoing work in the lab, and in this ongoing study, we seek to determine which DNA repair pathway(s) are affected by the loss of nuclear actin filaments and whether the presence of nuclear actin filaments during DNA repair corresponds to certain phases of the cell cycle. In this study, cells have been depleted of nuclear actin filaments by knock down of either the nuclear actin importer, Importin9, or an actin nucleator, Formin2, that we previously found to be required for DNA damage-induced nuclear actin filament formation. In these knock-down cells, DNA damage was induced and the extent of the damage was quantified by Comet Assay after one hour and then 24hr later in recovery. Cells unable to make nuclear actin filaments were found to exhibit significantly more DNA damage 24hr later than WT cells. An additional observation showed that nuclear actin filaments formed in ~20% of cells after DNA damage, which hints at a dependency on the cell cycle. We have imaged cell cycle synchronized cells in the presence and absence of nuclear actin filaments, due to the two major pathways of DNA repair being cell cycle dependent. DNA damage-induced nuclear actin filaments likely correspond to certain phases of the cell cycle, which appears to affect the DNA repair pathway that they are involved in.

Post-Golgi Trafficking

P3119
Board Number: B404
Golgi Fragmentation in Alzheimer's Disease.
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Amyloid beta (Aβ) peptide accumulation and amyloid formation are central events that mediate neurodegeneration in Alzheimer’s disease (AD). Aβ is produced by proteolysis of the amyloid precursor protein (APP). Trafficking and maturation of APP and its processing enzymes requires proper functioning of the Golgi apparatus, which depends on the highly ordered stacked structure. The Golgi apparatus is fragmented in the neurons of AD patient’s brain; however, the relationship between Golgi fragmentation and neurodegeneration in AD is largely unknown. Previous studies in AD transgenic mouse and tissue culture models suggests that Aβ accumulation induces Golgi fragmentation by activating cdk5, which phosphorylates the Golgi stacking factor GRASP65. Inhibiting cdk5 or expression of non-phosphorylatable mutants of GRASP65 restores Golgi morphology and reduces amyloidogenic trafficking and processing of APP in cell culture models of AD. These results suggest that Golgi fragmentation is an important mechanism through which Aβ may exert its toxic effects. A potential unrecognized source of Aβ toxicity may be that it compromises Golgi integrity and perturbs trafficking and processing of cell-surface proteins that are essential for neuronal function and viability. Therefore,
we hypothesize that Aβ-induced Golgi fragmentation disrupts trafficking and processing of APP and essential cell-surface proteins, which increases Aβ production and compromises the function and viability of neurons in AD. Preliminary analysis of AD transgenic mice of 1, 3, 6, 9, and 12 months indicates that Golgi defects are present as early as 3 months compared to age-matched controls. Interestingly, Golgi fragmentation does not correlate with proximity to plaques in AD mice with significant plaque deposition and oligomeric preparations of Aβ induces a more severe Golgi fragmentation phenotype compared to fibrilar preparations. Importantly, knockdown of Golgi structural proteins GRASP55 and GRASP65 increases Aβ secretion and impacts APP-CTF stability. Finally, proteomic analysis of cell-surface protein expression in response to Aβ-mediated Golgi defects indicates that Golgi structural abnormalities impacts the expression of numerous cell-surface proteins that are relevant to AD. Future studies will be aimed determining the mechanism of increased Aβ secretion in response to Golgi defects and exploring how Golgi defects influence cell-surface expression of AD-related proteins that were identified in our proteomic studies.

P3120
Board Number: B405
The ESCRT-III protein Chmp1a mediates secretion of Sonic Hedgehog on extracellular vesicles.
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Sonic Hedgehog (Shh) is a secreted hydrophobic morphogen with essential roles in tissue patterning and differentiation during vertebrate development. To date, the molecular machinery and underlying mechanisms regulating Shh release in the extracellular environment remain poorly characterized. Here, we provide in vivo and in vitro evidence for a critical role of the ESCRT-III family member Chmp1a in Shh secretion on small extracellular vesicles (sEVs) during brain development. Chmp1a loss-of-function mutations cause microcephaly in newborn patients. To better characterize the role of Chmp1a in vivo, we generated Chmp1a null mice that recapitulate the phenotype observed in patients, i.e. microcephaly and severe cerebellar hypoplasia. These features correlate with reduced levels of secreted Shh in the cerebrospinal fluid. We further show that Chmp1a depletion affects the release of Shh-loaded sEVs in cell culture supernatant, and that Shh co-purifies with the exosome fraction, together with CD63, CD81, CD9, TSG101 and Syntenin, but not actin nor GP96. Accordingly, transmission electron microscopy analyses in Chmp1a null embryos revealed that multivesicular bodies (MVBs) contain a reduced number of intraluminal vesicles compared to control littermates. However, immunoisolation of exosomes co-purified residual amounts of Shh. Mass-spectrometry analysis and immunoblotting of Shh-positive sEVs
identified specific protein markers, enriched in Shh-positive vesicles but absent in CD63-positive exosomes. Our data highlight a critical function of Chmp1a in brain development by mediating the secretion of Shh on a novel type of sEVs with a unique protein signature.

**P3121**
**Board Number: B406**
**GMAP-210 protein controls traffic of signaling vesicles at the immune synapse and T lymphocyte activation.**
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Signaling by the T cell receptor (TCR) is key in establishing an adaptive immune response. It takes place in a specific zone of the contact between T lymphocyte and antigen presenting cell: the immune synapse. We have previously shown that TCR signaling is regulated by the polarized transport of vesicles containing the adaptor molecule LAT1. Moreover, we and others have also shown that intraflagellar protein 20 (IFT20 a protein controlling cillum formation) and the SNARE VAMP7 protein regulate T-cell activation and LAT transport to the immune synapse\(^1,2,3\). Since that the golgin protein GMAP-210 anchors IFT20 to the golgi\(^4\), we are now investigating the role of the GMAP-210 in T cell activation. We found that the golgin protein GMAP-210 was present in LAT containing vesicles and that silencing of GMAP-210 inhibited the polarized delivery of LAT at the immune synapse. Moreover, silencing of GMAP-210 impairs the localization of VAMP7 and IFT20 in the golgi apparatus. The impaired recruitment of LAT and the lack-off in the localization of IFT-20 and VAMP7 in the golgi resulted in impaired TCR-induced signaling and T cell activation. To our knowledge this study shows for the first time the role of the GMAP-210 golgin in T lymphocyte activation and reinforces previous studies pointing to the similarities between the primary cilium and the immune synapse.


**P3122**
**Board Number: B407**
**The Arl3 and Arl1 GTPases cooperate with Cog8 to regulate selective autophagy via Atg9 trafficking.**
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The Arl3-Arl1 GTPase cascade plays important roles in vesicle trafficking at the late Golgi and endosomes. Subunits of the conserved oligomeric Golgi (COG) complex, a tethering factor, are important for endosome-to-Golgi transport and contribute to the efficient functioning of the cytoplasm-to-vacuole (Cvt) pathway, a well-known selective autophagy pathway. According to our findings, the Arl3-Arl1 GTPase cascade cooperates with Cog8 to regulate the Cvt pathway via Atg9 trafficking.
arl3cog8Δ and arl1cog8Δ exhibit profound defects in aminopeptidase I maturation in rich medium. Additionally, the Arl3-Arl1 cascade acts on the Cvt pathway via dynamic nucleotide binding. Furthermore, Atg9 accumulates at the late Golgi in arl3cog8Δ and arl1cog8Δ cells under normal growth conditions but not under starvation conditions. Thus, our results offer insight into the requirement for multiple components in the Golgi-endosome system to determine Atg9 trafficking at the Golgi, thereby regulating selective autophagy.

P3123
Board Number: B408
Mechanism of action of GTPase-activating protein Gcs1 in modulating GTP hydrolysis of Arl1. W. Chiu1, J. Hsu1, Z. Chen1, Y. Liu1, F.S. Lee1,2; 1Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan, 2Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan

Small GTPase ADP-ribosylation factors (Arf) are key regulators of membrane trafficking and their activities are determined by guanine nucleotide-binding status. Arl1 localizes to the trans-Golgi network (TGN) and participates in multiple membrane trafficking pathways in both mammalian cells and yeast. In yeast, Arl1p is activated by the guanine nucleotide exchange factor (GEF) Syt1 and inactivated by its GTPase activating protein (GAP), Gcs1. Activated Arl1p localizes to the TGN and then recruits the golgin protein Imh1 to the Golgi and activates phosphatidylserine (PS) flippase Drs2, thereby promoting vesicle transport from the Golgi. Previously, we found that Imh1 prolongs Arl1 activity through positive feedback regulation via a steric effect that modifies Gcs1 binding to Arl1. By altering the lipid environment, Drs2 facilitates Gcs1 targeting to the TGN and hence its interaction with Arl1, leading to Arl1 inactivation. These studies showed that Imh1p and Drs2 modulate the activity of Gcs1 by timing its interaction with the Golgi membrane and Arl1, hence providing feedback regulation of Arl1 activity. However, the regulation involved in the timing of Gcs1 to access the Golgi membrane for promoting Arl1 inactivation is unclear. Gcs1 is a highly phosphorylated protein. In addition to the residues identified from the prior large-scale screening, we found several new phosphorylation sites on Gcs1 by mass spectroscopy. Several phosphorylation sites of Gcs1 are highly regulated in response to glucose starvation. Interestingly, three of these phosphorylation sites are located at the lipid packing sensor (+ALPS) motif of Gcs1. We generated phospho-mimetic and phospho-deficient Gcs1 mutants to examine whether Gcs1 phosphorylation may regulate its function. We showed that phosphorylation at ALPS of Gcs1 is involved in Gcs1 function, which may for Gcs1-stimulated inactivation of Arl1. We infer that spatial and temporal regulation of Gcs1p phosphorylation might contribute to the Arl1p inactivation.

P3124
Board Number: B409
AKAP12-mediated PKA phosphorylation of NKD2 S223 facilitated TGF-α cell surface delivery and EGFR transactivation. Z.J. Cao1, B. Singh1, C. Li1, R.J. Coffey1; 1Epithelial Biology Center, Vanderbilt University, Nashville, TN

EGF receptor (EGFR) transactivation induced by GPCR agonists is mediated by metalloprotease-dependent cell surface cleavage of the EGFR ligand TGF-α where upon this soluble ligand binds to and activates the receptor. We previously showed that NKD2 is a short-lived, myristoylated basolateral sorting adaptor for the EGFR ligand TGFα. Herein, we show that this transactivation procedure is likely regulated by NKD2 phosphorylation at S223 through PKA signaling pathway, which is greatly boosted by
forskolin and the GPCR agonist vasoactive intestinal peptide (VIP). The S223 phosphorylation of NKD2 enhances TGFα precursor delivery to the cell surface for its subsequent cleavage by metalloproteases and EGFR activation. PKA-mediated NKD2 phosphorylation is facilitated by the scaffold protein, AKAP12. Mapping of interaction domain was between NKD2 (283-357aa) and AKAP12 (834-947aa). Thus, TGFα delivery regulated by PKA/AKAP12 complex to the cell surface represents a new mechanism of EGFR transactivation that occurs proximal to ligand cleavage by metalloproteases.

P3125
Board Number: B410
Iterative Sorting of Apical and Basolateral Cargo in Mardin-Darby Canine Kidney Cells.
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Epithelial tissues rely on their cells’ ability to generate and maintain surface polarity for transport and barrier functions. Keeping the cells polarized requires initial segregation of apical and basolateral domains on the cell surface, followed by continuous and regulated secretion of appropriate membrane proteins to the apical and basolateral domains. For several decades, the trans-Golgi network (TGN) was considered the most distal stop and hence – the ultimate protein sorting station for the distinct apical and basolateral transport carriers that reach their respective surface domains via the direct biosynthetic pathway. However, recent reports of apical and basolateral cargoes traversing post-Golgi compartments accessible to endocytic ligands, prior to their arrival at the cell surface, as well as of the post-TGN breakup of large pleomorphic membrane fragments that exit the Golgi region toward the surface, raised the possibility that compartments distal to the TGN either mediate or contribute to biosynthetic sorting. Here, we describe the development of a novel assay that quantitatively distinguishes different cargo pairs according to the degree of their correlation at the TGN and to the evolution of this correlation during their TGN-to-surface transport. Key to the high resolution capacity of our approach are: 1) the conversion of dense perinuclear clustering of organelles in 3D into a two-dimensional sample of spread-out microsomal fluorescence, and 2) the identification of TGN and post-TGN membrane protein cargo without the need for a TGN marker that would universally co-segregate with all types of cargo. Utilizing our assay, we provide the first evidence that in MDCK cells, apical NTRp75 and basolateral VSVG cargo proteins continue to undergo progressive sorting after they exit the TGN toward the cell surface. Our study contributes to the emerging view that intracellular sorting in the biosynthetic pathway is an iterative process rather than a series of crossroad decisions at discrete compartments.

P3126
Board Number: B411
A kinesin-3 motor transports newly synthesized basement membrane proteins specifically to a basal subregion of the lateral plasma membrane in epithelial cells.
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Basement membranes (BMs) are sheet-like extracellular matrices (ECMs) that play many important roles in tissue organization. In addition to providing attachment sites to cells, these structures act as a reservoir for growth factors, provide polarity information to cells, and provide instructive cues for tissue
morphogenesis. Polarized secretion of BM proteins ensures that the BM matrix assembles exclusively along the basal surface of epithelial cells. However, BM proteins rely on a poorly understood polarized secretion pathway that is distinct from that used by general basolateral cargo. The follicular epithelium of the Drosophila egg chamber secretes all major BM proteins and assembles a BM along its basal surface on the outside of the tissue. This geometry places the trafficking events involved in BM secretion near the tissue’s exterior and allows high-resolution imaging of the secretion process in a living, intact organ. We previously identified the GTPase Rab10 as a central regulator of BM polarized secretion. Here we show that a surprisingly small region of the basolateral membrane is targeted for BM secretion and that a kinesin-3 family motor is required for transport to this location. To identify the site of BM protein secretion, we blocked the final tethering stage of exocytosis using mutations in the exocyst complex, a known Rab10 interaction partner. In exocyst mutant cells, BM proteins accumulate just inside the basalmost ~0.5 um of the lateral plasma membrane (PM). To understand how this small region is targeted for secretion, we investigated the dynamics of Rab10+ vesicles leaving the Golgi. Rab10+ vesicles move rapidly and processively on microtubules (MTs) and accumulate near the basal-most region of the lateral PM. Since MT plus-ends are enriched basally, we performed an RNAi screen against plus-end-directed kinesin motors and identified the kinesin-3 family member Khc-73 as a novel regulator of BM trafficking. Khc-73 colocalizes with Rab10, and loss of Khc-73 reduces the basal accumulation of Rab10. We therefore propose that Khc-73 transports Rab10+ vesicles from the Golgi to the basalmost region of the lateral PM for secretion. To test the importance of this targeted secretion, we examined the structure of the BM after loss of Khc-73. In this mutant condition, BM proteins accumulate in between cells along the entire lateral domain and form an intercellular network of BM proteins that disrupts tissue organization. Altogether, this work highlights the importance of precisely targeting BM protein secretion, as this precise targeting ensures that the BM is assembled in the correct location to properly organize a developing tissue.

P3127
Board Number: B412
Microtubule-interaction with apical transport vesicles is mediated by the large GTPase Mx1.
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In epithelial cells apical proteins are delivered by specific transport carriers to the correct membrane domain. These vesicular carriers are composed of a heterogeneous population of proteins like motor proteins, annexins, lectins, Rab-GTPases and cargo molecules. Recently, association of the dynamin-related polypeptide Mx1 with apical post Golgi vesicles was described. Downregulation of Mx1 using Mx1-specific small interfering RNA significantly decreased the delivery of apical cargo in Madin Darby Canine Kidney (MDCK) cells, suggesting that this large GTPase plays crucial roles in the transport of distinct post-Golgi vesicles to the apical membrane of polarized cells.

We now demonstrate by co-immunoprecipitation that Mx1 associates with the tubulin cytoskeleton and Kif5B, a motor protein of the kinesin-1 family. As assessed by immunofluorescence microscopy, Mx1-positive vesicles are in close proximity to microtubules and co-localize with kinesin motors.

Furthermore, depolymerisation of microtubule in the presence of nocodazole significantly reduces the long distance transport of Mx1-positive vesicles. This supports the idea that Mx1 is involved in directed vesicle-movement along microtubules to the apical membrane.
P3128
Board Number: B413
The biogenesis and structure of a unique class of large pleomorphic transport intermediates arising from the Golgi apparatus is intricately tied to its cisternal architecture.
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The components of the secretory pathway in the eukaryotic cell are known to exhibit distinct morphological and biochemical fingerprints. The complex cisternal and tubular morphology of the ER gives way to the unique stacked cisternal morphology of the Golgi apparatus (GA) via the vesiculo-tubular structure of the ERGIC. Operating in-between these organelles and from these organelles to the plasma membrane, are transport intermediates (TI) that are either vesicular (50-100nm in diameter) or large (up to 2 μm) non-vesicular and pleomorphic in nature. While much is known about biogenesis, structure and mode of operation of the former class of TI’s, information on the latter is scarce. Trafficking of large cargoes that neither enter vesicular carriers nor leaves the Golgi cisternae have been shown to take place via these non-vesicular TI’s operating between the GA and plasma membrane. This implicates the conversion of large subdomains of the progenitor organelle into these transport intermediates directly by its cargo. Although, light and traditional electron microscopy has provided us some idea about the pleomorphic nature of these TI’s, their biogenesis, details of their elaborate 3D structure and their physical relationship to their progenitor organelle remains unknown. In this work, we use electron tomography in conjunction with a peroxidase tagging technique to dissect the mechanism of biogenesis, architecture and maturation of a system of unique alphavirus-induced TI’s from the GA that plays a role in alphavirus egress. We show that the biogenesis of the unique structure of these TI’s is tied mechanistically both to the structure of the GA as well and the nature of interaction of the viral proteins with the Golgi membrane. Our statistical analysis exhibits a good correlation between the size of the cargo with the size of the TI’s indicating that the size of the cargo, in this case, determines the size and shape of its carrier. Temporal snapshots from infected cells using peroxidase-tagged sub-compartment markers show that the TI-system exhibits a morphological flux that correlates with the structural and biochemical identity of the subdomain of the GA that it originates from, as the virus gradually transforms the GA into pleomorphic TI clusters. In summary, our work, for the first time, dissects the intricate and elaborate three-dimensional structure of a system of unconventional TI from the GA and pieces together its mechanism of biogenesis, mode of transport and its function. We further show that the morphological variations within this system of TI is intricately tied to the unique architecture of the Golgi apparatus.

P3129
Board Number: B414
Multiple PLA2 Enzymes Contribute to Membrane-Tubule Mediated Export from the TGN.
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Export of cargo from the trans Golgi network (TGN) occurs via the formation of multiple carriers including coated vesicles, non-coated vesicles, and membrane tubules. The molecular mechanisms involved in the formation of membrane tubules remain poorly understood, but may be linked to specific types of cargo molecules. Previous studies have established that membrane tubules emanating from the TGN require the activity of cytoplasmic phospholipase A2 (PLA2) enzymes; however, the specific enzyme(s) have not been identified. Here we report that membrane tubule-mediated export of certain
cargoes from the TGN is facilitated by a specific cytoplasmic PLA₂, platelet activating factor acetylhydrolase lb (PAFAHlb). PAFAHlb consists of a homo- or heterodimer of two catalytic subunits (alpha 1 and alpha 2) together with Lis1, and CRISPR-mediated knockout of alpha 1 and alpha 2 in HeLa cells significantly reduced the formation of TGN membrane tubules that transport newly synthesized gp130, a protein destined for the plasma membrane. Moreover, siRNA-mediated knockdown of another Golgi-associated phospholipase, cPLA₂alpha, further reduced the formation of TGN membrane tubules in PAFAHlb knockout cells. Inhibition of PLA₂-dependent TGN membrane tubules may have downstream effects on other organelles because PAFAHlb knockout cells were found to have enlarged lysosomes that were redistributed to the juxtanuclear region. This redistribution was exacerbated in cells knocked down for cPLA₂alpha. These results show that multiple Golgi-associated PLA₂ enzymes contribute to the formation of TGN membrane tubules, which in turn may contribute to downstream trafficking pathways. This work supported by NIH grant GM101027 to WJB.

P3130
Board Number: B415
Manganese-induced trafficking and turnover of GPP130 is mediated by sortilin.
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Elevated, non-toxic doses of manganese (Mn) protect against Shiga toxin-1 induced cell death via down regulation of GPP130, a cycling Golgi membrane protein that serves as an endosome-to-Golgi trafficking receptor for the toxin. Mn binds to GPP130 in the Golgi, causes GPP130 to oligomerize/aggregate and the complexes are diverted to lysosomes. In fact, based on experiments using the self-interacting FM domain, it appears generally true that aggregation of a Golgi protein leads to its lysosomal degradation. How such oligomers are selectively sorted out of the Golgi is unknown. Here we provide evidence that Mn-induced exit of GPP130 from the trans Golgi network (TGN) towards lysosomes is mediated by the sorting receptor sortilin interacting with the lumenal stem domain of GPP130. In contrast, FM-induced lysosomal trafficking of the Golgi protein galactosyltransferase was sortilin independent and occurred even in the absence of its native luminal domain. Thus, sortilin-dependent as well as sortilin-independent sorting mechanisms target aggregated Golgi membrane proteins for lysosomal degradation.

P3131
Board Number: B416
Characterization of vacuolar membrane trafficking pathways in fission yeast.
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Membrane trafficking is critical for transport of lipids and membrane proteins throughout the endomembrane system. In particular, defects in trafficking pathways that transport proteins and lipids to the lysosome have been linked to a number of human diseases, including Hermansky-Pudlak syndrome (HPS), a disease characterized by oculocutaneous albinism and pulmonary fibrosis. In eukaryotic cells, lysosomal trafficking is largely regulated by activation of small GTPases called ADP Ribosylation Factors (Arfs). Arf activation by Guanine Nucleotide Exchange Factors (GEFs) controls the formation of transport vesicles by recruiting specific coat proteins to the membrane. These coat proteins in turn recruit the cargo molecules to be trafficked. In Schizosaccharomyces pombe, membrane

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trafficking pathways that deliver proteins to the vacuole, the yeast equivalent of the lysosome, are likely regulated by the AP-1 and AP-3 coat protein complexes and the single fission yeast class I/II Arf, Arf1p. However, the GEFs that facilitate recruitment of these complexes and their downstream cargoes have not yet been defined in fission yeast. To begin to define these pathways, we explored the phenotypes of cells lacking the GEFs sec71 or sec72 or the coat protein subunits apl4 (AP-1) or apl6 (AP-3). Analysis of vacuolar morphology and pH, salt sensitivity, FM4-64 uptake, and localization of potential vacuolar cargoes revealed both distinct and overlapping phenotypes for these GEF- and coat-deficient strains. Additionally, we also observed different degrees of impaired septation in GEF- and coat-deficient strains, suggesting that these post-Golgi transport pathways may regulate trafficking of enzymes required for septum degradation. Collectively, our results begin to define the membrane trafficking pathways that mediate vacuolar transport in fission yeast and suggest the presence of both distinct and overlapping functions for sec71p and sec72p.

P3132
Board Number: B417
AP-1 mediated trafficking of the lysosomal vitamin B12 transporter ABCD4.
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A variety of human diseases are caused by improper protein trafficking within the trans-Golgi network (TGN)-endosome-lysosome system. Several bacterial toxins and viruses also employ this transport system in order to infect cells. Fundamental understanding of the mechanisms regulating this trafficking pathway is therefore essential to the development of novel therapeutic approaches. MEDNIK syndrome, a rare genetic disease characterized by mental retardation, enteropathy, deafness, peripheral neuropathy, ichthyosis and keratodermia, is caused by mutations in the AP1S1 gene encoding for the α1A subunit of the clathrin adaptor complex AP-1. This heterotrimeric clathrin adaptor complex is involved in recruiting cargo proteins and initiating the formation of transport vesicles between the TGN and endosomes-lysosomes. Cytosolic AP-1 is recruited by the small GTPase Arf1 to the membranes of the TGN where it interacts with specific motifs within the cytosolic tail of transmembrane proteins while also recruiting clathrin and inducing the formation of clathrin-coated vesicles (CCVs). Although defective trafficking of the ATP7A copper pump and the copper-dependent enzyme PAM have been shown to contribute to the pathogenic mechanism of MEDNIK syndrome, the severity and complexity of the disease, coupled with the broad function of AP-1, suggest that aberrant transport of other proteins also contribute to the syndrome. Mutations in the lysosomal transmembrane protein ABCD4 block vitamin B12 transport to the cytosol and cause a disease sharing clinical manifestations (neuropathy, hypotonia) with MEDNIK syndrome. Using co-immunoprecipitation and bioluminescence resonance energy transfer (BRET), we found that ABCD4 interacts with AP-1 in living cells. Mutations of the classical tyrosine (YXXØ) or dihydrophobically ([DE][XX][L/I]) based motifs in the c-terminal tail of ABCD4 did not lead to a loss of interaction with AP-1, suggesting a novel mode of binding. BRET experiments in an Arf1-KO CRISPR generated HEK293T cell line display reduced levels of interaction between ABCD4 and the µ1 and β1 subunits of AP-1. Moreover, ABCD4 accumulates in the Golgi apparatus in a γ-KO CRISPR generated cell line as determined by fluorescence microscopy. Finally, fluorescence microscopy experiments using bodipy-conjugated vitamin B12 show lysosomal accumulation of vitamin B12 in fibroblasts isolated from a MEDNIK patient. Together, these results suggest that ABCD4 transport to the lysosomes requires AP-1 and that aberrant vitamin B12 metabolism could contribute to MEDNIK syndrome pathogenicity.

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P3133

Board Number: B418

Analysis of Myosin 5A recruitment to endothelial Weibel Palade bodies.

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Endothelial cells store Von Willebrand-factor (vWF) in secretory granules called Weibel-Palade bodies (WPB). vWF trafficking and secretion depends on the interplay between WPBs and the cell cytoskeleton. Interaction with actin filaments is thought to be mediated through a Rab27A-MyRIP complex involving two elements; a direct MyRIP-actin interaction and an indirect actin interaction mediated through recruitment of Myosin 5A (Myo5A). In other cell types Myo5A can bind Rab3 isoforms, Slp4-a, or other effectors (e.g. rabphilin-3A) to mediate organelle-actin interactions. Because WPBs can also recruit Rab3 isoforms and Slp4-a the possibility exists that multiple mechanisms may operate for Myo5A recruitment to WPBs. In this study we aimed to examine the contribution of Rab27-MyRIP, Rab3 isoforms and Slp4-a to Myo5A recruitment to WPBs.

To investigate the role of secretory Rabs and their effectors in WPB-Myo5A recruitment HUVEC were transfected with specific siRNAs to deplete expression of Rab27A, MyRIP, Rab3B and Slp4-a or treated with the potent RabGGTII inhibitor 3-(3-Pyridyl)-2-hydroxy-2-phosphonopropanoic acid (3-PEHPC) to disrupt Rab-protein membrane localization. Depletion was assessed by quantitative PCR, western blot and immunocytochemistry and the subcellular localisation of Rabs, effectors and Myo5A determined by immunocytochemistry.

Myo5A immuno-localised to WPBs in HUVEC. Pre-absorption of the Myo5A antibody with the peptide antigen against which it was raised abolished WPB-Myo5A immunoreactivity. Analysis of endogenous Rab27A, MyRIP and Myo5A localisation on WPBs revealed 1) that WPBs in some cells showed Myo5A but not Rab27A or MyRIP localisation and 2) Myo5A immunoreactivity was associated with newly formed WPBs at the trans-Golgi network which lack Rab27A, MyRIP or Slp4-a. siRNA depletion of Rab27A removed Rab27A and MyRIP from WPBs but failed to deplete Myo5A immunoreactivity. siRNA depletion of MyRIP also failed to deplete WPB-Myo5A. Dual siRNA mediated depletion of Rab27A and Rab3GEP removed all detectable immunoreactivity to Rab27A, Rab3B or D, but failed to deplete Myo5A from WPBs. siRNA mediated depletion of Slp4-a, or Slp4-a and MyRIP failed to deplete Myo5A from WPBs. Selective inhibition of RabGGTase II to block all Rab prenylation and thereby remove all Rab proteins (confirmed by complete removal of all detectable Rab27A, MyRIP and Rab3 isoforms) from WPBs failed to deplete Myo5A from WPBs. PCR and RNA seq analysis showed that rabphilin-3A, is not expressed in the HUVEC cultures used here.

Our results point toward the existence of a Rab- and rabphilin-3A independent mechanism for Myo5A recruitment to WPBs. Future studies aim to determine what the mechanism might be.
P3134
Board Number: B419
Select alpha-arrestins control surface abundance of the mammalian Kir2.1 potassium channel in a yeast model.
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Protein composition at the plasma membrane is tightly regulated, with rapid protein internalization and selective targeting to the cell surface in response to environmental changes. For example, ion channels are dynamically relocalized to or from the plasma membrane in response to physiological changes, allowing organisms to maintain osmotic and salt homeostasis. To identify new factors that regulate the selective trafficking of one ion channel, we used a yeast model for a mammalian potassium channel Kir2.1. Kir2.1 maintains potassium homeostasis in heart muscle cells and defects in Kir2.1 lead to human disease. By examining the ability of Kir2.1 to rescue the growth of yeast lacking endogenous potassium channels, we discovered that specific alpha-arrestins regulate Kir2.1 localization. Specifically, we found that the Ldb19/Art1, Aly1/Art6, and Aly2/Art3 alpha-arrestin adaptor proteins promote Kir2.1 trafficking to the cell surface, increase Kir2.1 activity at the plasma membrane, and raise intracellular potassium levels. To better quantify the intracellular and cell surface populations of Kir2.1, we created fluorescence-activating protein (FAP) fusions and for the first time used this technique to quantify the cell surface residency of a membrane protein in yeast. We also identified two other Kir2.1 regulators, which are known effectors of alpha-arrestins: In particular, both the Rsp5 ubiquitin ligase and that the protein phosphatase calcineurin facilitated the alpha-arrestin-mediated trafficking of Kir2.1. We are currently using our yeast model to further define the regulatory elements needed for alpha-arrestin-mediated trafficking of Kir2.1 to the cell surface. We have deleted fourteen genes needed for various protein trafficking pathways in our Kir2.1 reporter system and are currently assessing the impact of each of these mutations on Kir2.1-mediated growth on low potassium and Kir2.1 localization using our FAP technology. Together, our findings implicate alpha-arrestins in regulating an additional class of plasma membrane resident proteins and describe a new tool to dissect the trafficking itinerary of any membrane protein in yeast.

P3135
Board Number: B420
The Role of PKC-ε in Focal Exocytosis.
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Focal exocytosis, the targeted docking and fusion of intracellular vesicles, delivers vesicle contents to specific sites and expands the membrane for processes such as directed cell migration, cell division, neurosecretion, and, in innate immune cells, phagocytosis. Although the mechanisms underlying focal exocytosis in neural cells have been studied, how it occurs in non-polarized cells is not well understood. FcγR-mediated phagocytosis in macrophages (MØ) is a physiologically relevant model of focal exocytosis in non-polarized cells. During phagocytosis, vesicles fuse into the forming phagosome to provide membrane for pseudopod extension in order for MØs to efficiently internalize bound pathogens. We
established that protein kinase C-epsilon (PKC-ε) mediates the addition of membrane during IgG-mediated phagocytosis. These studies probe the mechanism underlying that finding. During phagocytosis, PKC-ε concentrates at the forming phagosome (phagocytic “cup”). PKC-ε binds PI4P. In resting MØ, ~20% of PKC-ε is concentrated at the Golgi, an organelle rich in PI4P. The PI4P reporter, P4M, colocalizes with PKC-ε at the Golgi; P4M competes with PKC-ε for PI4P binding, reducing PKC-ε levels in P4M expressing MØ. Expression of the Golgi-directed PI4 phosphatase, hSac1-K2A, decreases PKC-ε at the Golgi in resting cells, and PKC-ε concentration at the cup during phagocytosis, suggesting that PKC-ε is being trafficked from the Golgi to the cup. TIRF microscopy of MØ attaching to IgG surfaces revealed PKC-ε puncta fusing into the plasma membrane; fusion was confirmed by whole cell patch clamping. PKC-ε+ vesicles co-localize with VAMP3 and TNF-α, a marker of focal exocytosis during phagocytosis, and a cytokine delivered to the cup, respectively. Notably, neither TNF-α nor VAMP3 are delivered to the membrane in PKC-ε knockout MØs; TNF-α is retained in the Golgi. Confocal imaging with 3D rendering shows PKC-ε puncta aligned along microtubules. Together these data support a model in which Golgi-associated PKC-ε orchestrates the delivery of VAMP3/TNF-α+ vesicles to the phagocytic cup. We are currently developing a technique to follow PKC-ε+ vesicle trafficking from the Golgi to the phagocytic cup in real time.

P3136
Board Number: B421
Identification of a barrier to soluble dendritic secretory cargo in the proximal axon of chemosensory neurons in Caenorhabditis elegans.
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Neurons are polarized cells with distinct functional compartments called axons and dendrites. Polarity in neurons arises from localization of specific membrane and cytosolic proteins to the axon or dendrite, respectively. The mechanisms by which membrane proteins achieve and maintain a polarized distribution in neurons are relatively well-understood. How soluble secretory proteins, such as growth factors, are sorted and trafficked into specific neuronal compartments, however, remains an open question. We recently found that the soluble TGF-β protein DAF-7 localizes specifically to and is secreted from the dendrite in a chemosensory neuron in C. elegans while being completely excluded from the axon. Using this model, we find at least some machinery that sorts and traffics dendritic membrane proteins is shared by the soluble secretory cargo. Under these conditions, DAF-7 was mislocalized to and accumulated at a specific, predictable region in the proximal axon. Interestingly, DAF-7 never entered the distal, synaptic portion of the axon. These data indicate the presence of a barrier in the proximal axon that can recognize soluble dendritic cargo and prevent its improper entry into axons in C. elegans, much like the barrier within the axonal initial segment (AIS) in mammalian neurons. We are using this model to identify the mechanisms responsible for sorting soluble dendritic proteins and determine the specificity and composition of this axonal barrier.
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P3137
Board Number: B422
Imaging the molecular architecture of the protein network that regulates clathrin-mediated endocytosis.
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Clathrin-mediated endocytosis involves a growing list of over 30 helper proteins. It is critical for membrane homeostasis, receptor regulation, and intercellular communication. Clathrin triskelia have three long arms which bind to the arms of other triskelia to form a honeycomb-shaped lattice on the inner side of the eukaryotic cell plasma membrane. A lattice grows and bends with the membrane to create a small vesicle that is taken up into the cell. The edge of the clathrin lattice has previously been studied mostly during vesicle scission, when dynamin is known to cut the neck of membrane to release a vesicle. But in earlier stages of clathrin pit maturation, the edge likely plays an important role in capturing receptors and regulating lattice growth and curvature. What proteins are present at the edge of the lattice during these early stages? Here, we use correlative super-resolution localization microscopy and platinum replica electron microscopy to map 19 different endocytic proteins with respect to clathrin lattices. We find that while some proteins are specifically located at the edge of the lattice and others are present throughout the lattice, some proteins previously assumed to be throughout the lattice, like AP-2, have a distinctly higher density at the edge. The relative nanoscale positions of these proteins and the biochemical composition of the lattice edge and center are a starting point in mapping and understanding the architectural network driving the endocytic machinery.

P3138
Board Number: B423
Unique cargo-specific response landscapes underpin the complex and nuanced role of galectin-glycan interactions on clathrin-independent endocytosis of MHCI and CD59.
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Glycosylation of membrane proteins is an often overlooked post-translational modification unique in its complexity and variety. In fact, altered glycosylation is a hallmark of cancer. Although these changes have been viewed as passive by-products of cancer metabolism (often used as biomarkers for detection), more recent studies have shown that these glycans can serve important functional roles. The role that glycan interactions can play in clathrin-independent endocytosis (CIE) is an area of increasing interest especially because CIE is a form of endocytosis which, unlike clathrin mediated endocytosis (CME), does not have well defined cytoplasmic machinery. Therefore, studying the role of glycans working from the outside could help elucidate some of the regulatory mechanisms that govern these trafficking pathways. In this study we show that the perturbation of global cellular glycosylation patterns by metabolic flux modulation affects CIE. Interestingly, these changes in glycosylation have cargo-specific effects. For example, treatment of cells with N-Acetyl Glucosamine (that corresponds to an increase in production of branched glycans) leads to an increase in major histocompatibility complex Class I (MHCI) internalization while at the same time mediating a decrease in endocytosis of CD59, a GPI-anchored protein. In order to better understand the mechanism behind these glycan-mediated effects we focused on the role of Galectin 3. Galectin3 is a secreted galactose binding pentameric lectin that is
essential in forming the galectin lattice which can be a major determinant of membrane characteristics. We found that knocking-down the expression of Galectin 3 led to a small decrease in MHCI uptake and an increase in CD59 uptake. Whereas, the inhibition of all glycan-galectin interactions by lactose treatment inhibits the uptake of both CIE cargo. Our results suggest that glycans play an important role in CIE that is quite nuanced, with each cargo affected in a specific manner and with galectin-driven effects existing on a continuum from stimulatory to inhibitory depending on the extent of galectin-glycan interactions. These treatments did not affect the uptake of transferrin, a CME cargo protein, further implying a CIE-specific role for glycosylation. The galectin lattice was also found to tailor membrane fluidity and cell spreading with disruption leading to more rapid protein diffusion and impaired spreading. The elucidation of the role glycans play in CIE could lead to a better understanding of how these still mysterious pathways are regulated and could offer insight into how altered glycosylation can alter the progress of cancer via the CIE pathway.

P3139
Board Number: B424
Assessing the function of adaptor-clathrin and adaptor-cargo interaction in endocytic progression.
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Clathrin mediated endocytosis (CME) is crucial to eukaryote cell biology. CME is initiated by the recruitment of clathrin, specialized adaptors and early endocytic factors to the plasma membrane. Importantly, clathrin is incapable of binding lipids or integral membrane proteins, and its recruitment requires interactions with endocytic adaptor proteins. Following site formation, the plasma membrane invaginates and a coat of endocytic proteins and clathrin forms around the lipid surface until the vesicle is released into the cytoplasm. While basic aspects of this process have been well documented, the regulatory mechanisms that control coat formation, and the role integral membrane protein cargo plays in adaptor recruitment and endocytic progression are incompletely understood. This work focuses on determining a role for the interaction between the adaptor Sla1 and clathrin in endocytosis and the role Sla1-cargo binding plays in adaptor recruitment. By using fluorescent microscopy, electron microscopy, biochemical approaches, and yeast as a model system, we show interaction with Sla1 is necessary for proper clathrin recruitment. We also show Sla1-clathrin binding plays a role in maintaining normal protein dynamics and progression of late stage mechanisms in endocytosis, specially the initiation of actin polymerization. Our results also suggest a role for clathrin in the regulation of the endocytic invagination shape. Interestingly, diminished clathrin recruitment at CME sites due to impaired interaction with Sla1 results in additional actin polymerization perhaps as a compensatory effect. Furthermore, we show endocytic protein cargo plays an active role in Sla1 adaptor recruitment to the plasma membrane via interactions between the Sla1 SHD1 and SH3-3 domains and cargo containing the NPFxD and ubiquitin sorting signal, respectively. Intriguingly, interaction with endocytic cargo regulates Sla1 nuclear localization, a less well-understood localization/function observed for Sla1 and several trafficking and cytoskeletal proteins.
P3140
Board Number: B425
A membrane trafficking screen to identify Clathrin-independent endocytosis machinery: A role for ROCK2 and Cofilin in CIE.
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Endocytosis is an important event that cells utilize to internalize cell surface proteins and fluid into the cell. There are two main forms of endocytosis: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE), amongst which CME is the most studied. While CME is important for the internalization of specific surface proteins, bulk trafficking of fluid and membrane occurs primarily in a clathrin-independent manner. While we have established the trafficking itinerary of many classical CIE cargo proteins, such as CD59 and Major Histocompatibility Complex Class I (MHCI), little is known about the cellular machinery involved in CIE. To identify proteins essential for the internalization of the GPI-anchor protein CD59 and the cell surface protein MHCI in Hela cells, we designed an siRNA screen utilizing the DhharmaconTM Membrane Trafficking library, which contains siRNA targeting 140 established membrane trafficking genes. Expression of target genes were individually knocked down and cells were allowed to internalize primary antibodies against the CIE cargo proteins of interest. Following internalization, the antibody bound proteins were visualized by immunofluorescence, the amount of internalized protein was determined and positive and negative hits were scored. Interestingly, there was little overlap among the list of proteins identified as important for internalization of these two cargo proteins, which suggests that CD59 and MHCI internalization has distinct requirements. Among these candidates was the kinase ROCK2, which upon depletion preferentially inhibited CD59 internalization, a result which was further confirmed using small molecule inhibitors of ROCK2. This phenotype was specific to ROCK2, as knockdown of the homologous protein ROCK1 had no effect on the internalization of either cargo. ROCK2, through the phosphorylation and activation of the LIMK kinase family, regulates the phosphorylation and subsequent deactivation of the actin severing protein Cofilin and thus ROCK2 can be viewed as a negative regulator of Cofilin. Interestingly, knockdown of Cofilin stimulated the internalization of both MHCI and CD59 and resulted in the accumulation of cargo in enlarged vesicles located in a dense actin network at the cell periphery. This phenotype was abolished in ROCK2/Cofilin double knockdown cells, which might suggest a requirement for ROCK2 mediated regulation of Cofilin in CIE. Further elucidating the interplay between ROCK2 and Cofilin and their role in CIE will be the focus of future studies.

P3141
Board Number: B426
Membrane tension regulates the recruitment of membrane bending protein epsin in clathrin-mediated endocytosis.
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The ability for plasma membrane to deform and bend is necessary for the establishment of cellular architecture and mediation of molecular transport across the bilayer. Membrane bending is integral during clathrin-mediated endocytosis (CME) where clathrin-coated pits (CCPs) invaginate. Membrane tension has been shown to have regulatory effect on CME. However, which endocytic component is involved and how it might be differentially regulating CCP dynamics under different tension environments are unknown. We hypothesize that membrane tension has a regulatory effect on

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recruitment of membrane bending protein epsin. In order to test this, we generated cells stably expressing EGFP-epsin and mCherry-clathrin light chain a (CLCa) and performed live cell imaging by total internal reflection fluorescence microscopy. Under hypo-osmotic shock, which elevate membrane tension, we found an increase in recruitment of epsin into the CCPs. We reason that recruitment of epsin reduces the energy threshold to bend membranes. Initiation density was higher for CCPs with epsin recruitment compared to the ones without epsin recruitment. Irrespective of membrane tension conditions, CCPs containing epsin showed a smaller proportion of short-lived CCPs, which are considered abortive, compared to CCPs that did not contain epsin. However, the decreased proportion of short-lived CCPs is even greater in cells under high tension, which have recruited more epsin. This may allude to the additional stabilization effect of epsin that present more clathrin binding domains which enable the continued formation of mature CCPs. Together, our results highlight a novel mechanosensitive role for epsin that facilitate CCP formation under different tension environments.

P3142
Board Number: B427
The role of membrane curvature in topography-induced cellular signaling.
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Many biomedical applications require direct contact between the cells and non-biological materials. For example, medical implants inserted into the patient’s body have intimate contact with adjacent cells and tissues. Synthetic materials are recognized by cells through their chemical compositions as well as their physical properties. The importance of physical properties in modulating cellular behavior, such as surface topography and material rigidity are increasingly recognized. In particular, studies show that surface topography in the scale of tens of nanometers to a few micrometers significantly affect cell adhesion and tissue integration. As topographic features are stable over long-term and easier to control, they offer unique advantages for modulating cell responses for tissue engineering. Despite a large body of observations, little is known about the origin or underlying mechanisms of the effect of topographical cues on cellular behavior. We explore the underlying mechanisms by employing advanced nanotechnology that generates precisely controlled nanotopography. Our results show that local membrane curvature induced by nanoscale topographical features significantly affect the distribution of curvature-sensitive proteins and stimulate several cellular processes in live cells including clathrin-mediated endocytosis and actin dynamics. We propose that local membrane curvature is the critical player in topography-induced intracellular signaling. Furthermore, our studies show a strong interplay between biological cells and nano-featured surfaces, which is an essential consideration for future development of interfacing devices.

P3143
Board Number: B428
Dynamin independent endocytosis responds to and regulates membrane tension via a negative feedback loop.
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Cells regulate their membrane area and tension by finely tuned mechanisms. However, the cellular processes and molecular mechanisms behind this regulation are poorly understood. Exo-endocytic processes undoubtedly play a major role in controlling these vital physical properties of the membrane. Here we have explored the role of multiple endocytic pathways in the maintenance of membrane homeostasis and its mechanism. We have used multiple ways to modulate membrane tension, including a PDMS based cell stretching device, osmotic shocks or deadhering of substrate attached cells, followed by and optical or magnetic tweezers to measure membrane tension.

We find that a specific endocytic pathway, the dynamin-independent CLIC/GEEC (CG) endocytic pathway is transiently up regulated during change of membrane tension by stretch-induced relaxation or by multiple other means. By contrast caveolar or clathrin-mediated endocytic pathways are not modified. The CG pathway is a high capacity pathway with properties giving it the potential to be a core regulator of membrane dynamics that can rapidly respond to changes in membrane tension. Modulating the CG pathway via perturbation of key regulatory genes correspondingly modifies the resting membrane tension and the rate of membrane retrieval after strain-induced relaxation. Further, we find a mechanotransduction machinery regulates GBF1 activity at cell surface thus modulating CG pathway in response to changes in tension. These features suggest that the CG pathway could be a vital component of the cellular machinery that sets resting membrane tension and maintains area homeostasis.

P3144
Board Number: B429
CD13 is a critical regulator of beta1 Integrin recycling, cell migration and focal adhesion turnover.
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CD13 is a multifunctional cell-surface adhesion molecule that is constitutively expressed on a variety of cells. Our recent studies demonstrated that CD13 regulates dynamin-mediated internalization of receptors of disparate classes to control downstream signal transduction pathways, implicating a fundamental role for CD13 in cellular endocytic processes. In the present study, we investigated mechanisms of CD13-mediated endocytic regulation in the context of the well-characterized program of integrin trafficking. Integrins are trans-membrane receptors mediating interactions between the extracellular matrix (ECM) and the actin cytoskeleton via focal adhesions (FAs), which are dynamic structures that form and dissolve in tightly regulated steps to enable cell migration. In Murine Embryonic Fibroblasts (MEFs), CD13 localizes with β1-integrin at FAs and at cell-cell junctions in epithelial cancer cells, prompting our exploration of potential contributions of CD13 in integrin function. Phenotypically, FAs in CD13KO fibroblasts are elongated and irregular with displaced FA accessory proteins, markedly reduced actin stress fibers and fewer microtubule extensions, indicating that FA formation and cytoskeletal organization is defective in the absence of CD13. This disrupted cytoskeletal organization in CD13KO cells was accompanied by reduced FA kinase phosphorylation, consistent with a link between CD13 and the control of FA dynamics. Importantly, in WT MEFs, CD13 and β1-integrin co-internalize, traffic to EEA1+ and Rab5+ early endosomes and recycle to the plasma membrane together.
via Rab11a+ recycling endosomes. Conversely in CD13KO MEFs, internalized β1-integrin is again found in early endosomes but rather than recycling, integrin aberrantly traffics to Rab7+ late endosomes/lysosomes. Pulse-chase assays confirmed that CD13 is necessary for β1-integrin recycling to the cell surface. Functionally, lack of CD13 led to reduced cell spreading and cell-ECM migration in a wound-healing assay. While CD13 accumulated at the cell leading edge of migrating cells and co-localized with scaffolding protein IQGAP1, deficiency of CD13 led to reduced accumulation of these proteins at the migrating front, suggesting that CD13 is necessary for guiding IQGAP1 localization to regulate directional migration. Mechanistically, CD13 is in a complex with the IQGAP recycling regulator ARF6 GTPase and this interaction is necessary for proper integrin trafficking. This study defines a critical role for CD13 in controlling β1 integrin focal adhesion dynamics and actin cytoskeletal organization in association with ARF6 and its regulator IQGAP1, thereby directing the fundamental cellular processes of FA turnover, receptor trafficking and cell migration.

P3145
Board Number: B430
Clathrin-independent endocytosis coordinated with filopodial formation in the growth cone, revealed by superresolution microscopy.
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The growth cone is a highly motile structure at the growing axonal tip at the time of the neuronal development/regeneration. The continuous reorganization of actin cytoskeletons and membrane trafficking should be performed in its leading edge. These two events are essential to the precise navigation of an axon, however, the relationship between them is not clearly understood. To observe their coordinating movements, we analyzed the dynamics of clathrin, a BAR domain protein, endophilin A3 (Endo3), and F-actin in the growth cone of NG108-15 cells using a superresolution microscopy 3D-SIM, and TIRFM (ref. 1). Whereas clathrin mainly accumulated in the basal membrane in the central domain, Endo3 emerged in the dorsal surface at its leading edge. Accumulation of Endo3 coincided with the F-actin bundling. When actin polymerization was inhibited by cytochalasin B or CK-666, intensity of Endo3 reduced markedly. Knockdown of fascin, a protein F-actin cross-linker, also reduced the numbers of Endo3 at the leading edge. Synaptophysin (Syp), like Endo3, arose near the filopodial bases, and most of them were retrogradely moving along the actin bundles. The retrogradely moving Syp puncta were colocalized with Endo3 and dynamin 1 (Dnm1), at the leading edge, but not with clathrin. Endo3 knockdown reduced Syp puncta from the leading edge whereas a clathrin inhibitor, Pitstop2, did not affected the Syp distribution. Interestingly, in the mouse cortical neurons, Endo3-RNAi showed the inhibited axonal growth, the reduced growth cone size, and the diminished intensity of its F-actin. We also showed the lipid raft domains, in which signaling proteins are enriched with cholesterol and sphingolipids, were endocytosed with Syp, and Gpm6a, a lipid raft protein involved in neuronal polarity determination (ref. 2), were specifically detected on the apical surface from epifluorescence images acquired using TIRFM. These results suggest that the Syp(+)-vesicles by Endo3-, Dnm1-dependent and clathrin-independent endocytosis, occurring at the apical membrane of the leading edge, coincides with filopodial formation. Refs. 1) Nozumi et al.: Cell Rep 18: 2203 ['17]; 2) Honda et al.: J Neurosci 37: 4046 ['17]
P3146
Board Number: B431
Actin control of endosomal sorting in neurons: impact on Alzheimer's disease.
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Endosomal sorting has been implicated in Alzheimer’s disease (AD). β-amyloid (Aβ) is generated in endosomes depending on the endocytic trafficking of the β-amyloid precursor protein (APP) and of APP cleaving enzymes, mainly of BACE1. We previously established that Aβ causes dysfunction of endosomal sorting for degradation (Almeida et. al, J Neurosci 2006). Moreover, we and others found that genetic risk of AD is associated with deregulation of endosomal sorting potentiating Aβ generation. One of these genes, CD2AP, is an actin-capping protein. Actin dynamics controls intracellular trafficking mainly by shaping cellular membranes. Actin helps to drive membrane invagination for endocytosis and shape membrane tubules for secretory trafficking (eg. Almeida et al, NCB 2011). However, a role for actin dynamics in endosomal sorting is not well established. We are investigating endosomal actin function in neurons, its (de)regulation by an Alzheimer’s risk factor, and its role in endosomal Aβ generation. We recently established that CD2AP is polarized to neuronal dendrites, where it controls the translocation of APP away from the endosomal limiting membrane during early endosome maturation into multivesicular endosomes (Ubelmann et. al, EMBO Reports 2017). Now we have found by dSTORM that perinuclear actin foci are more associated with early than late endosomes in neuronal cells. CD2AP localizes to endosomes independent of endosomal F-actin. Loss of function of CD2AP leads to a decrease in actin-positive endosomes and to the disruption of endosomal actin dynamics in neurons. Interference with a specific subunit of the F-actin polymerizing Arp2/3 complex depletes endosomal actin and increases Aβ. These results indicate that endosomal actin regulation by CD2AP is implicated in endosomal sorting. Dysfunction of endosomal CD2AP and actin potentiates amyloidogenesis and potentially contributes to Alzheimer’s disease development.

P3147
Board Number: B432
Parkinson Sac Domain Mutation in Synaptojanin 1 Impairs Clathrin Uncoating at Synapses and Triggers Dystrophic Changes in Dopaminergic Axons.
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Synaptojanin 1 (SJ1) is a major presynaptic phosphatase that couples synaptic vesicle endocytosis to the dephosphorylation of PI(4,5)P2, a reaction needed for the shedding of endocytic factors from their membranes. While the role of SJ1’s 5-phosphatase module in this process is well established, the contribution of its Sac phosphatase domain, whose preferred substrate is PI4P, remains unclear. Recently a homozygous mutation in its Sac domain was identified in early-onset Parkinsonism patients. We show that mice carrying this mutation developed neurological manifestations similar to those of human patients. Synapses of these mice displayed endocytic defects and a striking accumulation of

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clathrin coated intermediates strongly implicating Sac domain's activity in endocytic protein dynamics. Mutant brains had elevated auxilin (PARK19) and parkin (PARK2) levels. Moreover, dystrophic axonal terminal changes were selectively observed in dopaminergic axons in the dorsal striatum. These results strengthen evidence for a link between synaptic endocytic dysfunction and Parkinson's disease.

P3148
Board Number: B433
Cell repulsion driven by EphB2 is regulated by Eps15R-mediated endocytosis.
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EphB receptors and their ligands are important for formation of tissue boundaries during development and in adult tissue, and are also involved in cancer cell invasion. This form of contact-mediated cell-cell repulsion is dependent on removal of EphB receptors from the cell surface. The molecular mechanism for trans-endocytosis of EphB/ephrinB is largely uncharacterised and we therefore set out to identify novel components of this mechanism. First we investigated the role of clathrin-mediated endocytosis in EphB2-mediated cell repulsion using overexpression of dominant negative constructs of AP180 and dynamin-1. To assess EphB2-mediated cell repulsion we quantified the patterning of EphB2- and ephrinB1-expressing cells in a co-culture assay. Both expression of AP180 C terminus and dynamin1-T65A significantly inhibited EphB2-mediated patterning. Secondly, we identified Eps15R and Eps15 as binding partners of Numb, a known adaptor protein for EphB2. In the co-culture assay knockdown of Eps15R, but not Eps15, resulted in an inhibition of EphB2-mediated patterning. To investigate the function of Eps15R further, the lifetime of AP2sigma2-EGFP was assessed using live cell imaging in BSC1 cells. The mean lifetime was 47s in the Eps15R knockdown cells compared to 26s in the control, suggesting a significant slowing of clathrin-coated pit maturation. Morphological analysis of clathrin-coated pits using electron microscopy showed that Eps15R knockdown cells had fewer but larger pits. In particular the neck diameter of the pits was significantly increased compared to control. This led us to investigate whether Eps15R associates directly with clathrin using biochemical techniques. Immunoprecipitation and pull-down assays showed that the motif domain of Eps15R bound clathrin heavy chain terminal domain directly. The binding motif was mapped using alanine scanning mutagenesis and pull-downs and found to be, DPFxLDPF. An additional LDPF motif in the motif domain contributed further to clathrin binding. Finally, we used the EphB/ephrinB co-culture assay as a functional assessment of the Eps15R-clathrin interaction. Knockdown of Eps15R resulted in a reduced EphB2-patterning. This was rescued by expression of wild type Eps15R, but not by the clathrin-binding mutant, thus demonstrating the significance of the Eps15R-clathrin interaction for EphB trans-endocytosis. As a control wild type Eps15 was expressed, which does not contain a clathrin-binding motif. Eps15 could not rescue the loss of Eps15R in the EphB/ephrinB co-culture assay. These results provide the first evidence that Eps15R together with clathrin control EphB/ephrinB trans-endocytosis and thereby cell repulsion.
P3149
Board Number: B434
Tetraspanins TSP-12 and TSP-14 function redundantly to regulate the trafficking of the type II BMP receptor in Caenorhabditis elegans.
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Tetraspanins are a unique family of four-pass transmembrane proteins that play important roles in a variety of cell biological processes. One of their roles is to regulate the trafficking of their associated proteins. We have previously shown that two paralogous tetraspanins in C. elegans, TSP-12 and TSP-14, function redundantly to promote bone morphogenetic protein (BMP) signaling [1]. To dissect the functions of TSP-12 and TSP-14 in BMP signaling, we used the CRISPR/Cas9 system to tag the endogenous TSP-12 and TSP-14 proteins with different colored fluorescent proteins. These analyses showed that TSP-12 and TSP-14 share overlapping expression and localization patterns in multiple tissues, including the hypodermis, where the BMP receptors function to regulate one of the phenotypic outputs of the BMP pathway, worm body size. TSP-12 and TSP-14 are both localized to the cell surface and intracellular vesicles. We found that inside the cell, TSP-12 and TSP-14 are partially co-localized and that TSP-14 primarily localizes to the early endosomes, with some TSP-14 proteins also present in the late endosomes. We further showed that TSP-12 and TSP-14 are required for the intracellular recycling of the type II BMP receptor DAF-4, but not the type I receptor SMA-6. Together with previous findings by Gleason et al. showing that the type I receptor SMA-6 is recycled via the retromer complex [2], our work demonstrates the involvement of distinct recycling pathways for the type I and type II BMP receptors, and highlights the importance of intracellular trafficking in the regulation of BMP signaling in vivo.
1. Wang, L. et al. (2017) Two paralogous tetraspanins TSP-12 and TSP-14 function with the ADAM10 metalloprotease SUP-17 to promote BMP signaling in C. elegans. PLoS Genetics DOI:10.1371/journal.pgen.1006568
2. Gleason, R. J. et al. (2014) BMP signaling requires retromer-dependent recycling of the type I receptor. PNAS DOI:10.1073/pnas.1319947111

P3150
Board Number: B435
The Tail Waves the Dog: Differential Regulation of Expression of the Long and Short BMPRII Isoforms by Translation and Endocytosis.
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The expression and function of transforming growth factor-beta superfamily receptors are regulated by multiple molecular mechanisms. The type II BMP receptor, BMPRII, is expressed as two alternatively spliced forms, the long and short forms (BMPRII-LF and -SF), which differ by a ~500 amino acid C-terminal extension, unique among receptors from the TGF-beta superfamily. Whereas this extension was proposed to modulate BMPRII signaling output, its contribution to the regulation of receptor expression was not addressed. To map regulatory determinants of BMPRII expression we compared the synthesis, degradation, distribution and endocytic trafficking of BMPRII isoforms and mutants. Our findings identify translational regulation of BMPRII expression and the contribution of a 3' terminal
coding sequence to this process. BMPRII-LF and -SF differed also in their steady state levels, kinetics of degradation, intracellular distribution and internalization rates. A single di-leucine signal in the C-terminal extension of BMPRII-LF accounted for its faster clathrin-mediated endocytosis relative to BMPRII-SF, accompanied by mildly faster degradation. Higher expression of BMPRII-SF at the plasma membrane resulted in enhanced activation of Smad signaling, stressing the potential importance of the multi-layered regulation of BMPRII expression at the plasma membrane.

P3151
Board Number: B436
Mutations of the N-terminal TIR domain tyrosine result in loss of TLR9 function by directing autophagic elimination of the mutant protein.
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Nucleic acid sensing Toll-like receptors (TLRs) are important for host defense against pathogens but are also potent immunomodulators, thus help shape innate and adaptive immune responses. The cytosolic TIR domain of the TLRs acts as the platform for assembling the signalosome that translates ligand binding by pathogen motifs to intracellular transcriptional events. In the case of TLR9, the ligand is hypomethylated double-stranded DNA from pathogens, and results in the production of inflammatory cytokines such as TNFα and IL-6. Prior studies in TLR4 suggested that the ligand-mediated phosphorylation of the N-terminal tyrosine of the TIR domain was critical mediating downstream TLR signals. In this study, we examined the role of the N-terminal TIR domain tyrosine in TLR9 function, which unlike TLR4 requires an additional step of cleavage from a 160 kDa proprotein to an 80 kDa active form in the endosome prior to signaling. We substituted the N-terminal TIR domain Y870 of TLR9 with phenylalanine (Y870F) or with alanine (Y870A), and expressing them retro-virally in the TLR9 knockout bone marrow derived dendritic cells (BMDCs), and measured cleavage of the TLR9 pro-protein into active form, intracellular localization of TLR9, and ligand induced TLR9 cytokine production. Y870F, which still possessed the aromatic ring of tyrosine but lacks the phosphorylation site, had diminished TNFα and IL-6 production upon ligand binding, while Y870A had complete loss of cytokine production. These results correlated with a partial loss of the active 80 kDa in Y870F such that the diminution of cytokine production could be completely accounted for by the amount of 80kDa protein made suggesting the phosphorylation at this residue is not needed for signaling. Accordingly, there was a total loss of 80kDa protein in the Y870A mutant. Y870A TLR9 was able to bind to its normal chaperoning proteins GRP94 and Unc93b1. It was also capable of forming dimers with itself and wild type TLR9 as normally expected. However, while, wild type TLR9 was found within Lamp1+ endosomes within 4 hours of expression, Y870A TLR9 instead localized with LC3BII positive structures consistent with autophagic vesicles that become Lamp1+ after 24 hours, suggesting evolution into an autolysosome to presumably degrade and dispose of the mutant protein. These results suggest that the N-terminal TIR domain tyrosine is not important for phosphorylation as previously reported, but rather for proper targeting of the蛋白 to its correct subcellular location. Furthermore, binding to the normal ER chaperones, GRP94 and Unc3b1, is insufficient to target TLR9 to the endosome in the presence of such a mutation. Mutations of the N-terminal TIR domain tyrosine rather appear to result in targeting of mutant
**P3152**

**Board Number: B437**

**Catalytic activation cycle of β-arrestins by GPCRs.**

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How membrane trafficking and cellular signaling are interconnected is a fundamental question in cell biology. We recently discovered a clear example of such reciprocal control between signaling and endocytosis through the study of β-arrestin, a critical player in the signaling and trafficking of G protein-coupled receptors (GPCRs), the largest signaling receptor family. All of the many known functions of β-arrestin were previously thought to require β-arrestin to be scaffolded with its activating GPCR and remain in a stable physical complex. Using live-cell quantitative fluorescence imaging, we recently found that β-arrestin, previously thought to exclusively function in an obligate physical complex with its activating GPCR, can also function as an independent downstream transducer molecule by trafficking to clathrin-coated structures (CCSs) after dissociating from its upstream activating GPCR (Nature Cell Biology, March 2016). This requires an alternate cycle of β-arrestin activation in which the GPCR acts as a catalyst rather than co-scaffold, but how this is possible remains unknown. We now show that catalytic activation of β-arrestin is widespread, occurs for both β-arrestin isoforms and is triggered by a variety of GPCRs. Formation of the catalytic GPCR / β-arrestin complex does not require the GPCR cytoplasmic tail nor tail phosphorylation, distinguishing it from classical complex formation that requires both. Catalysis instead uses the GPCR core structure and involves destabilization of a salt bridge between the base of the β-arrestin finger loop and C-lobe that acts as an ‘ionic lock’ stabilizing the inactive state. Once dissociated from the catalytic complex, β-arrestin can operate apart from receptor by binding to plasma membrane phosphoinositides and to the CCS lattice. We propose a β-arrestin activation cycle catalyzed by transient interaction with the GPCR core and energetically driven through a network of non-receptor allosteric interactions. These results define a new framework for understanding cellular β-arrestin function and the signaling consequences of clathrin-mediated endocytosis.

**P3154**

**Board Number: B439**

**Molecular mechanisms and of microdomain-dependent protein trafficking.**

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Eukaryotic cells are organized into spatially and functionally distinct membrane-bound organelles, whose functions are defined by their lipid and protein composition. Accurate and robust sorting of membrane components between these compartments is necessary for the maintenance of organelle identity. For most membrane proteins, the determinants of their steady-state subcellular localization remain unknown. Lateral membrane domains known as lipid rafts provide an ideal platform for membrane sorting processes, and have been widely implicated in post-Golgi sorting and endocytosis/recycling. However, the structural determinants of protein association with such domains are almost entirely unknown. We have developed and characterized a robust experimental system for
direct, quantitative measurements of raft affinity in intact plasma membranes and used it to explore the determinants of transmembrane protein recruitment into raft domains and the consequences of this recruitment on subcellular traffic. We identified several structural features associated with raft affinity, and established a quantitative and functional relationship between raft association and subcellular protein localization. Specifically, we observed that raft association is fully sufficient for PM recycling of certain proteins, and that abrogation of raft partitioning for these proteins led to their degradation in the lysosomes. These findings identify structural determinants of raft affinity for transmembrane proteins and support the conclusion that ordered membrane domains mediate recycling of specific membrane components from the endosomal compartments to the PM. We have proceeded to define the molecular machinery that mediates raft lipid and protein sorting and recycling to the PM. Using a set of orthogonal transmembrane proteins as probes of raft and non-raft domains, we developed a high throughput siRNA screen to dissect the molecular machinery and dynamics for raft-mediated sorting. We identified a number of validated hits including known players of the early endocytic traffic (Rab5 and EEA1), but also novel players that appear to define a distinct class of trafficking mediators specific for raft-associated proteins. This pathway is not dependent on the classical recycling pathways defined by Rab4 and Rab11, rather defining a novel route for PM recycling of raft-prefering cargo.

**P3155**

**Board Number: B440**

Endocytosed PAM, MPR and EGF traverse a dynamic multivesicular body network together.

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In the endocytic pathway the epidermal growth factor (EGF)/EGF receptor complex is conveyed to lysosomes for degradation while the secretory granule membrane protein peptidylglycine α-amidating monoxygenase (PAM) and the lysosomal enzyme transporter cation independent mannose-6-phosphate receptor (MPR) are recycled to the trans-Golgi network and evade degradation. We studied the conserved mechanisms for endocytic trafficking of PAM by overexpressing PAM in HEK293 cells and comparing the endocytic trafficking of PAM, MPR and EGF. As expected, all three cargoes accumulated on intralumenal vesicles in multivesicular bodies. 10 and 20 min after uptake of labeled PAM and MPR or EGF more than 80% of labeled endosomes contained all three cargoes, at least partially on the same intralumenal vesicles. Live video imaging of the uptake of labeled cargoes demonstrated frequent contacts between endosomes: 20 min after uptake a PAM containing endosome made an average of 5.2+0.4 contacts/min lasting 2.9+0.4 s with other PAM containing endosomes of the same age, thus being connected to another endosome of the same age for approximately 25% of the time. In addition, uptake of Alexa 555 conjugated PAM antibody versus Alexa 488 conjugated PAM antibody introduced 10 min later showed frequent contacts and material exchange between early and late endosomes. Prelabeling of lysosomes with fluorescent dextran revealed contacts between PAM containing endosomes and lysosomes with approximately the same frequency (5.8+0.4 contacts/min of 2.3+0.2 s duration). Similar contacts were not seen by conventional electron microscopy. With the use of high pressure freezing tubular contacts and direct membrane apposition between multivesicular bodies and between multivesicular bodies and lysosomes could be observed. In conclusion, the same population of multivesicular bodies transports PAM, MPR and EGF, at least partially on the same intralumenal vesicles. The endosomal pathway is highly dynamic; a PAM containing endosome is most of the time briefly connected to another endosome or a lysosome. The mechanism of further sorting of cargo from intralumenal vesicles is obscure and probably involves back fusion of intralumenal vesicles with the limiting membrane of the multivesicular body.

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Board Number: B441
Proteomics reveals novel protein associations with early endosomes in an EGF-dependent manner.
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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is an integral component of proliferative signaling. When activated by a ligand at the plasma membrane, EGFR dimerizes with another ErbB family receptor, leading to kinase domain activation and transphosphorylation of C-terminus tyrosine residues. These phosphotyrosines act as crucial regulators of EGFR signaling as effector proteins dock to the receptor at these sites. The receptor undergoes clathrin-mediated endocytosis into early endosomes, where it can then be trafficked to a lysosome for degradation. However, the kinase domain of EGFR retains its activity during trafficking, suggesting that EGFR can continue to elicit signaling cascades after internalization. Unfortunately, there is no consensus as to how EGFR spatial regulation affects its signaling or interaction with downstream effectors. We hypothesize that EGFR localization in early endosomes permits unique interactions with downstream effectors. In an effort to identify proteins that uniquely associate with the internalized EGFR, we have developed a strategy for isolating early endosomes and analyzed the protein make-up of these compartments. HeLa cells were stimulated with and without EGF (10 ng/ml) for 15 min, and the post-nuclear supernatant (PNS) was loaded onto a 17% Percoll gradient which separates endosomes based on density. The gradient was fractionated, and fractions containing early endosomes were pooled and immunoprecipitated with an EEA1 monoclonal antibody. The morphology of isolated compartments was monitored using transmission electron microscopy, which revealed intact vesicles with an average diameter of 68.63 ±26.74nm. Endosomes were subjected to liquid chromatography/tandem mass spectrometry (LCMS) for proteomic analysis. The purification protocol yields a highly enriched population of early endosomes, as evidenced by immunoblot and LCMS analyses. The isolation method precipitates early endosome marker proteins, but not marker proteins specific to other organelles. Five proteins were detected in endosomes in a ligand-dependent manner: EGFR, RUFY1, STOML2, PTPN23, and CCDC51. We have developed a rapid and high-throughput isolation technique to collect early endosomes from HeLa cells that can be analyzed by LCMS to detect a distinct proteome. These data provide evidence that endocytic trafficking of the activated EGFR changes the protein composition and signaling potential of the early endosome.
Endosomes, Lysosomes, and Lysosome-related Organelles 2

P3157
Board Number: B442
A molecular mechanism to recruit galectin-3 into multivesicular bodies for polarized exosomal secretion.
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Galectin-3 (Gal3) plays a role of fundamental importance in the extracellular space, however the exact secretion pathway remained elusive. Lacking a canonical signal sequence for translocation into the endoplasmic reticulum (ER), Gal3 is exported independently of the secretory pathway by a not yet defined non-classical mechanism.

Here, we found Gal3 in the lumen of exosomes. Super-resolution and electron microscopy studies visualized Gal3 recruitment and sorting into intraluminal vesicles (ILVs). Computer simulations mimicking the experimental conditions depicted identical Gal3 recruitment to multivesicular bodies (MVBs), confirming the ability to discriminate between distinct Gal3 sorting stages into ILVs. Exosomal Gal3 release depends on the ESCRT-1 component Tsg101 and functional Vps4a. Either Tsg101 knockdown or expression of dominant-negative Vps4a¹²28Q causes an intracellular Gal3 accumulation at MVB formation sites. In addition, we identified a highly conserved tetrapeptide P(S/T)AP motif in the amino-terminus of Gal3 that mediates a direct interaction with Tsg101. Mutation of the P(S/T)AP motif results in a loss of interaction and a dramatic decrease in exosomal Gal3 secretion. We also found evidence for a functional role of Gal3 in the sorting process of exosomal proteins and ILV biogenesis. We conclude that Gal3 is the first member of endogenous ESCRT-independent proteins which are P(S/T)AP-tagged for exosomal release. In summary, this study identifies a functionally relevant PSAP-mediated direct Tsg101-interaction with an endogenous cargo molecule and provides a unique model of how proteins destined for exosomal secretion can be recruited and opens new avenues for polarized unconventional secretion of cytoplasmic proteins.

P3158
Board Number: B443
Ist1 regulates ESCRT-III assembly and function during multivesicular endosome biogenesis.
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Degradation of most integral membrane proteins is directed by the endosomal sorting complex required for transport (ESCRT) machinery, which selectively targets ubiquitin-modified cargoes into intraluminal vesicles (ILVs) within multivesicular endosomes (MVEs). To define mechanisms underlying ESCRT-mediated formation of ILVs, we exploited the rapid, de novo biogenesis of MVEs during the oocyte-to-embryo transition in C. elegans. In contrast to previous models suggesting that ILVs form individually, we demonstrate that they remain tethered to one another subsequent to internalization, arguing that they bud continuously from stable subdomains. Additionally, we show that membrane bending and ILV formation are directed specifically by the ESCRT-III complex in vivo in a manner regulated by Ist1, which promotes ESCRT-III assembly and inhibits the incorporation of upstream ESCRT components into ILVs.

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Our findings underscore essential actions for ESCRT-III in membrane remodeling, cargo selection, and cargo retention, which act repetitively to maximize the rate of ILV formation.

**P3159**

**Board Number: B444**

**Renitence vacuoles facilitate protection against phagolysosomal damage in activated macrophages.**

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As professional phagocytes, macrophages are susceptible to endolysosomal membrane damage inflicted by the pathogens and noxious particles they ingest. Whether macrophages have mechanisms for limiting such damage is not well understood. Previously we reported a phenomenon, termed “inducible renitence,” in which LPS activation of macrophages protected their endolysosomes against damage initiated by phagocytosis of silica beads. To gain mechanistic insight into the process, we analyzed the kinetics of renitence and morphological features of LPS-activated versus resting macrophages following silica bead-mediated injury. We discovered novel vacuolar structures that form in LPS-activated but not resting macrophages following silica bead phagocytosis. Because of their correlation with renitence and damage-resistant nature, we termed these structures “renitence vacuoles.” Renitence vacuoles formed coincident with silica bead uptake in a process associated with membrane ruffling and macropinoscyosis. However, unlike normal macropinosomes, which shrink within 20 minutes of formation, renitence vacuoles persisted around bead-containing phagosomes. Renitence vacuoles fused with lysosomes, and, after fusion, maintained their acidity. Phagosomes associated with renitence vacuoles, however, did not fuse with lysosomes. As damage-resistant, persistent macropinosomes, renitence vacuoles act as structural barriers to prevent fusion between damaged phagosomes and intact lysosomes and thereby preserve endolysosomal integrity.

**P3160**

**Board Number: B445**

**Phagocytic functions of human macrophages is impaired by infection with rhinoviruses.**

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Phagocytosis and degradation of microorganisms or debris by macrophages is crucial for pathogen clearance and resolution of inflammation. Alveolar macrophages are central for pulmonary host defense because they are responsible to survey the exposed airways. Alveolar macrophages from COPD (chronic obstructive pulmonary disease) patients demonstrate defective phagocytosis and their inability to clear respiratory pathogens could contribute to disease pathogenesis. However, the molecular drivers of this phagocytic defect are unknown. Human rhinovirus (HRV) and non-typeable Haemophilus influenzae (NTHi) are frequently isolated during COPD exacerbations. We hypothesised that HRV infection(s) of alveolar macrophages are key drivers of phagocytic defect observed in COPD. Alveolar macrophages isolated from bronchoalveolar lavage or monocyte derived macrophages (MDMs) were pre-challenged with HRV16. Following viral challenge, we analysed phagocytosis, phagosome maturation, reactive Tuesday-279
oxygen species generation and cytokine production. Alveolar macrophages or MDMs pre-challenged with HRV16 demonstrated deficient phagocytosis towards a range of targets. In AM derived from COPD patients, HRV16 further impaired phagocytosis. This inhibition was viral mediated and not observed with UV inactivated virus. We also performed RNA sequencing on HRV16 infected macrophages in the presence or absence of NTHi. We found that HRV16 treated macrophages were less able to respond to a second phagocytic trigger. Our results demonstrate that HRV16 impairs macrophage responses at multiple pathway levels.

**P3161**

**Board Number: B446**

**LC3B lipidation is required for macropinosome biogenesis.**

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Macropinocytosis allows the cell to non-selectively uptake large volumes of extracellular fluid through closure of plasma membrane protrusions and formation of large endocytic vesicles called macropinosomes. Macropinocytosis is critical for a range of diverse physiological processes, including antigen capture by macrophages and dendritic cells, and is a route of cell entry for a range of pathogens. Despite being the first endocytic vesicle seen by microscope, the molecular mechanism of macropinosome biogenesis is poorly understood. Using Ebola virus (EBOV) and the fluid-phase marker, dextran, both known to require macropinocytosis for trafficking into the cell, we demonstrate that lipidation of LC3B protein, originally described to be critical for autophagosome formation, is also required for biogenesis of macropinosomes. Depleting cells of LC3B or cellular machinery coordinating LC3B processing abolished internalization, but not cell binding of EBOV and uptake of dextran. The block to macropinosome formation was at the earliest detectable step when Ankfy1 protein and phospholipid PtdIns(3,4,5)P3 mark sites of macropinocytic cups. Both unprocessed and lipidated forms of LC3B interacted with Ankfy1 by immunoprecipitation and colocalization assays. This interaction appeared at the cell surface, suggesting that although various forms of LC3B possess an inherent ability to associate with forming macropinosomes, it is the lipidated form that drives closure of macropinocytic cups and, therefore, EBOV and dextran internalization. Based on these findings, we speculated that pharmacological inhibition of LC3B lipidation would interfere with macropinosome internalization. Indeed, PIK-III, a potent inhibitor of LC3B processing, efficiently blocked dextran uptake and replication-competent EBOV infection in human immortalized cell culture and blood-derived primary macrophages. Our findings demonstrate a clear role for LC3B in controlling macropinocytosis as well as identify novel targets for treatment of diseases dependent on it. This work was funded by the Douglass and Ewing Halsell Foundations.
P3162
Board Number: B447
Elucidation of mechanisms controlling phagolysosome resolution.
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Phagocytosis is a process that is critical for immunity, as well as for tissue development and repair. Circulating and tissue resident phagocytes continuously capture dead cells and invasive pathogens, which are degraded in phagolysosomes. The phagosome maturation pathways that convert phagosomes into microbicidal and hydrolytic compartments are highly regulated processes that have been extensively studied and consequently known in great detail. On the other hand, the fate of phagolysosomes, after their cargo is digested, is poorly understood. Utilizing phagosomal markers and fluorescent bacteria such as *E. coli* and filamentous *Legionella* as traceable and degradable phagosomal cargo, we followed mature phagolysosomes to their resolution. Unlike the assumption that phagolysosomes are exocytosed after particle degradation, our results indicate that phagolysosomes split and fragment into many vesicles. Combining pharmacological and genetic strategies, we found that this process is clathrin dependent. We then speculated that phagosome resolution is essential to recycle membranes, reform lysosomes and maintain the phagocytic appetite of macrophages. Indeed, macrophages could eat a larger number of particles 7 hours after a first round of phagocytosis. In marked contrast, interrupting the fragmentation of the phagolysosome blunted the ability of macrophages to undertake additional phagocytosis. Lastly, we provide evidence that phagolysosome vesicles seem indistinguishable from basal lysosomes in all functions examined including markers, acidification and fusogenicity with subsequent phagocytic and endocytic cargo. Thus, we propose that phagosome resolution is the mechanism by which macrophages reform lysosomes after particle degradation. Hence, the fragmentation of the phagolysosomes during the resolution phase may provide cellular resources for the continuous generation of new phagosomes and the prevention of this phase will critically impair the immune and housekeeping functions of the phagocytes.

P3163
Board Number: B448
Dissecting Atg27 Function in Budding Yeast Autophagy and Membrane Traffic.
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Eukaryotic cells use membrane trafficking to transport molecules, like lipids and proteins, to their designated location inside cells. Intracellular protein transport is dependent on the formation of membrane vesicles that carry these protein cargoes from a donor to a target compartment. Protein cargos have sorting signals that are recognized by adaptor proteins. Ultimately, this allows for the formation of membrane vesicles that contain the appropriate cargo. Under stress conditions, cells activate a specialized type of membrane transport known as autophagy. During this cellular process, cellular components that are damaged or unneeded are degraded and recycled. When autophagy is not functioning properly in humans, this can lead to a variety of diseases like cancer, heart, liver, and neurodegenerative diseases. Our lab uses baker’s yeast to better understand the membrane trafficking events underlying autophagy. More specifically, we are trying to understand the function and trafficking of the yeast membrane protein, Atg27 (autophagy-related protein #27). Atg27 is composed of 3...
domains: luminal, transmembrane, and cytoplasmic. While the cytoplasmic domain is known to contain at least one sorting signal important for proper Atg27 localization, the function of the luminal domain is unknown. Our work aims to map additional sorting signals on Atg27 and determine the functions of the luminal domain. To do this, we have generated yeast expressing fluorescent Atg27 molecules lacking parts of its cytoplasmic/luminal domains. We evaluate the effects of these mutations using fluorescence microscopy and autophagy assays. Atg27 is also a potential protein cargo receptor. We are also interested in identifying protein cargo recognized by Atg27. High-throughput experiments done by other labs have identified potential binding partners for Atg27. We assess whether these proteins require Atg27 for their trafficking and localization.

P3164
Board Number: B449
GRASP55 senses energy deprivation through O-GlcNAcylation to promote autophagosome-lysosome fusion.
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The Golgi apparatus is the central hub for protein trafficking and glycosylation in the secretory pathway. However, whether Golgi responds to glucose deprivation is so far unknown. Here, we report that GRASP55, the Golgi stacking protein located in medial- and trans-Golgi cisternae, is O-GlcNAcylated by the O-GlcNac transferase OGT in growth condition. Glucose deprivation does not disrupt the Golgi structure, but reduces GRASP55 O-GlcNAcylation. De-O-GlcNAcylated GRASP55 forms puncta outside of the Golgi area, which colocalize with autophagosomes and late endosome/lysosomes. GRASP55 depletion reduces autophagic flow and results in autophagosome accumulation. Biochemically, GRASP55 interacts with LC3-II on the autophagosomes and LAMP2 in late endosome/lysosomes, and functions as a bridge between LC3-II and LAMP2 for autophagosome and lysosome fusion; this function is negatively regulated by GRASP55 O-GlcNAcylation. Therefore, GRASP55 senses the glucose level through O-GlcNAcylation and acts as a tether to regulate autophagosome maturation.

P3165
Board Number: B450
A switch in the specificity of an endosomal CORVET tether underlies formation of regulated secretory vesicles in the ciliate *Tetrahymena thermophila*.
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In the endocytic pathway of animals, two related complexes, called CORVET (Class C Core Vacuole/Endosome Transport) and HOPS (Homotypic fusion and protein sorting), act as both tethers and fusion factors for early and late endosomes, respectively. Mutations in CORVET or HOPS lead to trafficking defects and contribute to human disease including immune dysfunction. HOPS and CORVET
are conserved throughout eukaryotes but remarkably, in the ciliate Tetrahymena thermophila, the HOPS-specific subunits are absent while CORVET-specific subunits have proliferated. As uncovered via forward genetics, a single VPS8 paralog in Tetrahymena (VPS8A) is required to synthesize prominent secretory granules called mucocysts. More specifically, Δvps8a cells fail to deliver a subset of cargo proteins to developing mucocysts, instead accumulating that cargo in vesicles also bearing the mucocyst sorting receptor, Sor4. Surprisingly, although this transport step relies on CORVET, it does not appear to involve early endosomes. Instead, Vps8a associates with the late endosomal/lysosomal marker Rab7, indicating target specificity switching occurred in CORVET subunits during the evolution of ciliates. Mucocysts belong to a markedly diverse and understudied class of protist secretory organelles called extrusomes. Our results underscore that biogenesis of mucocysts depends on endolysosomal trafficking, revealing parallels with invasive organelles in apicomplexan parasites and suggesting that a wide array of secretory adaptations in protists, like in animals, depend on mechanisms related to lysosome biogenesis.

P3166
Board Number: B451
The effect of endomembrane transport inhibitors on octanoic acid resistance in Drosophila.
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Drosophila sechellia, a species of fruit fly that is endemic to the Seychelles islands has evolved dietary specialization to the fruit of Morinda citrifolia. The fruit from M. citrifolia produces octanoic acid as a defense mechanism at levels that are lethal to dietary generalist species of Drosophila including sister taxa D. melanogaster and D. simulans. Drosophila sechellia has developed resistance to octanoic acid allowing it to specialize on M. citrifolia and eat its fruit without lethality. The cellular mechanisms involved in D. sechellia resistance to M. citrifolia are unknown, but genetic analysis has alluded to a difference in endomembrane function between octanoic acid resistant D. sechellia and the more susceptible Drosophila species. To elucidate the potential cellular mechanisms that provide D. sechellia resistance to octanoic acid, D. simulans, D. melanogaster, and D. sechellia were given several drugs that inhibit specific routes of endomembrane transport. After exposure to each drug, the flies were introduced to food containing octanoic acid and death rates were measured and compared within and between species. Exposure to Retro-2, an inhibitor of retrograde transport from the early endosome to the endoplasmic reticulum through the trans-Golgi network, increases survivorship of individuals upon exposure to octanoic acid in all three species tested. These experiments are beginning to provide insight into the cellular mechanisms that confer toxin resistance in D. sechellia.

P3167
Board Number: B452
Major Facilitator Superfamily Domain-Containing 12 (MFSD12) regulates melanin synthesis from lysosomes.
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Mammalian hair, skin, and eye color arise from the presence and distribution of melanin pigments. Melanins are synthesized by pigment cell melanocytes in specialized subcellular organelles, named
melanosomes. Humans produce both eumelanin (brown and black) and pheomelanin (yellow and red) pigments. Melanin synthesis is disrupted in a number of human genetic diseases that cause skin and eye hypopigmentation, often associated with vision defects, for example oculocutaneous albinism (OCA). Variations in gene products that control melanin synthesis affect melanin quantity and distribution in humans and animal models. While hundreds of pigmentation genes have been identified, few of these are directly linked to normal, physiological pigment variation in ethnically diverse humans. Through genome-wide association studies, our collaborators have identified a novel gene that correlates with pigmentation levels in humans, Major Facilitator Superfamily Domain-containing 12 (MFSD12). Our preliminary data show that depletion of MFSD12 from immortalized mouse melanocytes increases melanin content. MFSD12 is a putative membrane transporter of unknown function, and our preliminary data show that it localizes predominantly to lysosomal membranes, not to melanosomes, and may regulate maintenance of lysosomal pH. This suggests that MFSD12 regulates melanin synthesis indirectly from lysosomes.

P3168
Board Number: B453
Studying human ATG4 homologs using ATG4 quadruple knockout (QKO) cells.
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Macroautophagy is a conserved cellular degradation process hallmarked by the covalent attachment of the ubiquitin like LC3/GABARAP family proteins to phosphatidylethanolamine (PE) on growing autophagosomes. One of the important regulators of this reaction is ATG4. It first primes LC3/GABARAP to expose the conserved carboxyl-terminal glycine to facilitate lipidation, while it also delipidates to release LC3/GABARAP for recycling. How does one protein family control both the production and removal of LC3/GABARAP-PE to ensure functional autophagy has been a central question in studying LC3/GABARAP and ATG4. Here, by utilizing a ATG4 quadruple knockout (QKO) HEK293 cell line generated by CRISPR/Cas9 in our lab, we aim to understand how the four human ATG4 homologs cooperate to exert priming and delipidation activities on seven LC3/GABARAP family proteins to regulate autophagy activity.

We first validated the ATG4 QKO by immunoblotting all four ATG4 homologs and saw complete loss of protein levels. We next examined four LC3/GABARAP homologs and observed complete loss of priming and lipidation, further supporting functional KO of all ATG4s. Notably, we can now robustly identify the pro-forms of LC3B and GABARAPL1 in our QKO. By rescuing the QKO with individual ATG4 homologs, we found that ATG4B alone can restore priming and lipidation of LC3/GABARAP family proteins when expressed at low levels comparable to endogenous levels. QKO rescued with a mutant ATG4B depleted of its carboxyl-terminal LC3 interacting region (LIR) showed accumulation of lipidated LC3/GABARAP, strongly suggesting it as an important delipidation-specific regulatory motif. In contrast, ATG4A is more specific to the GABARAP subfamily and can only partially restore GABARAPL1 priming when overexpressed. Lipidated GABARAPL1 also seems to accumulate, indicating ATG4A cannot fully support GABARAPL1 delipidation. In addition, lipidated GABARAPL1 does not accumulate after lysosomal inhibition, suggesting ATG4A alone cannot support functional autophagy. Lastly, ATG4C and ATG4D only showed very little priming activity towards GABARAPL1 when overexpressed individually. In summary, by systematically analyzing individual ATG4s in QKO, together with previous efforts examining their activity in vitro, we establish the framework of how different ATG4 homologs act on various LC3/GABARAP substrates to regulate autophagy.
P3169

Board Number: B454

Delipidation of mammalian LC3 proteins by each of the four Atg4 proteases.
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Autophagy is a bulk cellular degradation process where material to be degraded is encapsulated in an organelle called the autophagosome which grows de novo. The covalent conjugation of LC3 proteins to lipids in the growing membrane is required for proper autophagosome maturation. During this process, LC3 proteins undergo two proteolytic cleavage events. First, pro-form LC3 is cleaved at the COOH-terminus to reveal a glycine residue required for the lipidation reaction. The second event releases LC3 from the membrane, allowing for recycling of the LC3 and to facilitate fusion of the autophagosome with the lysosome. The Atg4 family of proteases, containing Atg4A, Atg4B, Atg4C, and Atg4D, drives both cleavage events but how Atg4 proteases are able to distinguish between soluble and lipid-anchored LC3 is not well-understood. By reconstituting the lipidation reaction in vitro, we are able to produce lipidated LC3 allowing us to directly test the delipidation activities of these four proteases. We show that Atg4B cleaves soluble LC3 orders of magnitude faster than the lipidated form, which is directly impacted by the anchoring of LC3 into the membrane, rather than the attachment of a lipid moiety. Further, although Atg4B is very efficient at cleaving soluble LC3, Atg4A displays more activity on lipidated substrates. Additionally, Atg4C and Atg4D can be activated by the removal of their expanded N-terminal domains to process lipidated LC3, which may also suggest roles for these homologs in specialized forms of autophagy. These results show that while all four Atg4 family members can be activated to similar enzymatic activities on lipidated substrates, only Atg4B efficiently processes the soluble form. Further, through mutagenesis of Atg4B, we have shown that the recognition of the lipidated form, but not the soluble form, is sensitive to a COOH-terminal LC3-Interacting Region. This differentiation is particularly notable with members of the LC3 substrate family. We suggest a model whereby Atg4B drives very fast priming of LC3 proteins while delipidation is inherently slow and regulated by Atg4 homologs.

P3170

Board Number: B455

Temperature-dependent sorting of fluorescent protein-tagged tyrosinases to the melanosome.
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Tyrosinase is a type I integral membrane glycoprotein that catalyzes melanin biosynthesis in mammals. Oculocutaneous Albinism Type 1B is caused by mutations that render human tyrosinase temperature-sensitive (ts). We used the murine tyrosinase Himalayan mutant as a model to study ts tyrosinase variants. Our main objective was to characterize the temperature-sensitive melanosomal targeting of full length murine tyrosinase-FP chimeras. To this end, we epistope-tagged the protein with a C-terminal EGFP or monomeric RFP and used epitope-directed antibodies for biochemical detection. Non-pigmented COS-7 and melanized 1011-mel human melanoma cells were transfected and selected for stable constitutive expression of FP-tagged Himalayan and wild type tyrosinases. Indirect immunofluorescence microscopy results showed that, while the wild type tyrosinase-FPs sort efficiently to the lysosome or mature melanosome in non-pigmented and pigmented cells respectively, both Himalayan tyrosinase-EGFP and -mRFP accumulate within the endoplasmic reticulum (ER) when
expressed at the nonpermissive temperature (37°C), as evidenced by coincident staining with ER markers. In contrast, the mutant variant is able to exit the ER and be transported to the lysosome/melanosome at the permissive temperature (31°C). Endoglycosidase treatment followed by immunoblotting with FP-directed antibodies showed that Himalayan tyrosinase-EGFP retained high mannose oligosaccharides, failing to reach maturity, which confirms ER retention at 37°C. We then assessed whether this ER retention of Himalayan-FP tyrosinase expressed at the nonpermissive temperature is reversible or irreversible. Cells were grown at 37 °C and then incubated with cycloheximide for 30 min, limiting subsequent analysis to polypeptides accumulated at the nonpermissive temperature by blocking new protein synthesis. Cells were then either maintained in cycloheximide at 37°C or shifted to 31°C, and analyzed by western blotting and indirect immunofluorescence microscopy. Our data suggest that some of the Himalayan-tyrosinase-EGFP variant may be rescued by a subsequent reduction in temperature. The reversible nature of this potential misfolding is consistent with increased substrate-induced melanization seen in Himalayan-expressing melanocytes and may open new avenues for the development of molecular therapeutics targeting the molecular changes associated with temperature-sensitive albinism. In addition, this FP-tagged tyrosinase may be used as a tool for kinetic experiments in live cells aimed at the further dissection of membrane trafficking pathways involved in the biogenesis of lysosome-related organelles.

P3171
Board Number: B456
The role of canonical and non-canonical autophagy in bone resorption by osteoclasts.
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Osteoclasts are cells specialized in the degradation of bone. To do this, osteoclasts form a lysosome-like membrane domain called the ruffled border (RB) that is contained within an actin-rich attachment site, called the sealing zone (SZ). Lysosomal vesicles fuse to the RB, thereby releasing their content into the extracellular space to degrade the underlying bone matrix. LC3, a marker of autophagic vesicles, localises to the RB, alluding to a potential role of autophagy in the resorptive function of osteoclasts. In vitro, 25% of osteoclasts from mice deficient in autophagy, through FIP200 deletion, still expressed GFP-LC3 at the RB. Through live cell imaging, GFP-LC3 was found to localise to the RB in a time-dependent manner, with osteoclasts in earlier stages of SZ formation having a strong concentration of GFP-LC3 within the SZ, which eventually dissipated as the SZ collapsed. The localisation of LC3 to the RB may be analogous to LC3-associated phagocytosis (LAP) in macrophages, an autophagy-independent pathway whereby LC3 is recruited to phagosomes to enhance phagosome maturation. In mice, autophagy deficiency through FIP200 deletion led to a modest decrease in bone density, which corresponded to an increase in bone resorption as measured in vitro.

LAP is critically dependent on the protein, Rubicon. In osteoclasts generated from the RAW 264.7 macrophage cell line, siRNA knockdown of Rubicon resulted in a modestly increased (13%) resorption of mineralized substrate compared to controls. Studies in Rubicon knockout mice are required to confirm this leads to a change in bone mass. Using live cell imaging with LysoTracker and GFP-ezrin, we examined lysosomal vesicle targeting to the RB and sealing zone dynamics, respectively. We observed no significant differences between Rubicon knockdown osteoclasts and controls.

Earlier studies in bone diseases, such as Paget’s disease of bone, have illustrated that dysregulation of autophagy in osteoclasts can result in serious bone pathology. Our data add to this by suggesting that
both canonical autophagy and LAP-like pathways modulate the resorptive capacity of osteoclasts during normal bone homeostasis.

**P3172**  
**Board Number: B457**  
**Host cells degrade the*H. pylori*pore-forming toxin, VacA, to resist cell death.**

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Helicobacter pylori persistently colonizes the gastric mucosa of more than half of the world population, with prevalence as high as 90% in developing nations. Infection with*H. pylori*causes chronic gastric inflammation and is the leading cause of stomach ulcers and gastric cancer. Both the ability of*H. pylori*to colonize the stomach and the risk of developing*H. pylori*-mediated disease is directly associated with the secretion of a pore-forming virulence factor called vacuolating cytotoxin A (VacA). VacA has been reported to trigger a wide range of cellular responses including cellular vacuolation, plasma membrane permeability, disruption of mitochondrial membrane permeability, and apoptosis. The mechanisms by which these processes occur are not yet fully understood. During our investigation into VacA toxicity, we observed that host cells recover from VacA-induced vacuolation and grow to confluency. Furthermore, we found that VacA-induced cell death is dependent on the presence of supplemental ammonium chloride (NH₄Cl), a commonly used autophagy inhibitor. Therefore, we hypothesized that host cells are able to degrade VacA via autophagy. By assessing the intracellular levels of VacA both by western blot and light microscopy, we have shown that host cells degrade VacA and that supplemental NH₄Cl and chloroquine inhibit VacA degradation. We found that, over time, VacA colocalizes with late endosomes and autophagosomes, suggesting that VacA is trafficked to autophagosomes. Additionally, in contrast to a proposed model where VacA is trafficked to mitochondria to induce apoptosis, we do not observe VacA localization to mitochondria. We propose a model in which host cells traffic VacA to autophagosomes to be degraded by autophagy, thereby permitting cellular recovery from VacA-induced toxicity.

**P3173**  
**Board Number: B458**  
**Single Cell Analysis of Vacuolar pH Using Confocal Microscopy.**

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Vacuole and lysosome functions—including ion storage and protein degradation—play large roles in human diseases such as lysosomal storage disorders and Alzheimer’s. In order for these functions to occur properly, vacuolar pH must lie within a specific range. Due to the breadth of the vacuole’s function, pH regulation has direct implications in cellular age. Despite this, the relationship between vacuolar pH and the size and shape of the vacuole has remained understudied. The proton pump for vacuoles, the V-ATPase, is a protein complex that spans the vacuole membrane. An increase in the vacuole’s membrane surface will increase the number of proton pumps present on the vacuole, and should therefore lower the pH. To test our hypothesis about the relationship between vacuole...
morphology and vacuolar pH, we use confocal microscopy to collect single cell data comparing the surface area and volume of yeast vacuoles to their pH. The dye, BCECF will be used to measure pH within yeast vacuoles. At the same time, the lipophilic dye, FM-4-64 will be used to stain the vacuole membrane. Then, using computational image analysis, we will reconstruct three-dimensional models of vacuoles to measure their volume and surface area. If our hypothesis is accurate, we predict that there would be an inverse relationship between vacuolar pH and the surface area of the vacuole.

Polarity in Development

P3174
Board Number: B460
Rab11/Fip5 regulates formation of the terminal web and is necessary for microvilli stabilization during zebrafish intestinal development.
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Formation of a hollow tube of cells, or lumen, is an essential morphogenetic event that occurs repeatedly throughout development and results in the formation of many organ types, including the gut, kidneys, trachea, and neural tube. A crucial first step in lumen formation is establishment of apical-basal cell polarity, such that opposing cellular domains are specialized for unique functions. In the gut, the apical side of intestinal cells are lined with specialized membrane protrusions called microvilli that enhance the cell surface area and aid in nutrient absorption. These microvilli are anchored in an actin- and intermediate filament-rich cytoskeletal network, known as the terminal web, located just below the apical cell surface. Despite the importance of microvilli in intestinal cell function, the molecular machinery that governs apical microvilli formation remains largely unclear. Here we use zebrafish as a model system to determine the role of a Rab11 effector, Fip5, in apical lumen formation and microvilli establishment during gut development. Using CRISPR gene editing, we created loss-of-function mutations for both fip5a and fip5b (zebrafish have two paralogs of fip5). Alone, neither fip5a nor fip5b homozygous mutant fish show a strong phenotype, suggesting that they can compensate for one another. However, transmission electron microscopy analysis on the gut of homozygous fip5a and fip5b double mutant fish revealed severe defects in apical trafficking and microvilli stability. At 3 days post fertilization, the gut cells of embryos show structures that resemble microvilli inclusion bodies, which are cleared by 6 days post fertilization and replaced with an accumulation of enlarged apical endosomes up to several microns in size. By 11 days post fertilization, the fish have progressively worsened, now showing large gaps between cells, suggesting defects in cellular junction integrity and deformed apical cell caps. These defects appear to be caused by a disruption in targeting Rab11-endosomes to the apical plasma membrane since Rab11 is mislocalized to the basolateral side in fip5a;fip5b double mutant fish. Significantly, we also found that cytokeratin, a major component of the terminal web, is mislocalized from the apical side of cells suggesting that Rab11/Fip5 endosomes may play an essential role in formation of the terminal web and stabilization of microvilli throughout gut development.
P3175
Board Number: B461
Vangl1/2 function in neural tube convergence and extension.
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Anterior-posterior elongation of the embryo is fundamental for early vertebrate development and body plan formation. Tissue elongation is driven by convergence and extension (CE), in which the tissue undergoes narrowing along one axis and extension along the perpendicular axis. In the mouse embryo, morphogenesis of the early neural tube involves CE, driven by epithelial cell intercalation where neighbor cells move between each other in a polarized way, as well as bending and closure of the neural plate into a tube, driven by epithelial cell shape change and cytoskeletal reorganization. At the genetic and molecular level, components of the planar cell polarity (PCP) pathway have been shown to regulate these polarized cell behaviors. Mutations of the Van Gogh like 2 (Vangl2) gene, lead to mice with a short body axis and open neural tube in the hindbrain and spinal cord regions, known as craniorachischisis (CRN). Previous data from our lab showed that the Vangl2 Loop tail (Lp) mutant, which corresponds to a point mutation in the C-terminal end of Vangl2, affects cell intercalation efficiency but not polarity of neural plate cells. As the close homolog Vangl1 has a similar localization to Vangl2 in the neural plate, our hypothesis is that Vangl1 may compensate to maintain normal polarity in the absence of Vangl2. Using a line carrying knockout alleles of both Vangl genes we find that loss of Vangl1 and 2 (Vangl1gt/gt;Vangl2ko/ko) affects the polarity and the efficiency of cell intercalation, while the presence of one copy of Vangl1 (Vangl1gt/+;Vangl2ko/ko) rescues both polarity and efficiency. In contrast, the polarity of intercalation is lost in embryos lacking Vangl1 (Vangl1gt/gt;Vangl2ko/+), and one copy of Vangl2 is not sufficient to rescue this effect. The apical area of neural cells is significantly increased in both Vangl1gt/+; Vangl2ko/ko and double mutant embryos, compared to the double het (Vangl1gt/+; Vangl2ko/+), and the actin filament organization is disrupted, suggesting a role of Vangl2 in apical constriction. These data show that Vangl1 and Vangl2 together cooperate to maintain cell behavior and to drive neural tube morphogenesis. Furthermore, they suggest that the polarity of CE is primarily regulated by Vangl1 while Vangl2 is responsible for regulate the apical changes that drives neural tube bending and closure.

P3176
Board Number: B462
Perturbations of intracellular flows explain and invert the transport-dependent PAR polarization of C. elegans zygotes.
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Throughout the last decades, access to genetic and chemical perturbations boosted our molecular-level understanding of cell biological processes. However, it was suggested that the spatio-temporal organization of cells and developing embryos also depends on physical transport processes such as diffusion, cytoplasmic streaming, and cortical flows. And thus far, it remains a challenge to unravel the function of physical transport during morphogenesis due to the lack of suitable perturbation methods for in vivo systems.
Here, we exploit thermoviscous pumping (Weinert & Braun, J. Appl. Phys. 2008) to induce intracellular flows in single cells and developing embryos. This firstly allows to systematically dissect the role of flows during PAR polarization. We find that i) cytoplasmic flows towards the membrane drive PAR loading locally, ii) cytoplasmic flows parallel to the membrane efficiently couple to the actomyosin cortex, causing long range cortical flows. iii) These induced cortical flows move pre-established PAR domains. iv) While small displacements of PAR domains are being self-corrected and cells divide normally, asymmetric cell division is inverted as soon as PAR domains were rotated across the anterior-posterior axis.

Taken together, these perturbation experiments directly show that intracellular hydrodynamics are essential for PAR polarization and reveal a robust mechanism by which flows alter the entire body axis of the embryo. Furthermore, our findings suggest that asymmetric cell division is mediated by a self-amplifying, binary decision process with high robustness against imperfect PAR positioning. Finally, we emphasize more general opportunities to dissect the principles of transport-dependent organization of living systems in space and time.

P3177

Board Number: B463

Counter-rotational cell flows drive morphological and cell fate asymmetries in mammalian hair follicles.
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Organ morphogenesis is a complex process dependent on the interplay between cell fate specification, epithelial-mesenchymal interactions, and tissue polarity. The emergence of the regularly spaced, globally aligned pattern of hair follicles across the mammalian skin is a striking example of organ morphogenesis that arises through coordinate epidermal-dermal signaling and planar polarized morphogenesis controlled by the planar cell polarity (PCP) pathway. Here, using a combination of live imaging, automating cell tracking, mouse genetics, and laser ablation, we discover a novel program of PCP-dependent cell rearrangements that drive planar polarization and polarized cell fate specification of mammalian hair follicles. Upon specification, hair placode epithelial cells undergo dramatic cell rearrangements that generate a counter-rotational pattern of cell flows. These movements reposition placode cells along the anterior-posterior axis such that posterior cells converge toward the center of the placode and flow anteriorly, while anterior and lateral cells flow outward and posteriorly toward the placode rear. Counter-rotational cell flows are driven by myosin-dependent, spatially polarized cell neighbor exchanges where asymmetric PCP protein localization correlates with junction shrinkage and disassembly. Strikingly, this pattern of cell rearrangements generates not only the morphological polarization of the hair follicle, transforming the placode from vertical to anterior-directed growth, but also directs the planar polarized organization of progenitor fates upon which the future hair follicle is built. Specifically, Shh-expressing hair germ progenitors shift from a central to anterior location while an outer ring of Sox9-expressing stem cell progenitors moves posteriorly toward the placode rear. Cell fate specification also feeds into polarized morphogenesis, as counter-rotational movements are lost in the absence of radially symmetric cell fates. Further, we show that PCP-dependent cell rearrangements displace a crucial stromal signaling center - the dermal condensate - toward the anterior, where it is necessary for the maintenance of polarized hair germ and stem cell progenitor fates. Our results define the cellular mechanisms that generate planar polarity in complex multicellular structures and demonstrate how a simple pattern of local cell rearrangements directs both morphological and cell fate asymmetries.
P3178
Board Number: B464
The ciliary GTPase Arl3 maintains tissue architecture by directing Planar Cell Polarity (PCP) dependent mitotic spindle orientation during epidermal morphogenesis.
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Planar cell polarity (PCP) can modulate mitotic spindle orientation in the context of tissue morphogenesis, but the underlying regulatory mechanisms are largely unknown. During mammalian skin development, oriented cell divisions are biphasic. Well-established polarity pathways drive asymmetric cell division (ACD), positioning the mitotic spindle perpendicular to the tissue plane in conserved process essential for epidermal differentiation. Basal stem cells (SCs) must also undergo self-renewing planar cell divisions during skin development and regeneration, but it is unknown whether defects in this process can have detrimental consequences for the maintenance of tissue architecture. Using in utero gene targeting in developing mouse embryos, here we show that depletion of ciliary GTPase Arl3 from basal SCs causes severe defects during skin development: abnormal expansion of progenitor cell populations, loss of epidermal integrity and skin barrier deficiency. Interestingly, Arl3 KD resulted in unexpected defects to mitotic spindle orientation: planar cell divisions, specifically along the anterior-posterior (A-P) tissue plane, were significantly diminished. Loss of self-renewing planar cell divisions in basal SCs depleted of Arl3 was balanced by increased ACD, hyper-proliferation of progenitor cell populations and expansion of suprabasal cell layer. These observations suggest that an Arl3-dependent mechanism maintains cell division polarity along the A-P tissue axis, and that disruption of planar spindle orientation has detrimental consequences to epidermal architecture. We hypothesized that loss of planar mitotic spindle orientation could be a consequence of defective PCP signaling. In Arl3 KD epidermis, PCP signaling molecule Celsr1 fails to maintain its polarized distribution at cellular junctions across the A-P tissue plane. This resulted in defective hair follicle angling, a hallmark of disrupted PCP during skin development. Clonal analysis in mosaic embryos revealed that Arl3 is required for the maintenance of Celsr1 polarity and transduction of PCP to neighboring basal cells. In the absence of Celsr1, Frizzled 6 loses its asymmetrical distribution in the plane of the tissue and becomes abnormally segregated to the apical cortex of basal SCs. Celsr1 and transferrin internalization experiments suggest that Celsr1 is normally endocytosed during mitosis, but that Arl3-dependent recycling of PCP components may be required for their polarized membrane segregation. We propose that Arl3 regulates polarized endosomal trafficking of PCP components to orient cell divisions in the plane of the epithelium. Cell-cell communication via small GTPase signaling likely plays a crucial role specifying planar mitotic spindle orientation during tissue morphogenesis.

P3179
Board Number: B465
TRANSCRIPTIONAL DYNAMICS OF SINGLE-CELL REGENERATION IN THE CILIATE STENTOR COERULEUS.
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In many multicellular organisms, the ability to regenerate tissue is lost through the course of development. Some organisms—including flatworms, salamanders and zebrafish—retain regenerative potential throughout their lifetimes and are powerful systems for uncovering mechanisms of tissue

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regeneration, particularly in regards to cell-to-cell signaling. Repair at the single cell scale is also critical for an organism’s wound healing response; however, little is known about regeneration at the level of an individual cell. Here, I present our efforts to develop a unicellular model for studying regeneration and wound repair in the ciliate, Stentor coeruleus. In addition to its remarkable size (cells can reach up to 1 mm in length) and subcellular complexity, Stentor has incredible regenerative abilities: almost any portion of the cell, when removed through excision, will give rise to a normally proportioned cell with intact subcellular organization. Pioneering studies of Stentor elucidated many morphological principles of its regeneration (Morgan, 1901; Tartar, 1961), but we have much to learn about the molecular basis of Stentor’s regenerative and healing abilities. To gain a molecular foothold into the process, we have recently sequenced and annotated the genome of Stentor coeruleus. Now, using RNA-seq I am describing the transcriptional dynamics underlying the regeneration of a key organelle in the cell -- the oral apparatus (OA). This complex organelle is one of the most prominent features of the cell, composed of thousands of cilia. Cells can be induced to shed and regenerate the OA upon osmotic shock. Preliminary results indicate that after shedding, conserved genes involved in centriole production are expressed in two temporal waves, separated by an hour. Using this approach, we can identify novel genes involved in the process of centriole and cilia biogenesis. The detailed transcriptional time course, in combination with RNAi manipulations, will further reveal the genetic networks that regulate OA regeneration, and will help elucidate the fundamental principles of cell regeneration and healing at the scale of a single cell.

P3180
Board Number: B466
The DEP domain protein LET-99 regulates spindle positioning downstream of multiple polarity cues in C. elegans.
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Asymmetric divisions produce daughter cells with different fates, and thus are critical for animal development. During asymmetric divisions, the mitotic spindle must be positioned on a polarized axis to ensure the differential segregation of cell fate determinants into the daughter cells. Spindle positioning is also important in symmetrically dividing cells to produce specific cell arrangements. In many cell types a cortically localized complex consisting of Gα, GPR, and LIN-5 (Gα/Pins/Mud, Gα/LGN/NuMA) mediates the recruitment of dynein, which exerts pulling forces on astral microtubules to physically position the spindle. In the C. elegans one-cell embryo, the conserved PAR polarity proteins organize into anterior and posterior domains, which regulates the asymmetry of cortical GPR/LIN-5 to align the spindle on the anterior-posterior (AP) axis. In contrast, at the 4-cell stage in the endomesodermal precursor cell (EMS), the PAR proteins are localized to inner/outer domains. Nonetheless, EMS divides asymmetrically with its spindle oriented on the AP axis, and this alignment depends on partially redundant Wnt and MES-1/SRC-1 signaling pathways initiated from a neighboring cell. The mechanisms connecting cell signaling to the spindle positioning machinery remain to be determined. We previously identified LET-99, a DEPDC1 family protein, as a key intermediate for spindle positioning. LET-99 is localized to the cortex/membrane and is restricted to a posterior lateral band by the PAR proteins in the one-cell; genetic and biochemical data suggest that LET-99 may be a target of the PAR kinases PAR-1 and PKC-3. LET-99 in turn inhibits the cortical recruitment of GPR/LIN-5 to Gα and is essential for the dynamic asymmetric localization of GPR/LIN-5. More recently, our analyses of fast-inactivating temperature sensitive mutants revealed that LET-99 also acts in the MES-1/SRC-1 pathway for EMS
spindle positioning; LIN-5 is required for EMS spindle positioning as well, potentially in a Ga independent manner. To further elucidate the MES-1/SRC-1 pathway and test the hypothesis that PAR proteins regulate spindle orientation in the EMS cell, we examined division patterns using temperature sensitive par-1 and pck-3 mutations. The data suggest that PAR-1 may act in the Wnt pathway, but we found no role for PKC-3. In addition, we found that the PAR-1 related kinase PIG-1 acts in the MES-1/SRC-1 pathway, as does the small G protein Rac. LET-99 is also needed (redundantly) for membrane furrowing during cytokinesis. Rac regulation is important during cytokinesis and LET-99 has a domain with weak homology to RhoGAP proteins. We are currently testing the hypothesis that LET-99 associates with Rac and that this is important for cytokinesis and/or EMS spindle orientation.

P3181
Board Number: B467
Par3 interacts with Prickle3 to maintain planar cell polarity (PCP) in the vertebrate neural plate. I. Chuykin1, O. Ossipova1, S. Sokol1;
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The planar cell polarity (PCP) coordinates cell orientation in embryonic epithelia and controls vertebrate neural tube closure. In the Xenopus neural plate, the core PCP proteins Prickle3/Vangl2 localize to the anterior apical domain of each cell, but the significance of this apical localization has been unclear. We show that the apical polarity protein Par3/Pard3 is also planar-polarized in the neural plate, pointing to a possible role of Par3 in PCP. Supporting this view, interference with Par3 function inhibited neural plate PCP and neural tube closure. Mechanistically, Par3 physically associated with Prickle3 and promoted its apical localization. Overexpression of a Prickle3-associating Par3 fragment disrupted PCP in the neural plate. Moreover, a Par3 construct lacking Prickle3 binding lost the ability to inhibit body axis elongation commonly associated with PCP defects. Based on these results, we propose a new mechanistic link between Par and PCP components that is essential for neural tube closure.

P3182
Board Number: B468
Protein palmitoylation as a mechanism of asymmetric protein localization during cell division. E. Stypulkowski1, E. Witze1;
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Asymmetric cell division results in two distinctly fated daughter cells to generate cellular diversity during development and tissue homeostasis. A major molecular hallmark of an asymmetric division is the unequal partitioning of cell-fate determinant proteins. However, the mechanisms driving the asymmetric targeting and localization of these determinants to the membrane remains unclear. Protein palmitoylation is a dynamic post-translational modification which regulates protein localization between the cytosol and membrane domains. We have previously established that Wnt5a-mediated signaling promotes protein depalmitoylation to foster polarized protein localization. We present protein palmitoylation as a key mechanism for the asymmetric partitioning of the cell-fate determinants Numb (Notch antagonist) and β-catenin (canonical Wnt regulator). Using point mutants, we show specific palmitoylated residues on proteins are required for asymmetric membrane localization. Furthermore, we find that palmitoylation components can restrict Notch and Wnt transcriptional activity to one daughter cell. Moreover, we show altering the expression of the depalmitoylating enzyme, APT1, changes the transcriptional signatures of human breast cancer cells to those resembling that of Notch and β-catenin. Together, these findings demonstrate that palmitoylation is a key mechanism of
asymmetric cell division regulating Notch and Wnt-associated protein dynamics, gene expression, and cellular functions.

P3183
Board Number: B469
Rapid diffusion state switching underlies stable cytoplasmic gradients in the C. elegans zygote.
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Shortly after fertilization of the C. elegans embryo, the cytoplasmic RNA-binding proteins MEX-5 and PIE-1 rapidly form opposing concentration gradients that span the 50-um length of the cell. PAR-1, a conserved cell polarity regulator localized on the posterior cortex, phosphorylates MEX-5 and increases its diffusion in the posterior cytoplasm, leading to the preferential retention of MEX-5 in the anterior (Tenlen 2008, Daniels 2010, Griffin 2011). Through mechanisms that are not understood, high concentrations of MEX-5 in the anterior increases the diffusion of PIE-1, giving rise to posterior-rich concentration gradient of PIE-1 (Daniels 2009, Wu 2015).
In this study, we use Near-TIRF imaging and single-particle tracking to characterize the reaction/diffusion dynamics that maintain the MEX-5 and PIE-1 gradients. We find that both proteins interconvert between fast-diffusing and slow-diffusing states on time scales that are much shorter (seconds) than that of gradient formation (minutes). We have estimated the kinetics of MEX-5 and PIE-1 switching between slow and fast-diffusing states from the appearance rate of slow-diffusing particles and by smPReSS analysis (Robin 2014). We find that the kinetics of diffusion-state switching are strongly polarized along the A/P axis by the PAR polarity system such that fast-diffusing MEX-5 and PIE-1 particles are ~symmetrically distributed whereas slow-diffusing particles are highly enriched in the anterior and posterior cytoplasm, respectively. Using mathematical modeling, we demonstrate that our estimates for the kinetics of MEX-5 and PIE-1 diffusion-state switching are sufficient to account for their respective concentration gradients. The reaction/diffusion mechanisms characterized here provide a quantitative framework for understanding how polarity cues at the cortex control the partitioning of factors in the cytoplasm.

P3184
Board Number: B470
Basal cell shape influences division orientation in the mammalian epidermis.
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The ability to control cell fate through oriented cell division is imperative for the proper development of many organs, such as the stratified epidermis. Basal stem cells of the epidermis can divide in two ways: 1) perpendicularly to the epithelial plane to produce one basal and one suprabasal daughter that goes on to differentiate, and 2) parallel to the epithelial plane, which generates two basal daughter cells and expands the stem cell pool. While mechanisms leading to perpendicular divisions are known, those orienting planar divisions have not been explored. Late in embryonic skin development, apical-basal polarity factors align the spindle to promote perpendicular divisions. Thus, we hypothesized that parallel cell divisions might be controlled by planar cell polarity (PCP), which relies on a set of cortical “core” transmembrane components that are asymmetrically localized along the epithelial plane. The goal of this project was to determine a potential role for planar cell polarity in orienting basal cell divisions that occur within the plane of the epidermis. In the absence of PCP, such as in the Vangl2Δp/Δp mutant,
embryos exhibit increased perpendicular, asymmetric divisions at the expense of planar, symmetric divisions. This defect was not due to increased proliferation rates, nor to mislocalization of cortical spindle anchoring proteins like LGN. Rather, we link the reduction in planar divisions to alterations in cell shape, which are indirectly caused by the neural tube defects characteristic of PCP mutants. We demonstrate that early in epidermal stratification, there is a close relationship between interphase cell height and mitotic spindle orientation. Moreover, failure of the epidermis to close over the neural tube in PCP mutants leads to cell crowding and a lower frequency of planar cell divisions. We propose a model in which basal epidermal cells utilize shape, rather than cortical PCP cues, to inform planar division orientation.

P3185
Board Number: B471
Oligomerization mediates self-stabilizing cortical asymmetry of the keystone polarity protein PAR-3.
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The PAR proteins constitute a highly conserved biochemical module that forms and stabilizes cellular asymmetries in a wide variety of contexts and in response to a wide variety of cues. During polarization of the C. elegans zygote, a transient cue triggers the dynamic segregation of PAR proteins into complementary anterior and posterior domains. Mutual antagonism between anterior and posterior PAR proteins plays a central role in stabilizing this pattern. However, the anterior PAR protein PAR-3 is unique in that it is required for all other PAR asymmetries, but remains stably asymmetric when all other PAR proteins are either absent or uniformly distributed. A key property of PAR-3 is its ability to oligomerize, which is required for its cortical localization. Here we show that oligomerization mediates dynamically self-stabilizing PAR-3 asymmetries in the absence of mutual antagonism. Combining single-molecule imaging and particle tracking, we show that PAR-3 monomers bind weakly to the membrane where they assemble rapidly and reversibly into oligomers. We show that oligomer dissociation rates decrease sharply with oligomer size. We further show that recruitment rates of PAR-3 monomers are five-fold higher on the anterior cortex where PAR-3 oligomers are enriched and that this bias depends strongly on the ability of PAR-3 to oligomerize implying positive feedback in which cortical PAR-3 oligomers directly bind/recruit cytoplasmic monomers. Using a quantitative model with parameter values constrained by experimental measurements, we find that weak membrane binding of PAR-3 monomers, reversible assembly into slowly-dissociating cortical oligomers, and positive feedback on monomer recruitment is sufficient to explain the stable persistence of a PAR-3 enriched cortical domain. Our model predicts that the PAR-3 domain will self-focus over time through dynamic competition among PAR-3 oligomers for a finite pool of PAR-3 monomers. However, the domain size will be effectively stable over the timescale of polarization given a sharp decrease in oligomer mobility with oligomer size and sufficiently slow exchange of oligomer subunits, which we confirm experimentally. Our results reveal a novel mechanism that underlies the dynamic stabilization of cortical polarity in the C. elegans zygote. We hypothesize that variants of this mechanism, involving dynamic exchange and oligomerization of membrane-associated proteins, may operate in other cells to stabilize polarized states.
In the vertebrate retina, the different neuronal cell types required for visual perception are organized within specific layers. Light is detected by rod and cone photoreceptors (PRs) located in the outer nuclear layer (ONL), which then convey visual information to interneurons located in the Inner Nuclear Layer (INL) via synapses that form in the outer plexiform layer (OPL). Amacrine cells located in the basal-most region of the INL modulate the stimulation of ganglion cells of the Ganglion Cell Layer (GCL) mediated by bipolar cells. How each neuron is stereotypically segregated in specific layers and wires into a functional circuitry remains largely unknown. Here we hypothesized that regulators of cell polarity might be involved. Among the major polarity pathways, the partitioning defective (Par) proteins have the broadest function in a multitude of cell types and organisms. Since some Par proteins, such as Par3, are sufficient to induce polarization in isolated cells, they are classically referred to as cell intrinsic regulators of polarity. In the mammalian retina, Par3 localizes in tight junction at the apical membrane of retinal progenitor cells (RPCs) during development, and in tight junctions formed between PRs and Müller cells in the adult retina. To investigate the mechanisms of spatial segregation of neurons, we conditionally knocked-out Par3 in RPCs using the alpha-Pax6:Cre mouse line. Par3 cKO showed an early disruption of the retinal apical membrane and displaced mitosis at E14 and P0. By P14, Par3 cKO displayed a disrupted apical membrane and OPL. Apical distribution of PR, bipolar and horizontal cell nuclei was disturbed but, surprisingly, Par3 inactivation did not affect lamination of the basally-located amacrine cells, Müller glia, or Ganglion cells, or the formation of the inner plexiform layer (IPL), suggesting that the molecular mechanism regulating lamination of the inner retina is different and independent from outer retina lamination. Although the retina was dramatically disorganized, cell fate determination did not seem affected, but PRs rapidly degenerated, with a 90% decrease between P14 and P60, indicating that Par3 is required for photoreceptor survival. To refine the cellular mechanism mediating Par3 function, we lineage-traced Par3 KO RPC clones using the GlastCreERT2 mouse line induced at E14.5. Surprisingly, clonal deletion of Par3 did not lead to PR, bipolar or horizontal cell displacement, indicating that Par3 functions cell non-autonomously to regulate lamination in the outer-retina. Together, these results identify Par3 as a regulator of retinal lamination and photoreceptor survival.

**Neuronal Degeneration and Regeneration**

**P3187**

**Board Number: B474**

Peripheral nervous system changes during senescence in MDX mice.

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Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by mutations in the dystrophin gene, generating critical protein malfunction. The dysfunctional dystrophin leads to degeneration of skeletal muscle fibers which causes retrograde changes that affect the peripheral...
nervous system (PNS). Further degeneration may occur with the aging process, what has been poorly investigated. In this sense, the present work evaluated the motor function and sciatic nerve microenvironment during senescence in MDX mice. C57BL/10 mice were used as the control strain. Thus, we used fifteen mice from each strain (control and mutant), which were evaluated at six, twelve, and eighteen months old. The motor function was monitored using the walking track test (Catwalk system). The animals were sacrificed and the sciatic nerves processed for immunohistochemistry (neurofilament and p75NTR antisera). The results showed that MDX mice present a significant motor deficit (two-fold decrease) as compared to six months old control animals. Such decreased SFI, although less intense, was detected at twelve months (172%) and eighteen months (117%). The Max Contact Max Intensity decreased 13%, 19% and 21% in MDX mice as the result of aging (6, 12 and 18 months old). p75NTR immunolabeling increased 77%, 79% and 72% in MDX mice and Neurofilament immunolabeling decreased 30%, 46% and 56% in MDX mice throughout the lifespan. Overall, the results herein indicate that the constant cycles of muscle degeneration/regeneration in MDX mice cause retrograde structural changes in the sciatic nerve microenvironment, directly affecting the homeostasis of the PNS during the course of DMD.

P3188
Board Number: B475
Protective effects of pregabalin in the spinal cord microenvironment in MDX mice submitted to sciatic nerve axotomy.
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Duchenne muscular dystrophy (DMD) causes degeneration of skeletal muscle fibers resulting in retrograde changes that affect the central nervous system (CNS). In turn, spinal motoneurons show the typical signs of chromatolysis and loss of synapses in the early stages of the disease. Pregabalin was designed as a potent successor to a related drug, gabapentin. Pregabalin binds to the alpha2-delta subunit of the voltage-gated calcium channel in the CNS. Among its neuroprotective effects are modulation of excitatory neurotransmitters, anti-apoptotic and anti-inflammatory effects and downregulation of astrogliosis and microglial reaction in the central nervous system. In this sense, the present work studied the effects of pregabalin in the spinal cord microenvironment in MDX mice (experimental model for the study of DMD) during disease. C57BL/10 mice were used as the control strain. MDX (n=10) and C57BL/10 (n=5) were submitted to the axotomy of the left sciatic nerve in the sixth week of life, stimulating retrograde reaction and glial reaction in the spinal cord microenvironment. The mice were treated with 30 mg/kg/day of pregabalin i.p, seven days before the nerve injury and seven days after the injury until euthanasia. Vehicle counterparts received saline injections. The spinal cords were obtained and processed for immunohistochemistry (GFAP and synaptophysin antisera). The results showed a 60% reduction in GFAP expression in the MDX group treated with pregabalin relative to the MDX saline group (MDX+pregabalin contralateral – 9,082±599, MDX+saline contralateral – 15,070±487, p<0.005; MDX+pregabalin ipsilateral – 12,293±535, MDX+Saline ipsilateral – 19,195±360, p<0.005 – integrated density of pixels at spinal cord lamina IX of Rexed). Also, an increased expression of synaptophysin was obtained (MDX+pregabalin contralateral – 9,836±122, MDX+saline contralateral – 7,979±131, p<0.005; MDX+pregabalin ipsilateral – 7,447±271, MDX+saline ipsilateral – 5,153 ± 212, p<0.005). The comparison of MDX and C57BL/10 mice showed that pregabalin ameliorates the effects of DMD on motoneurons. Overall, the present results indicate that a pregabalin can be used to protect the CNS from the chronic degenerative processes during the course of DMD.
Prions are unconventional infectious agents that cause invariably fatal neurodegenerative diseases including Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, and chronic wasting disease in deer and elk. These diseases are caused when a host-encoded GPI-anchored glycoprotein, mammalian prion protein (PrP), misfolds from its cellular form (PrP\(^c\)) into a disease-associated, aggregation prone, self-replicating conformation (PrP\(^\Delta\)). While prions (PrP\(^\Delta\)) are unorthodox infectious agents since they lack coding nucleic acids, like traditional infectious agents, interspecies transmission of prions via natural routes occurs efficiently and is characterized by high infectivity titers. Interestingly, the efficiency of interspecies prion infection varies widely among mammalian species even though the primary sequence of PrP\(^\Delta\) is highly conserved between species. At the extreme ends of this spectrum, rabbits are nearly resistant to infection, while the bank vole (Myodes glareolus) appears to be a uniquely universal recipient. Previous work has shown that the conversion of native PrP\(^\Delta\) into PrP\(^\Delta\) in vitro requires species-specific glycosylation patterns and cofactor molecules. Here, we test the hypothesis that specific glycosylation and cofactor requirements play an important role in determining the host range of prion replication using the bank vole model.

Approximately 1.5 million Americans suffer from traumatic brain injury (TBI), which may lead to Alzheimer’s disease (AD) like condition. An estimated 56 billion dollars are spent as a result of TBI and about 90,000 Americans suffer from long-term disability. Uprising stress, busy lifestyle and unhealthy food habits are the major causes of traumatic brain injury. The majority of the treatment strategies are based on the improvement of cholinergic function in the brain and one of the emerging therapeutic target is to enhance the acetylcholine level in the brain. Standardized botanical extracts including Huperzia serrata, Convolvulus pluricaulis (Shankhapushpi; SP) and Celastrus paniculatus (Jyotismati; JY) are long been known to attenuate brain function and well-being. Research studies have demonstrated the versatile roles of these novel phytochemicals as natural acetylcholinesterase inhibitors and neuroprotectants, and exhibited their efficacy in the management of neurological or memory impairment, dementia, Parkinson’s and Alzheimer’s disease. In the recent past, we developed a novel, standardized Huperzia serrata extract, CogniUp\textsuperscript{TM} enriched with 1% Huperzine A, which demonstrated broad spectrum safety and neuroprotective abilities. In this study, we developed a unique
combination of CogniUp, SP and JY (MZ001), which can effectively and synergistically inhibit acetylcholinesterase (AChE) inhibition. Based on our initial assessment, concentration-dependent AChE inhibition kinetics was assessed individually using 0, 3.0, 6.0, 9.0, 12.0 and 24.0 mg SP/ml, while AChE inhibition kinetics was assessed individually using 0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg JY/ml. Simultaneously, concentration-dependent AChE inhibition kinetics was assessed individually using 0, 0.0625, 0.125, 0.25 and 0.5 μg CogniUp/ml. Several combinations were assessed and ultimately, we found that a combination of SP, JY and CogniUp (12 mg/ml, 4 mg/ml and 0.125 μg/ml) provided the most efficacious and synergistic AChE inhibition. No significant adverse events were observed. Further studies are in progress to establish the therapeutic efficacy of our unique proprietary combination- MZ001 containing SP, JY and CogniUp.

P3191
Board Number: B478
RARβ agonist induces endodermal differentiation by inhibiting Hoxa1 in differentiating mouse ES cells.
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Mouse Embryonic Stem (ES) cells are pluripotent in nature and can be differentiated in culture into all three cell lineages: ectoderm, mesoderm, and endoderm. In ES cells, retinoic acid (RA, a derivative of vitamin A) induces differentiation acting through the Retinoic Acid Receptors (RARs) and their isoforms (RARα, RARβ, RARγ). In previous research, RARβ has been shown to repress the expression of Hoxa1, a homeobox Transcription Factor (TF) required for ES neuronal differentiation, and induce endodermal differentiation in mouse ES cells. However, the mechanism by which endodermal induction interferes with neuronal differentiation has yet to be determined. Therefore, this work is aimed at identifying the RARβ target genes induced by a commercial RARβ agonist, AC55649 (AC), that may interfere with the neuronal differentiation. For this purpose, we performed transcriptome analyses on mouse ES cells treated with RA versus RA plus 5 micromolar AC for 48 hours. Our results indicate that about 80 genes were differentially expressed by 2-fold or more in mouse ES cells treated with RA plus AC as compared to cells treated with RA only. Validation by quantitative Polymerase Chain Reaction (qPCR) was performed for RARβ targets such as Zscan4, Coro6 and Mettl26. Zscan and Coro6 were found to be downregulated by AC plus RA compared to RA alone, while Mettl26 was found to be upregulated by AC. Analyses at the protein level were performed by Western blot and immunofluorescence. The overall result indicates that RARβ activation promotes endodermal differentiation by acting as repressors of neuronal differentiation.

P3192
Board Number: B479
HSV-1 triggers paracrine fibroblast growth factor 4 secretion from astrocytes: potential role for astrocyte reactivity and repair in herpes simplex encephalitis.
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Herpes simplex virus -1 (HSV-1) infections of the central nervous system (CNS) may result in devastating encephalitis characterized by altered astrocyte reactivity and activation. Regulation of the astrocyte secretome critically influences the outcome of a brain injury. Inflammatory cytokines are initially beneficial for clearance of virally infected cells but become detrimental if activated for too long. Neurotrophic factors secreted by activated astrocytes are crucially involved in regulation of inflammatory response and repair mechanisms. In this study, we employed primary murine cortical cultures containing astrocytes and neurons to study neurotrophic factor signaling in HSV-1 infected CNS cells. Interestingly, fibroblast growth factor 4 (FGF-4), a paracrine neurotrophic factor, was dramatically up-regulated by a switch-on mechanism and induced neurotrophic signaling in neighboring cells. This effect was specific to HSV-1 infection and independent of innate immunity signaling indicating a virus induced mechanism. We thus propose that during the course of an HSV-1 brain infection, FGF-4 shifts astrocytic responses from inflammation to repair, thereby promoting viral spread in the CNS.

P3193
Board Number: B480
Mutation of the Drosophila RNA-binding protein Muscleblind, causes accumulation of rhodopsin, ER stress and retinal degeneration.
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Many inherited retinopathies result from the loss-of-function mutations in genes required for phototransduction, such as the gene encoding the light-sensitive receptor rhodopsin (Rh). Defects in the biosynthesis, folding, and/or trafficking of Rh result in insufficient protein levels in the photoreceptor cell membrane, which can lead to retinal degeneration and blindness. As in humans, mutations that reduce functional Rh levels in the fruit fly, Drosophila melanogaster, recapitulate clinical manifestations of retinopathies. Through a genetic screen for Drosophila genes required for phototransduction, we identified a mutation in one isoform of the evolutionarily conserved muscleblind-like (MBL) family of proteins. MBL is a member of a family of RNA-binding proteins that regulates the splicing, transport and stability of tissue-specific RNAs. In humans, the loss of function of either one of two isoforms of MBL (MBNL1/2) cause myotonic dystrophy. We found that mutations in one Drosophila Mbl isoform results in defects in phototransduction, and in progressive degeneration of the photoreceptor cells. The mutant flies also display a profound reduction in the levels of Rh1 and other phototransduction proteins, such as the TRP and TRPL cation channels, without discernible effects on their respective mRNA levels. We found that the Rh1 protein accumulated in the endoplasmic reticulum (ER) of the photoreceptor cells of mutant animals. Moreover, there was a significant increase in the mRNA levels of the transcription factor Xbp1, a major regulator of the ER stress response. Our results suggest that Mbl regulates the biosynthesis, folding, transport, or stability of Rh1. We suggest that the retention of Rh1 in the ER results in unmitigated ER stress, which consequently leads to photoreceptor cell death and retinal degeneration. We are currently conducting analyses aimed at revealing the specific mechanisms by which this Mbl mutation affects ER function in fly photoreceptor cells. By circumventing the developmental defects associated with the complete loss of Mbl, the mutation we identified allows us
to dissect the homeostatic functions of Mbl in adult photoreceptor cells, and the specific molecular mechanisms that result in photoreceptor degeneration.

**P3194**
Board Number: B481

**Neurons survive and regenerate after simultaneous injury to axons and dendrites.**
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After axon injury, neurons recover function by regenerating the damaged process. It has recently been shown that neurons can also survive dendrite severing and can regenerate these processes. It is not known how well neurons in vivo survive simultaneous injury to axons and dendrites, even though this scenario is likely to occur during stroke and traumatic brain injury. If neurons can survive simultaneous injury to both compartments it is not known whether they would initiate one type of regeneration first, axon or dendrite, or would regrow both types of processes simultaneously. To address these questions we used an in vivo system in which we can use a pulsed UV laser to induce controlled injury to specific regions a neuron. Axons, dendrites, or all processes of ddaE sensory neurons in whole living larvae were severed with a laser and responses were tracked over time. As previously described removing the axon or all the dendrites resulted in regeneration. However, when the axon and all the dendrites were removed to leave an isolated cell body very little regrowth was observed although the cells did survive over the 72h period analyzed. We considered two hypotheses that could account for this failure after complete severing: 1) loss of contact between the neuron and surrounding cells made it difficult to regenerate, or 2) the axon and dendrite regeneration programs are mutually inhibitory and cannot run at the same time. To distinguish between these hypotheses, we cut the axon and all of the dendrites except for one, which would allow the cell to remain in contact with surrounding cells. In this case rapid outgrowth was initiated, and our initial analysis suggested that both axonal and dendritic compartments were regenerating. To elucidate whether induction of either regeneration pathway would halt regeneration that has already begun, we performed the injuries 24h apart. Again, our results suggested that both pathways are able to run in parallel. We conclude that, at least this neuron type, can survive injury of both axons and dendrites and can regenerate both types of processes simultaneously.

**P3195**
Board Number: B482

**Microglia activation in animal model of post-traumatic stress disorder.**
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The central nervous system has historically been considered immune-privileged; however this privileged position mostly consists of adaptive immune responses with restricted access of infiltrating lymphocytes into the brain parenchyma, while cells of the innate immune system — microglia — are abundant in the brain. Microglia is also considered a key player in many neuroinflammatory conditions. Microglial cells respond to infectious agents such as LPS with reactive phenotype and changes in expression of certain markers such as Iba1. Reactive microglia are also found in the brain during neuroinflammatory processes in depression, bipolar disorder, post-traumatic stress disorder (PTSD). We used animal model of PTSD in order to test how chronic stress affects the neurogenesis, reactivity of microglial cells as well as their density in the dentate gyrus of the hippocampus and whether hippocampal volume is changing during PTSD. According to our results, 10 days after stress onset the
number of Iba1+ microglial cells in the dentate gyrus of the hippocampus increased substantially compared to the control group (Mann–Whitney, p=0.028). However, we did not see any inflammatory foci, i.e. microglial nodules. The intensity of Iba1+ staining of as well as the size and shape of cells did not differ from the control group. The hippocampal volume did not change significantly. We propose that neurotoxic or neuroprotective role of microglia cells can change depending on the microenvironment, such as in presence of certain cytokines, interleukins, hormones that lead to corresponding changes in the molecular profiles of glial cells. The particular mechanisms of microglia activation and its role in neurogenesis is discussed. This work was supported by Contract №14.575.21.0036, unique identifier of applied scientific research and experimental development RFMEFI57514X0036.

P3196
Board Number: B483
Novel Single nucleotide polymorphism in the exon 3 of MYOC gene enhance the risk of Glaucoma.
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Genetic polymorphism on MYOC gene alters the myocilin protein leading to the interruption in normal regulation of Intraocular pressure (IOP) that ultimately causes glaucoma. The present study was conducted with an aim to identify the polymorphism on exon 3 of MYOC gene in glaucoma patients of Lahore division. For this purpose a case control study was conducted with 100 patients and 100 controls subjects. DNA was extracted from each blood samples and targeted DNA fragment was amplified by PCR. Polymorphisms were identified through sequencing. It was observed that allelic and genotypic frequency of rs74315341 and rs879255525 were significantly associated with glaucoma in studied population. The polymorphism on rs74315341 polymorphic site led to the change of Arginine into Serine whereas rs879255525 transforms Lysine into Asparagine. Haplotype TGAAGCCATTTC was found to be significantly associated with disease onset whereas the haplotype GGAAGCCATTTC was found to be protective against disease development. In conclusion MYOC gene polymorphisms were identified as susceptible regions for glaucoma onset in population of Lahore division. Our paper is the first report to identify a novel mutation rs879255525 for glaucoma on exon 3 of MYOC gene.

P3197
Board Number: B484
The fusogen EFF-1 drives phagosome sealing during cell process clearance.
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Programmed cell death and cell process pruning are common in metazoan development and homeostasis. Dismantling of morphologically complex cells, sporting long processes, poses a particularly interesting problem, as different regions of a cell are often in different microenvironments, and contact different cells. We have used the *C. elegans* tail-spike cell (TSC) to understand the death and clearance of a morphologically complex cell. The TSC extends a microtubule-laden process towards the tail tip during embryonic morphogenesis and then dies. Tracking of death using still images and long-term light-sheet microscopy reveals that the TSC undergoes three distinct degeneration/clearance...
events. The proximal TSC process is dismantled first, and undergoes Wallerian degeneration-like beading. The cell soma then dies and is cleared in a manner resembling other apoptotic cells. The distal process retracts and accumulates in a distinct varicosity. All three mechanisms depend independently on CED-3/caspase. Importantly, a similar sequence accompanies the demise and clearance of embryonic and sexually dimorphic CEM neurons. Thus, the TSC death program may represent a general mechanism for dismantling morphologically complex cells, including neurons.

Clearance of the proximal and distal TSC fragment is independent of known apoptotic engulfment proteins, except for SAND-1/MON1, which promotes phagosome maturation, suggesting that novel mechanisms are at play. From a genetic screen for mutants disrupting TSC clearance, we identified two mutants with lesions in eff-1, encoding a previously-described C. elegans fusogen. These mutants and one carrying a canonical allele, are defective in TSC distal process varicosity clearance, and do not display cell body or proximal process clearance defects. EFF-1 is expressed in and functions in hyp10 cells, which surround and engulf TSC process remnants. While hyp10 appears to recognize the distal process varicosity in eff-1 mutants, the phagosome that is formed remains open, suggesting that EFF-1 may mediate the fusion event required for membrane scission and phagosome sealing. Supporting this idea, the phagosome is labeled with PLCδ-PH::mKate2, a reporter for unsealed phagosomes. FRAP data also suggest that the eff-1(−) phagosome is open. Fusion-dead EFF-1 fails to rescue distal process clearance. EFF-1 also localizes to the tips of phagosome arms, consistent with a function in phagosome sealing.

Direct mediators of membrane scission that promote the formation of endosomes, phagosomes, and other plasma membrane-derived organelles are not known. Our data reveal a novel paradigm for complex cell dismantling that may be broadly conserved, and suggest a novel role for EFF-1 in the scission event promoting phagosome sealing.

P3198
Board Number: B485
Cell biology of functional axon regeneration.
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Functional axon regeneration requires a regenerating neuron to form new synapses onto relevant targets, thus rebuilding an effective circuit. To model this process in vivo, we developed an assay in C. elegans that links regeneration of a single, specific axon (DA9) with regained activation of its post-synaptic targets and recovery of relevant behavior. By imaging DA9 axon morphology and synaptic components during functional axon regeneration, we can analyze the process of rebuilding circuits in molecular detail. DA9 normally has synapses only in a specific region of its axon in the dorsal nerve cord. Surprisingly, we find that axon injury initially results in the formation of transient synaptic vesicle clusters in the DA9 dendrite. These clusters form independently of the injury-sensing DLK pathway. DA9 microtubule polarity is not altered by axotomy, suggesting that injury may trigger minus end-directed transport of vesicle clusters. Optogenetic stimulation of DA9 during this early phase of regeneration results in dendritic vesicle release, activation of ventral cells, and aberrant behavioral response. Later, as the DA9 axon regenerates into its former dorsal synaptic area, new synapses are formed in the correct dorsal region. This remodeling enables activation of dorsal cells when DA9 is stimulated, restoring largely normal circuit function and behavior. Together, these data reveal a surprising amount of circuit plasticity during functional axon regeneration, and establish an in vivo system to investigate its mechanisms.
P3199
Board Number: B486
Pharmacologically increasing microtubule acetylation corrects stress-exacerbated effects of organophosphates on neurons.
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Many veterans of the 1990-1991 Gulf War contracted Gulf War Illness, a multi-symptom disease that primarily affects the nervous system. Here we treated cultures of human or rat neurons with diisopropylfluorophosphate (DFP), an analog of sarin, one of the organophosphate toxicants to which the military veterans were exposed. All observed cellular defects produced by DFP were exacerbated by pretreatment with corticosterone or cortisol, which, in the rat and human neurons respectively, serves in our experiments to mimic the physical stress endured by soldiers during the war. To best mimic the disease, DFP was used below the level needed to inhibit acetylcholinesterase. We observed a diminution in the ratio of acetylated to total tubulin that was correctable by treatment with tubacin, a drug that inhibits HDAC6, the tubulin deacetylase. The reduction in microtubule acetylation was coupled with deficits in microtubule dynamics, which were correctable by HDAC6 inhibition. Deficits in mitochondrial transport and dopamine release were also improved by tubacin. Thus, various negative effects of the toxicant/stress exposures were at least partially correctable by restoring microtubule acetylation to a more normal status. Such an approach may have therapeutic benefit for individuals suffering from GWI or other neurological disorders linked to organophosphate exposure. (AP and ANR are co-first authors. ANR is supported by an NRSA from the NIH. This work was supported by grants from the DOD to PWB and KAS, and a grant from the NIH to PWB.)

P3200
Board Number: B487
TRPV4 mediated neuronal hyperexcitability and disrupted mitochondrial axonal transport in a Drosophila model of inherited neuropathy.
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Dominant missense mutations in the gene encoding the cation channel transient receptor vanilloid, family member 4 (TRPV4) cause inherited neuropathies including Charcot-Marie-Tooth disease 2C (CMT2C). in vitro, mutations in TRPV4 that cause CMT2C cause a gain of TRPV4 channel function and increased intracellular calcium which subsequently leads to cellular toxicity. However, the mechanisms by which CMT2C causing mutations in TRPV4 lead to neuronal dysfunction in vivo remain poorly understood. We generated transgenic Drosophila that express either wild-type or a CMT2C causing TRPV4 mutant (TRPV4R269C) to assess the effect of TRPV4R269C on neuron function in vivo. Selective expression of TRPV4R269C in Drosophila CCAP neurons (NCCAP) results in a failure of Drosophila wing expansion that is blocked by genetically inactivating the channel pore, demonstrating the requirement of channel function in mediating this phenotype. Perforated patch clamp analysis of NCCAP reveals that TRPV4R269C causes a calcium-dependent increase in NCCAP neuronal excitability which is rescued by application of a TRPV4 selective antagonist. High level expression of TRPV4R269C causes synaptic and dendritic degeneration, both of which are rescued genetically by inactivating the channel pore or

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pharmacologically by feeding larvae a TRPV4 selective antagonist. We conducted a genetic screen in NCCAP and found that CaMKII knockdown potently suppresses the TRPV4R269C mediated wing expansion phenotype and selectively rescues degeneration of synapses but not dendrites. We also find that TRPV4R269C, but not controls, inhibits the processivity of both anterograde and retrograde mitochondrial transport in axons. Induction of TRPV4R269C expression after eclosion causes progressive locomotor impairment, suggesting TRPV4R269C can disrupt adult neuron function leading to a progressive neuropathy. Our data demonstrate that TRPV4R269C causes neuropathy through elevated neuronal intracellular calcium which disrupts mitochondrial transport and mediates neurodegeneration through compartment-specific calcium-mediated signaling pathways and supports further investigation of TRPV4 antagonists as potential therapeutics for the treatment of CMT2C.

P3201
Board Number: B488
Nanoparticle delivery of fidgetin siRNA as a microtubule-based therapy to augment nerve regeneration.
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Microtubule-stabilizing drugs have gained popularity for treating injured adult axons, the rationale being that increased stabilization of microtubules will prevent the axon from retracting and fortify it to grow through inhibitory molecules associated with nerve injury. We have posited that a better approach would be not to stabilize the microtubules, but to increase labile microtubule mass to levels more conducive to axonal growth. Recent work on fetal neurons suggests this can be accomplished using RNA interference to reduce the levels of fidgetin, a microtubule-severing protein. Methods to introduce RNA interference into adult neurons, in-vitro or in-vivo, have been problematic and not translatable to human patients. Here we show that a novel nanoparticle approach, previously shown to deliver siRNA into tissues and organs, enables siRNA to gain entry into adult rat DRG neurons in culture. Knockdown of fidgetin is partial with this approach, but sufficient to increase the labile microtubule mass of the axon, thereby increasing axonal growth. The increase in axonal growth occurs on both a favorable substrate and a growth-inhibitory molecule associated with scar formation in the injured spinal cord. The nanoparticles are readily translatable to in-vivo studies on animals and ultimately to clinical applications. (TOA and AJM are co-first authors. PWB and DJS are co-senior authors. This work was supported by a grant to PWB from the Craig H. Neilsen Foundation, a grant from the DOD to DJS, and NIH grants to PWB and DJS. TOA is supported by an NSF GRFP.)

P3202
Board Number: B489
Differential effects of oxidative stress on neurons cultured from different regions of the embryonic chick brain.
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It has been suggested that oxidative stress-induced damage to proteins and lipids within neurons may underlie the cellular etiology in neurodegenerative diseases such as Alzheimer’s or Multiple Sclerosis.
Due to the number and complexity of interactions among cytosolic proteins involved with neuronal function, neurons are particularly susceptible to the effects of oxidative stress. For example, oxidative stress-induced hyperphosphorylation of microtubule-associated protein tau causes their aggregation into non-functional clumps, and eventual degradation of the microtubule network; thus aggregates of hyperphosphorylated tau have commonly been seen in post-mortem tissue from individuals afflicted with Alzheimer’s disease. However, these effects appear to vary in different brain regions. Our laboratory has therefore employed neurons cultured from different regions of the embryonic chick brain at embryonic day 10 through 14 to study the differential effects of oxidative stress at the cellular level. Neurons isolated from the different brain regions were grown in mixed-cell cultures and treated with hydrogen peroxide or tert-butyl hydroperoxide (from 1 to 100 μM) for up to five days. In vitro, cerebral neurons generally grow longer neurites, while neurons derived from the cerebellum or optic tectum grow shorter processes but with more extensive branching. Oxidative stress induces more extensive process retraction and detachment in the cerebellar and tectal neurons, as well as decrease in overall cell adhesion protein expression and localization to the cell membrane. In contrast, cerebral neurons subjected to oxidative stress show decreased expression and localization of the cytoskeletal support protein neurofilament-68. In untreated cerebral neurons, tau protein tightly colocalizes with the microtubule network. Increasing levels of oxidative stress induces a lower correlation between tau and the cytoskeleton, coupled with reductions in neural process length. In addition, oxidative stress induces redistribution of phospho-tau from dispersal through axonal processes to localization in the cell body. These results suggest that oxidative stress induces hyperphosphorylation of tau protein in live cells, causing them to form aggregates that lead to cytoskeletal degradation and neuronal retraction. Collectively, our results suggest differential susceptibility of neuronal subpopulations to oxidative stress, which may offer potential avenues for investigation for the cellular mechanisms affected by different neural disorders.

**P3203**

**Board Number: B490**

**A new microtubule-based approach for augmenting nerve regeneration.**

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Most studies on microtubules in the nervous system have focused on the dynamic properties of the microtubules, with stabilization by taxol and related drugs being proposed as a therapeutic for nerve injury. However, while initial indications were hopeful, more recent observations have called into question whether such drugs directly help nerves to regenerate or if they only provide a small advantage by reducing proliferation of the glial scar. In particular, there is concern that microtubule-stabilizing drugs will impede the ability of the regenerating axon to navigate to its target, given that microtubule dynamics are known to be important for growth cone guidance. Even so, microtubules remain a worthy target, given that they are downstream of many of the growth factors and cell signaling pathways that have already been shown to be relevant to nerve regeneration. We hypothesize that a better approach than microtubule stabilization may be to boost the levels of the more dynamic component of the microtubule mass, which is more akin to how axons grow and navigate during development. Previously we showed in work on fetal neurons that fidgetin is a microtubule-severing protein that pares down the dynamic portions of microtubules in the axon, such that depleting fidgetin results in an increase in dynamic microtubule mass and faster axon growth. Using AAV5 to transduce adult dorsal root ganglion neurons with fidgetin shRNA, we have now tested whether fidgetin knockdown assists axon regeneration in a novel in-vitro system in which the cells are plated on a laminin...
P3204

Board Number: B491

HIV-1 Nef induced inflammation, astrogliosis, and chromatolysis can be reversed by inhibiting TGFβ.

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Astrocytes and neurons play a major role in the maintenance and proper function of the brain. Learning and memory can be compromised if either of these cell types are affected. The human immunodeficiency virus (HIV) has the capacity of damaging the nervous system. Although viral replication in astrocytes is limited and neurons are not infected, early viral proteins can be produced in astrocytes and affect nearby cells. Keeping the virus under detectable levels does not exempt patients from developing HIV-associated neurocognitive disorders (HAND) which still affect near 50% of the people living with HIV. When viral particles enter the brain, cytokines can induce an inflammatory state leading to neurotoxicity. The transforming growth factor β (TGFβ) is a cytokine important for several cell characteristics and neuron viability. Our group has demonstrated that the HIV early protein Nef causes learning impairment when expressed by astrocytes. We have seen that Nef by itself can increase trafficking of immune cells into the brain and promote inflammation as well as increased expression of TGFβ. In this study we aim to understand how astrocytes expressing HIV-1 Nef promote inflammation, astrogliosis, and affect neuron viability. To demonstrate this, astrocytes were transfected with Nef or GFP (control) plasmids and treated with rTGFβ or a TGFβ inhibitor. Lysates were collected at 24, 48, and 72 hours to measure protein expression. Sprague Dawley rat brains were infused with astrocytes expressing Nef and treated daily by giving an oral dose of either placebo or a TGFβR inhibitor. Immunofluorescence for target proteins was performed in tissues collected at days 1 and 5 after surgery. Our results show that astrocytes expressing Nef have increased levels of TGFβ and GFAP. In rat brain tissues, Nef inhibits astrocyte proliferation, increases astrogliosis and chromatolysis. These effects seem to be reversed as soon as one day after surgery with the use of the TGFβR inhibitor. We also have preliminary data suggesting a reduction in tight junction protein in the Nef treated group when compared to naïve rats. Previously, we have also seen changes in spine density related to Nef expression. Thus, our future studies include looking at the blood brain barrier (BBB) permeability and measuring pre and post synaptic proteins. Immune responses in the brain can vary depending on the insult. A better characterization of the effect of Nef in the brain and the role of TGFβ pathway in these responses is relevant for understanding neuropathologies currently disturbing HIV patients.
P3205
Board Number: B492
Investigating the neuroprotective functions of TLDc proteins.
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Neurodegenerative disorders are becoming more prevalent with increased life-expectancy, yet the mechanisms of disease initiation and progression are still to be unraveled. Oxidative stress (OS) is one of the key factors implicated in the neurodegenerative process and organisms have developed multiple mechanisms to neutralise the potential damage that reactive oxygen species can cause. Oxidation resistance 1 (Oxr1) has been identified by our group as one of the proteins responsible for neuronal cell protection against OS. We discovered that mice lacking Oxr1 display progressive ataxia, death of cerebellar granule neurons and only survive for 24 days. Oxr1 can effectively reduce cellular OS levels, although the molecular mechanisms of action remain unclear. Oxr1 is a member of a protein family that share a highly conserved C-terminal TLDc domain. Importantly, mutations in TLDc domain-containing protein TBC1D24 are associated with seizures, deafness and neurodegeneration in humans. Therefore, understanding the function of TLDc proteins will shed light on mechanisms of cellular stress response and role of these proteins in disease.

In order to identify pre-symptomatic pathways in Oxr1 knockout mouse, transcriptomic analysis has been carried out. These data revealed that genes involved in neuroinflammation were up-regulated when Oxr1 was deleted in vivo, but interestingly no genes related to the OS response were significantly altered. This led us to hypothesise that Oxr1 might be regulating the activity of antioxidant machinery at the protein rather than mRNA level. Using an unbiased protein interaction screen in the brain, several key antioxidant enzymes were identified as putative interactors of Oxr1. Using a combination of in vitro methods and in vivo assays in neuronal cells we are currently investigating the functional interaction between Oxr1 and these enzymes in the regulation of cell sensitivity to OS. Our results indicate that Oxr1 may be influencing important post-translational modifications of antioxidant proteins to modulate their function. Further investigation will provide a better understanding of neuronal OS defense system and role of TLDc proteins in this process.

P3206
Board Number: B493
The NINDS Repository: Publicly available DNA and Cell Lines sampled from individuals diagnosed with neurological disorders.
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Neurological disorders present serious health concerns and challenges to health and health-related quality of life in the United States. Most neurological disorders are complex diseases involving genetic and environmental risk factors, many of which are not yet clearly understood. The identification of reliable genetic risk markers will, therefore, contribute to preventive efforts, earlier diagnoses, disease management options, and new targeted therapeutics. To this end, The National Institute of Neurological Disorders and Stroke (NINDS) Repository offers a public resource containing DNA and lymphoblastoid cell lines with corresponding de-identified clinical and demographic data representing a diverse set of neurological disorders and neurologically normal controls. To date, NINDS Repository samples have been used in over 300 peer-reviewed scientific publications, including genome-wide association studies,
case-control studies, whole-genome and whole-exome sequencing studies, structural variation studies, and candidate gene studies. The genetic and genomic data collected by these studies have been deposited in the Database of Genotypes and Phenotypes (dbGaP), a NIH/NLM sponsored repository for restricted-access data from studies investigating the contributions of genetic variation to phenotypic variation and disease (http://www.ncbi.nlm.nih.gov/gap). Since its establishment, specimens from over 37,000 individuals have been successfully banked and can be accessed through an online catalog (http://catalog.coriell.org/NINDS): individuals diagnosed with cerebrovascular diseases (N>10,800), Parkinsonism (N>5,600), motor neuron diseases (N>2,500), epilepsy (N>6,000), Tourette syndrome (N>4,100), Dystonia (N>2,200), and neurologically normal controls (N>6,100).

P3207
Board Number: B494

Hyperglycemia upregulates calpains expression and promotes sodium channels proteolysis in rat brain.

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Calpains play an important role in neurological pathologies, cardiovascular diseases and diabetes. In several populations it has been observed that single nucleotide polymorphisms in the calpain 10 gene increases the risk of developing diabetes, and its expression in increased in pancreas of diabetic patients. Sustained hyperglycemia triggers cellular damage in neurons, including alterations in voltage-dependent sodium channels (NaV) function that are related to chronic pain. Interestingly, in animal models of chronic pain calpain 1 proteolysed NaV. Despite that neuropathic pain is a common comorbidity of diabetes, it is unknown whether calpains expressed in neurons (calpains 1, 2, 5, 7 and 10) are susceptibles to glucose up rise and if that may affect sodium channels integrity. We analyzed the expression of calpains in normo and hyperglycemic rats by sqRT-PCR and western blot and studied the proteolysis pattern of sodium channels, using whole brain total RNA and protein samples. Hyperglycemia was induced by a single injection of streptozotocin (STZ, 60 mg/kg) and glucose concentration was measured 72h afterwards; CN rats were injected with vehicle. Half of each group was also exposed to the calpain inhibitor MDL28170 (3mg/ml), applied intraperitoneally daily for 3d after hyperglycemia onset (>250 mg/dL). We did not find differences on mRNA levels for CAPN 1, 2, 5, 7 and 10 between groups. However, at protein level we found that hyperglycemia induced increases of calpain 1, 2 and 5 of 2-fold or more, which were reverted by MDL28170 to control levels. Calpain 10 and calpain 7 expressions were insensitive to treatment with STZ or MDL28170 alone. However calpain 10 became sensitive to MDL28170 after hyperglycemia onset. Regarding the proteolysis of sodium channels, we found that hyperglycemia increases proteolysis of several NaV isoforms, including NaV1.1, NaV1.2 and NaV1.8. Isoform NaV1.6 was not affected by hyperglycemia. By immunofluorescence assay we found that calpains 1 and 10 colocalize with sodium channels in hippocampus and there is a tendency to increase his colocalization in hyperglycemic brains. All together our results suggest that calpains expression is stimulated by hyperglycemia and can be partially responsible for degradation of NaV1.2 of sodium channels. They also suggest that other proteases may be involved. Proteolysis of NaV by calpains may alter the kinetics of NaV, accounting for the misfunction of these channels in chronic pain. Alteration in NaV function or expression may also be involved in development of neurological comorbidities of diabetes such as cognitive dysfunction. Supported by PAPCA 2013-38 FESI-UNAM and PAPIIT-DGAPA-UNAM IN223116.
P3208
Board Number: B495
Investigating a role of TOR-2 in controlling ER export of AMPA receptors to regulate synaptic excitability in C. elegans.
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Human torsinA is an ER-resident protein, which is a member of large and structurally diverse family of AAA+ proteins that hydrolyze ATP to release energy for remodeling substrates. Mutation or deletion in DYT1 gene, is responsible for a dominantly inherited human movement disorder called early-onset torsion dystonia. DYT1 dystonia is an autosomal disorder, characterized by painful, involuntary sustained muscle contractions and abnormal postures. While clinical manifestations are well-documented, underlying molecular mechanisms by which torsinA modulates neuronal activity remain to be fully elucidated. Biochemical and in vivo evidence suggest that torsinA, like other AAA+ proteins, has molecular chaperone activity (Caldwell et al., 2003; Burdette et al., 2010). A quantitative readout for endoplasmic reticulum (ER) stress response in the nematode, Caenorhabditis elegans, indicates that torsinA functions as a homeostatic regulator of ER stress (Chen et al., 2010). Likewise, overexpression of human wild-type (WT) torsinA and related invertebrate orthologs can suppress the accumulation of misfolded proteins (McClean et al., 2002; Caldwell et al., 2003). Based on torsinA chaperone activity at the ER, we are interested in exploring its role in neurotransmitter receptor trafficking from ER to plasma membrane in C. elegans. C. elegans tor-2 is a homologue of human torsinA that is expressed in a few neurons in the adult hermaphrodite, including the AVE interneurons. These neurons are responsible for driving backwards movement which is regulated by AMPA type glutamate receptors. Changes in synaptic AMPA receptor levels have been proposed to be a key regulatory event in synaptic plasticity (Wang et al., 2010). Our preliminary data show that tor-2 mutation or pan-neuronal RNAi knockdown of tor-2 in C. elegans caused defects on backwards movement and nose touch response, a phenotype indicative of glutamatergic involvement. We also found that pan-neuronal tor-2 RNAi in C. elegans caused a significant decrease in abundance of the AMPA type glutamate receptor, GLR-1 in the ventral nerve cord. Interestingly, the Human Liver Proteome Project determined that human cornichon 4 physically interacts with torsinA. Cornichons are a family of ER-localized transmembrane proteins) that directly interact with AMPARs immediately after translation in the ER to promote export from the ER and Golgi complex (Haering et al., 2014; Drummond et al., 2012). We propose that TOR-2 activity modulates the trafficking of AMPA-type glutamate receptors and further regulate synaptic excitability in C. elegans.

P3209
Board Number: B496
Expression and distribution pattern of Pnn in ischemic cerebral cortex and cultured neural cells exposed to oxygen–glucose deprivation.
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Pnn, a multifunctional protein, participates in embryogenesis as well as in apoptosis, proliferation, and metastasis in tumor cells through regulating gene transcription and mRNA alternative splicing. However,
the role of Pnn in non-dividing cells, particularly regarding stress response, remains unknown. In the present study, we applied primary cultured rat neural cells and mice with middle cerebral artery occlusion (MCAO)-induced ischemic stroke to examine the alternation of Pnn in the stress response to ischemic insults. In cultured primary cortical neurons with normoxia, Pnn is mainly localized in the nuclear speckle. The expression level of Pnn was increased immediately after exposure to oxygen-glucose deprivation (OGD) and then declined in the reoxygenation period. Moreover, the nuclear speckle Pnn was gradually translocated to the cytosol during the reoxygenation period in a time-dependent manner. The apoptosis-associated proteins in the primary cortical neurons were also found to be increased with OGD/reoxygenation. In primary astrocytes, Pnn is localized mostly in the nuclear speckle both under the normoxia and the OGD condition. Unlike that in neurons, the Pnn expression in astrocytes was decreased immediately after OGD and then gradually up-regulated during the reoxygenation period. Three days post induction of ischemic stroke in mice, the neuronal expression of Pnn in the peri-ischemic region was reduced. However, the Pnn expression in astrocytes was not altered. Moreover, the nuclear speckle distribution of Pnn in neurons was also diminished following ischemic stroke. In conclusion, the Pnn expression and distribution after OGD and during reoxygenation showed distinct manner in neurons and astrocytes, implying that Pnn may play different roles in different neural cells in the stress response to ischemic injury.

P3210
Board Number: B497
Human tryptophan hydroxylase-2 expression in S. cerevisiae results in cytotoxicity.
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Major depressive disorder is a debilitating neuropsychiatric illness that has been projected to affect approximately one in six Americans in their lifetime. Conventional methods of pharmaceutical treatment, most recently including selective serotonin reuptake inhibitors (SSRIs), have been known to ameliorate symptoms; however, these treatments are slow-acting and present a range of side effects. Thus, it is necessary to reevaluate how depression is managed in an effort to develop rapid-acting and robust methods of treatment. Tryptophan hydroxylase-2 (TPH2) has been discovered to be responsible for the majority of serotonin biosynthesis in the mammalian brain, and developing an understanding of this enzyme, its molecular structure, and regulation would be the prime target for novel therapeutic methods. Intriguingly, 300 single nucleotide polymorphisms (SNPs) have been identified in human TPH2 and proven to induce the onset of select neuropsychiatric illnesses, including uni- and bipolar depression. These disease-associated mutations enhance the natural aggregation propensity of TPH2, reducing the pool of functioning TPH2, and consequently, serotonin, in the brain. In order to assess if TPH2 misfolding underlies serotonergic dysfunction, we seek to use S. cerevisiae as a model to screen for cytotoxicity upon exogenous expression of TPH2. We are actively screening three TPH2 galactose-inducible constructs, 303, 416, and 426. The 303 construct is integrating, whereas 416 and 426 are non-integrating. Our yeast spotting assay results thus far have indicated that 416 and 426 yield the greatest cytotoxicity in yeast. We anticipate conducting further study with known TPH2 mutations in order to screen for their cytotoxicity. Developing the molecular characterization of TPH2 will allow us to harness its enzymatic capacity and discover ways to enhance serotonin production in individuals who experience depression and other debilitating neuropsychiatric disorders.
P3211
Board Number: B498
Disruption of Tuba1a leads to neurodegeneration that can be rescued by Mg++.  
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In neurons, the microtubule network stabilizes axons and dendrites, and facilitates protein trafficking. Mutations that disrupt the TUBA1A α-tubulin isotype cause developmental brain malformations. Indeed, we showed that an Asparagine to Aspartic acid substitution at residue 102 (Tuba1aND) results in developmental brain abnormalities and perinatal death in homozygous Tuba1aND mice. Our data from yeast and mice showed that the Tuba1aND mutation compromises the stability of microtubules. Interestingly, mice heterozygous for this mutation (Tuba1aND/) develop abnormal motor phenotypes as adults, suggestive of neurodegeneration, despite apparently normal brain development. Microtubule networks are lost and disorganized in many neurodegenerative diseases, but it is not clear whether microtubule dysfunction causes neurodegeneration or is a result of loss of neuron health. Our data suggests that compromising microtubule function can cause neurodegeneration. The Tuba1aND/+ mutation is located adjacent to a magnesium (Mg2+) binding site in alpha tubulin and Mg2+ stabilizes αβ-tubulin heterodimers. We demonstrate that Mg2+ supplementation can partially attenuate the Tuba1aND/+ motor phenotypes. Therefore, we can manipulate the scale of microtubule dysfunction within the brain at different developmental and post-developmental time points using Mg2+. The Tuba1aND allele allows us to dissect the mechanism of microtubule function in neurodegeneration.

Synaptic Cell Biology

P3212
Board Number: B499
Multi-color STORM in neurons reveals molecular organization of the LGI1 synaptic complex.  
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Leucine rich glioma activated 1 (LGI1) is a neuronal protein that forms a trans-synaptic bridge linking pre- and post-synaptic transmembrane proteins (ADAM22 and ADAM23) and helps to organize a multimeric complex at the synapse including AMPAR and voltage-gated potassium channels [1]. LGI1 autoimmune encephalitis is a severe neuropsychiatric disorder related to epilepsy. It is an antibody-mediated pathogenesis where the patients produce autoantibodies against LGI1, which alter synaptic plasticity. However, the molecular mechanisms that lead to the observed problems in patients still remain largely unknown. To elucidate early molecular changes at the synaptic level in LGI1 encephalitis, we used multi-color super-resolution microscopy (STORM) [2]. Using well-characterized synaptic markers (Homer and Bassoon) as molecular standards, we determined the positioning of LGI1 and 4 other related proteins (AMPA, ADAMS and voltage-gated potassium channels) within the synaptic space at nanoscale resolution. Further, we compared this molecular architecture in healthy neurons.
versus neurons treated with antibodies from patients suffering from LGI1 autoimmune encephalitis. Our results show that LGI1 auto-antibodies impact the nanoscale organization of pre-synaptic proteins, while the post-synaptic protein organization is more subtle. These results suggest a loss of LGI1 interaction with pre-synaptic proteins upon antibody binding and give further insight into early changes in pathology.

**P3213**

**Board Number: B500**

**Nanoscale redistribution of NMDA receptors subunits in anti-NMDA receptor autoimmune encephalitis.**

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The anti-N-methyl-D-aspartate receptor (NMDAR) autoimmune encephalitis is a neuropsychiatric disorder mediated by NMDAR autoantibodies. Patients’ antibodies cause NMDAR internalization but the early events at the synaptic level that lead to the depletion of surface NMDARs are poorly understood. Here, using super-resolution microscopy and Monte Carlo simulations, we studied the effects of NMDAR autoantibodies on the nanoscale distribution of NMDAR subpopulations. Our results show an early, antibody-induced clustering of synaptic and extrasynaptic receptors. This clustering is subunit specific and mainly affects GluN2B-containing NMDARs. Following receptor internalization, the remaining surface NMDARs return to control clustering levels but are preferentially retained at the synapse. These results are recapitulated by Monte Carlo simulations if a model is considered by which antibodies induce NMDAR cross-linking and disruption of NMDAR-protein interactions within and outside the synapse. Finally, activation of ephrin-B2 receptor partially restores the nanoscale surface distribution of NMDARs.

**P3214**

**Board Number: B501**

**Cytoskeletal Regulation of Neurodevelopment in a Human iPSC-derived Autism Model.**

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Autism is a genetically complex neurodevelopmental disorder in which patients exhibit social deficits in verbal and non-verbal forms of communication and display restricted and repetitive behaviors. Emerging evidence suggests that altered neural connectivity, particularly at the level of synaptic connections, contributes to disease pathology. Dynamic rearrangements of the actomyosin cytoskeleton drive neural circuit formation, including neurite extension and the development of actin-enriched spines at excitatory synapses. Actomyosin regulatory pathways are also one of the major molecular mechanisms disrupted by both Autism-associated copy number variants (CNVs) and de novo mutations. Yet, it is still unknown how actomyosin regulation shapes developing cortical circuits and the impact of specific actomyosin regulatory pathways on Autism pathology. To understand the cytoskeletal
mechanisms that lead to altered neural circuitry in Autism, we develop cortical brain organoids from patient induced pluripotent stem cells (iPSC). Our Autism-derived cortical organoids exhibit increased excitatory synapse formation, similar to post-mortem patient samples and mouse models of Autism. To address whether myosin-II activity contributes to this observed increased in excitatory synapse area, we acutely treated 3-month old neurotypic cortical organoids with the RhoA kinase (ROCK) inhibitor, Y-27632. We confirmed that Y-27632 treatment reduced ROCK-driven myosin-II activation in the cortical organoids through reduced phosphorylation of the myosin regulatory light chain (RLC) at Ser19. Intriguingly, acute Y-27632 treatment mimicked Autism pathology by significantly increasing excitatory pre-synaptic surface area as labeled by vGlut-1. We hypothesize that a corresponding increase in synaptic Rac1 activity mediates this increase in excitatory synaptic surface area. In support of this hypothesis, we observe increased phosphorylation of the Rac1 downstream target, coflin, at Ser3 in Y-27632-treated cortical organoids. These results suggest that Rac1 activity dysregulation could be a driving mechanism underlying Autism synaptic pathology, leading us to investigate which Rac1 activity regulators are present during cortical development. For example, in neurotypic cortical organoids, we found that the Rac1 inactivator, ArhGAP23, is enriched at excitatory synapses. Through the use of a human cortical organoid model, we demonstrate that coordinated myosin-II and Rac1 activity underlie excitatory synapse development, and that alterations in the balance between these actomyosin pathways can promote Autism pathology.

**P3215**

**Board Number:** B502

**Remodeling of the postsynaptic plasma membrane during neural development.**

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Neuronal synapses are the fundamental units of neural signal transduction, and must maintain exquisite signal fidelity while also accommodating the plasticity that underlies learning and development. To achieve these goals, the molecular composition and spatial organization of synaptic terminals must be tightly regulated; however, little is known about the regulation of lipid composition and organization in synaptic membranes. Here, we quantify the comprehensive lipidome of rat synaptic membranes during post-natal development and observe dramatic developmental lipidomic remodeling during the first 60 post-natal days, including progressive accumulation of cholesterol, plasmalogen, sphingolipids, and lipid containing w-3 polyunsaturated fatty acids (e.g. DHA). Further analysis of membranes associated with isolated post-synaptic densities (PSDs) suggests the PSD-associated post-synaptic plasma membrane (PSD-PM) as one specific location of synaptic remodeling. We analyze the biophysical consequences of developmental remodeling in reconstituted synaptic membranes and observe remarkably stable microdomains, with the stability of domains increasing with developmental age. Remarkably similar changes were observed during in vitro development of cultured hippocampal neurons, suggesting a cell-autonomous program for developmental lipidomic remodeling in neurons. Disruption of lipidomic remodeling leads to reduced efficiency of synaptogenesis. We rationalize the developmental accumulation of microdomain-forming lipids in synapses by proposing a mechanism wherein palmitoylation of the immobilized scaffold protein PSD-95 nucleates domains at the postsynaptic plasma membrane. These results reveal developmental changes in lipid composition and palmitoylation that facilitate the formation of post-synaptic membrane microdomains, which may serve key roles in function of the neuronal synapse.
P3216
Board Number: B503
Competition in the postsynaptic density for PDZ domains of PSD-95.
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Molecular mechanisms of synaptic plasticity are of great interest because derangement of synaptic plasticity contributes to neural diseases such as autism, schizophrenia, cognitive impairment, neuropathic pain, epilepsy, and stroke. This work addresses the molecular mechanisms underlying NMDA-type glutamate receptor-triggered plasticity at excitatory synapses. A critical step in this process is a change in the rate of trapping of AMPA-type receptors (AMPARs) in the postsynaptic density (PSD), which increases the number of AMPARs and strengthens the electrical signal at the synapse. Our work aims to determine whether trapping of AMPARs in the PSD is mediated by rearrangement of the PSD scaffold caused by changes in the affinity of different PSD proteins for the PDZ domains of the major synaptic scaffold protein, PSD-95. Our earlier publication (1) supports this notion by showing that: 1. Phosphorylation of the abundant PSD protein synGAP by CaMKII reduces its affinity for the PDZ domains of PSD-95; and 2. The composition of the PSD is altered in synGAP deficient mice such that AMPAR-binding proteins with PDZ ligands, including TARPs and LRRTM2, are increased in concentration relative to PSD-95. We have now replicated these finding in synGAP-deficient rats. These findings suggest that the extent of binding of particular synaptic proteins to the PDZ domains of PSD-95 is regulated by activity-dependent phosphorylation of synGAP. We are testing this hypothesis in cultured rat neurons. We have isolated PSDs from neuronal cultures before and after induction of synGAP phosphorylation by pharmacological activation of NMDARs. The ratios of AMPAR-associated proteins to PSD-95 in the PSDs are determined by quantitative immunoblotting. We have found that the ratio of TARPs to PSD-95 is consistently increased in PSDs after chemical activation of synaptic NMDARs. We are using cultures from synGAP-deficient rats to determine if synGAP deficiency alters the composition of the PSD in rat cultures. We plan to transfect with a variety of synGAP mutant proteins in order to determine which domains or phosphorylation sites on synGAP are important for regulating PSD composition.


P3217
Board Number: B504
Nuclear Factor One (NF1)-Dependent Developmental Program Directs the Timing of Gene Expression in Maturing Neurons.
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Developmental timing mechanisms play an essential role in nervous system development, and their disruption during synapse maturation is implicated in neurodevelopmental disorders. To examine how the timing of gene expression related to synapse formation is regulated during neuronal maturation, we used cerebellar tissues from gene knockout mouse and lentiviral transduction of mouse cerebellar
granule neuron (CGN) cultures together with microarray, qRT-PCR, chromatin immunoprecipitation, immunocytochemistry and immunohistochemistry assays. We found that Nuclear Factor One (NFI) regulates the temporal expression of genes required for dendrite and synapse formation via its delayed binding to their promoters in developing CGNs. Further, several other regulatory factors of NFI-dependent developmental program were also identified. (1) Calcineurin/NFAT signaling pathways suppress the program at early development stage via preventing NFI occupancy of late mature genes. (2) Transcription factor ETV1 direct binds to and is required for the NFI occupancy and expression of target genes, including ETV1 itself. This sequential transcription factor interactions and subsequent binding to downstream promoters form an auto-regulatory loop to rapidly upregulate mature gene expression at a critical developmental window. (3) Neurotrophin BDNF induced phosphorylation and accelerated the departure of the trans-repressor NFATc4 from NFI late-gene promoters, accelerating the initial onset of NFI binding in early maturing CGNs. BDNF dismissal of NFATc4 from late-genes was linked to MEK5/ERK5-dependent sequestration of NFATc4 in the cis–Golgi. These results reveal a developmental timing mechanism for dendrite/synapse gene expression in maturing neurons. NFI temporal occupancy together with Calcineurin/NFAT signaling pathways, ETV1, BDNF and NFATc4 extra-nuclear sequestration form an auto-regulatory gene network and precisely regulate the temporal gene expression in maturing neurons.

P3218
Board Number: B505
Determining the molecular basis of ultrafast endocytosis.
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Ultrafast endocytosis is a novel clathrin-independent endocytic pathway that recycles synaptic vesicles under physiological conditions. In mouse hippocampal neurons, vesicle membrane is retrieved within 100 ms after fusion via this endocytic pathway. However, molecular mechanism underlying ultrafast endocytosis remains elusive. To reveal molecular mechanisms, we coupled molecular perturbation with flash-and-freeze approach that visualizes membrane dynamics in electron micrographs with millisecond temporal resolution. Our data have shown that actin polymerization is required for the initial membrane curvature. The transition from shallow to deep pits is mediated by synaptojanin, phosphatidylinositol phosphate phosphatase. This transition requires the conversion of plasma membrane PI4,5P2 to PI4P. The steep negative curvature at the base of the endocytic pit is then stabilized and further constricted by endophilin. Finally, Dynamin-1 severs the vesicle from the plasma membrane. Furthermore, pharmacological inhibition of lipid binding of Dynamin-1 does not abolish ultrafast endocytosis, suggesting that the PH domain may be dispensable for ultrafast endocytosis. Thus, despite its speed, ultrafast endocytosis is mediated through coordinated actions of multiple proteins.
P3219
Board Number: B506
Long-term adaptation of G-protein signalling in the brain is facilitated by active G-proteins' feedback control of the amount of RGS proteins.
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Regulation of G-protein signaling is essential for the brain. This regulation is mediated primarily by a class of proteins called Regulators of G-protein Signaling (RGS). With two dozens of RGSs regulating thousands of G-protein signaling cascades, this regulation mechanism is involved in virtually every known physiological process and plays a major role in neural signal processing. Much of what we know about RGS is derived from the retina. In the first stage of visual processing, RGS9 accelerates the recovery of photo-transduction in both rods and cones. In the second stage, RGS7 and RGS11 accelerate the light-on response of majority of bipolar cells—the Depolarizing Bipolar Cells. For a given receptor activity, decreasing or increasing the amount of RGS proteins leads to more or less active G-proteins, respectively. This allows the signal transduction to change the overall gain without the need to change other components of the cascade. This regulation is believed to be especially important during long-term adaptation such as from daylight to night vision. Much less known is what mediates the change of RGS levels during long-term adaptation. Recent data on dark adaptation in rods and light adaptation in rod bipolar cells indicate that a long-term decrease in a ligand's stimulation of a G-protein signaling cascade should lead to a decrease of RGS protein level in the cascade, and vice versa. Using knockout mouse models, we showed that the decrease of the amount of active G-proteins lead to the decrease of RGS7 and RGS11 level in rod bipolar cells. Therefore the RGS protein level and the active G-protein level reach a steady state through their feedback interactions, after a long-term adaptation to a given receptor activity. We developed a computational model of the feedback regulation of G-protein signaling cascades, which provides an over arching principle that reconciles a lot of controversial data in the literature and explains G-protein-dependent regulation in a variety of biological systems.

P3220
Board Number: B507
The role of the sub-complex α-syntrophin and α-dystrobrevin on the stability of postsynaptic AChR dynamics at the NMJ.
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The sub complex α-syntrophin and α-dystrobrevin, components of the dystrophin glycoprotein complex, is critical for the stability of the neuromuscular junction. Mice deficient in either α-syntrophin (α-syn-/-) or α-dystrobrevin (αdbn-/-) exhibit severe abnormal synaptic phenotypes, including a dramatic decrease in the postsynaptic receptor density (more than 70% of AChRs are lost), high turnover rate of AChRs, and abnormal synaptic structure. In contrast to muscles deficient in α-syntrophin, muscles deficient in α-dystrobrevin show ~ 50% of muscular dystrophy. Here, we wanted to investigate whether both genes could act in parallel or within similar signaling pathways on the maturation and maintenance of synaptic structure. Analyses of NMJs of asyn+-/- αdbn+-/- heterozygous mice showed that the number/density of AChRs, turnover rate of AChRs, and synaptic structure are normal, similar to wild
type NMJs. Analyses of homozygous double mutant αsyn/-/αdbn/- showed that the abnormal synaptic phenotype of NMJs (abnormal synaptic structure, ~ 70% of AChRs are reduced with a high turnover rate) is no more severe than the synaptic phenotype of NMJs deficient in either α-syn/- or α-dbns/- alone. When α-dbns-GFP was electroporated into mice deficient in α-syntrophin, the synaptic phenotype was partially restored. However, electroporation of α-syntrophin into mice deficient in α-dystrobrevin has no effect on the abnormal synaptic phenotype. Altogether these results suggest that α-syntrophin and α-dystrobrevin work within the same pathway and that α-syntrophin acts upstream of α-dystrobrevin in the sequential recruitment of proteins involved in postsynaptic AChR stability.

P3221
Board Number: B508
Spontaneous rhythmic electrical activity of the tentacular apparatus of Mnemiopsis leidyi.
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Ctenophores have attracted considerable attention regarding their role in the evolution of nervous systems (JEB 218:598); yet we know little about their nervous systems due to limited electrophysiological data. Previous studies of Pleurobrachia pileus (Phil Trans Roy Soc B339:1) demonstrated an afferent integrative center within the tentacular bulb. The present study used extracellular recording to examine the electrical activity of tentillae and tentacular bulb of Mnemiopsis leidyi. We found spontaneous slow trains of robust biphasic action potentials (0.1-0.3 mV, 0.25-1 spike s⁻¹) in tentilla where they arise from the tentacular bulb of adult animals. Similar rhythmic firing (0.02-0.04 mV, 0.5-1 spike s⁻¹) was observed in tentacles of 4 mm pre-metamorphic cydippid larvae, i.e. bearing only two tentacles and no lobes. Lesion analysis of excised bulb/food groove complexes mapped distributed bioelectrical activity in the oral region of adults based on electrophysiological recordings from multiple locations. This effort reveals that spontaneous rhythmic spiking originated in the aboral end of the bulb. We further demonstrate through paired electrode recording, that rhythmic spiking is conducted into the food groove tentillae at approximated speed of 0.149 m s⁻¹. To our surprise, to date we have not been able to evoke propagated action potentials by application of food (Artemia nauplii), mechanical or electrical stimulation in this region of the nervous system, in adults. In conclusion, we report here for the first time that spontaneous rhythmic action potentials likely originate inside the tentacular bulb of M. leidyi and propagate into the embedded tentillae. Surprisingly, our results are in contrast to those of Moss and Tamm with P. pileus: P. pileus bulb does not exhibit spontaneous firing, yet responded to the application of food and electrical stimulation to the tentacle with trains of short duration volleys in the tentacle, that in turn drove oscillatory slow wave activity in the bulb. Although Moss and Tamm showed that oscillator activity occurs in the P. pileus bulb that can be driven by exogenous input, our data here indicate that in M. leidyi, there is a central oscillator that drives spontaneous rhythmic activity in the bulb. Future efforts will focus on the cellular source(s) of the signal, the local circuit activity, the signal conduction pathway, its chemical neurotransmitter sensitivity and its sensory and/or motor functions. Support: NSF EPS-0447675 and Chinese Scholarship Council (GD).
Sleep is an evolutionarily conserved and vital behavior. For a period of inactivity to be defined as sleep, it must be regulated by both circadian and homeostatic factors, result in an increased arousal threshold, and occur when the organism is in a restful posture. Drosophila melanogaster exhibits sleep-like states both during the day and at night making it an ideal genetic model to investigate the mechanisms of sleep. To investigate potential glial requirements for sleep and rhythmicity, a Translating Ribosome Affinity Purification (TRAP) screen was performed to identify astrocyte-enriched genes, which were then tested for those that affected rhythmicity. One of the identified genes is CG14141, hereafter referred to as Noktochor (Nkt, sleepless in Bengali). Nkt has both a neuronal and a glial requirement for night sleep. NKT protein has a single Ig domain and is predicted to be secreted. Based on genetic experiments, the neuronal requirement can be localized to the αβ' Kenyon cells of the mushroom body, an anatomical locus known to be required for memory and sleep. Knockdown of Nkt expression in the αβ' Kenyon cells in an Rdl1 GABA receptor mutant background enhanced the night sleep phenotype, suggesting that GABA signaling is involved in the neuronal requirement of NKT for night sleep. In contrast, Rdl1 partially suppressed the glial Nkt knockdown phenotype, suggesting that glial and neuronal NKT act on distinct sleep circuits. Current studies are focused on the signaling mechanisms through which NKT regulates sleep.

Ctenophores have recently attracted much attention because several multigenetic molecular analyses proposed Phylum Ctenophora to be the sister taxon to all other animals (Science 342:1242592; Nature 510:109). The common Western Atlantic ctenophore Mnemiopsis leidyi is currently the object of intense ecological and physiological study. The tentacular apparatus of ctenophores is critical to feeding and very likely bears a diversity of sensory functions. The tentacular apparatus is composed of the tentacular bulb and affiliated tentacles or tentilla, which undergoes profound changes during metamorphosis during development of the adult ctenophore. Previous studies of the cydippid Pleurobrachia pileus suggest that the tentacular bulb is an integrative center for afferent signals arising from the distal tentacle (Trans Roy Soc B 339:1). EdU analyses for cell proliferation in P. pileus revealed a broadly diffuse flow of cells from an aboral proliferation zone (Dev Biol 350:183). The tentacular apparatus of adult cydippid and lobate ctenophores are superficially similar but as we show here, are morphologically distinct. Here, we use correlative light and electron microscopy in conjunction with fine extracellular probe recording (c.f. Dong et al., this meeting) to demonstrate structure/function relationships within the M. leidyi tentacular bulb. All preparations were fixed by modification of the method of Tamm and Tamm (Cell Tiss Res. 264:151) with careful attention to osmolarity, followed by infiltration and embedment in Spurr’s Resin. Cryosectioning (Reichert 2800N) established the general outline, thereby providing a noncollapsed scaffold against which to overlay images from 0.25 μm semi-thick light microscopy and TEM of an entire tentacular bulb (~3 mm long). This method revealed several surprising
features throughout the bulb. Selected major features include: 1) unattached oral end of the bulb, including the canal; 2) what we interpret as a food absorptive layer of cells, confirming a recent report (Curr Biol 26:2814); 3) A distinct structural feature that we interpret to be bulb neuropile; 4) A region of tentillar longitudinal fission and growth (TLFG) that moves laterally to either side of the bulb to ‘feed’ tentillar structure within the feeding groove; 5) fluorescence microscopy of Hoechst-stained live tentilli reveals numerous nuclei along the length of each tentillum, providing support for the concept that the tentilli are multicellular structures; and 6) a variety of unknown cell types at different stages of differentiation. Support: NSF EPS-0447675, Auburn Undergraduate Research Fellowship (DM), AU-CMB Undergraduate Summer Research Scholarship, AU Biosciences Fund for Excellence (ZT), and the Chinese Scholarship Council (GD).

P3224
Board Number: B511
The role of DNA repair factors in developing and maintaining proper neurotransmission in Caenorhabditis elegans.
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In eukaryotes, genetic information is stored in DNA and thus, genome stability is essential for health of the cells. In reality, DNA is constantly attacked by various sources such as free radicals, UV, and chemicals. Each mutagen can cause damage to nucleotides in DNA and may produce mutations. To repair DNA damage and avoid accumulation of mutations, numerous DNA repair enzymes and pathways exist in eukaryotic cells.

DNA repair defects result in genome instability, carcinogenesis, accelerated aging and cell death. In addition, mutations of DNA repair genes are known to cause neurological disorders in humans. Based on our findings in yeast DNA repair, we began C. elegans research project to explore novel roles of DNA repair in neurogenesis and neurodegeneration. Our neuropharmacological assays revealed that four DNA repair factors and nucleases are indispensable for proper neurotransmission. Because these factors are highly conserved throughout evolution, our research outcome would contribute to understanding mechanisms of human neurological diseases and disorders.

P3225
Board Number: B512
Investigation of the FSHR-1 Receptor as a Potential Substrate of the Anaphase-Promoting Complex at the C. elegans Neuromuscular Junction.
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Healthy nervous system function requires a balance of excitatory and inhibitory (E:I) signaling. E:I balance relies upon ubiquitination, a process performed by the ubiquitin signaling system (USS). The USS employs a series of enzymes, including E3 ubiquitin ligases, which recognize and transfer ubiquitin molecules onto bound substrates, marking them for degradation or altering their localization or function. However, substrates of the hundreds of E3 ligases remain unknown. Proper function of neuronal ubiquitin ligases and their substrates is needed for regulation of E:I balance. Our previous data showed that one highly conserved ubiquitin ligase, the Anaphase Promoting Complex (APC) regulates E:I balance at the C. elegans neuromuscular junction (NMJ), a specialized synapse where muscle

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contraction is regulated by excitatory motor neurons that release acetylcholine and inhibitory motor neurons that release GABA. At the C. elegans NMJ, the APC acts in GABA motor neurons to promote GABA release and prevent excess muscle contraction. However, substrates of the APC mediates its effects at this synapse are unknown. Here, we are testing the hypothesis that the conserved G protein-coupled receptor, Follicle Stimulating Hormone Receptor 1 (FSHR-1), is a substrate of the APC critical for normal NMJ signaling in C. elegans. FSHR-1 is found in the intestines and nervous system and regulates germline cell differentiation, stress responses, and innate immunity. FSHR-1 possesses a D-box sequence, a motif often recognized by the APC. Loss of function (LoF) fshr-1 mutants exhibit decreased muscle contraction and suppress the increased muscle contraction seen in APC LoF mutants, indicating FSHR-1 may act downstream of the APC at the NMJ. Current work is focused on analyzing the biochemical relationship between FSHR-1 and the APC and determining the cell types in which FSHR-1 is endogenously expressed and where it acts to control NMJ signaling. Preliminary imaging identified FSHR-1 expression in one pharyngeal neuron subclass, but further imaging is required. Rescue experiments indicate FSHR-1 expression in GABA or cholinergic neurons is sufficient to restore near wild type muscle contraction. We are now generating C. elegans strains expressing fluorescently tagged versions of FSHR-1, in which we will quantitatively measure changes in neuronal FSHR-1 concentrations when the APC is rendered non-functional. If FSHR-1 is an APC substrate at the NMJ, then APC LoF mutants should exhibit increased levels of neuronal FSHR-1 compared to wild-type worms. E:I imbalances are responsible for many neurological diseases, so a better understanding of the APC and its substrates may provide insight into the mechanisms behind diseases like Huntington’s and epilepsy.

P3226
Board Number: B513
RhoA activity regulates spine morphology and memory associated behavior.
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A dendritic spine is assumed to play an important part in formation of memory, for it changes its shape or size depending on synaptic input. Rho family GTPase is a small GTP-binding protein, which regulates cytoplasm through actin cytoskeleton. Rho family is assumed to mediate spine morphology from studies in vitro. However it is still unclear what Rho family GTPase actually do to spine morphology in vivo. To elucidate this problem, we establish in vivo experimental system to express targeted protein in specific cell types by combining Cre-Flex system with Adeno Associated Virus (AAV) injection. We succeeded to express RhoA and compare dendritic spine morphology in D1 receptor expressing-medium spiny neuron (MSN) at NAc (Nucleus Accumbens). Dendritic spines shaped into broad head and short neck in constitutively active RhoA (RhoA-CA) expressing MSN, on the other hand, spines shaped into long neck in dominant negative RhoA (RhoA-DN) expressing MSN. Then we investigated behavioral phenotype of RhoA using cocaine-induced conditioned place preference (CPP) test. We confirmed their place preference was increased in RhoA-CA expressing mouse. These results suggest that dendritic spine morphology affected by RhoA activity enhances place memory in vivo.
P3227
Board Number: B514
A molecular mechanism underlying retinogeniculate convergence in mouse visual thalamus.
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For decades, it has been thought that feature-specific visual information (e.g. color, direction, contrast, etc.) is transferred through unmixed, parallel channels from retina to visual thalamus, and then to the cortical visual centers. To avoid mixing such feature-specific information in the thalamus, each thalamic relay cell must receive inputs from only a small number of functionally similar retinal ganglion cells (RGCs). However, in contrast to this long-held belief, a high degree of RGC convergence onto the thalamic relay cells has recently been reported by several groups. This includes cellular level convergence, in which dozens of functionally distinct RGCs converge onto one relay cell, and a subcellular levels of convergence, in which axons from as many as a dozen distinct RGCs converge onto the same region of a relay cell dendrite, forming a synaptic structure called complex retinogeniculate (RG) synapse. Here, we sought to identify molecules that contribute to the development of complex RG synapses and retinal convergence in the visual thalamus. RNAseq analysis identified Leucine-Rich Repeat Transmembrane Neuronal 1 (LRRTM1) as target-derived synaptic organizer enriched in visual thalamus, but not other retinorecipient nuclei, during the emergence of complex RG synapses. To test its role in RG synapse development, we assessed RG synapses in targeted mutant mice lacking LRRTM1 (lrtrtm1/-) using serial block-face scanning electron microscopy (SBFSEM) and AAV-brainbow circuit tracing. Both analyses revealed a significant reduction in the number of complex RG synapses in visual thalamus of lrtrtm1/- mice. Since lrtrtm1/- mutants have reduced retinal convergence (without defects in other aspect of retinofugal circuitry), we used these mutants to address the functional significance of RG convergence. While these mutants exhibited normal responses to simple visual tasks in a two alternative forced swim task, they performed poorly in more complex tasks. These studies, therefore, reveal, for the first time, a significant role for retinothalamic convergence in visual function.

P3228
Board Number: B515
VER/VEGF receptor-related proteins regulate GLR-1/GluR1 surface levels and control behavior.
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The dynamic restructuring of glutamate synapses provides the cellular basis of learning and memory. Failure to properly form or maintain glutamate synapses can lead to a variety of neurological disorders, and controlling their abundance and activity is critical for maximizing functional recovery after stroke. However, the molecular mechanisms that regulate the formation and function of these connections in vivo during normal nervous system development are not well understood. Expression of channelrhodopsin in the glutamatergic sensory neuron ASH\textsuperscript{1} of C. elegans enabled us to combine optogenetic control of a simple glutamate-dependent behavior, the nose-touch response (NOT), with a focused RNAi screen to identify genes important for glutamate synapse function. RNAi knockdown of \textit{ver-1} or \textit{ver-4}, VEGF receptor homologs\textsuperscript{2}, resulted in a strong defect in ASH-stimulated glutamate-dependent locomotor reversals, but did not affect overall glutamate-independent locomotion based on thrashing assays. Analysis of loss of function mutants confirmed these RNAi results. The intensity and density of the presynaptic vesicle marker RAB-3::mCherry, expressed specifically in ASH, was unaltered...
in ver mutants. In contrast, GLR-1::GFP glutamate receptor puncta in the nerve ring and ventral nerve cord of ver mutants was altered, consistent with a defect in GLR-1 clustering. Confocal imaging of GLR-1 tagged with both mCherry and pH-sensitive superecliptic pHluorin (SEP::mCherry::GLR-1)\(^3\) indicated that ver mutants exhibit a preferential loss of cell surface GLR-1 and likely act downstream of UNC-11/AP180-mediated endocytosis to control receptor recycling. Both the NOT behavior and GLR-1 trafficking defects in ver mutants were restored by specific expression of VERs in GLR-1 expressing interneurons. Mutants lacking the VER ligand PVF-1\(^4\) also exhibited NOT defects and similar reductions in cell surface GLR-1 levels, suggesting that ligand binding may be required for VER activity in this system. Together, our data identify VER-1, VER-4 and their ligand as novel regulators of GLR-1 trafficking and signaling in C. elegans. Future experiments will identify the subcellular site of action of VERs and the mechanism by which PVF-1/VER signaling regulates GLR-1 trafficking.

References:

P3229
Board Number: B516
Calcium and Calcineurin-Dependent Regulation of CaMKII Targeting to Inhibitory Synapses.
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Calcium/calmodulin dependent kinase II (CaMKII) plays a central role in regulating synaptic strength in the central nervous system (CNS) and is an essential mediator of processes underlying learning and memory. It has been well-established that translocation of activated CaMKII to excitatory synapses drives NMDA receptor-dependent long term potentiation at those sites. Our previous studies have found that CaMKII can also translocate to, and potentiate, inhibitory synapses; although under different stimulus conditions than those inducing excitatory LTP. An unresolved question remains as to how CaMKII can be selectively targeted to inhibitory, and not nearby, excitatory synapses upon activation. This study examined the role of two factors controlling CaMKII targeting and modulation of inhibitory synapses: the nature of the calcium stimulus driving selective translocation and the role of calcineurin in regulating this translocation. Pharmacological stimuli that have been found to drive CaMKII to excitatory synapses generated larger calcium elevations in cortical and hippocampal neurons than those that enhanced CaMKII localization at inhibitory synapses. To examine whether differences in the magnitude of calcium stimuli alone are sufficient to differentially target CaMKII to synapses, photolytic calcium caging was employed. Stimuli that generated larger calcium signals were found to localize CaMKII to excitatory synapses, with lesser calcium signals driving CaMKII to inhibitory synapses. More physiological stimuli were also found to drive CaMKII to inhibitory synapses. One Hz field stimulus-driven neuronal activity induced a moderate rise in calcium that enhanced CaMKII localization at inhibitory synapses and potentiated inhibitory currents. Additional studies examined the role of the phosphatase calcineurin, also known to affect both excitatory and inhibitory synaptic transmission, on CaMKII targeting to inhibitory synapses. Results suggest that calcineurin activity acts to restrict CaMKII from localizing to inhibitory synapses and may serve to help maintain selective targeting of the kinase to synapses.
P3230  
Board Number: B517  
Tenectin recruits integrin to stabilize boutons and regulate vesicle release at the Drosophila neuromuscular junction.  
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Assembly, maintenance and function of synaptic junctions depend on extracellular matrix (ECM) proteins and their receptors. Here we report that Tenectin (Tnc) is an ECM component required for the structural and functional integrity of synaptic specializations at the Drosophila neuromuscular junction (NMJ). Tnc, a large Mucin-type protein with RGD motifs, is secreted from motor neurons and from striated muscles and accumulates in the synaptic cleft. Using genetics, electrophysiology, histology and electron microscopy, we found that Tnc selectively recruits the \(\alpha PS2/\beta PS\) integrin at synaptic terminals. In \(tnc\) mutant larvae, the levels of \(\alpha PS2/\beta PS\) integrin are significantly reduced at perisynaptic locations, adjacent to the postsynaptic densities; however, the \(\alpha PS2/\beta PS\) integrin are not disrupted at the muscle attachment sites. Our tissue specific knockdown and rescue studies revealed that the Tnc/integrin complexes have distinct pre- and postsynaptic functions: the presynaptic complexes control neurotransmitter release, while postsynaptic complexes ensure the architectural integrity of synaptic boutons. Specifically, the \(tnc\) deprived NMJs have less individualized synaptic boutons with drastically reduced levels of postsynaptic integrin, as well as \(\alpha\)-Spectrin. Electron micrographs captured a sparse SSR at \(tnc\) mutant NMJs, similarly to that observed in mutants with reduced postsynaptic \(\alpha\)-Spectrin. These results suggest that Tnc stabilizes the boutons architecture partly engaging integrin and spectrin complexes. Our ongoing work focuses on Tnc function in the organization of presynaptic active zones. We found that a mutation that abolishes spectrin dimerization, hence the formation of the spectrin-based membrane skeleton (SBMS), recapitulates the vesicle release defects seen at \(tnc\) mutant NMJs. This indicates that the SBMS is critical for the normal release of synaptic vesicles. We are currently investigating a role for Tnc/integrin complexes in the recruitment and/or anchoring of SBMS in the vicinity of presynaptic active zones. These complexes may provide a structural network that organizes the presynaptic active zones and regulates neurotransmitter release.

P3231  
Board Number: B518  
Neto - the obligatory subunit of glutamate receptors, functions in both pre- and post-synaptic compartments to enable synapse development and homeostasis at the Drosophila neuromuscular junction.  
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Synapse development is a highly orchestrated process coordinated by intercellular communication between the pre- and postsynaptic compartments, and by neuronal activity itself. Here we use the Drosophila NMJ as a model system for glutamatergic synapse development to dissect the mechanisms of synapse assembly and homeostasis. In flies as in mammals, neuron arrival at its target muscle triggers formation of large synaptic aggregates of ionotropic glutamate receptors (iGluRs), and promotes expression of more iGluRs to permit synapse maturation and growth. We have recently discovered the obligatory auxiliary protein, Neto, essential for iGluRs clustering and NMJ functionality. Neto belongs to a family of highly conserved auxiliary proteins that regulate glutamatergic synapses. Drosophila neto encodes two isoforms, Neto-\(\alpha\) and Neto-\(\beta\), with different cytoplasmic domains, generated by alternative

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splicing. The cytoplasmic domains are rich in putative phosphorylation motifs and docking sites and are highly divergent among Neto proteins probably reflecting cell/tissue specific roles. Our previous studies revealed that Neto isoforms (i) engage in extracellular interactions that stabilize iGluRs at synaptic sites and trigger postsynaptic differentiation, and (ii) differentially modulate the iGluRs function and gating properties. In addition, Neto-β, the predominant isoform at the fly NMJ, mediates intracellular interactions that anchor postsynaptic density (PSD) components and sculpt iGluRs postsynaptic composition. In the absence of Neto-β, the NMJs are short, with enlarged boutons, and drastically reduced levels of type-A iGluRs. Nonetheless, these mutants have normal evoked potentials due to a robust presynaptic compensatory response. In contrast, our recent studies indicate that loss of Neto-α (which represents <10% of the total Neto pool) induces mild NMJ defects, but significantly reduced basal neurotransmission. We report that Neto-α functions in both pre- and postsynaptic compartments at the Drosophila NMJ. In the absence of Neto-α, the glutamate receptors fields/ PSDs appear enlarged and the sharp boundaries between PSDs and Dlg/PSD-95 are lost. Knockdown and rescue experiments indicate that presynaptic, and not postsynaptic, Neto-α modulates neurotransmitter release. In addition, neuronal Neto-α is required for synapse homeostasis. Chronic or acute/pharmacological reduction of postsynaptic iGluRs activities trigger increased presynaptic release in control, but not in neto-α null mutants, or in animals where Neto-α is perturbed in the presynaptic compartment. Our studies demonstrate that Neto proteins function in both motor neurons and muscles to enable synapse assembly and to coordinate synapse development and function.

P3232
Board Number: BS19
Vesicular transporters heterogeneity regulates vesicle dynamics, localization and synaptic transmission in mouse central synapses.
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At excitatory synapses, vesicular glutamate transporters (VGLUTs) are essentially thought to play a role in the refilling of neurotransmitter into synaptic vesicles (SVs). We have recently demonstrated that over-expression of 2 different isoforms, VGLUT1 and VGLUT2, can confer distinct dynamic properties to SVs in cultured giant presynaptic terminals. However, the functional significances of these different vesicle dynamics remained to be clarified. Here we show that upon co-expression of fluorescently labelled VGLUT1 and VGLUT2 in same terminals, SVs can be sorted into at least 2 different pools: VGLUT1-containing vesicles with high mobility and VGLUT2-containing vesicles with lower mobility. In physiological conditions, proximity ligation assay also revealed that VGLUT1-containing vesicles localized more efficiently to release sites than VGLUT2-containing vesicles. Electrophysiological recording and synapto-phluorin imaging finally demonstrated that VGLUT1 over-expression increases synaptic transmission and vesicle recycling compared to VGLUT2 over-expression. Thus, we propose that VGLUTs heterogeneity might contribute to the regulation of synaptic transmission by modulating vesicle dynamics and trafficking, and controlling their accessibility to active zones.
Neuronal Development, Structure, Mechanics and Motility

P3233
Board Number: B520
A role for the calcium-activated protease calpain in the regulation of netrin-1/DCC-mediated cortical axon outgrowth.
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During embryonic development, neurons extend axons towards their appropriate synaptic targets to establish functional neuronal circuits. The growth cone, a highly motile structure at the axon tip, is capable of recognizing extracellular guidance cues and translating them into directed axon outgrowth through modulation of the actin cytoskeleton. The netrin family of guidance cues is vital for proper neuronal pathfinding. In particular, netrin-1 mediates its attractive function through the receptor deleted in colorectal cancer (DCC), which recruits proteins to mediate axon outgrowth and guidance. The calpain family of cysteine proteases is well known for its role in cleaving cytoskeletal proteins leading to cell death while playing a vital role in adhesion turnover during cell migration. Less is known about its role during brain development although some studies have highlighted its importance in the formation and maintenance of dendritic spines, and axon outgrowth. Here we identified DCC as a novel calpain substrate and we analyzed its role in the netrin-1/DCC signaling pathway during axon outgrowth. We found that calpain was able to cleave DCC in vitro. Calpain proteolysis of cytoskeletal targets is a mechanism of regulation of neurite consolidation and protrusive activity in neurons. We assessed calpain-specific spectrin cleavage, and found that netrin-1 activated calpain in embryonic cortical neurons in an Erk1/2-dependent manner. Furthermore, we demonstrated that netrin-1 stimulation promoted cleavage of DCC within the same time-frame of calpain and ERK1/2 activation. Interestingly, netrin-1-mediated Erk1/2 activation was abolished in calpain-1/2-deficient cortical neurons dissociated from Nestin-Cre;Capns1fl/fl embryos compared to control neurons. However, DCC expression was not unaffected in calpain-deficient cortical neurons. Using another calpain-specific substrate t-BOC that links calpain activity to fluorescence intensity in live cortical neurons, we showed that netrin-1 stimulated calpain activity in live cortical neurons. Interestingly, cortical neurons overexpressing calpastatin displayed longer neurites. Using an siRNA approach to diminish both calpain-1 and calpain-2 expression, neurons with reduced calpain expression also displayed longer axons and were unresponsive to netrin-1 stimulation. Altogether, we propose a novel model whereby netrin-1/DCC-mediated axon outgrowth is modulated by calpain-mediated proteolysis of DCC and cytoskeletal targets.

P3234
Board Number: B521
Calcineurin substrate protein supports reproduction and neurite caliber development by acting like the KSP domain of human neurofilament medium subunit (NEFM) in Caenorhabditis elegans.
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Repeats in amino acid sequence confer proteins specialized structure and function, playing a critical role in evolution of proteins. The KSP repeats of neurofilament medium subunit (NEFM) regulate axonal diameter and neuronal conduction in myelinated axons through molecular extension mediated by phosphorylation. The number of the KSP repeats exhibits a positive correlation with larger axonal diameter in larger animals among some mammalian species. However, how different numbers of KSP repeats determine cellular structure and function has not been fully examined. Here, we report that a calcineurin substrate protein CNP-2 is contains a KSP-like repetitive sequences rich in Lys (K), Arg (R), Ser (S), Thr (T), Pro (P), and Glu (E), which are frequently found in proteins that display flexible architectural features. cnp-2 is highly expressed in extensively elastic tissues such as the spermatheca, the spermathecal-uterus valve, and male tail. Also, cnp-2 is critical for both hermaphrodites and male reproductive processes. Bioinformatics analyses and experiments in transgenic worms revealed that the KSP repeat domain of unc-89/obscurin (a giant sarcomeric signaling protein) is functionally homologous with CNP-2, although it is distantly related and shows low sequence similarity and identity. In addition, expression of KSP repeats from human NEFM reversed reproduction defects in cnp-2 worm mutants in a repeat-number-dependent manner; a greater number of KSP repeats showed more efficient effects with respect to morphology and activity. Taken together, the data suggest that cnp-2 be a KRSTPE-rich repeat protein that share common features with the KSP repeats of NEFM, including regulatory molecular extension to support inter-molecular interactions, and contractile and elastic cellular structures. This study also provides insight into how alterations in the number of amino acid repeats may play a role in the determination of cellular morphology and function as a molecular mechanism contributing to the evolution of species-specific traits.

P3235
Board Number: B522
Neurofilament transport impairment precedes microtubule-neurofilament segregation in axons treated with 3,3'-iminodipropionitrile (IDPN).
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Neurofilaments are space-filling protein polymers that are particularly abundant in large axons, where they are known to increase nerve conduction velocity by expanding axonal caliber. In addition to this structural role, neurofilaments are also cargoes of axonal transport, moving forwards and backwards along microtubule tracks in a rapid but intermittent manner. This movement is significant clinically because disruptions in neurofilament transport are thought to give rise to the abnormal neurofilament accumulations observed in neurological diseases such as amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth disease, and hereditary spastic paraplegia, as well as in toxic neuropathies such as those induced by 2,5-hexanedione and 3,3'-iminodipropionitrile (IDPN). Intriguingly, ultrastructural studies on axons in these neuropathies have revealed a striking cytoskeletal segregation in which neurofilaments are displaced to the axon periphery surrounding a central bundle of microtubules and organelles, in sharp contrast to healthy axons in which neurofilaments and microtubules are intermixed. In addition, radioisotopic pulse labeling studies on laboratory rats have shown that IDPN selectively impairs neurofilament transport without affecting the transport of membranous organelles. Using computational modeling, we showed previously that this segregation can be explained by a disengagement of neurofilaments from their microtubule tracks, combined with a “zippering together” of the microtubules by moving organelles that engage simultaneously with two or more microtubules. This model predicts that neurofilament transport impairment precedes segregation. To test this, we
have characterized the time course of neurofilament segregation and transport impairment in adult mice exposed to IDPN. Segregation was analyzed by immunofluorescence microscopy of axonal cross-sections and transport was analyzed using the pulse-escape fluorescence photoactivation technique in peripheral nerves of Thy1-PAGFP-NFM transgenic mice, which express a photoactivatable fluorescent neurofilament protein in neurons. Our data confirm the time-dependent and dose-dependent segregation of axonal neurofilaments and microtubules induced by IDPN and show that neurofilament transport impairment precedes the segregation. This indicates that the impairment of neurofilament transport by IDPN is not simply a consequence of neurofilament separation from their microtubule tracks. These studies establish IDPN treatment as an experimentally tractable model of neurofilament accumulation in disease, and pave the way for future studies that will address the mechanisms of neurofilament transport disruption and neurofilament accumulation in disease.

P3236
Board Number: B523
Chemical and mechanical signals interact to direct axon guidance.
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During brain development, growing neurons navigate through a highly complex environment as they extend towards their synaptic targets. This process of axon pathfinding is controlled by a complex suite of guidance cues. Studies of axon pathfinding have focused primarily on chemical guidance signals; however, neurons also sense and respond to local tissue stiffness. To test how chemical and mechanical cues are integrated, we studied how substrate mechanics affects the response of Xenopus laevis CNS neurons to the chemical guidance cue Semaphorin3A (Sema3A). Neurons grown on soft substrates similar in stiffness to Xenopus brain tissue exhibited significantly less Sema3A-induced collapse as well as a reduced turning angle in response to a Sema3A gradient compared with neurons grown on stiffer substrates. Both calcium dynamics and cyclic GMP levels - critical regulators of Sema3A signalling - were elevated on soft substrates, and pharmacological studies further indicate that cGMP levels play an important role in the stiffness-dependent modulation of the Sema3A response. Investigating how neurons integrate different signals in complex environments will help us to better understand axon guidance during development and regeneration.

P3237
Board Number: B524
ALS-linked mutations increase the viscosity of liquid-like axonal TDP-43 RNP granules in neurons.
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Ribonucleoprotein (RNP) granules, liquid-like non-membrane bound structures, are enriched in specific RNAs and RNA binding proteins (RBPs) and mediate critical cellular processes. Purified RBPs can form liquid droplets in vitro through liquid-liquid phase separation. Mutations in human RBPs, TDP-43 and FUS, are linked to ALS/FTD, but the biophysical properties of these proteins have not been studied in neurons. Whether neuronal TDP-43 RNP granules are liquid-like, and how their liquid droplet properties contribute to physiological and pathologic processes is unknown. Here we show that TDP-43 RNP
granules in the axons of primary cortical neurons display liquid-like properties, including fusion events with rapid relaxation to circular shape, shear stress-induced deformation, and rapid fluorescence recovery after photobleaching. Moreover, wild type (WT) neuronal TDP-43 RNP granules exhibit distinct motility characteristics and biophysical properties depending on their location along the axon. Mid axonal TDP-43 RNP granules are highly dynamic, motile, and susceptible to disruption by 1,6-hexanediol, suggesting they are held together by weak hydrophobic interactions. Accordingly, mid axonal TDP-43 granules show a high degree of internal molecular mobility as well as rapid exchange with the soluble pool of TDP-43. In contrast, proximal TDP-43 granules show decreased motility and partial fluorescence recovery after photobleaching, suggesting that they have a more limited mobile fraction. Unlike mid axonal TDP-43 granules, proximal granules are not susceptible to disruption of weak hydrophobic interactions. Thus, proximal TDP-43 granules appear to be more biophysically complex and display some liquid-like properties but also may have more viscous and/or stabilized regions. These data demonstrate diversity of neuronal RNP granules that may reflect different maturational states. Furthermore, disease-linked TDP-43 mutant granules display slower half-bleach recovery and increased viscosity compared to TDP-43 WT, indicating that the mutations alter the biophysical properties of the granules in neurons. TDP-43 M337V and G298S granules show slower tau values and incomplete fluorescence recovery compared to TDP-43 WT granules. In addition, TDP-43 mutant granules show an order of magnitude increase in viscosity compared to WT granules; these altered biophysical properties may confer toxic gain of function through increased propensity for pathologic transformation. Our data suggest that RNP granules must be studied within the complex morphology of neurons in order to fully appreciate their physiologic roles and the pathologic transitions of these structures in neurodegenerative disease.

**P3238**

**Board Number: B525**

*Nox2 is involved in retinotectal connections in developing zebrafish embryos.*

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The generation of precise neuronal connections during both development and regeneration of the nervous system requires growing axons to find their correct synaptic targets. This process is mediated by the neuronal growth cone that is located at the tip of growing axons and senses the environment through molecular and physical cues. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox) are multi-subunit transmembrane proteins that generate superoxide and secondarily hydrogen peroxide (H₂O₂), which both have important signaling functions. These reactive oxygen species (ROS) regulate central nervous system development, including neuronal progenitor cell maintenance, neuronal differentiation, cerebellar development, neuronal polarity, and neurite outgrowth. However, it is unclear whether Nox derived-ROS regulate axonal growth and guidance in *vivo*. To address this problem, we have investigated Nox function in the development of retinotectal connections in zebrafish embryos. Inhibition of Nox enzymes in zebrafish embryos by a chemical inhibitor, Celastrol, decreased optic nerve thickness and tectal innervation by retinal ganglion cell (RGC) axons while increasing the width of the ganglion cell layer (GCL). Next, we have established Nox isoform-specific knockouts by CRISPR/Cas9 genome editing in order to identify the specific isoform required for axonal growth and guidance along the retinotectal pathway. *nox2* mutant embryos exhibited an increased and disorganized GCL as well as mistargeted RGC axons in optic tecta. Imaging embryos expressing the H₂O₂ -specific biosensor roGFP2/orp1 revealed that *nox2* mutant embryos have higher basal ROS levels in the eye compared to wild type embryos, suggesting that *nox2* deletion could be over-compensated by other Nox isoforms. Lastly, *in vitro*, cultured RGCs exhibited Nox2-dependent axonal outgrowth.
controlled by slit2 and netrin-1. In summary, these results suggest an important role for Nox2 function in the development of retinotectal connections.

P3239
Board Number: B526
TRIMming neurons: TRIM9 and TRIM67 modulate neuronal morphogenesis.
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We identified the closely related neuronally-enriched E3 ubiquitin ligases TRIM9 and TRIM67 as key regulators of the membrane trafficking and cytoskeletal dynamics underlying neuronal morphogenesis. In particular TRIM9 and TRIM67 are individually critical for embryonic cortical neurons to respond appropriately to the extracellular axon guidance cue and morphogen netrin-1. Our published studies also show that TRIM9 is required for appropriate morphogenesis, migration, and synaptogenesis in adult-born neurons in the dentate gyrus of the hippocampus. As deletion of Trim9 or Trim67 results in striking behavioral deficits, including spatial learning and memory deficits, these results indicate that TRIM9 and TRIM67 are master regulators of the form and function of developing embryonic and adult-born neurons. We hypothesize this involves their ligase activity; identification of their interacting partners and/or ubiquitination substrates will better reveal how cytoskeletal dynamics and membrane trafficking are regulated downstream of the ligases. We have found that the actin polymerase VASP and the netrin receptor DCC are ubiquitinated in a TRIM9-dependent fashion, whereas the exocytic tSNARE SNAP25 interacts with TRIM9, but is not ubiquitinated in a TRIM9 dependent fashion. Here we describe a two-pronged unbiased proteomic approach to identify additional putative interaction partners and ubiquitination targets of TRIM9 and TRIM67 from murine embryonic cortical neurons. In the first, we exploit the proximity-dependent biotin identification (BioID) approach, a technique in which biotin is covalently ligated to proteins proximal to a protein of interest, such as substrates or interaction partners, which are then identified by affinity purification and mass spectrometry. The second strategy exploits the DiGly antibody, which recognizes an epitope on ubiquitinated peptides revealed by trypsin digestion, to identify proteins differentially ubiquitinated in the presence and absence of TRIM ligases or the axon guidance cue netrin-1. The overlap of proteins identified by these two distinct approaches: proteins in close proximity to the ligases, that are differentially ubiquitinated in their presence, represents high priority candidates for substrates. Results from these approaches have identified high priority candidate interaction partners and/substrates, that are potential regulators of cytoskeletal dynamics or exocytosis, which may function downstream of netrin-DCC signaling in developing neurons. We use immunoprecipitation and ubiquitination assays to validate candidates. Time-lapse TIRF microscopy of fluorescently tagged candidates then reveals alterations in candidate localization or behavior caused by netrin stimulation or deletion of Trim9 or Trim67.
Investigation of the Roles of Novel Endogenous Ligand of Aryl Hydrocarbon Receptor in Neural Development.

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Activation of aryl hydrocarbon receptor (AHR) by xenobiotic toxic chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin regulates a variety of cellular processes including embryogenesis, tumorigenesis and inflammation. However, the identity of endogenous AHR ligands remains elusive. A potential AHR ligand, compound X, was identified from zebrafish embryos using previous developed cell free bioassay for dioxin-like compounds. In this study, we aim to investigate the effect of compound X in AHR activation and further explore its physiological effects. Compound X induces AHR translocation into nucleus and upregulates downstream cytochrome P450 1A1 (CYP1A1) in vitro. It also promotes neuronal differentiation in neuroblastoma (NB) cells, which is consistent with our previous studies that overexpression of AHR leads to NB cells differentiation. Furthermore, compound X enhances zCyp1a, myelin-associated myelin basic protein (zMb) and SRY (sex determining region Y)-box 10 (zSox10) expression and improves the mobility of zebrafish larvae via Ah2 pathway. Therefore, our results suggest that compound X is an endogenous AHR ligand, and plays a critical role during neural differentiation.

Responses of cultured mouse cerebral cortical axons to netrin-1 depending on developmental stages: outgrowth and collateral branching starting with filopodial protrusion.

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Netrin-1, a multifunctional axon guidance cue, elicits axon outgrowth via one of its receptors DCC (deleted in colorectal cancer) in several types of neurons, including cerebral cortical neurons of embryonic mice. However, we and others have observed de novo formation of axon branches without axon outgrowth induced by netrin-1 in cortical culture of neonatal hamsters. These previous reports suggested the possibility that netrin-1 function might alter during development. We tested this possibility employing dissociated culture prepared from cerebral cortices of embryonic mice.</p>

Morphometric imaging analysis revealed netrin-1-induced outgrowth in embryonic day (E) 14 axons and netrin-1-induced branching in E16 axons. Morphometric analysis also showed netrin-1-evoked filopodial protrusions sprouted on the shafts of E16 axons in advance of branch formation. These netrin-1-dependent filopodial protrusions were better visualized by a novel method called atmospheric scanning electron microscopy. By contrast, netrin-1-independent protrusions were observed in E14 axons. Since E14 axons exhibited not only high density of pre-existing filopodial protrusions but also high density of spontaneously developed axon branches under basal conditions, and correlation analysis revealed that longer axons had lower density of branches in E14 axons with and without netrin-1 treatment, it is possible that netrin-1-dependent axon outgrowth is a factor in decreasing the density of branches in E14 axons. Treatment with an anti-DCC function-blocking antibody affected not only axon outgrowth but also axon collateral branching and preceding filopodial protrusion.
conclusion, we showed two distinct functions of netrin-1 in mouse cerebral cortical neurons in vitro—promotion of axon outgrowth, and promotion of axon collateral branching that involves filopodial protrusion from the shafts. As netrin-1 exhibited one of these two functions depending on the stage of embryos utilized for cortical culture, our study raised the possibility of a shift in netrin-1 function during cortical development. Our study also suggested that a netrin-1 receptor DCC may contribute to both of cortical axon outgrowth and collateral branching starting with filopodial protrusion. This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers JP23590225 and JP26460282 (Grants-in-Aid for Scientific Research (C)).

**P3242**

**Board Number: B529**

Unraveling the role of microenvironment topography on cortical interneuron migration using microfabricated substrates.

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During embryonic brain development, cortical interneurons (INs) migrate long distances before integrating into cortical circuits. This migration step is crucial since its dysfunction can lead to the abnormal positioning of INs and to the formation of defective cortical circuits, likely responsible for neuropsychiatric diseases at adulthood. During their migration, INs cross different territories with complex chemical and physical properties. Several diffusible molecules that guide the migration of INs have been identified, but little is known about the contacts and interactions of INs with their close physical microenvironment.

**The aim of this study is to better understand how physical parameters of the environment, especially its topography, can influence the migratory behavior of INs.** This process of contact guidance has already been reported in the migration of non-neural cells and in the navigation of growing axons, but never in migrating neurons.

To address this question, we used microfabrication techniques to build PDMS migration substrates with a precisely controlled micro-topography. On these substrates coated with adhesion molecules (laminin and n-cadherin), we were able to culture, observe and record embryonic INs exhibiting a physiological migration for several hours in between the nucleus-sized structures.

With this system, we first showed that the area of migration of INs cultured on a substrate of regularly spaced pillars, either squared or round, is significantly increased when compared with the area of migration of INs cultured on a flat substrate.

In addition, by precisely analyzing the dynamic behavior of individual cells, their orientation and morphology, we observed that the shape of the structures (either square or round pillars) specifically influenced the morphology and the behavior of migrating INs. In particular, we noticed a robust alignment of cells amongst square pillars, where INs grow long, straight and unipolar leading processes.

We showed that this topography was able to efficiently guide INs migration: directional phases are stabilized and cells are more dynamic. On the contrary, round pillars promoted growth cone splitting and leading process branching.

**Migrating INs are therefore able to respond precisely and differently to distinct geometries of their close microenvironment, by specific dynamic and morphological transformations.** These results highlight the importance of the spatial arrangement of adhesive cues in the microenvironment to achieve directed neuronal migration and overall unravel a new, important role of topographical cues in the developing brain.
P3243
Board Number: B530
HDAC6 inhibition puts the brake on axon growth and microtubule invasion into peripheral growth cone.
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In the peripheral domain of growth cones, interactions between F-actin and dynamic microtubules influence growth cone behavior and direction of axon advance. Our previous research suggested that decreasing acetylated microtubule (AcMT) levels in the axon accelerates growth cone advance by promoting invasion of dynamic MTs into the leading edge of growth cones. Here, we tested whether increasing AcMT levels in neurons would affect axon growth and microtubule dynamics. To increase the amount of AcMTs, we used tubacin, a cell-permeable inhibitor of HDAC6, an enzyme that removes acetyl groups from MTs. Analysis of cultured Helisoma neurons exposed to tubacin and subsequently stained with antibodies for acetylated tubulin showed inhibition of HDAC6 resulted in increased microtubule acetylation within the axon and cell body. Using time-lapse DIC microscopy, we found that tubacin treatment decreased the rate of growth cone advance, central domain advance, and axon advance compared to the same neuron prior to treatment. Treatment with niltubacin, an inactive analog of tubacin, did not affect growth cone behavior. Analysis of microtubule distribution within growth cones showed tubacin treatment decreased the percent of microtubules that extended into the growth cone periphery, suggesting tubacin suppressed microtubule dynamics. The inverse relationship between axonal AcMTs and dynamic growth cone MTs may reflect a role for AcMTs in regulating the distribution of MT plus end tracking proteins (+TIPS).

P3244
Board Number: B531
Activity-Dependent Modulation of Neuronal Proliferation and Differentiation in Cultured Chick Olfactory Neurons.
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The olfactory system regenerates and produces mature neurons from non-neuronal precursors through an endogenous differentiation pathway. However, we have previously shown that differentiation of olfactory neurons can be modulated by odor stimulation: exposure to stimulatory odorants induces acquisition of structural and functional neuronal properties in cultured embryonic chick olfactory neurons. The mechanism by which odors promote neuronal differentiation remains unclear. It is known that cyclic adenosine monophosphate (cAMP) serves as a second messenger for odor signal transduction, and is thus a prime candidate for pharmacological manipulation to directly activate the signal transduction machinery. Thus, to determine if differentiation can occur in the absence of odorant stimulation, elements of the cAMP signaling cascade were directly manipulated. Cultured olfactory neurons from an 18-day old chick embryo were exposed to either an adenyl cyclase activator (forskolin) or adenyl cyclase inhibitors (MDL-12,330A or SQ 22,536) for 12-hour periods daily for five days. Neuron differentiation was determined using immunocytchemistry to stain for olfactory marker protein (stains structurally mature neurons only), Golf (stains functionally mature olfactory neurons), and -tubulin III (stains both mature and immature neurons). Our data suggest that direct activation of adenyl cyclase with forskolin increases the number of neurons but does not drive structural or
functional maturation. Inhibition of the cyclase has no effect on neuron proliferation or differentiation. These results suggest that direct activation of adenylyl cyclase upregulates neuron production, but that modulation of olfactory maturation requires stimulation of the odorant receptors themselves, implying the involvement for the activation of additional cellular elements. Our results have important implications for the study of the environmental influence on neuron development and differentiation.

**Mitochondrial Dynamics, Movement and Turnover**

**P3245**
**Board Number: BS33**
The mitochondrial Rho-GTPase, Miro, is resident at peroxisomes and regulates peroxisomal trafficking.
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Peroxisomes are single-membrane bound organelles with a multitude of functions, including reactive oxygen species metabolism, fatty acid β-oxidation and lipid biosynthesis. Patients lacking peroxisomes (Zellweger syndrome) die within the first year of life, emphasising the requirement for functional peroxisomes in cellular health. To ensure optimal functionality of peroxisome-dependent processes throughout the cell they must be trafficked; however, how peroxisomes undergo intracellular transport remains poorly characterised. Here we identify Miro, an outer mitochondrial membrane protein that is critical for mitochondrial trafficking, as a novel regulator of peroxisomal motility. We find that further to their mitochondrial localisation, Miro1 and Miro2 are localised at peroxisomes and that signalling through the first GTPase domain of Miro1 is important for peroxisomal targeting. Additionally, we find that Miro interacts with the cytosolic peroxisomal-membrane-protein chaperone Pex19, suggesting direct insertion of Miro into the peroxisome membrane from the cytosol. Utilising Miro1 and Miro2 double knockout mouse embryonic fibroblasts we show that loss of Miro leads to a significant decrease in peroxisomal motility. Coupled to this, in the absence of Miro peroxisomes are less elongated leading to a reduction in peroxisome size. These results contribute to our fundamental understanding of peroxisomal trafficking and morphology and support a complex crosstalk between peroxisomes and mitochondrial biology.

**P3246**
**Board Number: BS34**
Cycling clouds of actin filaments regulate mitochondria size and distribution in mitotic cells.
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In mammalian cells, mitochondria form interconnected networks that fragment prior to cell division to allow for segregation of mitochondrial mass between daughter cells. Equitable partitioning of mitochondria is critically important to prevent bottleneck effects and the potential expansion of deleterious mitochondrial DNA mutations. To date, the precise mechanism regulating mitochondria inheritance remains controversial. Previous work from our lab identified a dynamic, rotating cloud of actin filaments propagating through interphase mitochondrial networks. We observed that actin clouds move in a continuous manner, cycling onto and off of subpopulations of mitochondria, transiently inducing fission. Here, using HeLa cells and primary epidermal keratinocytes, we find that mitochondrial...
actin cycling persists through all stages of the cell cycle, but is markedly upregulated at the G2/M transition. Specifically, we observe that mitochondrial actin clouds cycle nearly three times faster in mitotic cells (interphase: 1049±201 sec/cycle; m-phase: 384±87 sec/cycle). Consistent with this observation, we find that mitotic actin clouds are 2.86 times larger, 183% faster, and display a stronger bias for persistent, unidirectional motility as compared to interphase clouds. Enhanced cycling may be dependent on CDK1, as inhibition of the mitotic kinase with either ro3306 (20µM, 1h) or CGP74514a (10µM, 1h) robustly blocks actin cloud formation. We observe that rapidly transiting mitotic actin clouds have profound effects on mitochondrial network organization. In prometaphase, actin clouds promote and maintain mitochondrial fragmentation. In metaphase, cycling actin filaments induce microtubule-independent motility of mitochondria which ensures homogenous distribution of mitochondria in both xy and z planes. Finally, in telophase, we observe selective actin assembly on mitochondria localized to the cleavage furrow. Based on these observations, we hypothesize that mitochondrial actin clouds function as a mechanism to fragment and symmetrically partition mitochondria during cell division.

P3247
Board Number: B535
Understanding the enhanced mitochondrial fusion capabilities of a Mitofusin 2 mutant allele.
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A healthy mitochondrial network is vital for proper cellular function and is maintained through the opposing forces of mitochondrial fusion and division. While our understanding of mitochondrial division has been advanced by numerous studies, mitochondrial fusion occurs through a still largely unknown mechanism distinct from that of vesicle fusion or viral fusion. The protein machinery responsible for mitochondrial fusion includes the large GTPase proteins Mitofusin 1 and 2 (Mfn1 and Mfn2, respectively). This study aims to add to the understanding of the molecular mechanism of mitochondrial outer membrane fusion using disease-associated mutant alleles. Multiple disease-causing Mfn2 mutants are able to rescue mitochondrial fusion in Mfn2-null cells, indicating that a lack of fusion is not the only mechanism by which these alleles cause disease and leading to the question of how Mfn2 mutants may alter Mfn2 function without rendering the protein inactive. Previous studies have shown that mitochondrial fusion requires mitochondrial membrane potential, mitofusin proteins present on both opposing mitochondria, hydrolysis of GTP, and assembly of the mitofusin proteins. We have characterized one mutant form of Mfn2, Mfn2C390R, which appears to meet at least some of these requirements. When expressed with Mfn1, Mfn2C390R is able to fuse mitochondria in cells and in a cell-free in vitro mitochondrial fusion assay. Unexpectedly, this form of Mfn2 can fuse mitochondria in vitro even under restrictive conditions such as low temperature or no added nucleotide, which has not been reported before. We further assayed whether this enhanced fusion ability is due to a difference in assembly state of Mfn2. Indeed, our data suggest that Mfn2C390R forms different assemblies than Mfn2WT and that these assemblies may be due to increased affinity of Mfn2C390R for Mfn1. Although Mfn2C390R removes a cysteine from the protein, there does not appear to be any difference in disulfide bond formation between Mfn2WT and Mfn2C390R, indicating that changes in disulfide bonds do not contribute to the changes in assembly state of Mfn2C390R. We propose that this disease associated mutation makes fusion by Mfn2 more promiscuous, which may alter mitochondrial function by compromising quality control.
**P3248**

**Board Number: B536**

Mitofusin-2 mediated mitophagy and mitochondrial networking dynamics regulate the mesodermal differentiation of human pluripotent stem cells.

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Pluripotent stem cells reprogram their metabolism from predominantly glycolysis towards enhanced mitochondrial oxidative phosphorylation during differentiation in order to adapt to the metabolic needs of the mature differentiated state. To optimize mitochondrial function for this process, mitochondria elongate and form fused networks, thus allowing for the exchange of proteins and mitochondrial DNA. Mitochondrial damage results in mitochondrial autophagy or mitophagy during which mitochondrial networks fragment and damaged mitochondria marked for degradation. Importantly, a key mediator for both mitochondrial network formation and mitophagy is the GTPase Mitofusin 2 (Mfn2), located at the outer mitochondrial membrane. While Mfn2 has been shown to affect metabolism in heritable disease, its role in regulating the bioenergetics of stem cells is not fully understood. We hypothesized that Mfn2-mediated mitophagy and mitochondrial fusion is required for the differentiation of pluripotent stem cells.

Human induced pluripotent stem cells (hiPSCs) were treated with Bone Morphogenetic Protein 4 (BMP4) and CHIR99021 (Wnt activator) for 3 days to induce mesodermal differentiation as evidenced by marked upregulation of the mesodermal transcription factor Brachyury. After three days of differentiation, the mitochondrial networks demonstrated increased fusion as shown by immunofluorescent staining of the mitochondrial membrane protein TOM20 (assessed quantitatively using the Mitochondrial Fragmentation Count, p<0.001), and by a photoactivatable mitochondrial localized GFP in live cells.

To investigate the role of Mfn2 in the bioenergetics of iPSCs, an shRNA Mfn2 construct was transduced into cells. Using the ATP fluorescent biosensor Perceval HR, a significant decrease in the ATP/ADP ratio from 1.26 to 0.85 (p<0.001) was seen after Mfn2 depletion.

Because Mfn2 is also involved in the degradation of mitochondria, the ratiometric pH-sensitive fluorescent biosensor Keima was used and indicated a tripling of baseline autophagy from an autophagy index of 2.7 to 9.7 (p<0.01) within three days of differentiation. To confirm that Mfn2 mediated autophagy to occur, a shRNA construct of Mfn2 was used. Induction of autophagy in the hiPSCs with the mitochondrial inhibitors increased the autophagy index from 0.7 to 3.1. However, cells with shRNA exhibited the same autophagy index as baseline iPSCs showing that Mfn2 mediates autophagy in hiPSCs. These findings suggest that mitophagy and mitochondrial networking are Mfn2-mediated adaptive processes which are required for the metabolic reprogramming of differentiating human pluripotent stem cells.

**P3249**

**Board Number: B537**

Role of Septin 9 in mitochondrial fission.

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Mitochondria are fundamental eukaryotic organelles in which respiration and energy production occur. They are highly dynamic organelles that undergo fission and fusion to create interconnected networks. Regulation of fission and fusion is important for maintaining proper mitochondrial function, morphology...
and abundance, and is critical for cell survival. Though key players such as Drp1, Mff, INF2, and actin have been found to contribute to mitochondrial fission, the exact mechanisms which regulate fission are still being defined. Recently, depletion of SEPT2, which belongs to the Septin family of cytoskeletal GTP binding proteins, was shown to elongate mitochondria, suggesting that SEPT2 was required for fission. Using CRISPR to knockout specific septins in HeLa cells, we have found that knockout of SEPT9 results in a more significant elongation phenotype than SEPT2 KO. SEPT9 localizes to constriction sites along mitochondria where fission occurs. In addition, we have found that p114RhoGEF is activated by binding to SEPT9 and that p114RhoGEF and RhoA, in conjunction with SEPT2, form a complex detectable by co-immunoprecipitation. Moreover, depletion of p114RhoGEF by siRNA, or inhibition of the RhoA substrate ROCK by Y27632, in wild type cells phenocopies SEPT9 knockout, suggesting that activation of ROCK by SEPT9 is critical for fission. In support of this hypothesis, the elongated mitochondrial phenotype in the SEPT9 knockout cells can be rescued by treating cells with RhoA Activator. Together, our results indicate that septins, and particularly SEPT9, regulate mitochondrial fission by local activation of RhoA and ROCK, likely to activate myosin to thereby facilitate the constriction of mitochondria necessary for complete fission.

P3250
Board Number: B538
Mutational analysis of Mitofusin1 and Mitofusin2 reveal distinctive functional properties required for mitochondrial outer membrane fusion.
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A dynamic mitochondrial network is essential for both mitochondrial function and a variety of critical cellular pathways including response to stress and apoptotic cell death. In vertebrates, mitochondrial outer membrane fusion is mediated by Mitofusin1 and Mitofusin2 (Mfn), large GTPases of the dynamin related protein family. To gain insight into the poorly understood mechanism of Mitofusin-mediated membrane fusion, we have characterized the activity of Mitofusin mutations etiologically linked to the neurodegenerative disease Charcot-Marie-Tooth (CMT) Syndrome. Here we have characterized the mutation of a highly conserved residue in the GTPase domain that is found in both Mitofusin1 & Mitofusin2 and in the Drosophila homolog, MARF. Despite this level of conservation, the effect of the mutation on mitochondrial fusion activity is highly dependent on context. Cells lacking Mfn1, Mfn2, or both, have a highly fragmented network compared to wild type controls. To determine the effect of the mutation on mitochondrial fusion, we stably express the mutant in null fibroblast cells at levels similar to wild type and score mitochondrial morphology in multiple isolates. When expressed in wild type cells, mutant Mfn1 causes a striking mitochondrial hyperfusion phenotype. In contrast, when expressed alone or with wild type Mfn1, there is no observable change in mitochondrial structure, consistent with no fusion activity. We also observe mitochondrial hyperfusion when the mutant Mfn1 is expressed with wild type Mfn2. These data indicate that the functional differences between Mfn1 and Mfn2 are important to support increased fusion activity. Surprisingly, our analysis of the equivalent mutation in Mfn2 indicate that the mutant does not support hyperfusion, but restores mitochondrial morphology in null cells in a manner that is indistinguishable from wild type. In the mitochondrial outer membrane, Mitofusins form dimers and higher order structures visualized as foci in light microscopy. Although this Mfn1 mutant is also a dimer as determined by sucrose gradient centrifugation, we observe an increased number of foci compared to wild type Mfn1. This is more dramatic when the mutant is expressed in the absence of either wild type Mfn1 or Mfn2. Together, these data indicate that the highly conserved
Mitofusin GTPase domain confers distinct activities on Mfn1 and Mfn2, which can significantly affect the overall rate of fusion in cells.

**P3251**
**Board Number: B539**
A close-up view of mitophagy using mt-keima and super-resolution microscopy.

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Mitophagy is a cellular process that selectively removes damaged, old or dysfunctional mitochondria. Defective mitophagy is thought to contribute to normal aging and to various neurodegenerative and cardiovascular diseases. Previous methods used to detect mitophagy in vivo were cumbersome, insensitive and difficult to quantify. We created a transgenic mouse model that expresses the pH-dependent fluorescent protein mt-Keima in order to more readily assess mitophagy. Keima is a pH-sensitive, dual-excitation ratiometric fluorescent protein that also exhibits resistance to lysosomal proteases. At the physiological pH of the mitochondria (pH 8.0), the shorter-wavelength excitation predominates. Within the acidic lysosome (pH 4.5) after mitophagy, mt-Keima undergoes a gradual shift to longer-wavelength excitation. We describe how to apply mt-Keima with stimulated emission depletion (STED) microscopy to visualize mitophagy in various living tissues including skeletal muscle, heart, liver, adipose tissue, and kidney, obtained from mt-Keima transgenic mice. Thus, we can assess this process at nanoscale resolution (50nm) in normal living tissues but also how tissues mitophagy is altered following changes under genetic perturbations, or aging. In addition, we show how to monitor mitophagic flux in living cells exploring mitophagy relationships with other cellular compartments by Hyvolution microscopy via high resolution confocal microscopy combined with deconvolution to achieve resolution of ~120nm of multiple cellular compartments. In conclusion, this approach enable to explore mitochondrial dynamics and mitophagy interconnections in health and disease conditions.

**P3252**
**Board Number: B540**
A novel role for RalA during PINK1-Parkin mitophagy.

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Mitochondria have numerous functions that are vital for cellular health, ranging from regulating intracellular calcium levels to generating ATP. Over time, mitochondria become damaged, so they lose their metabolic function and can release pro-apoptotic proteins. Thus, maintaining a healthy pool of mitochondria is critical for overall cellular health. One mechanism of mitochondrial quality control is mitophagy** - the selective removal of damaged mitochondria from the cell. Impaired mitophagy is associated with various pathological conditions, including cancer and neurodegenerative diseases such as Parkinson’s disease. The PINK1-Parkin pathway is one of the best-characterized signaling pathways that regulates mitophagy. Following mitochondrial damage, the kinase, PINK1, accumulates on the outer mitochondrial membrane (OMM). PINK1 accumulation triggers the recruitment of Parkin, an E3 ubiquitin ligase, from the cytosol to the OMM. Parkin recruitment leads to the ubiquitination of outer mitochondrial proteins and recruitment of the autophagy machinery, resulting in mitochondrial clearance. Although PINK1 accumulation is important for Parkin recruitment, it remains unclear how
Parkin relocates to damaged mitochondria. A previous study found that the small GTPase, RalA, recruits Drp1 to facilitate mitochondrial fission during mitosis. Given that RalA recruits Drp1 during mitosis, we hypothesized that RalA functions in a similar manner during mitophagy to mediate Parkin recruitment. We find that RalA relocates to mitochondria following mitochondrial damage and colocalizes with Parkin. To determine if RalA is involved in Parkin recruitment, we examined shRalA HeLa cells and found that they exhibit impaired Parkin recruitment. We also find that clathrin-mediated endocytosis (CME) facilitates RalA and Parkin localization to mitochondria during mitophagy. Collectively, these data support a novel role for RalA during PINK1-Parkin mitophagy.

P3253  
Board Number: B541  
Nerve Growth Factor Induces Mitochondrial Fission Which is Required for Axon Branching.  
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Nerve Growth Factor (NGF) induces branching through activation of phosphoinositide 3-kinase (PI3K). Recently, mitochondria have emerged as major determinants of the sites of axon branching. NGF treatment decreased the length and increased the number of axonal mitochondria labeled with mitotracker green after 15 min of acute treatment, indicative of fission. Consistently, live imaging of mitochondria following 5 minutes of NGF treatment revealed mitochondria fission. The effect of NGF in mitochondria length was reversed after 3.5 hour of NGF withdrawal. Direct activation of PI3K using a cell permeable peptide in the absence of NGF copied the effects of NGF. Conversely, inhibition of PI3K using LY294002 blocked the effects of NGF on mitochondria. Pharmacological and peptide-mediated inhibition of dynamin related protein 1 (Drp1), a required component of mitochondria fission, blocked NGF induced axon branching. Live imaging of axons in the presence of NGF revealed that YFP-Drp1 accumulated at sites of mitochondria fission and endogenous Drp1 was detected in axons through immunocytchemistry. NGF promoted phosphorylation of Drp1 at the activating site S616 trough ERK activation independently of the PI3K pathway. Additionally, inhibition of ERK signaling blocked the effect of NGF in mitochondria fission and Drp1 accumulation along the mitochondria. Inhibition of actin polymerization using Latrunculin-A also blocked the effect of NGF in mitochondria fission and Drp1 accumulation. Furthermore, live imaging of axons indicates that actin patches strongly co-localize with sites of mitochondria fission. Expression of DRP1 dominant negative mutant, significantly decreased the number of branches in neurons that were raised in the presence of NGF. Finally, BDNF and NT3 also decrease the length and increase the number of mitochondria. Collectively, these observations indicate: 1) NGF mediates phosphorylation of DRP1 through ERK signaling, and drives mitochondria fission through the activation of both the ERK and PI3K pathways; 2) mitochondria fission is required for NGF-induced branching. As PI3K signaling is not involved in Drp1 phosphorylation, PI3K might contribute to fission through regulation of the actin cytoskeleton independently of changes in Drp1 activity. This work was supported by the NIH NS078030.
P3254

Board Number: B542

Cells compartmentalize their mitochondrial population to serve different metabolic purposes. P. CHANDRIS\textsuperscript{1}, C. GIANNOU\textsuperscript{1,2}, H. SHROF\textsuperscript{1}, G. PANAYOTOU\textsuperscript{1,2}, J. LONCAREK\textsuperscript{1}, D. Kong\textsuperscript{4}; 1NIBIB, National Institutes of Health, Bethesda, MD, 2NIDDK, National Institutes of Health, Bethesda, MD, 3Molecular Oncology, BSRC Al. Fleming, Athina, Greece, 4NCI, National Institutes of Health, Frederick, MD

Despite increasing interest regarding the role of the TCA cycle and its anaplerosis in cancer pathology and other diseases, valuable information gleaned from live cell imaging and the construction of genetically encoded fluorescent reporters for enzymatic activity related to the TCA cycle is missing from the field. Here we design and implement a set of fluorescently tagged chimeras to study the nanostructural organization of inner mitochondria membrane enzymes by means of fixed and live cell super resolution microscopy along with a new genetically encoded FRET reporter to monitor a-ketoglutarate metabolism upon selective depletion of energy sources. We further complement flow cytometry and live cell imaging data from our FRET reporter with biochemistry and metabolic activity assessment with the use of a Seahorse instrument and fixed and live super resolution imaging of inner mitochondria membrane and matrix enzymes. We present evidence that key enzymes involved in ketoglutarate metabolism alter their nanoarchitectural organization in a reversible and distinct manner in a subpopulation of mitochondria, as a response to selective energy source depletion. We confirm our live and fixed super-resolution data by performing correlative light – electron microscopy (CLEM) to further demonstrate the distinct, local organization of the mitochondrial matrix as a function of energy source challenge.

P3255

Board Number: B543

Mitochondrial-driven assembly of a cortical anchor for mitochondria and dynein. L.M. Kraft\textsuperscript{1}, L.L. Lackner\textsuperscript{1}; 1Molecular Biosciences, Northwestern University, Evanston, IL

Inter-organelle contacts facilitate communication between organelles and impact fundamental cellular functions. Here we examine the assembly of the mitochondria-ER-cortex anchor (MECA), which tethers mitochondria to the ER and plasma membrane. We find the assembly of Num1, the core component of MECA, requires mitochondria. Once assembled, Num1 clusters persistently anchor mitochondria to the cell cortex. Num1 clusters also function to anchor dynein to the plasma membrane, where dynein captures and walks along astral microtubules to help orient the mitotic spindle. We find that dynein is anchored by Num1 clusters that have been assembled by mitochondria. When mitochondrial inheritance is inhibited, Num1 clusters are not assembled in the bud and defects in dynein-mediated spindle positioning are observed. The mitochondrial-dependent assembly of a dual-function cortical anchor provides a mechanism to integrate the positioning and inheritance of two essential organelles and expands the function of organelle contact sites.
P3256
Board Number: B544
Investigating the molecular basis and regulation of the Mmr1-mitochondria association.
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During asymmetric cell division, many organelles have to be inherited by daughter cells and retained in mother cells through active transport and retention mechanisms. In order to better understand these mechanisms, we are using Saccharomyces cerevisiae as a model to study mitochondrial transport and retention in polarized, asymmetrically dividing cells. Mmr1 is a bud-enriched protein that functions as a mitochondrial adaptor for Myo2, which drives actin-based transport of mitochondria to the bud. Post-transport, Mmr1 is proposed to anchor mitochondria at the bud tip to ensure proper distribution of mitochondria. Although both functions require an interaction between Mmr1 and mitochondria, the molecular basis of the Mmr1-mitochondria interaction is not well understood. Our in vitro phospholipid binding assays indicate Mmr1 can directly interact with phospholipid membranes enriched in the mitochondrial specific phospholipid, cardiolipin (CL). We find that an unpredicted membrane binding site composed of amino acids 76-195 is both necessary and sufficient to interact with mitochondrial membranes in vivo and CL enriched liposomes in vitro. Furthermore, our work and that of others indicate that Mmr1 is a phosphoprotein. Our data suggest the phosphorylation of amino acids S81 and S83 within the identified membrane-binding domain regulate Mmr1’s interaction with the mitochondrial membrane. All together, our data provide insight into how phosphorylation may be used to spatially and temporarily regulate the Mmr1-mitochondria interaction and, consequently, the function of Mmr1 in mitochondrial inheritance.

P3257
Board Number: B545
Mitochondria-lysosome contacts regulate mitochondrial fission via Rab7 hydrolysis.
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Both mitochondria and lysosomes are critical for maintaining cellular homeostasis, and dysfunction of both organelles has been observed in multiple diseases. Mitochondria are highly dynamic and undergo fission and fusion to maintain a functional mitochondrial network which drives cellular metabolism. Lysosomes similarly undergo constant dynamic regulation by Rab7 GTPase, which cycles from active GTP-bound state into inactive GDP-bound state upon GTP hydrolysis. Here, we investigated the regulation of mitochondria-lysosome membrane contact sites in living cells using high spatial and temporal microscopy. Mitochondria-lysosome contacts dynamically formed in healthy untreated cells and were distinct from damaged mitochondria targeted into lysosomes for degradation. Contact formation was regulated by active GTP-bound lysosomal Rab7, while contact untethering was mediated by Fis1 recruitment of TBC1D15/Rab7-GAP to mitochondria to drive Rab7 GTP hydrolysis to release contacts. Functionally, lysosomal contacts marked sites of mitochondrial fission allowing for lysosomal regulation of mitochondrial network dynamics, while conversely, mitochondrial contacts regulated lysosomal Rab7 hydrolysis via mitochondrial-localized TBC1D15. Mitochondria-lysosome contacts thus allow for bidirectional regulation of mitochondrial and lysosomal dynamics, and may explain the dysfunction observed in both organelles in various human diseases.
P3258
Board Number: B546
Direct Detection of ER-Mitochondrial Contacts with Fully Quantified Fluorescence Microscopy.
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Inter-organelle contacts between the endoplasmic reticulum and the mitochondria are tightly regulated in cells. ER-mitochondrial contact sites have been painstakingly characterized through biochemical means, and yet much remains to be understood due to their small physical extent. As a result, even with super-resolution imaging, these inter-organelle contact sites are difficult to study through fluorescence colocalization measurements. Here, we have developed Fully Quantified Fluorescence Microscopy for use with nearly any fluorescence microscope, thereby enabling simple, rapid, and consecutive measurements of donor-quenched FRET efficiencies in biological samples. With this, we are working to characterize the spatial and temporal properties of ER-mitochondrial contact sites in live cells.

P3259
Board Number: B547
The polycystins are modulated by cellular oxygen sensing pathways and regulate mitochondrial function.
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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the development of fluid-filled renal cysts and is caused by mutations in the Pkd1 and Pkd2 genes that encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively. The polycystin proteins form a complex that participates in ciliary mechanosensation and that regulates calcium release from the endoplasmic reticulum (ER). Increasing evidence indicates that loss of PC1 leads to perturbations in energy production, although the mechanisms responsible for this effect are unclear. In an effort to find modulators of PC1 trafficking and function, we performed a Genome Wide siRNA screen and identified the oxygen-sensing Prolyl Hydroxylase Domain-containing protein PHD3 (or EGLN3) as a protein that influences PC1 trafficking. Moreover, we find that oxygen levels regulate the subcellular localization and the channel activity of the polycystin complex through its interaction with EGLN3, which hydroxylates PC1, thus providing a link between polycystin function and oxygen levels. Interestingly, we find that Pkd1\textsuperscript{−/−} cells that lack PC1 expression have a significantly lower oxygen consumption rate as compared to PC1-expressing cells and show less mitochondrial calcium uptake in response to bradykinin-induced ER calcium release. Inhibiting PHD3 function reduces mitochondrial oxygen consumption rate in PC1-expressing cells to the level that is measured in cells that lack PC1 expression. In support of a role for the polycystins in the regulation of mitochondrial calcium dynamics, we find that PC1 and PC2 co-fractionate with Mitochondria-Associated ER Membranes (MAMs). Taken together, our data identify a new regulatory pathway and suggests a novel role for the polycystins in sensing and responding to cellular oxygen levels.
P3260
Board Number: B548
Control of mitochondrial homeostasis by endocytic regulatory proteins.
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Mitochondria play essential roles in cellular energy processes, including ATP production, control of reactive oxygen species, and apoptosis. While mitochondrial function is regulated by the dynamics of fusion and fission, mitochondrial homeostasis remains incompletely understood. Recent studies implicate Dynamin-2 and dynamin-related protein-1 (Drp1), as GTPases involved in a step-wise mechanism for mitochondrial fission (Lee et al., Science, 2017). Here we identify the endocytic regulatory ATPase, EHD1, as a novel regulator of mitochondrial fission. Initially, we observed that EHD1-depletion induces a static and elongated network of mitochondria in the cell, suggesting that it either indirectly regulates or participates directly in the process of mitochondrial fission. To test whether EHD1 serves as a mitochondrial fission protein, we used a staurosporine-induced mitochondrial fragmentation assay. It has previously been demonstrated that upon depletion of Dynamin-2 or Drp1, cells are protected from staurosporine-induced fragmentation. However, when we depleted EHD1, we found that cells remained sensitive to staurosporine, suggesting that EHD1 likely functions differently than Dynamin-2 and Drp1, possibly in an indirect manner. Recent studies have demonstrated that VPS35 and the retromer complex influence mitochondrial homeostasis either by Mul1-mediated ubiquitination and degradation of the fusion protein Mitofusin2 (Mfn2), or by removal of inactive Drp1 from the mitochondrial membrane. Given that we have previously demonstrated that both EHD1 and its interaction partner, Rabankyrin-5, interact with the retromer complex, we thus hypothesized that these endocytic proteins might affect mitochondrial homeostasis by influencing retromer function. Indeed, we showed that EHD1-depletion caused a reduction in expression of the retromer subunit Vps35 subunit, while Rabankyrin-5 depletion induced sequestering of Vps35 in the Golgi region. These findings are consistent with a model proposed by Wang et al. (Nat. Med, 2016) in which Vps35 and the retromer is impeded in its ability to translocate to the outer mitochondrial membrane and remove inactive Drp1, thus facilitating localization of active Drp1 on the membrane and promoting fission. Currently we are exploring additional layers of regulation of the retromer by endocytic regulatory proteins and the impact on mitochondrial homeostasis. Overall, our studies provide new information on the previously uncharacterized relationships between endocytic regulatory proteins and mitochondria. Understanding the mechanisms underlying mitochondrial fission and fusion has important ramifications for cancer, cardiovascular diseases, and neurodegenerative disorders including Alzheimer’s disease and Parkinson’s disease.

P3261
Board Number: B549
Modulating mitochondria dynamics is a potential therapeutic strategy for MED13L syndrome.
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The mediator complex subunit 13-like protein (MED13L), is a component of the Cdk8 module (also containing cyclin C, Cdk8 and Med12) that associates with the RNA polymerase II holoenzyme. Loss of Med13L function results in several developmental defects. For example, MED13L mutation results in...
congenital heart disease including transposition of the great arteries, intellectual disability, muscular hypotonia and behavioral difficulties. One function of MED13 is to anchor cyclin C in the nucleus. Several stressors including oxidative damage interrupts this interaction resulting in cyclin C translocation from nucleus to mitochondria. At the mitochondria, cyclin C promotes mitochondrial fragmentation, an important step in early cell programmed death. In this report, we showed the skin fibroblast from a patient with carboxy-terminal truncated MED13L (HSF MED13L) exhibits released cyclin C and a high frequency of fragmented mitochondria. Our preliminary data suggests that aberrant mitochondrial fragmentation associated cytoplasmic localization causes a decrease in mitochondrial respiration of HSF MED13L. Seahorse analysis suggests that oxygen consumption of MED13L HSF cells is reduced suggesting that mitochondrial function is diminished in these cells. Finally, pharmacological inhibition of cyclin C re-localization partially restores mitochondrial function suggesting that mitochondrial fitness may be part of the pathogenesis associated with MED13 inactivation.

P3262
Board Number: B550
Carbon black nanoparticles disrupt mitochondrial dynamics in human lung cells.
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Carbon black (CB) is the primary nanoparticulate component of environmental pollution from fossil fuel combustion. Prior research has demonstrated that CB induces apoptosis, or programmed cell death, upon accumulation of CB via receptor-mediated endocytosis. The cellular consequences of CB incorporation are not well characterized. Human Bronchial Epithelial cells were used for all analyses described here. In evaluating CB incorporation into cells, we found a dose-dependent accumulation of CB that continues over several days. This identified CB as a stress dependent upon accumulation over time though acute affects have been noted in these studies. CB exposure causes an elevation of reactive oxygen species (ROS) within 8 hours of initial exposure. This result, along with observed abnormalities in mitochondrial dynamics in CB exposed cells garnered interest in mitochondria. CB concentrations ranging as low as 25 µg/ml induce a significant decrease in mitochondrial perinuclear localization at 36 and 48-hour time-points. Small/punctate mitochondrial numbers increased significantly with CB dosage over a period of 24-hours which is consistent with mitochondrial stress/dysfunction. Live cell imaging of mitochondrial motility revealed ER stress to disrupt mitochondrial motility, while CB showed no significant affect. Initial analysis of fission events showed that following 24-hours of stress, live cell capture of cellular behavior showed no elevation of fission rates in HBE cells. This suggest changes in mitochondrial numbers may occur at earlier timepoints. Future work with live cell imaging will assess mitochondrial fission events at different time points. This work reveals chronic CB exposure to impact mitochondrial dynamics in human lung epithelial cells.
P3263
Board Number: B551
Inhibition of mitochondrial fission and disruption of swimming behavior following Mdivi-1 treatment of Paramecium tetraurelia.
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Dynamin-related proteins are components of many cellular processes including mitochondrial fission, vesicle formation and programmed cell death. Previous studies in yeast and mammalian cells have shown that an inhibitor of yeast Dnm-1 protein activity (Mdivi-1) can block fission of mitochondria resulting in elongate mitochondrial networks. Effects on the mammalian homolog Drp-1 show similar effects and also reduce cytochrome C release, a trigger of apoptosis. Contrasting studies find that Mdivi-1 effects are more likely due to inhibition of electron transport chain complexes rather than fission interference. We have examined the effects of Mdivi-1 treatment on Paramecium tetraurelia. Paramecium are large, multinucleate cells covered in cilia that allow them to swim to areas rich in food. When Paramecium are starved, a programmed rearrangement of the macronucleus is triggered, likely by a caspase. The outcome is an easily observed, fragmented nucleus. Treatment with Mdivi-1 delayed entry into macronuclear rearrangement and also resulted in alterations in mitochondrial phenotype. Mitochondria became less punctate and showed denser fluorescence patterns, indicating increased networking. Cells also were significantly larger compared to control after treatment. Interestingly, when we treated cells during stationary phase, we noticed a decrease in swimming speed and increase in turning behavior. This could be due to inhibition of electron transport chain complexes. The effect was only transient, as cells tested 24 hours later showed no differences in swimming behavior. Our experiments indicate that both bodies of work related to mDiva effects on cellular processes related to mitochondria are likely valid.

P3264
Board Number: B552
Translocation of cyclin C during oxidative stress is regulated by interactions with multiple trafficking proteins.
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Eukaryotic cells take cues from their environment and interpret them to enact a response. External stresses can produce a decision between adjusting to behaviors which promote surviving the stress, or enacting a cell death program. The decision to undergo programmed cell death (PCD) is controlled by a complex interaction between nuclear and mitochondrial signals. The mitochondria are highly dynamic organelles that constantly undergo fission and fusion. However, a dramatic shift in mitochondrial morphology toward fission occurs early in the PCD process. We have identified the transcription factor cyclin C as the biochemical trigger for stress-induced mitochondrial hyper-fragmentation in yeast (Cooper et al., 2014 Dev. Cell) and mammalian (Wang et al., 2015, MCB) cells. In response to PCD stimuli such as oxidative stress, cyclin C is released from the nucleus and associates with the mitochondrial fission machinery. Loss of cyclin C prevents mitochondrial fission while its ectopic introduction into the cytoplasm induces complete fragmentation in the absence of stress. Many of the details of the control of cyclin C localization within the cell have not been elucidated.
To initially investigate the regulation of cyclin C translocation events in S. cerevisiae, a two hybrid screen was used. We have identified multiple conserved trafficking proteins which interact with cyclin C. These
include Gea1/2 and multiple components of the TRAPPII complex, both of which act as guanosine exchange factors (GEFs) for small GTPase regulators of vesicle traffic (Arf1/2 and Ypt31/32 respectively). Arf1 and its homolog Arf2 also mediate mitochondrial dynamics and contact with the ER. Gea2 interaction with cyclin C was confirmed by co-immunoprecipitation, and fluorescence microscopy data also indicates that the two proteins can colocalise. The use of conditional mutants has revealed that upon loss of functional Gea1/2 or Arf1/2, cyclin C is translocated to the cytosol without stress. Following from this we propose a model in which the protein trafficking machinery acts to keep cyclin C in the nucleus during healthy cell maintenance.

P3265
Board Number: B553
The single mitochondrion of *C. fasciculata* is a dynamic network.
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Mitochondria are dynamic organelles, undergoing fission and fusion in order to maintain their shape and function. Kinetoplastid parasites, including the causative agents of Chagas disease, African sleeping sickness and Leishmaniasis, possess unusual mitochondrial characteristics. For instance, each cell contains only one mitochondrion, which typically assumes the shape of a branched network extending throughout the cell. Also, all of the organism’s mitochondrial DNA is found in a unique structure called the kinetoplast. The cell cycle of these organisms must therefore be carefully orchestrated to coordinate division of the kinetoplast and mitochondrion with nuclear mitosis and cytokinesis. We are using the model kinetoplastid *Crithidia fasciculata* to investigate the timing of mitochondrial biogenesis and division within the parasite cell cycle. We have created a cell line expressing mitochondrial-targeted GFP and examined the fluorescence pattern in partially synchronized cultures. By measuring different parameters of the mitochondrial network during various cell cycle stages, we have determined that mitochondrial biogenesis occurs mainly in G1 and is correlated with cell growth. During mitosis, mitochondrial size remains constant before being divided symmetrically in a process coincident with cytokinesis. We also sought to observe mitochondrial growth and division in live cells. This analysis revealed that the mitochondrial network is surprisingly dynamic. Tubules branch, fuse and slide at a frequency of approximately one event every 5 minutes regardless of cell cycle stage. The role of such rapid mitochondrial remodeling in an organism with a single organelle is not yet clear, nor do we know the proteins that mediate these reactions. Interestingly, kinetoplastids lack homologs for most of the proteins involved in mitochondrial dynamics in yeast and mammals, suggesting that certain components of the machinery for fusion and fission may be novel. In exploring mitochondrial dynamics in an early-diverging eukaryote such as *C. fasciculata*, we hope to gain insight into the ancestral functions of these processes. In addition, our screen for molecules involved in maintenance of mitochondrial shape may reveal new proteins that also function in higher eukaryotes.
P3266
Board Number: B554
CoQ biosynthetic components form a supracomplex localized to ER-mitochondria contact sites.
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In eukaryotes, the conserved coenzyme Q biosynthetic pathway is housed within mitochondria and functions to enzymatically produce coenzyme Q (CoQ) - an essential electron transporting component of the respiratory chain. The pathway is comprised of at least 13 proteins, which build CoQ from 4-hydroxybenzoate and farnesyl-PP, derived from tyrosine and the extramitochondrial mevalonate pathway, respectively. Previously published biochemical and proteomic analysis indicate that a subset of CoQ pathway components interact together to form a dynamic, multi-subunit complex associated with the matrix side of the mitochondrial inner membrane. We examined the spatial organization and dynamics of the CoQ biosynthetic complex in vivo in budding yeast using endogenously expressed functional fluorescently-tagged Coq proteins. Our data indicate that components of the biosynthetic complex uniquely localized to multiple resolvable focal structures within mitochondria in cells. The relatively low cellular copy number of the Coq-labeled foci suggests that these structures represent supramolecular assemblies of the CoQ complex. Consistent with the high evolutionary conservation of this pathway, we also observed discrete Coq focal structures within mitochondria in human cells. In yeast, Coq foci formation was dependent on pathway components that are essential for the production of CoQ, suggesting that Coq substrates are required for supracomplex formation. To test a role for substrate in Coq supracomplex assembly, we utilized cells expressing an enzymatically inactive allele of Coq6 that can be bypassed by the addition of exogenous vanillic acid, a bihydroxybenzoic acid derivative. In these cells, Coq foci formation was strictly dependent on the presence of vanillic acid, indicating that supramolecular complex formation depends on substrate and/or substrate flux through the pathway. To gain insight into the function of the CoQ supracomplex, we examined its spatial localization in cells relative to other cellular structures. Our analysis indicates that Coq foci were highly enriched at ER-mitochondria contact sites. Thus, our data suggest a model where CoQ intermediates drive the formation of a supramolecular CoQ biosynthetic machine at ER-mitochondria contact sites to facilitate substrate influx and utilization as well as product distribution.

P3267
Board Number: B555
A HIGH-CONTENT LIVE IMAGING MITOPHAGY ASSAY TO EVALUATE SMALL MOLECULE MITOPHAGY ENHANCERS.
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Human genetic evidence has implicated mitochondrial dysfunction in the etiology of Parkinson’s disease, a neurodegenerative disorder characterized by motor dysfunction. Loss of function mutations in the PARK2 (encoding parkin) and PINK1 genes cause early-onset recessive Parkinson’s disease. PINK1, a protein kinase, and parkin, a ubiquitin ligase, act together to remove damaged mitochondria from the cell by mitophagy, a crucial process to maintain healthy mitochondria. These findings suggest that enhancing mitophagy may be a therapeutic strategy for Parkinson’s disease. To evaluate the effects of
small molecules on mitophagy, we developed a stable PARK2 overexpressing U2OS cell line with a tandem RFP+GFP reporter that is tagged to mitochondria by a MTS signal. RFP is acid-stable, whereas GFP is quenched in acid environments, such as the lysosome. Mitochondria tagged with this reporter appear orange (RFP+GFP) in the cytoplasm. Once they undergo mitophagy and end up in the lysosomes, the GFP fluorescence is quenched and only the red RFP fluorescence remains visible. High content live imaging of this cell line shows that administration of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) causes a dose-and time-dependent increase in mitophagy. These effects are mediated by parkin as confirmed by the absence of CCCP-induced mitophagy in control cells. Transient overexpression of USP30 decreases mitophagy as previously demonstrated by others. HBX 41,108, a non-selective USP30 inhibitor, dose-dependently enhances CCCP-induced mitophagy, whereas Nilotinib, a BCR-Abl inhibitor, shows little effect. Neither inhibitor has an effect in the absence of CCCP in this assay. A Tank-binding kinase 1 inhibitor BX795 inhibited CCCP-induced mitophagy. These data suggest that high content live imaging with the PARK2 and tandem RFP+GFP reporter overexpressing U2OS cell line is a suitable assay for evaluating small molecule enhancers of parkin-mediated mitophagy.

P3268
Board Number: B556
A novel pharmacological tool blocks physiological mitochondrial fission through specifically inhibiting the Mff-Drp1 protein-protein interaction.
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Within a single mammalian cell, thousands of mitochondria form a dynamic network. The morphological state of this network is tightly linked to its function. Cellular viability depends on maintaining a balance between fission and fusion of mitochondria within this network. An imbalance between these two processes is a hallmark of numerous pathologies. Excessive fission is found in cancers, cardiovascular disorders, and neurodegeneration. While mitochondrial fission poses as an attractive therapeutic target for these disorders, better tools are needed to differentiate physiological fission from excessive pathological fission or fragmentation. Four identified adaptor proteins recruit the main mediator of fission, dynamin related protein 1 (Drp1), to the mitochondrial outer membrane, yet the function of each adaptor is unknown. One adaptor Mff is primarily responsible for physiological fission. We hypothesized that a specific Mff-Drp1 protein-protein interaction inhibitor would differentiate physiological from pathological fission. Using a rational design approach, we identified a small peptide P259 that specifically inhibits Drp1’s interaction with Mff, but not with any of the other outer-membrane adaptors. Contrary to a pathological fission inhibitor, treatment with P259 disrupted mitochondrial network morphology and function under basal conditions. In WT mouse brains, P259 limited mitochondrial recruitment of Drp1, resulting in a 30% decrease in ATP production and enlargement of striatal mitochondria. Furthermore, treatment with P259 reduced the lifespan of R6/2 Huntington’s disease model mice and caused behavioral abnormalities in WT mice. Cumulatively, these results suggest that Mff orchestrates basal recruitment of Drp1 to the mitochondrial membrane thereby by facilitating physiological fission and maintaining mitochondrial network homeostasis. Results from this study suggest that adaptor specific recruitment of Drp1 mediates pathological versus physiological fission.
P3269
Board Number: B557
The size of the fission complex based on the FCS calibrated imaging of GFP-Drp1 protein in the HeLa cell line.
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One of the main players in the process of mitochondrial fission is the protein Drp1 which assembles into helical ring-like structures on the outer mitochondrial membrane. Using fluorescence microscopy we performed a quantitative analysis of the diffusion and distribution of the GFP-Drp1 protein expressed in the gene edited HeLa cell lines. The obtained distribution shows that around half of the endogenous GFP-Drp1 pool remains in the cytoplasm, predominantly as the tetramer. The mitochondrial pool of GFP-Drp1 forms many different oligomeric states with average size of the complex equal to 56 GFP-Drp1 monomers. We identified individual fission events on the recorded time lapse movies and estimated the average number of Drp1 molecules forming the functional fission complex to be around 100.

P3270
Board Number: B558
An optical method for detecting associations between the endoplasmic reticulum and mitochondria, and their relevance to motor neuron disease.
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Physical membrane interactions between the ER and mitochondria play a key role in a number of cellular processes including calcium homeostasis, lipid trafficking and cell death signalling. These organelles are tethered by the formation of protein complexes at specialised regions of ER membrane termed mitochondrial associated membranes or MAMs. Dynamic changes in these contact sites are observed in response to certain physiological stresses, and perturbations to the MAMs have been identified in both familial and sporadic forms of neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis (ALS). It has been suggested that dysfunction to ER-mitochondria contacts may be a common pathogenic mechanism underlying neuronal cell death. The integral ER membrane protein VAPB is enriched at the MAMs and binds to the outer mitochondrial membrane (OMM) protein PTPIP51 to regulate membrane associations. A missense point mutation vapB<sup>S565</sup> is associated with a familial form of ALS termed ALS type 8. Here, we have developed a bi-fluorescence complementation (BiFC) system for the visualisation of ER-mitochondria contacts in both fixed and living cells using complementary split Venus YFP fragments fused to the ER targeting domain of VAPB or the OMM targeting sequence of the mitochondrial protein TOMM20 respectively. Co-expression of these fusion proteins yields discrete fluorescent puncta at regions of close apposition between the ER and mitochondria (<10nm distance), consistent with a MAM localisation. We have validated the use of this approach by detecting an increase in ER-mitochondria contacts in response to tunicamycin-induced ER stress and serum deprivation as previously observed. By using this technique, we have detected a significant increase in the number of fluorescent puncta in NSC34 cells expressing the ALS-linked VAPB<sup>S565</sup> but not from overexpression of wild-type VAPB. Further work will allow us to directly correlate features of the MAMs with mitochondrial abnormalities and dysregulated calcium homeostasis as is observed in neurodegeneration.
**P3271**

**Board Number: B559**

A STANDARDIZED METHOD TO QUANTIFY ER-MITOCHONDRIAL INTERFACES IN ELECTRON MICROGRAPHS.

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The interaction sites between endoplasmic reticulum (ER) and mitochondria are signaling hubs in the cell implicated in calcium transfer, lipid metabolism, autophagy, and cell death among other functions. Transmission electron microscopy (TEM) is commonly used to visualize the geometry of these interfaces, but metrics used to describe and compare between conditions, as well as the techniques of measurement are not standardized. Here we describe a pair of scripts for ImageJ that allow for rapid, reproducible and flexible quantification of interface geometries. One script is focused on measuring the physical apposition of the membranes while the other considers the potential exposure of the outer mitochondrial membrane (OMM) to calcium released from the ER. In both cases, the user simply traces the OMM and nearby ER membranes; the script bins the interface distances and returns the length of the OMM that participates in an interface of a given gap width, as well as the total OMM and ER lengths. A ‘score’ based on the inverse-square of the distance is also generated as an attempt to make a single value representing ‘interface-ness’. The additive nature of these measures allows cell-wise totals or averages to be calculated for a given TEM section. While this approach standardizes the measurement technique for a number of parameters of ER-mitochondrial interfaces, it remains to be seen which parameters best correspond with physiological changes in different models.

**P3272**

**Board Number: B560**

Mechanistic insights into the regulation of mitochondrial fission by cyclin C.

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Cyclin C is a component of the mediator complex of RNA polymerase II that localizes to the nucleus under normal conditions. In response to stress, cyclin C translocates to the cytosol and mitochondria and mediates stress-induced mitochondrial fission and apoptosis. The molecular mechanisms by which cyclin C induces mitochondrial fission are unknown. Using in vitro experimental approaches, we sought to investigate the mechanistic basis of cyclin C mediated mitochondrial fission. We found that recombinant cyclin C and Drp1, the dynamic-like GTPase that produces mitochondrial scission, directly interact with each other without the requirement of any accessory proteins. This interaction requires the C-terminal 120 amino acids of cyclin C independent of the cyclin Box motif that directs Cdk binding. Upon heterologous expression, the GFP tagged carboxyl terminal domain localized to mitochondria and induced mitochondrial fission in cyclin C null MEF cells even in the absence of stress. On the contrary, the N-terminal 250 amino acid cyclin box domain is responsible for the nuclear retention of cyclin C under normal conditions and cannot induce stress-induced mitochondrial fission. The GTPase domain of Drp1 is an important site of interaction between Drp1 and cyclin C. Using size-exclusion chromatography and native-PAGE, we found that cyclin C reduces the oligomerization of Drp1 in solution. This is accompanied by a concomitant decrease in Drp1 GTPase activity in the presence of lipids. The ability of cyclin C to depolymerize Drp1 is enhanced by GTP and GTP analogs. We hypothesize that cyclin C
depolymerizes inactive multimeric Drp1 in solution enabling the interaction of Drp1 with the mitochondrial fission machinery to facilitate mitochondrial fission.

Receptors, Transporters, and Channels

P3273
Board Number: B561
The Role of Golgi Apparatus in Phagocytosis: Ca2+-Dependent Focal Exocytosis of Golgi-derived Vesicles Helps Uptake in Macrophages.
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During phagocytic uptake by macrophages, role of Golgi apparatus was previously ruled out. Notably all such reports were limited to Fcy-receptor mediated phagocytosis. Here we unravel a highly devolved mechanism for recruitment of Golgi-derived secretory vesicles during phagosome biogenesis, which was important for uptake of most cargos except IgG-coated ones. We report recruitment of Mannosidase-II positive Golgi-derived vesicles during uptake of diverse targets including latex beads, E. coli, Salmonella Typhimurium and Mycobacterium tuberculosis in human and mouse macrophages. The recruitment of Mannosidase-II vesicles was an early event mediated by focal exocytosis and coincided with the recruitment of transferrin receptor, VAMP3 and dynamin-2. Brefeldin A treatment inhibited Mannosidase-II recruitment and phagocytic uptake of serum coated or uncoated latex beads and E. coli. However consistent with previous studies, Brefeldin A treatment did not affect uptake of IgG-coated latex beads. Mechanistically recruitment of Mannosidase-II vesicles during phagocytic uptake required Ca2+ from both extra- and intra-cellular sources apart from PI3Kinase, microtubules and dynamin-2. Extracellular Ca2+ via voltage-gated Ca2+ channels establish a Ca2+-dependent local PIP3 gradient, which guides the focal movement of Golgi-derived vesicles to the site of uptake. We confirmed Golgi-derived vesicles recruited during phagocytosis were secretory vesicles as their recruitment was sensitive to depletion of VAMP2 or NCS1 whereas recruitment of recycling endosome marker VAMP3 was unaffected. Both VAMP2 and NCS1 depletion individually resulted in the reduced uptake by macrophages. Together the study provides a previously unprecedented role of Golgi-derived secretory vesicles in phagocytic uptake, the key innate defense function.

P3274
Board Number: B562
TRPM7 Ion Channel Regulates Magnesium Reabsorption in the Renal Proximal Tubule.
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Mg2+ deficiency can be identified in up to 60% of critically ill patients and contributes to many human diseases and conditions, including hypertension and diabetes. One cause of hypomagnesemia stems from phosphate depletion and hyperparathyroidism, but the mechanisms by which hypomagnesemia develops remains undetermined. Phosphate reabsorption in renal proximal tubule segment is mediated by sodium-phosphate cotransporters, which are bound to and regulated by NHERF1 and NHERF3 scaffolding proteins. The TRPM7 ion channel, which is associated with hypomagnesemia with secondary hypocalcemia (HSH) disease, is also expressed in the proximal tubule, but its functions in this nephron
segment remain unknown. To determine the impact of TRPM7 channel on Mg\(^{2+}\) reabsorption in the proximal tubule, we generated mice lacking the channel specifically in proximal tubule by crossing TRPM7\(^{−/−}\) mice with PEPCK-Cre transgenic mice. TRPM7\(^{−/−}\) (PEPCK-Cre) mice excrete more Mg\(^{2+}\) in urine than control TRPM7\(^{+/+}\) mice but have a lower serum Mg\(^{2+}\) level. This data indicates that TRPM7 is involved in controlling the renal Mg\(^{2+}\) reabsorption in the proximal tubule. We conducted a yeast two-hybrid screen using a mouse kidney library and identified NHERF3 as a potential TRPM7-interacting protein. Biochemical approaches were employed to confirm the interaction, and we found that the COOH-terminal six amino acids of TRPM7 constitute a PDZ binding motif, which is required for the channel binding to the 2nd PDZ domain of NHERF3. NHERF3 is co-localized with TRPM7 in proximal tubule epithelial cells, where it appears to regulate the concentration of the channel on the apical membrane. We previously showed that the TRPM7 channel is regulated by G protein coupled receptors signaling through the phospholipase C (PLC) and protein kinase C (PKC) pathway. Our experiments in the proximal tubule cell line have shown that PKC regulates internalization of the channel. We conclude that TRPM7 regulates Mg\(^{2+}\) reabsorption in the renal proximal tubule and that NHERF3 plays a critical role in TRPM7 channel regulation.

**P3275**

**Board Number: B563**

**STIM1-Induced Conformational Transition of Orai1 Leads to Channel Activation.**

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The immune response mechanisms at the cellular level are mostly triggered by a decrease in the concentration of Ca\(^{2+}\) within the endoplasmic reticulum (ER), followed by the opening of the calcium release-activated calcium (CRAC) channels that leads to an increase in the concentration of intracellular Ca\(^{2+}\). The stromal interaction molecule1 (STIM1) detects the decline of ER Ca\(^{2+}\) concentration and activates the channel via protein-protein interaction with Orai1 protein (the pore unit of CRAC channel). Mutations in Orai1 that impair its function cause severe immunodeficiency in humans. Although there are some experimental studies on the activation mechanism of Orai1 channel using STIM1, the molecular details of STIM1 interaction with Orai1 that results in channel opening are not yet understood. For example, there are two distinct STIM1 binding sites on Orai1, one in the N-terminus and one in the C-terminus. Yet, it is not known whether the C-terminus binding can cause Orai1 conformational changes and trigger channel gating by itself or requires N-terminus binding as well. To understand the molecular details of this signal transduction pathway, we developed all-atom molecular models of the STIM1/Orai1 complex and studied the binding of STIM1 to C-terminus of Orai1 followed by conformational changes in Orai1 that leads to channel activation. In this study, we compared Orai1 conformation after STIM1 binding with that in the control simulation, where STIM 1 was excluded. Our results showed an important conformational change in the Orai1 channel upon STIM1 binding in which M1 helices in the pore of Orai1 are bent. Furthermore, we observed an increase in the volume of the pore that most likely facilitates the gating process, while the hydrophobic part and glutamate ring remained fairly fixed. Thereby, our results indicated the mechanism by which STIM1 binding to the Orai1 C-terminal induces activation and suggests that such binding is most likely both necessary and sufficient for channel gating.
P3276
Board Number: B564
Lysosomal calcium signaling through channel TRPML1 is impaired by lipofuscin accumulation in RPE cells.
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The TRPML1 channel is a conduit for lysosomal calcium efflux, and channel activity may be affected by lysosomal contents. The lysosomes of retinal pigmented epithelial (RPE) cells are particularly susceptible to lysosomal accumulations as they must degrade the outer segments phagocytosed daily from adjacent photoreceptors, and incomplete degradation can lead to storage of waste material and lipofuscin in the cells. This study asks whether stimulation of TRPML1 can release lysosomal calcium in RPE cells and whether such release is affected by lysosomal accumulations. The TRPML agonist ML-SA1 raised cytoplasmic calcium levels in mouse RPE cells and ARPE-19 cells; this rise was rapid, robust, reversible, and reproducible. The rise was not altered by extracellular calcium removal or by thapsigargin, but was eliminated by lysosomal rupture with glycyl-L-phenylalanine-beta-naphthylamide (GPN). Treatment with desipramine to inhibit lysosomal acid sphingomyelinase or YM201636 to inhibit PIKfyve reduced the cytoplasmic calcium rise triggered by ML-SA1. Co-treatment with chloroquine and U18666A induced formation of neutral, autofluorescent lipid in RPE lysosomes and decreased lysosomal calcium release. Lysosomal calcium release was also impaired in RPE cells from the ABCA4/- mouse model of Stargardt’s retinal dystrophy. Neither TRPML1 mRNA nor total lysosomal calcium levels were altered in these models, suggesting a more direct effect on the channel. In conclusion, stimulation of TRPML1 elevates cytoplasmic calcium levels in RPE cells, but this response is reduced by lysosomal accumulations.

P3277
Board Number: B565
High Throughput Functional Characterization of the SLC-Transporters PEPT1 and OCT2 in Real Time.
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PepT1 and OCT2 are members of the SLC transporters family. Recently, they are of high interest in the development process of new pharmacological agents, due to their involvement in drug uptake and secretion. PepT1 is involved in absorption of oligopeptides in the small intestines, working as a proton/peptide symport into the cell, while OCT2 is involved in renal secretion of organic cations. Due to this growing interest in membrane transporters, there is an increasing demand for reliable high throughput systems, enabling the measurement of membrane transporter activity. Electrophysiological methods generate detailed and high quality data. However, conventional electrophysiology is in most cases not applicable or inefficient for the investigation of membrane transporters, due to their usually low turnover rates compared to ion channels.

Here we present dose responses based on single point or cumulative additions post repeated activations of both transport types under different pharmaceutical targets. Electrophysiological data of the SLC transporters PepT1 and Oct2 was generated with a fully automated high throughput platform, applying solid-supported membrane (SSM) based electrophysiology. This method, proven by about 100 peer-reviewed publications, allows the recording of a large number of individual transport proteins in parallel, ensuring a better signal to noise ratio compared to patch clamp.
Using this system, we were able to compare the kinetics (Km, vmax) of three different OCT2 substrates in a highly parallelized manner. By a competitive inhibitor assay we determined the IC50 of a PepT1 inhibitor. Furthermore, we performed several case studies of various targets, such as NaK-ATPase, mitochondrial transporters, channel rhodopsin, sugar transporters, and others.

P3278
Board Number: B566
Identifying Proteins that Interact with the Yeast Multidrug Transporter Pdr5 Through Genetic Suppression.
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Critical to an understanding of how ABC transporters function is the identification of interacting proteins and their biochemical function. We analyzed a mutation (E1013A) in the C-loop of the deviant ATP-binding site of Pdr5, a highly promiscuous multidrug transporter. This mutation causes a modest reduction in ATP hydrolysis and increased sensitivity to xenobiotic compounds. To identify proteins that interact with Pdr5, we transformed this mutant with a yeast genomic clone bank that overexpresses yeast genes and selected colonies with restored resistance to clotrimazole, a potent Pdr5 transport substrate. Of particular interest was a plasmid encoding the Sks1, serine/threonine kinase, which phosphorylates Ser-837 located in the linker between transmembrane helix 6 and nucleotide-binding domain 2. Overexpression of Sks1 increases drug resistance without increasing the level of Pdr5 in plasma membrane vesicles or restoring ATPase activity. It also further increases resistance in wild-type strains. The Sks1 kinase is known to be induced during glucose and nitrogen starvation. Our research demonstrates, however, that although Pdr5-mediated resistance is diminished under these conditions, it is not rescued by overexpressing Sks1.

P3279
Board Number: B567
Heparan Sulfate restricts BMP signaling and BMPR dynamics and interactions, mechanisms possibly altered in Hereditary Multiple Exostoses.
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The bone morphogenetic protein (BMP) signaling pathway has critical roles in many processes including skeletal development and growth, and its perturbations can lead to diverse pathologies. A case in point is Hereditary Multiple Exostoses (HME), a pediatric musculoskeletal disorder caused by loss-of-function mutations in the heparan sulfate (HS)-synthesizing enzymes EXT1 and EXT2. HME is characterized by benign cartilaginous tumors (exostoses) that form next to the growth plates of various skeletal elements, causing multiple health problems. The HS chains, and the proteoglycans of which they are part, regulate signaling by key HS-binding proteins including hedgehogs and FGFs, but their roles in BMP signaling remain unclear. Previously we showed that interference with HS function by genetic or pharmacological means rapidly increases canonical BMP signaling, suggesting that HS normally limits BMP signaling by restricting ligand availability, BMP receptor (BMPR) dynamics and/or BMPR-ligand interactions. To analyze such possibilities, we transfected cell lines with constructs encoding Snap-BMPRII and/or Snap-BMPRIa fusion proteins. Co-transfected cells rapidly responded to BMP-2 treatment with major increases in pSMAD1/5/8 and interestingly, the same was seen after treatment
with HS antagonist Surfen. To assess BMPR dynamics, we carried out fluorescence recovery after photobleaching assays and found that receptor mobility decreased significantly after treatment with BMP-2 or Surfen, suggesting that the receptors had transitioned to lipid rafts and/or had undergone oligomerization. To demonstrate that BMPRII population transitioned to lipid rafts, we carried out sucrose gradient ultracentrifugation to separate lipid raft and non-lipid raft cell surface domains. Indeed, treatment with Surfen recruited an abundant amount of BMPRII to the lipid raft domains compared to control-treated cells. Lastly, to show that BMPRIA and BMPRII undergo oligomerization during treatment with Surfen, we performed in situ proximity ligation assays. As indicated by increased number of cell surface fluorescent sites, treatment with Surfen did promote receptor-receptor interactions compared to the control-treated cells. Together, the data indicate that cell surface HS chains are important regulators of both BMP signaling and BMPR dynamics. The HS deficiency in HME may alter these important basic physiologic mechanisms, causing increases in local BMP signaling and promoting formation of exostoses.

P3280
Board Number: B568
Genetic improvement of iron content in Arabidopsis seeds through double Overexpression of IRP1 (Iron Responsive Protein 1) and VTL (Vacuolar Iron Transporter-Like) genes.
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Iron is an essential element that participates in numerous oxidation-reduction reactions in all organisms. Iron exist in two reversible oxidation states, Fe\(^{2+}\) and Fe\(^{3+}\), and chemical properties of these redox states determine the unique functions of Fe in electron transport chains in respiration and photosynthesis. Fe is also important in metabolic processes like DNA, lipid synthesis, removing ROS and nitrate assimilation. Recent evidence shows that in monocotyledon plants the re-mobilization of iron to the apoplast is a controlling factor in H2O2 production associated with the activation of defenses in plants. According to the WHO, 2002, about two billion people suffer from iron anemia. The strategies that the increase iron content in plants by biotechnological methods may be useful to decrease iron anemia in the world. In my research I am testing a new strategy for increasing the Fe content in the plant Arabidopsis. Our lab is focusing on the small family of vacuolar Fe transport proteins (5 VTL proteins) that we have recently shown to be involved vacuolar Fe homeostasis. Furthermore I am working on IRP1 gene that is involved in iron acquisition and homeostasis by an unknown mechanism. The results show that double over-expressing plants VTL1×IRP1 (4.3 nmol/mg), VTL2×IRP1 (3.7 nmol/mg), VTL3×IRP1 (4.8 nmol/mg), VTL5×IRP1 (4.0 nmol/mg) have higher amounts of iron compare with the wild type (2.7 nmol/mg). Localization of Fe in Arabidopsis seedlings was visualized using Perls’ staining with DAB intensification. For this study, six-day-old seedlings, which had been grown on ES solid medium with 40 μM iron, were stained. In all VTL-IRP1 OE lines the localization of Fe was stronger than the wild type, with some unique localization in the VTL4 and 5-IRP OE lines. These points may show accumulation of iron in the nucleus and/or vacuoles. Morphological analyzes support an increased iron content of the plant, Over-expressed lines were grown on agarose media containing different iron concentrations (40 μM and 0 μM Fe) and the length of primary roots was analyzed after 7 days. Results show that inVTL2- IRP1 and VTL5 - IRP1 plants in 0 μM Fe, the root length was the same as the root length in 40 μM iron and confirm that these plants do not suffer from iron deficiency, likely as a result of their genetic background. All these results establish a proof that gene manipulating of VTL and IRP1 is novel strategy to increase in bio-available seed Fe.
P3281

Board Number: B569

POST-STIM1 interactions differentially regulate PMCA4 splice variant function during T cell activation.

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T cells are dependent upon prolonged elevations in cytosolic Ca\textsuperscript{2+} to initiate NF-kB/NFAT gene expression signatures in response to antigen presentation. STIM1-dependent inhibition of Ca\textsuperscript{2+} extrusion through PMCA4 contributes to this Ca\textsuperscript{2+} signal. Recent investigations revealed the ER membrane protein Partner of STIM1 (POST) as a STIM1-binding protein proposed to negatively regulate PMCA4 function, although this was not studied in the context of T cell activation, the focus of the current study. Interestingly, while we confirmed that POST negatively regulates PMCA4 activity in resting T cells, this role changes in response to T cell activation, with POST opposing STIM1-mediated attenuation of PMCA4 activity. To further define this phenomenon, we co-expressed POST and/or STIM1 with either PMCA4a or PMCA4b splice variants in Jurkat T cells. Inhibition of PMCA4b-mediated Ca\textsuperscript{2+} clearance by STIM1 was independent of T cell activation or POST whereas PMCA4a exhibited a STIM1-induced activation-dependent delay in Ca\textsuperscript{2+} clearance that was enhanced by POST KD. Analysis of molecular interactions by colocalization and FRET studies revealed complex activation-dependent interactions between all 3 proteins, with STIM1 and POST stably associating but POST dissociating from PMCA4a upon activation. Finally, to understand the implication of POST-mediated control of Ca\textsuperscript{2+} clearance on T cell function, we examined control of Ca\textsuperscript{2+} oscillations and NFAT activation. Interestingly, significant Ca\textsuperscript{2+} oscillations were observed in both resting and stimulated T cells, with POST expression inversely related to oscillation frequency. These results reveal differential regulation of PMCA4a and PMCA4b by STIM1 and POST during T cell activation, with profound implications for the generation of Ca\textsuperscript{2+} oscillations and, perhaps, downstream activation of NFAT.

P3282

Board Number: B570

Super resolution localization and live-tracking analyzed by pair correlation and a novel powerspectral method reveal short-term cAMP regulation of the water channel aquaporin-3.

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Single particle localization and live tracking in cells are essential to reveal nanoscale regulation of proteins. The water channel aquaporin-3 (AQP3) is important for the renal ability to concentrate urine but little is known regarding plasma membrane regulation of AQP3 in response to short-term hormone
mediated urine concentration facilitated by increased levels of cAMP. Super resolution Photoactivatable Localization Microscopy (PALM) with quantitative Pair Correlation analysis (PC-PALM) revealed cAMP mediated nanoscale clustering of AQP3 in the plasma membrane upon cAMP stimulation. Novel power spectral analysis of live-PALM image sequences of tracks lasting 5 frames revealed that while the measured diffusion coefficients of AQP3 were identical between control and cAMP stimulated cells, the confinement radius increased significantly, in qualitative agreement with the results from PC-PALM analysis. Thus fixed and live PALM measurements revealed a change in AQP3 plasma membrane nano-organization upon cAMP stimulation, indicating short-term hormone regulation of AQP3 at the nanoscale level. This regulation of nano-organization may play an important physiological function in regulation of urine concentration and body water homeostasis.

P3283

Board Number: B571

Characterization of the membrane progesterone receptor beta (mPRβ) using the Xenopus laevis oocyte model.

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Membrane progesterone receptors (mPRs) are distinct from the classical nuclear progesterone receptors and have 7 membrane spanning domains and belong to the progestin and AdipoQ receptor (PAQR) family. Unlike the classical nuclear receptor which mediates its effects via genomic mechanisms, mPRs are cell surface receptors which rapidly alter cell signaling via non-genomic modulation of intracellular signaling cascades. Emerging physiological roles for mPRs have been documented in different tissues, including oocytes, testis, uterus, placenta, breast, brain and immune cells. This raises the profile of mPRs as potential therapeutic targets for different diseases linked to neurological disorders, cancer, fertility-related issues and type II diabetes. However, the structure of mPRs and their immediate downstream intracellular signaling pathways remains ill defined. Here we use progesterone (P4)-dependent release of meiotic arrest in the Xenopus laevis oocyte to characterize mPR structure and function. We show that mPR topology is opposite to that of GPCRs with an extracellular C-terminus and a cytosolic N-terminus. We further show through deletions of the N- or C-terminus that the N-terminal domain is essential for mPR signaling, whereas the C-terminus is crucial for XmPR trafficking and plasma membrane residence. Since mPR shares sequence similarity with alkaline ceramidase, we have initiated studies to test whether mPR in the oocytes has ceramidase activity. We used metabolomics and pharmacological approaches and showed that the ceramidase inhibitor, ceranib1, negatively regulates P4-induced oocyte maturation, whereas preliminary metabolomics data revealed changes in free fatty acids in response to P4 treatment. Collectively, our data provide new insights into the structure-function relationship of membrane progesterone receptors with physiological implications in different tissues.
P3284
Board Number: B572
CYSTEINE-LESS ISOFORMS OF GLUT1 AND GLUT2 ARE FUNCTIONAL AND SUITABLE FOR BIOCHEMICAL AND PHYSICOCHEMICAL STUDIES.
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GLUT transporters are a family of transmembrane proteins that facilitate the translocation of various sugars across membranes. Because these carriers are involved in metabolic diseases and cancer, understanding the molecular details of sugar transport is critical for the rational design of drugs targeting these diseases. The recent publication of crystal structures of some members of this family provides a starting point for assembling the mechanism of sugar transport, but further biochemical and physicochemical characterization is needed. Here, we generate and functionally characterize constructs of GLUT1 and GLUT2 devoid of their six cysteine residues (C-less) as platforms for biochemical and physicochemical studies, i.e. cysteine scanning mutagenesis and site-specific labeling. To test the functional expression of the C-less proteins, we used a combination of overexpression in cells (HEK 293T and CHO-K1 cells, and Xenopus oocytes) followed by kinetic assays of radiolabeled 2-deoxyglucose (DOG) uptake. Our results indicate that both constructs retain native-like functionality, as judged by the minor changes in kinetic parameters when compared to the wild-type carriers. Similarly, both proteins preserve their sensitivity to the GLUT inhibitors cytochalasin B and quercetin, which confirms kinetic properties similar to those of the wild-type transporters. As proof of concept, we introduced cysteine residues in GLUT2 C-less and test accessibility of MTSET, an alkylating thiol-reactive agent, by following changes in DOG uptake. While MTSET did not affect DOG import into Xenopus oocytes expressing the C-less protein, it blocked the activity of GLUT2 wild-type and that of mutants carrying single cysteine residues at selected positions. Together, our results suggest that replacement of the cysteines residues of GLUT1 and GLUT2 causes minimal structural and functional alterations. We propose that these constructs are suitable platforms for biochemical and physicochemical studies aimed at understanding the interactions of the transporter with its substrate and immediate environment. Funding: FONDECYT 3160734 (MV-U), and FONDECYT 1130386 and FONDEF D11i1131 (AMR)

P3285
Board Number: B573
A novel inactivation mechanism of STIM1 involving the interplay of multiple cytosolic domains in a resting state.
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Store-operated Ca2+ entry (SOCE) is the main supervisor of calcium signaling pathways in various cellular processes like the gene expression, differentiation. Two key components of the store-operated calcium channel (SOC) or calcium released activated Ca2+ channel (CRAC) complex are stromal interaction molecule 1 (STIM1), an ER Ca2+-sensor, and Orai1, a pore-forming channel protein in the plasma membrane. When ER store is depleted, STIM1 undergoes conformational changes that unveil CAD (CRAC channel-activating domain). CAD binds and activates Orai1. STIM1 is known to have its inhibitory domain within a cytosolic domain called IDstim right after CAD region. However, the molecular mechanism by which the inhibitory domain induces rearrangement of functional domains of STIM1 by shielding CAD in the closed state before store depletion remains largely unclear. Here we identified a linker between CAD and the inhibitory domain (IDstim) of STIM1 is another functional
determinant and essential for stabilizing the closed state of STIM1, which might arrange the interactions between IDStim and CAD associated domains. Surprisingly, structural distortion of the linker of CAD and IDStim activates the CRAC channel without store depletion by altering the segments by the mutation and insertion of a different length of ELP linker. Also, STIM1 deleted IDStim is capable of constitutively activating Orai1, verifying an inhibition effect of IDStim. Furthermore, we found the deletion of CC1 causes loss of IDstim inhibition effect. Especially, leucines (L251 or L258) in CC1 which is critical in CC1-CC3 interaction, are important for IDstim-related inhibition of CAD activity. These studies propose, establishes a detail STIM1 resting/inactivation mechanism by which IDstim rearranges CAD by stabilizing the interaction of CC1-CC3 domain in the closed state.

P3286
Board Number: B574
K⁺ Channel Tetramerization Domain 5 (KCTD5) protein is a novel TRPM4-associated protein that regulates channel activity and cell migration.
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Cell migration is a fundamental process involved in physiological and pathological events such as wound healing, embryonic development and cancer metastasis. Cell migration regulation depends on a variety of mechanisms such as cytoskeleton rearrangements, focal adhesions turnover and local Ca²⁺ oscillations. TRPM4 is a Ca²⁺-activated non-selective cationic channel that conducts monovalent but not divalent ions. We previously demonstrated that TRPM4 channels regulate cell migration, contractility and is required for focal adhesion disassembly. Moreover, increased TRPM4 expression has been related to pathologies in which cytoskeletal rearrangement and cell migration are altered, such as fibrosis and cancer. Thus, the elucidation of the mechanisms that regulate TRPM4 activity might contribute important information for therapeutic strategies. We used a mass spectrometry-based proteomics approach to identify TRPM4-associated proteins. These studies revealed K⁺ Channel Tetramerization Domain 5 (KCTD5), a putative adaptor of cullin-3 E3 ubiquitin ligase, as a novel TRPM4-interacting protein. Therefore, we hypothesized that KCTD5 is a novel regulatory protein of TRPM4, modulating focal adhesion dynamics and cell migration. Here, we demonstrate that KCTD5 interacts with TRPM4 and regulates its activity. Moreover, we show that KCTD5 induces the ubiquitination of the channel and that KCTD5 silencing diminishes maximal TRPM4 currents. Moreover, we demonstrate that KCTD5 regulates the number of focal adhesions, cell spreading, migration and contractility. Together, these data suggest that KCTD5 interacts with TRPM4, promotes its ubiquitination, and regulates its activity, probably leading to an increase in cellular migration.
P3287

Board Number: B575

Tunneling of Ca\(^{2+}\) downstream of SOCE specifically signals to downstream effectors and subcellular domains.

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The function of Store-Operated Calcium Entry (SOCE) extends beyond the replenishment of Endoplasmic Reticulum (ER) Ca\(^{2+}\) stores following agonist stimulation, to controlling directly and indirectly the activity of multiple intracellular Ca\(^{2+}\) effectors and as such cellular processes. The point source Ca\(^{2+}\) entry through SOCE channels is restricted to a small sub-plasmalemal (PM) microdomain due to biochemical and physical barriers. However, some Ca\(^{2+}\)-dependent effectors downstream of SOCE do not localize to the SOCE microdomain. Ca\(^{2+}\) tunneling has been well characterized in pancreatic acinar cells and frog oocytes and shown to extend the spatial spread of SOCE Ca\(^{2+}\) signals while maintaining specificity, by using ER tunnels to activate distant effectors. Ca\(^{2+}\) in the SOCE microdomain is taken up into the ER and released at distal sites to activate effectors such as the Ca\(^{2+}\)-activated Cl channel Ano1. Herein, we show that Ca\(^{2+}\) tunneling is functional in non-excitatory cells and that it encodes significant specificity in the SOCE signal. By dissecting the sensitivity and dependence of multiple SOCE downstream effectors and subcellular domains, including the mitochondria, Ca\(^{2+}\)-activate K channel, bulk cytosol, sub-PM domain, and NFAT, we show that Ca\(^{2+}\) tunneling defines spatio-temporal Ca\(^{2+}\) coding downstream of SOCE. While mitochondria readily perceive Ca\(^{2+}\) release when stores are full, SOCE shows little effect in raising mitochondrial Ca\(^{2+}\), while Ca\(^{2+}\)-tunneling is completely inefficient. In contrast, Ca\(^{2+}\)-activated potassium channels display a similar sensitivity to Ca\(^{2+}\) release and Ca\(^{2+}\) tunneling, while the activation of NFAT1 is exquisitely sensitive to SOCE and not to Ca\(^{2+}\) release. Those results establish Ca\(^{2+}\) tunneling as a mechanism for encoding specificity in the cellular responses downstream of SOCE. They further show that Ca\(^{2+}\) tunneling is most efficient in specifically activating activators in the sub-PM domain thus expanding the spatial scope of the limited SOCE microdomain.

P3288

Board Number: B576

The yeast H\(^{+}\)-ATPase Pma1 promotes Rag/Gtr-dependent TORC1 activation in response to H\(^{+}\)-coupled nutrient uptake.

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The yeast Target of Rapamycin Complex 1 (TORC1) plays a central role in controlling growth. How amino acids and other nutrients stimulate its activity via the Rag/Gtr GTPases remains poorly understood. We now report that the signal triggering Rag/Gtr-dependent TORC1 activation upon amino-acid uptake is the coupled H\(^{+}\) influx catalyzed by amino-acid/H\(^{+}\) symporters. H\(^{+}\)-dependent cytosine uptake, ionophore-mediated H\(^{+}\) diffusion, and inhibition of the vacuolar V-ATPase also activate TORC1. As the increase in cytosolic H\(^{+}\) elicited by these processes stimulates the compensating H\(^{+}\)-export activity of the plasma membrane H\(^{+}\)-ATPase (Pma1), we have examined whether this major ATP-consuming enzyme might be involved in TORC1 control. We found that when the endogenous Pma1 is replaced with an H\(^{+}\)-ATPase from tobacco plant, H\(^{+}\) influx or increase fails to activate TORC1. Our results show that H\(^{+}\) influx coupled to nutrient uptake stimulates TORC1 activity and that Pma1 is a key actor in this mechanism.
Kinases and Phosphatases 3

P3289
Board Number: B578
The inhibitory effects of haptoglobin on osteoclast differentiation through TLR4 signaling.
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Haptoglobin (Hp), a member of the acute phase proteins, is known to be a major hemoglobin (Hb)-binding protein that plays a protective role against Hb-induced cytotoxicity in various organs. However, the involvement of Hp in bone-related cells has not been fully understood. In this study, we investigated the effects of Hp on osteoclastogenesis. Histomorphometric analyses with Hp gene-deficient mice showed significant bone loss due to increased osteoclast formation. Administration of exogenous Hp in these mice recovered bone volume. Consistent with the in vivo results, Hp inhibited osteoclastogenesis by suppressing expressions of major transcription factors such as c-Fos and NFATc1. Hp-induced suppression of c-Fos expression was mediated by an increase in interferon beta (IFN\textbeta) levels, a well-known c-Fos inhibitor. Hp-induced inhibition of osteoclastogenesis was recovered by treatment with IFN\textbeta-specific neutralizing antibody. Taken together, these results demonstrate that inflammation-induced Hp secretion from osteoblast plays a protective role against excessive osteoclastogenesis via IFN\textbeta augmentation.

P3290
Board Number: B579
Mitotic phosphorylation of Hsp72 uncouples ATP binding from substrate release and clusters amplified centrosomes in cancer cells.
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The function of protein phosphorylation in cell regulation is perhaps best understood in terms of regulating protein-protein interactions with reader modules that directly recognize the phosphorylated side chain. Another major role for phosphorylation is in the regulation of protein kinases, through stabilization of the activation loop to stabilize a catalytically-competent state. We have discovered a new role for phosphorylation in the regulation of a molecular chaperone, Hsp72, a member of the 70kDa Heat shock family of molecular chaperones (Hsp70s). These proteins comprise a nucleotide binding domain (NBD) and substrate-binding domain (SBD) connected by a linker that couples exchange of ADP for ATP with release of protein substrate. Mitotic phosphorylation of Hsp72 by Nek6 at Thr66 located in the NBD targets Hsp72 to the mitotic spindle. We determined the crystal structure of Hsp72 NBD containing a genetically encoded phosphoserine at position 66. Mutations in the residues that link phosphorylation to subdomain interactions restore the connection between ATP binding and substrate release. Thus, phosphorylation of Thr66 is a reversible mechanism that decouples the allosteric connection between nucleotide binding and substrate release, providing a new paradigm in our understanding of regulation in the Hsp70 family. Cancer cells frequently possess extra amplified...
centrosomes and the Nek6-Hsp72 is critical for the clustering of excess centrosomes into two poles. Inhibition of Hsp72 in acute lymphoblastic leukaemia cells resulted in increased multipolar spindle frequency that correlated with centrosome amplification, while loss of Hsp72 or Nek6 function in non-cancer derived cells disturb neither spindle formation nor mitotic progression. Hence, the Nek6-Hsp72 module represents a novel actionable pathway for selective targeting of cancer cells with amplified centrosomes.

P3291
Board Number: B580
Cell size-dependent regulation of Wee1 localization bursts by Cdr2 cortical nodes.
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Cell size control requires mechanisms that link cell growth with the cell cycle machinery. In eukaryotes, the key driver of the cell cycle is the cyclin-dependent kinase Cdk1. How information about cell size and geometry is relayed to Cdk1 is poorly understood. In many eukaryotes, complex cell geometry and non-cell autonomous signaling complicate studies of cell size signal transduction. The fission yeast Schizosaccharomyces pombe is an excellent model organism for size control research because of their simple rod-shaped geometry, stereotyped growth pattern, and conserved cell cycle machinery. Fission yeast cells enter mitosis at a defined size due in part to mysterious multiprotein punctate structures termed “nodes.” These nodes form a belt around the medial cell cortex, and their components include the Cdk1 inhibitor Wee1, along with two Wee1-inhibitory kinases Cdr1 and Cdr2. The number of nodes scales with cell size, but how nodes are organized to facilitate cell-size signal transduction is unclear. Here, we addressed node organization and function by combining TIRF microscopy, photobleaching, super-resolution molecule counting, and biochemical fractionation. We show that Cdr2 forms the stable core of megadalton-sized nodes, and nodes are enriched for catalytically active Cdr2, which increases in level during interphase growth. While Cdr2 and Cdr1 remain constant at nodes over time, Wee1 localizes to nodes in short bursts with lifetimes of several seconds. Recruitment of Wee1 to nodes requires Cdr2 kinase activity and a short domain within the N-terminus of Wee1. These localization bursts are required for progressive phosphorylation of Wee1 as cells grow, and burst frequency increases over 10-fold as cells approach division size. Cell size-dependent burst frequency is altered in cells lacking the protein kinase Pom1, which couples Cdr2 kinase activity to cell size. Our results indicate that stable Cdr2 nodes become progressively active as cells grow during interphase. Increasing node activity leads to more frequent bursts of Wee1 localization to nodes, where the combined activities of Cdr1 and Cdr2 kinases lead to inhibitory phosphorylation of Wee1. Our work suggests a mechanistic model for how Cdr2 nodes integrate cell size signals for dynamic regulation of Wee1 during cell growth.

P3292
Board Number: B581
Ubiquitin- dependent maturation of the PP1 phosphatase that opposes yeast Aurora B during mitosis.
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Ubiquitylation of proteins regulates the fidelity of mitotic chromosome segregation through both proteolytic and non-proteolytic mechanisms. In this study, we report a novel mechanism by which ubiquitylation affects chromosome segregation. The Aurora B - Protein Phosphatase 1 pair (Ipl1 and Glc7...
in S. cerevisiae) maintains the fidelity of chromosome segregation by ensuring correct bipolar attachment of spindle microtubules prior to anaphase. We show that the Glc7/Ipl1 balance is affected by a single point mutation in the gene encoding the E1 ubiquitin activating enzyme, Uba1 (uba1-W928R). This mutation increases Histone H3-S10 phosphorylation in a temperature-sensitive ipl1-2 strain, indicating that uba1-W928R acts by increasing Ipl1 activity and/or reducing phosphatase activity. Consistent with this, Ipl1 protein levels are higher in the uba1 mutant, indicating that uba1-W928R acts in part by increasing Ipl1 protein levels. However, Glc7 phosphatase activity is reduced in uba1-W928R mutant even though Glc7 protein levels remain constant. Two GLC7 mutations that suppress the temperature-sensitivity of ipl1-2 (glc7-127 and glc7-S99L) cause lethality in the uba1-W928R mutant background. Furthermore, uba1-W928R alters the localization of Glc7- most cells possess a Glc7 spot close to the nucleolus. It is known that Glc7 maturation requires the activity of the two highly conserved PP1 regulators Sds22 and Ypi1, as well as the AAA ATPase Cdc48 and its adaptor Shp1. Loss of any one of these proteins results in the aggregation of Glc7. We show that uba1-W928R does not prevent the sds22-6-induced aggregation of Glc7, but it blocks the formation of cytoplasmic Glc7 aggregates in a YPI1 mutant. Similarly, while mutations in sds22 exacerbate growth defects of the uba1-W928R mutant, a mutation in YPI1 does not cause additive growth defects in the presence of uba1. Thus, the uba1-W928R mutation is epistatic to mutations in YPI1 but not SDS22. Together, our work indicates that one or more steps in the folding or maturation of Glc7 is dependent upon ubiquitylation. Furthermore, although Sds22 and Ypi1 can form a ternary complex with Glc7, they appear to have separate roles in Glc7 maturation that are differentially influenced by a defect in ubiquitylation. Cdc48 (also known as VCP or p97) is a hexameric AAA ATPase that acts as a segregase to extract (segregate) ubiquitylated proteins from macromolecular complexes. Glc7, known to be ubiquitylated in vivo, or another regulator, such as Sds22 or Ypi1, could be the Cdc48 client(s) necessary for Glc7 maturation.

P3293

Board Number: BS82

Cell-type-specific isolation of 14-3-3 associated phosphoprotein from complex brain tissues.
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Understanding the general properties of signal transduction processes in the nervous system make it possible to clarify how these processes work to mediate specific functions in the brain. The major molecular mechanism underlying signal transduction is protein phosphorylation. Previously, phosphoproteomics on striatal slices treated with forskolin are performed and more than 200 phosphorylated proteins are obtained. However, this method is still limited by the fact that brain tissues are composed of multiple cell types that are each unique in their patterns of signal transduction. In order to pursue protein phosphorylation at the specific cell type that is excited in a particular stimulation, in vivo phosphoproteomic analysis is needed. Here we developed Cre-dependent AAV vector expressing 14-3-3 proteins. When this AAV vector is injected to a cell-type-specific Cre recombinase expressing mouse, Cre recombinase activates the expression of 14-3-3 proteins to enrich phosphorylated proteins. When crossed to a variety of Cre recombinase expressing mouse lines, this methodology is generalizable and useful for the identification of molecular changes in any cell type in response to specific stimulus.
P3294
Board Number: B583
Low molecular-weight gel fraction of Aloe vera exhibits protective effects by inhibition of matrix metalloproteinase-9 activity.
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To ascertains whether the inhibitory activity of MMP-9 induced by the low molecular-weight gel fraction of Aloe vera (lgfAv) affects ethanol-induced gastric lesions, we investigated the gastroprotective effects of oral (p.o.) administration of lgfAv (molecular weight cut-off < 50.0 KDa, 150.0 mg/kg body weight) in an ethanol-induced gastritis using mice for 1 h exposure. By measuring ulcer index, we compared the anti-ulcerative activity of the fraction by mRNA Expression and immunohistochemical analysis. As a result, the lgfAv-treated mice exhibited drastically fewer ulcer lesions than the untreated control mice did. It featured that lgfAv lessened the ulcer lesions than their relevant controls. Moreover, the transcriptional level of MMP-9 was completely alleviated by lgfAv treatment. The transcriptional level of MMP-9 was significantly alleviated by lgfAv treatment of the model. However, RT-PCR and immunohistochemistry experiments revealed that lgfAv treatment in mucosal tissues had the potential to inhibit the mRNA and protein expression levels of MMP-9, respectively. The protein expression of MMP-9 was closely associated with lgfAv-induced gastroprotection against ethanol-induced gastric lesions. Overall, the present results suggest that lgfAv has the potential to alleviating ethanol-induced acute gastric lesions, which is mediated partly by the suppression of the mRNA expression of MMP-9.

P3295
Board Number: B584
The with no lysine [K] kinase pathway regulates the localization of inward rectifier potassium channels.
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The with no lysine [K] (WNK) pathway regulates ion transport across cell membranes and regulates cellular processes such as blood pressure control, sodium reabsorption in kidney and neuronal function. It consists of protein kinase WNK (WNK1-4 in mammals), its downstream target kinases oxidative-stress responsive 1 (OSR1) and Ste20-related proline-alanine-rich kinase (SPAK), and downstream cation chloride cotransporters. The deregulation of these components results in human diseases such as hypertension \cite{1}. The objective of our study was to identify novel interactions of the WNK pathway components and subsequently elucidate novel functions of the pathway. Using a bioinformatics approach to identify potential OSR1/SPAK binding proteins, we discovered several proteins of the inward rectifier potassium channel (Kir) family to contain the OSR1/SPAK recognition motif. Our electrophysiological studies showed that constitutively active OSR1 increased the activities of Kir2.1 and Kir2.3 which contain the OSR1/SPAK binding motif. However, it did not alter the activity of Kir4.1 which does not contain that motif. The co-expression of WNK1 with wild-type OSR1 also activated Kir2.1, suggesting that WNK pathway is involved in controlling the Kir channels. Then we explored possible mechanisms of how the Kir channels are regulated by the WNK pathway. We depleted OSR1 in cells by siRNA-mediated gene knockdown and expressed epitope-tagged Kir2.3, followed by the treatment of cells with or without sodium chloride (NaCl) that induces WNK pathway activation. We discovered that Kir2.3 is localized primarily in the perinuclear region of the cells. Treatment of cells with NaCl decreased
the perinuclear Kir2.3 localized cells and increased cell membrane-associated Kir2.3 containing cells and cells in which Kir2.3 is dispersed throughout the cytoplasm. In addition, it also drove OSR1 towards the cell membrane. Interestingly, the depletion of OSR1 prevented the sodium chloride-induced localization changes of Kir2.3, indicating that OSR1/SPAK regulates its localization. The expression of Kir2.3 was not affected by the depletion of OSR1. The inhibition of WNK kinase activity by a small molecule WNK inhibitor (WNK463) also decreased the sodium chloride-induced cellular localization changes of Kir2.3, indicating that OSR1 activation by WNK is necessary for the regulation of Kir channel localization. In addition, it also prevented the localization change of OSR1occured with NaCl treatment. Therefore, these data indicate that WNK pathway regulates the Kir channel cellular localization via the activation of OSR1. 1. Dbouk, H.A., C.L. Huang, and M.H. Cobb, Hypertension: the missing WNKs. Am J Physiol Renal Physiol, 2016. 311(1): p. F16-27.

P3296
Board Number: BS85
The Role of Glycolytic ATP Generation for the Maintenance and Restoration of Vascular Endothelial Barrier Function.
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Acute Lung Injury (ALI) is a disease which causes pulmonary edema leading to severe morbidity and mortality in critically ill patients, usually occurring as a consequence of severe pneumonia or sepsis. ALI is characterized by endothelial barrier dysfunction of the lung vasculature, leading to disassembly of endothelial adherens junctions, increased vascular permeability and fluid accumulation in the lungs. While the mechanisms of assembly and disassembly of endothelial adherens junctions have been extensively studied, little is known about the bioenergetics of the barrier repair process, or barrier maintenance.

Endothelial ATP generation primarily relies on glycolysis, and PFKFB3 has recently been shown to act as a key glycolysis regulatory enzyme in endothelial cells, but little is known about its role in regulating the endothelial barrier.

To quantitatively determine the role of PFKFB3 in endothelial barrier permeability, we performed a transendothelial electrical resistance (TER) assay. Human lung microvascular endothelial cells (HLMVECs) were stimulated with the inflammatory mediator thrombin to induce barrier leak. During the recovery phase, endothelial cells were subsequently treated with PFK15, a specific inhibitor of PFKFB3. PFK15 significantly attenuated barrier restoration after thrombin treatment (p<0.05). Using live cell confocal microscopy of endothelial cells expressing the ATP biosensor Perceval HR, we assessed intracellular ATP levels following thrombin stimulation and found an initial decrease followed by rapid recovery, indicative of enhanced glycolytic ATP generation. Interestingly, pharmacological inhibition of PFKFB3 even in the absence of thrombin stimulation resulted in a drastic increase in barrier permeability.

These data suggest that glycolytic ATP generation as regulated by PFKFB3 is important for restoring the endothelial barrier after injury and for homeostatic maintenance of barrier function. A better understanding of the endothelial barrier bioenergetics could allow for the development of novel therapeutic approaches which enhance barrier function in severe diseases such as acute lung injury.
P3297
Board Number: B586
Regulatory roles of weak encounters between proteins.
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Weak encounters between proteins are conventionally attributed to the transition state intermediates. This view shares the spirit of the similar, but better understood, exchange between proteins and DNA where the low-affinity interactions are instrumental in accelerating the search for a promoter site. The Gram-negative bacterium Escherichia coli (E. coli) is a unique survivalist equipped to handle a myriad of external stress conditions. Recent structural work has shown the presence of weak protein-protein interactions between two paralogous, albeit functionally distinct pathways in E. coli: one that regulates sugar uptake and carbon metabolism, and the other one involved in nitrogen metabolism and a diverse array of other cellular processes. We built a stochastic, theoretical model of these weak, transient encounters between three proteins from these pathways. We find that the futile encounters by the third protein can have non-trivial outcomes for the formation of a specific complex between the two cognates. We argue that the bacteria could exploit such interactions for signaling purposes to complement conventional gene regulation, particularly under complex starvation conditions. The emerging picture hints toward an elegant stress response machinery that reflects a tight balance between competing metabolic demands of the cytosol.

P3298
Board Number: B587
The glycolytic enzyme phosphofructokinase-1 assembles into filaments.
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Despite abundant knowledge of the enzymology, regulation, and biochemistry of glycolytic enzymes, we have limited understanding of how they are spatially organized in the cell. Emerging evidence indicates that non-glycolytic metabolic enzymes regulating diverse pathways can assemble into polymers. We show here the tetramer- and substrate-dependent filament assembly by phosphofructokinase-1 (PFK1), considered the “gatekeeper” of glycolysis because it catalyzes the step committing glucose to breakdown. The recombinant liver isoform of PFK1 (PFKL) but not the platelet or muscle isoforms (PFKP and PFKM, respectively) assemble into filaments, initiated by the substrate fructose-6-phosphate (F6P). Negative stain electron micrographs reveal that PFKL filaments are apolar and made of stacked tetramers oriented with exposed catalytic sites positioned along the edge of the filament. We confirmed activity-dependent filament assembly with an inactive but tetrameric mutant, PFKL-His199Tyr, that does not form filaments in the presence of F6P, as determined by transmission electron microscopy and 90° light scattering. Electron micrographs and biochemical data with a PFKL/PFKP chimera indicate that the PFKL regulatory domain mediates filament assembly. To assess filament formation by PFK1 in cells, we expressed EGFP-tagged PFKL in MTLn3 rat mammary carcinoma cells and used live-cell imaging to examine PFKL-EGFP localization and dynamics. TIRF microscopy shows that PFKL-EGFP form dynamic
punctae throughout the cytoplasm. A small subset of PFKL-EGFP particles remained stationary at the ventral plasma membrane for several seconds, suggesting temporary docking events. Neither glucose starvation nor incubation of cells with 2-deoxyglucose, a glucose analogue that cannot be metabolized, had a discernable impact on punctae size, dynamics, or membrane docking. However, addition of citrate, an allosteric inhibitor of PFK1, induced the formation of large PFKL-EGFP-positive structures resembling stress granules. Our findings reveal a new behavior of a key glycolytic enzyme with insights on spatial organization and isoform-specific glucose metabolism in cells, including possibly a mechanism for localized ATP production. Moreover, our findings open a clinically relevant new direction for determining whether PFKL filaments regulate metabolic programming in diseases such as cancer.

**P3299**

**Board Number: B588**

**Proliferation of Immortalized Schwann Cells and Cyclic AMP Levels in Response to Forskolin.**

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Schwann cell growth in vitro is stimulated by heregulin, a neuronal growth factor and mitogens that activate cyclic AMP, the universal cellular second messenger. Schwann cell proliferation versus myelination is orchestrated by optimal concentration of mitogens and promyelinating factors. While the concentration of heregulin and forskolin that stimulates growth has been well characterized in primary cultures, the levels of forskolin that stimulate proliferation in Schwann cell lines are not known. This study was undertaken to determine the concentration of forskolin and corresponding levels of cAMP that promotes optimal Schwann cell proliferation. It was hypothesized that an increase in forskolin dosage will elicit a dose-dependent rise in cell proliferation and cAMP levels. Immortalized Schwann cells were cultured with no growth factors (control media N2), 12.5 ng/ml heregulin, various doses of forskolin at 0.5 µM, 1 µM, 2 µM or 3 µM and heregulin + forskolin for 4, 6, 12 or 24 hours. Using the MTT proliferation assay, preliminary studies show that a time-dependent cell growth was observed in cultures incubated with 0.5 µM forskolin or heregulin + forskolin. Cells incubated with 1 µM forskolin exhibited a decrease in proliferation after 12 hours while cells treated with heregulin and forskolin continued to grow for 24 hours. A similar pattern was observed for cells treated with 2 µM forskolin and heregulin + forskolin. At concentrations of 3 µM forskolin, a significant decrease in proliferation was observed after 12 hours. Levels of cAMP were increased in cultures incubated for 12 hours with 1 µM forskolin (184+/−10.51 pmol/300,000 cells) compared to N2 (14.34+/−5.3 pmol/300,000 cells) and heregulin (14.31+/−3.67 pmol/300,000 cells); while cells incubated with heregulin+2 µM forskolin showed a considerable increase (225+/−4.88 pmol/300,000 cells). Secretion of cAMP decreased in cultures incubated with 3 µM forskolin (107.52+/−43.72 pmol/300,000 cells) or forskolin + heregulin (135.67+/−39.32 pmol/300,000 cells). These preliminary findings indicate that increasing concentration of forskolin along with incubation time promotes Schwann cell growth. However, increasing forskolin dosage beyond 2 µM limits Schwann cell proliferation and concentrations of cAMP.
P3300
Board Number: B589
Protein kinase CK2 regulates skeletal muscle differentiation.
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CK2 is an extremely conserved tetrameric protein kinase implicated in a plethora of cellular processes due to its terrific pleiotropy and constitutive activity. Despite the growing knowledge about its functions, little is known about its involvement in muscle differentiation. In this study, we dissect the CK2 role in myogenic differentiation using in vitro C2C12 myoblast cell line and primary muscle derived stem cells (MDSCs) as well as two different in vivo systems. Our data show that the CK2 subunits are differently regulated during the differentiation process, namely the protein-level of the regulatory β-subunit significantly increases, while the amount of the catalytic α and α’ subunits is unchanged. Moreover, the inhibition of CK2 activity by the specific compound CX-5011 dramatically inhibits myotube formation in both C2C12 and MDSC cells. In particular, CX-5011 does not affect the initiation of the myogenic program, as revealed by the expression of the early myogenic markers, while it dramatically dampens the expression of late contractile (troponins) and fusion (caveolin-3 and myomaker) differentiation markers. Finally, we demonstrate that CK2 plays an essential role also in vivo, since its kinase activity is required for efficient muscle differentiation in both mouse and zebrafish animal models. Concluding, our findings clearly reveal a crucial role of CK2 in the progression of in vitro and in vivo myogenic program.

P3301
Board Number: B590
Protein tyrosine phosphatase alpha positively regulates invadopodia-mediated cancer cell motility.
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Cell migration plays a critical role in many physiological processes including wound healing. Aberrant cell migration is implicated in cancer metastasis, the leading cause of cancer-related mortality. In normal cells, migration is a dynamic process characterized by cycles of cell adhesion to and release from the extracellular matrix (ECM), and this involves cellular structures known as focal adhesions. Tumour cells often gain the ability to migrate through tissues, and this invasive migration involves cellular structures called invadopodia. Invadopodia are dynamic actin-based protrusions of the plasma membrane that mediate ECM degradation, and they are related to but distinct from focal adhesions. Protein tyrosine phosphatase alpha (PTPα), a widely expressed transmembrane protein, acts in normal cells to promote focal adhesion formation and migration. However, little is known about the role of PTPα in cancer cell motility. We hypothesize that PTPα regulates invadopodia formation and function to promote the invasive motility of malignant cells.

MDA-MB-231 breast cancer cells are a classic model for invadopodia research, and express PTPα. Small interfering-RNA (siRNA) was used to deplete PTPα in MDA-MB-231 cells, and PTPα can be reintroduced into the depleted cells using a siRNA-resistant PTPα-expressing plasmid. Transwell migration and invasion assays were used to investigate the role of PTPα in tumour cell motility. An invadopodia-mediated ECM-degradation assay was then used to determine the degradation ability of control vs.
PTPα-depleted cells. Lastly, MDA-MB-231 cells were transfected with GFP-PTPα, immunostained for invadopodial markers and imaged to determine if PTPα localizes to invadopodia. We found that PTPα-depleted cells had reduced abilities to migrate and invade. Matrix degradation assays revealed that PTPα-depleted cells are impaired in their ability to degrade ECM and this defect was rescued by restoring PTPα expression. Interestingly, PTPα-depleted cells and control cells formed equivalent numbers of invadopodia, and PTPα co-localized with cortactin, actin, and MT1-MMP to punctate invadopodia-like structures. Together, these findings indicate that PTPα, present within mature invadopodia, positively regulates invadopodia-mediated ECM degradation while not affecting invadopodia formation. Our ongoing research is investigating the molecular basis of PTPα-dependent invadopodia function. An improved understanding of invadopodia assembly, maturation, and function may reveal new mechanistic targets for therapeutic intervention to prevent cancer metastasis and limit mortality.

P3302
Board Number: B591
Regulation of Renal Proximal Tubule Na,K-ATPase by Creb Regulated Transcriptional Coactivators and Salt Inducible Kinase 1.
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The kidney plays a central role in blood pressure regulation, controlling Na+ reabsorption via locally produced natriuretic, and anti-natriuretic factors. Previous studies indicated that natriuretic and anti-natriuretic factors, including dopamine and norepinephrine acutely alter Na+ reabsorption through the phosphorylation of Na,K-ATPase in the renal proximal tubule (RPT). However chronic regulation at the transcriptional level has not been studied. Our investigations indicate that protein Kinase A and calcium-mediated signaling pathways are involved in transcriptional control by dopamine and norepinephrine, as well as at the post-transcription level. Our studies have focused in transcriptional regulation of the gene encoding the Na,K-ATPase beta subunit gene (atp1b1) in primary rabbit kidney proximal tubule (RPT) cell cultures. In this report, evidence is presented that (1) recently discovered cAMP-regulated transcriptional coactivators (CRTC), and Salt-Inducible Kinase 1 (SIK1) contribute to the transcriptional regulation of atp1b1 in RPT cells and (2) renal effectors, including norepinephrine, dopamine, prostaglandins, and Na+, play a role. Evidence for the role of CRTC includes the loss of transcriptional regulation of atp1b1 by a dominant-negative CRTC, and as well as by a CREB mutant with an altered CRTC binding site. In a number of experimental systems, SIK1 phosphorylates CRTC, which are then sequestered in the cytoplasm, preventing their nuclear effects. Consistent with such a role of SIK1 in primary RPT cells, atp1b1 transcription increased in the presence of a dominant-negative SIK1 (which could not phosphorylate CRTC). Regulation by dopamine, norepinephrine, and monensin was also disrupted by a dominant-negative SIK1. These latter observations can be explained if norepinephrine phosphorylates and inactivate SIK1, resulting in CRTC mediated nuclear effects. In contrast, the effects of monensin very likely are mediated by activated SIK1 which phosphorylates class IIa Histone Deacetylases (HDACs), leading to increased transcription by MEF2c transcription factors. Our results support the hypothesis that Na,K-ATPase in the RPT is regulated at the transcriptional level via SIK1 and CRTCs, in addition to the previously reported control of the phosphorylation of basolaterally oriented Na,K-ATPase.
Signaling from the PM/cytoplasm to the Nucleus

P3303
Board Number: B592
Stat3 Regulates Primary Embryonic Erythroid Cell Maturation and is Activated Independently of Erythropoietin Signaling.
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Stat3, when phosphorylated (pStat3), is a common transcriptional regulator and often a drug target to treat many cancers that use its signaling to modify cellular respiration and evade apoptosis. We have found that pStat3 is present during embryonic (primitive) erythropoiesis and have used this system as a model to better understand the role of Stat3 in primary blood cells. Primitive erythropoiesis arises on embryonic day 7.5 in the murine embryo and is required for embryonic survival. Using imaging flow cytometry to facilitate quantitative protein and morphometric analysis at the single cell level, we find pStat3 in primitive erythroblasts throughout maturation. Importantly, pStat3 is also observed in human stem cell-derived primitive erythroblasts. pStat3 inhibition results in increased apoptosis and decreased proliferation of primitive erythroblasts, and is associated with delayed erythroblast maturation and increased cell size. Stat3 binds and regulates several caspase related genes and caspase inhibition also results in delayed erythroblast maturation. The cell cycle checkpoint regulator, Gadd45g, is bound by Stat3 and its expression is increased following pStat3 inhibition, suggesting that Stat3 repression of Gadd45g may be a mechanism to increased cell division and terminal maturation. Known Stat3-targeted mitochondrial and oxidative phosphorylation genes are highly expressed in primitive erythropoiesis, suggesting that Stat3 may also regulate these general processes. Indeed, chemical inhibition of Stat3 results in rapid depolarization of the mitochondrial membrane. Though pStat3 levels increase in response to erythropoietin (Epo), surprisingly, normal pSTAT3 levels were observed in EpoR-null mice, indicating that other pathways activate Stat3 in primitive erythroblasts. Our preliminary studies suggest that the elevated level of ROS found in primitive erythroid cells may increase pSTAT3. Taken together, our data support a model where by Stat3 acts as a general regulator of the apoptotic machinery required for erythroid maturation, proliferation, and mitochondrial respiration. Additionally, this signaling is likely achieved through an endogenous pathway that is independent of cytokine signaling, a default “on-switch” to allow for rapid establishment of primitive erythropoiesis in the murine embryo. Finally, our data suggest that cancer cells may have co-opted Stat3 roles normally functional in primary embryonic erythroid cells.

P3304
Board Number: B593
The Semaphorin receptors, Neuropilins and Plexins, promote Hedgehog signaling through distinct cytoplasmic mechanisms.
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Hedgehog (HH) signaling is essential for embryonic and postnatal development, while perturbation of HH pathway function can lead to a variety of developmental diseases, birth defects, and cancers. Neuropilins, which have well-characterized roles in Semaphorin and VEGF signaling, have recently been implicated in the regulation of HH signaling. Neuropilins contain short, catalytically inactive cytoplasmic
domains, requiring Plexin co-receptors to regulate small intracellular GTPases during Semaphorin signal transduction. However, the mechanism of Neuropilin function in HH signal transduction remains unclear, and a role for Plexin proteins in HH signaling has not been explored. Here we report that Neuropilins and Plexins both promote HH signaling through distinct cytoplasmic mechanisms. Using HH-dependent luciferase reporter assays in NIH/3T3 cells, we show that the Neuropilin-1 cytoplasmic and transmembrane domains are both necessary and sufficient to regulate HH pathway activity, independently of Plexin and Semaphorin binding. We also show that Neuropilin-1 localizes to the primary cilium, a key platform for HH signal transduction, although this localization does not correlate with HH signaling promotion. Instead, our data suggest that Neuropilin-1 selectively regulates GLI activator function through a novel 12-amino acid cytoplasmic motif. Strikingly, we also find that multiple Plexin family members promote HH signaling, independent of their ability to interact with Neuropilins. Instead, point mutations in the GTPase activating domain of Plexins prevent HH pathway promotion, suggesting that GAP function is required for Plexin-dependent HH regulation. Furthermore, deletion of the autoinhibitory Plexin-A1 extracellular domain significantly increases HH pathway activity, providing additional evidence that Plexin GAP activity regulates HH signaling. Together, our data suggest that Neuropilins and Plexins regulate HH signaling downstream of ligand activation through distinct cytoplasmic mechanisms. Therapeutic approaches targeting Semaphorin receptors may be useful to regulate overactive HH signaling in cancer and other diseases.

P3305
Board Number: B594
Novel hypomorphic **Smoothern** allele causes skeletal and craniofacial defects.
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Sonic hedgehog (Shh) signaling is a critical developmental pathway best known to regulate cell fate specification and proliferation. Shh signals through a tightly regulated signaling cascade that requires the G-protein coupled receptor Smo (Smo) and the primary cilium. Smo acts as the obligate transducer of the Shh signal and is normally enriched in cilia when the pathway is stimulated. Without Smo development ceases at embryonic day (E)9.0 and the embryo dies, signifying the necessity of the pathway during development. While the Shh pathway and Smo are well studied, the biological mechanisms surrounding Smo have yet to be fully characterized. Using an ENU-induced forward genetic screen in mice we identified a novel recessive allele of Smo carrying a missense mutation, called **AW4**. Smo**AW4** mutants display craniofacial defects by E10.5 and subsequent limb anomalies before dying shortly after birth. To test allelism, we generated E9.5 embryos heteroallelic for Smo**AW4** and Smo**bentbody** (known null); these alleles failed to complement indicating Smo**AW4** is an allele of Smo. Moreover, Smo**AW4** only partially restored the Smo null phenotype; the Smo**AW4/bentbody** embryo still appeared developmentally delayed but survived until E10.5. The less severe phenotype indicates Smo**AW4** is a hypomorphic Smo allele. In the developing neural tube Shh signaling is important for the patterning of ventral cell fates. Smo**AW4** embryos display diminished ventral cell fates and decreased Shh activity in the neural tube. Consistent with this, we found transcription of Shh-dependent gene expression was lower in Smo**AW4** mouse embryonic fibroblasts (MEFs) stimulated with Shh conditioned media than in their wild type controls. Additionally, we found Smo**AW4** MEFs were insensitive to Smo agonist (SAG), which binds to the Smo binding pocket to directly activate the protein. Through immunofluorescence we determined Smo fails to properly localize to the primary cilium in Smo**AW4** neural tube and MEFs. Taken together, our data argue Smo**AW4** is a novel Smo allele that perturbs Shh signaling, leading to aberrant craniofacial and limb development. Despite a reduction in Shh signal transduction, the Smo**AW4** embryo
survives longer than the Smo null. It is widely believed that Smo activation occurs in the primary cilium, therefore it is unclear how the Smo\textsuperscript{AWd} mutant signals as well as it does despite a cilia localization defect.

P3306
Board Number: B595
Single-molecule tracking study of β-catenin nucleocytoplasmic translocation and regulation thereof by Custos.
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The purpose of this study is to determine the translocation dynamics of wild-type (wt) β-catenin at the single-molecule level and their changes over a time course of exposure to wnt3a ligand in HeLa cells, and to characterize the interaction with this process of the novel protein Custos. Single-particle tracking (SPT) was conducted via single-point edge-excitation subdiffraction (SPEED) microscopy, measuring translocation efficiency and mapping the route of the molecule through the nuclear pore complex (NPC). Results indicate that β-catenin translocation follows a route similar to that of transport receptors and is regulated via wnt3a such that the import efficiency increases with ligand stimulation to a peak at 4 hours after introduction of the ligand and decreasing thereafter, with export efficiency decreasing in comparison with control to a nadir at 4 hours and rising back to a level approximating control within 8 hours. It is shown via single-molecule FRET (SMF) that Custos interacts with β-catenin on both sides of the nuclear envelope, providing evidence that Custos is involved with the translocation of β-catenin; however, the exact nature of this relationship remains to be elucidated in further study. It is concluded thus far that β-catenin is regulated via wnt3a via translocation efficiency, enters the nuclear envelope via the nuclear pore in the manner similar to that of a transport receptor, and interacts with Custos during the translocation process. Study continues to characterize the interaction between β-catenin and Custos at the NPC and between β-catenin and the phenylalanine-glycine (FG)–repeat regions of NPC central channel nucleoporins curing translocation.

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1144462 to S.J.S. and by grants from the National Institutes of Health (NIH GM097037, GM116204 and GM122552) to W.Y. and (NIH GM115929) to R.H.

P3307
Board Number: B596
Comparison of YAP translocation in primary and immortalized cells.
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Objective: Yes-associated protein (YAP) is a transcriptional co-activator that has been implicated in a wide range of cell functions: proliferation, survival, fibrosis, differentiation, apoptosis, and senescence. As indicated by such diverse functional outputs, YAP’s activity and function is regulated by a host of scaffolding, transcriptional, and structural proteins which are likely cell, tissue, and disease dependent. Our previous work has demonstrated the expression and active function of YAP in primary human trabecular meshwork (HTM) cells. In this study, we wished to test whether immortalized HTM cells (TM1) were an appropriate model for studying the regulation of YAP. To this end, we used two YAP
stimuli that have been well characterized in other model systems, cell density and oxidative stress, and assayed the nuclear localization of YAP.

**Methods:** Primary (isolated from 7 donors) and immortalized HTM cells were routinely cultured in DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics. To test the effect of cell density, cells were plated at low or high density on glass coverslips and serum starved for 3 days to remove the effects of serum. To test the effect of oxidative stress, cells were handled similarly and exposed to 250 μM hydrogen peroxide in media for 1 h. In all cases, cells were fixed and standard immunofluorescent techniques were used to assay the intensity of YAP within the cell nucleus. All values are expressed as percentage of control and shown as mean ± SEM.

**Results:** In all cases, YAP demonstrated a mixed expression pattern, with signal in both the cytoplasm and the nucleus. Increased cell density reduced the average nuclear intensity of YAP in primary (79.6% ± 2.8%; p<0.05; n=3) HTM while increasing it in immortalized cells (113.6% ± 2.0%; p<0.05; n=2). Further, the decrease observed in primary HTM cells is modest compared to reports in other cell types. After oxidative stress, primary cells exhibited a subtle drop in nuclear intensity (84.8% ± 5.4%; p<0.05; n=4), however, this was not observed with immortalized cells (102.0 ± 1.6%; p=0.33; n=2).

**P3308**

**Board Number: B597**

*Osthole and nitric oxide attenuate advanced glycation end products-induced NF-kB and MAPK activation in renal tubular cells.*

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Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin, a natural coumarin derivative extracted from Chinese herbs, reportedly possesses various therapeutic properties and applications. To explore whether osthole was linked to altered advanced glycation end products (AGE)-mediated diabetic nephropathy, the molecular mechanisms of osthole responsible for inhibition of AGE-reduced nitric oxide (NO) bioactivity in renal proximal tubular cells were examined. We found that raising the ambient AGE concentration causes a dose-dependent decrease in NO generation. Osthole significantly reverses AGE-inhibited NO generation and induces high levels of cGMP synthesis and PKG activation. Treatments with osthole, the NO donor S-nitroso-N-acetylpenicillamine, and the MAPK inhibitor U0126 markedly attenuated AGE-inhibited NOS protein levels and NO generation. Moreover, AGE-induced NF-kB/MAPK activation, RAGE/p27Kip1/collagen IV protein levels, and cellular hypertrophy were reversed by osthole. The ability of osthole to suppress NF-kB activation was also verified by the observation that it significantly decreased IkB protein degradation. These findings indicate for the first time that in the presence of osthole, the suppression of AGE-induced biological responses is probably mediated by blocking NF-kB and MAPK signaling cascades or activating the NO pathway.

**P3309**

**Board Number: B598**

DETECTION OF NUCLEAR AND CYTOPLASMIC PROTEIN-PROTEIN INTERACTIONS OF THE CD44-INTRACYTOPLASMIC DOMAIN WITH RUNX2 BY PROXIMITY LIGATION ASSAY.

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CD44 is a transmembrane receptor involved in signal transduction. CD44 undergoes a proteolytic cleavage by presenilin within the gamma secretase complex, which produces the CD44 intracytoplasmic...
domain or CD44-ICD. This 72 residues peptide can be translocated into the nucleus where it can regulate transcription. The transcriptional regulatory mechanism(s) is not well understood but published EMSA and chromatin immunoprecipitation data have found the CD44-ICD in a complex with Runx2 in the MMP-9 promoter.

We hypothesize that the CD44-ICD directly interacts with Runx2 in the nucleus. To test this hypothesis we carried out Proximity Ligation Assays (PLA) using MCF-7/vector (CD44 negative) and MCF-7/CD44 (CD44 positive) cells grown on chambered slides. An anti-CD44-ICD antibody (Abcam) and an anti-Runx2 antibody (Santa Cruz Biotechnology) were used to generate the PLA data. This assay not only detects protein-protein interactions (PPI) but also the subcellular localization of such interactions. The PLA data collected as confocal microscopy images indicate that the CD44-ICD interacts with Runx2 in the nucleus. This data validates our hypothesis. However, we also detected an unexpected similar interaction in the cytoplasm. This cytoplasmic localization for the CD44-ICD/Runx2 PPI interaction suggests a potential novel CD44-mediated mechanism of gene regulation. Future PLA and co-immunoprecipitation experiments will be carry out to confirm the cytoplasmic CD44-ICD/Runx2 PPI interaction.

**P3310**  
**Board Number: B599**  
Particulate matter and its effect on the aryl hydrocarbon receptor (AhR)/CYP1A1 pathway in adherent THP-1 cells.  
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**INTRODUCTION/OBJECTIVE:** Air pollution and Particulate Matter (PM) have recently been classified as human carcinogens. PM is a mixture of compounds including soil, heavy metals and organics like polycyclic aromatic hydrocarbons (PAH). The objective of this study is to evaluate the molecular mechanisms by which the PAH content in PM10 (PM with mean aerodynamic diameter ≤10 μm) may influence cell responses. Specifically, we are interested in the Aryl hydrocarbon Receptor (AhR)/CYP1A1 pathway in adherent THP-1 cells.

**METHODS:** THP-1 cells were cultured in suspension and transformed to macrophages with phorbol-myristate-acetate. The cells were then treated with eight different PM10 samples of known soil and PAH content collected from an “industrial” (IND) and a “business” (BUS) area in Mexico City. Q-PCR was conducted to measure gene expression levels of AhR and CYP1A1. The intracellular localization of AhR and CYP1A1 was evaluated by immunofluorescence microscopy. We also assessed the production of TNFα and IL-6 by ELISA. All experiments also included the use of the following: 1) Polymyxin A against endotoxins, 2) α-naphthoflavone as an AhR inhibitor, and 3) N-acetyl cysteine as a free radical scavenger. One-way ANOVA was used to determine differences between groups. We assessed correlations between PM10 soil and PAHs content and the cell responses using Pearson’s analysis.

**RESULTS:** After exposure to PM10, THP-1 cells exhibited a significant increase in the expression of CYP1A1 compared to the unexposed cells. A similar effect was not observed in the case of AhR gene expression. In this instance, however, PM10 induced the translocation of the AhR receptor to the nucleus accompanied by the expression of CYP1A1. Only α-naphthoflavone significantly decreased the level of CYP1A1 expression (p<0.01). The PM10 PHA content correlated with an increased trend in the expression of AhR when using the samples with the highest content of PAHs (IND) (r=0.934, p<0.06). PM10 -induced cytokines production correlated with the soil content of the samples (IL-6; r=0.492, p<0.004, TNFα: r=0.669, p=0.000).
CONCLUSIONS: CYP1A1 gene and protein expression are AhR dependent after PM10 exposure. These data demonstrate that PM participates as a xenobiotic in an AhR-mediated signal transduction pathway mediated by the PM10 PAH content.

P3311
Board Number: B600
Diverse patterns of phosphatase activation and localization: unique implications for the control of interferon-gamma signaling in CNS neurons.
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The immune cytokine interferon-gamma (IFN-g) is required for the elimination of numerous central nervous system (CNS) viral infections. Mediated by JAK/STAT signal transduction, the cellular response to IFN-g involves the expression of genes that result in the establishment of the antiviral state, and is controlled by multiple negative feedback mechanisms. Whether these control mechanisms, previously characterized in non-neuronal cells, operate similarly in CNS neurons is not well-understood. We previously demonstrated that the control of the neuronal IFN-g pathway is distinct from that in non-neuronal cells, as the duration of STAT1 phosphorylation and IFN-g responsive gene expression in IFN-g treated neurons was markedly extended as compared to mouse embryonic fibroblasts (MEF). We also showed that compared to MEF, STAT1 dephosphorylation was delayed in IFN-g treated neurons, providing a mechanism for the extended kinetics of neuronal STAT1 phosphorylation. The current study investigated the expression and subcellular localization of two STAT1 phosphatases, TC45 and SHP-2, to address the hypothesis that mislocalization of these phosphatases could result in the observed delay in neuronal STAT1 dephosphorylation. Although no differences in overall expression levels of TC45 were observed between neurons and MEF, neurons demonstrated an approximately 3-fold lower level of SHP-2 activation as compared to MEF, regardless of IFN-g exposure. To examine the subcellular localization of these phosphatases, neurons and MEF were exposed to IFN-g, and subcellular fractions were purified at 1, 24, and 48h post-IFN-g treatment. Our results showed that while SHP-2 was found strictly in the cytoplasmic fraction of both cell types, TC45 was found primarily in the cytoplasmic fraction of the neurons, whereas it was equally distributed between nuclear and cytoplasmic fractions in MEF. These results indicate that as compared to non-neuronal cells, neurons lack TC45 in the nucleus, and demonstrate a lower level of SHP-2 activation, which provides a likely explanation for the observation that neuronal STAT1 is not dephosphorylated as rapidly as it is in MEF. Understanding the control of the IFN-g response in CNS neurons will ultimately aid in the characterization of antiviral immune mechanisms of the CNS.
P3312
Board Number: B601
Changes in corticotrope gene expression upon increased expression of peptidylglycine alpha-amidating monooxygenase, an evolutionarily conserved secretory pathway enzyme.
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A key enzyme in the biosynthesis of biologically active peptides is peptidylglycine alpha-amidating monooxygenase (PAM: E.C. 1.14.17.3). This Type I integral membrane enzyme uses a 2-step reaction to convert peptidylglycine intermediates, which are negatively charged at physiological pH, into bioactive peptides with an uncharged alpha-amide. Identification in Chlamydomonas reinhardtii of a gene encoding CrPAM, whose properties closely resemble those of vertebrate PAM, suggests that this enzyme was present in the last eukaryotic common ancestor. In all species, PAM requires Cu, Zn, O2 and ascorbate, functioning best at low pH, making it dependent upon systems that regulate luminal pH and metal levels. In C. reinhardtii and rodent, PAM cycles through the secretory pathway and is found on ciliary membranes. Analysis of PAMKO zebrafish and mouse cell lines over-expressing PAM revealed its effects on cytoskeletal organization and secretion. The gamma-secretase mediated cleavage of PAM releases a soluble cytosolic fragment which localizes to the nucleus, raising the possibility of gene regulation by this fragment of PAM. We examined corticotrope tumor cells in which PAM expression could be induced following exposure to doxycycline to levels seen in the pituitary and atrial myocytes (Ciccotosto et al, 1999, J. Cell. Biol. 144:459). RNaseq analysis was used to identify transcripts altered upon induction of PAM. Differential gene expression analysis was performed on biological triplicates using Cuffdiff, Limma and DESeq2. A set of ~60 transcripts was identified by all 3 analytical methods, and another 200+ transcripts were identified by 2 of the 3 methods. Quantitative polymerase chain reaction (qPCR) was used to validate the differentially expressed transcripts; of the 45 transcripts tested, 23/45 (29) were validated by qPCR. To eliminate transcripts associated only with the doxycycline-inducible system, these transcripts were assessed in wildtype AtT-20 cells vs. two independent, stably transfected AtT-20 lines expressing similarly elevated levels of PAM, trimming the list further. The set of genes whose expression was consistently increased as a consequence of increased PAM expression included a peptidyl-prolyl isomerase (Fkbp4), 4-hydroxyphenylpyruvate dioxygenase (Hpd), and myotilin, an actin cross-linking protein. Genes consistently decreased as a consequence of increased PAM expression included peptide precursors (adrenomedullin, cholecystokinin) and a peptidase inhibitor (Serpine1). Additional studies are in progress to determine whether these proteins contribute to the effects of PAM on cytoskeletal organization and regulated secretion. Support: DK032949 (NIH).

P3313
Board Number: B602
WNT5A Isoforms A and B are differentially regulated during osteogenesis and early mouse development.
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WNT5A is a secreted ligand that activates primarily non canonical Wnt signaling pathways. It has important roles in morphogenesis (i.e., anterior-posterior axis elongation and limb formation) and differentiation (i.e., bone and cartilage). WNT5A functions in proliferation, adhesion, migration, and cell...
polarity. Altered WNT5A expression is associated with various human diseases, particularly cancer, but has been linked with the inflammatory response. Our lab is interested in the functional distinctions between the WNT5A isoforms that we term A and B. These isoforms are derived from distinct promoters and the processed proteins differ by 19 AA on their N-terminus. One possibility is that the isoforms display distinct patterns of expression in particular cells, during differentiation and in development, as a consequence of their unique promoters. To begin addressing this hypothesis we analyzed the expression of the WNT5A isoforms during differentiation of osteoblast cells using the cell line hFOB1.19. The cells were plated and grown to confluence at 33.4°C at which point the cultures were transferred to 39°C and treated with differentiation medium. At Days 0, 3, 7, and 10 RNA was isolated from the cells and converted to cDNA. qPCR was conducted using primers for actin, WNT5A isoform A, WNT5A isoform B, RUNX, and OSTERIX and relative-fold change for each target determined. Isoform A and isoform B transcript numbers were also quantified using qPCR generated standard curves. Both isoform A and isoform B transcripts increase by Day 3 of differentiation. At Day 0 there were approximately 1.8X more isoform A than B transcripts. By Day 10 isoform B transcripts levels were slightly higher than isoform A. Isoform B transcripts show a greater fold-change relative to Day 0, increasing by 16-fold, whereas isoform A was found to have a 6-fold increase. RUNX and OSTERIX transcripts both increased and their patterns of expression confirmed that the hFOB1.19 osteoblasts were differentiating. We also examined the expression of WNT5A isoforms A and B during early mouse embryogenesis. Analysis of mouse embryo RNA at stages 10, 11, 12 13, and 14 dpc revealed that promoter A and B transcripts levels are equal at dpc 10 but that promoter A transcripts increase during the period of limb formation and remain higher than promoter B at the other stages. Together, these results suggest that the WNT5A isoform A and B promoters are differentially regulated.

P3314
Board Number: B603
WNT5A isoforms A and B display differential protein function and promoter activity.
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WNT5A is a secreted ligand that activates primarily non-canonical Wnt signaling pathways. It has important roles in morphogenesis (i.e., anterior-posterior axis elongation and limb formation) and differentiation (i.e., bone and cartilage). WNT5A functions in proliferation, adhesion, migration, and cell polarity. Altered WNT5A expression is associated with various human diseases, particularly cancer, but has been linked with the inflammatory response. Our lab is interested in the functional distinctions between the WNT5A isoforms that we term A and B. These isoforms are derived from distinct promoters and the processed proteins differ by 19 AA on their N-terminus. One possibility is that the isoforms have differential affinity for non-canonical receptors, selectively affecting particular signaling pathways. Another possibility is that the isoform promoters are differentially regulated. The genomic sequences likely corresponding to the promoter and gene regulatory regions of each isoform were individually cloned into a luciferase reporter vector. Our experimental models are hFOB1.19, normal human osteoblasts that can be induced to differentiate, and HCT-116, a colorectal carcinoma cell line that doesn’t express either isoform A or B proteins. Isoform A and B luciferase promoter constructs were transfected into HCT-116 and hFOB1.19 cells. The patterns of expression in the two cell types were similar for Promoter A but differed more for Promoter B. The cell lines were co-transfected with isoforms A and B expression vectors and luciferase reporters for the transcription factors AP-1 and NFAT, targets of the Wnt Ca2+ and PCP/CE non-canonical signaling pathways. Results show that in HCT-116 the AP-1 reporter is up-regulated when isoform A is transfected but down-regulated when isoform B is transfected. In hFOB1.19 cells both isoforms A and B down-regulate the AP-1 reporter. NFAT
reporter expression was down-regulated in HCT-116, whereas there was no effect in hFOB1.19 cells. Next, AP-1 and NFAT transfected HCT-116 cells were treated with purified isoform B protein for 24 hrs. Our results suggest that the purified isoform B protein up-regulates AP-1 and down-regulates NFAT. Overall, these results suggest that WNTSA isoform proteins have differential effects on the non-canonical signaling pathways in different cell types and that the isoform promoters are differentially regulated.

P3315
Board Number: B604
Size-Dependent Protein Segregation Triggers Macrophage Activation and Phagocytosis.
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Macrophages clear their host of pathogens, cellular debris, and compromised cells through a process of engulfment and digestion known as phagocytosis. One pathway leading to phagocytosis is initiated by macrophage Fc receptor (FcR) recognition of the Fc region of antibodies immobilized on a target. Phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM)-containing protein associated with the FcR is known to precede phagocytosis, but the mechanism by which FcR binding leads to ITAM phosphorylation remains unclear. The kinetic segregation model of immune signaling proposes that segregation of a phosphatase can lead to sustained ITAM phosphorylation. We tested whether this model holds for antibody-dependent cell phagocytosis by making a set of size-variant synthetic antigens to modulate the distance between a macrophage and a model membrane. We attached these antigens to lipid bilayer-coated beads to act as minimal target cells, and we observed a significant decrease in phagocytosis of targets with large intermembrane distances compared to those with smaller distances. Using TIRF imaging of a live-cell ITAM phosphorylation sensor, we confirmed that ITAM phosphorylation in macrophages decreases with increasing interface distance. By isolating plasma membrane vesicles from macrophages, we were able to show that the tall CD45 phosphatase is segregated from interfaces with short intermembrane distances but present in interfaces with taller intermembrane distances. Taken together, our findings show that shortening the intermembrane distance of a macrophage/target interface to exclude CD45 will lead to increased macrophage activation. This result indicates that the kinetic segregation model applies to antibody-dependent cell phagocytosis and may aid in the design of future antibody therapies.

P3316
Board Number: B605
Heparin-induced BMP6 expression and localization changes in vascular smooth muscle cells.
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Bone morphogenetic protein 6 (BMP6) has been shown to bind heparin and influence the activity of bone morphogenetic proteins, which are members of the transforming growth factor-β (TGF-β) superfamily of proteins that regulate proliferation, differentiation, pattern formation, and apoptosis. It can be predicted that BMP6 upregulation might play a role in the anticoagulant functions of heparin; however, the anti-inflammatory mechanisms of heparin are not well characterized, and the role of BMP6 is under investigation. There is an established link between BMP6 and smooth muscle cell physiology, but it is unclear how BMP6 affects this physiology. Microarray data collected from serum-
deprived vascular smooth muscle cells treated with heparin for 24 hours indicated a 1.899-fold increase in BMP6 compared to untreated control cells. Fluorescent microscopy using antibodies specific for BMP6 shows that short-term heparin treatment (less than 40 minutes) resulted in decreased cytoplasmic BMP6 staining, suggesting that heparin could induce BMP6 release from proliferating vascular smooth muscle cells. Current research looks to validate these findings by observing localization and expression changes of BMP6 in response to heparin treatment in vascular smooth muscle cells cultured in serum-containing media and serum-free media.

P3317
Board Number: B606
Signal-Regulated Nuclear Transport and Accumulation of Smad Proteins studied by High-Speed Single-Molecule Microscopy.
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Nuclear translocation of active Smad protein complexes is a critical step in signal transduction of transforming growth factor β (TGF-β) from transmembrane receptors into the nucleus. Specifically, normal nuclear accumulation of Smad2/Smad4 protein complexes induced by TGF-β1 plays critical roles in preventing cancers. However, the relationship between nuclear accumulation and the nucleocytoplasmic transport kinetics of Smad proteins in the presence of TGF- β1 remains obscure. By combining a high-speed single-molecule tracking microscopy and Förster resonance energy transfer (FRET) techniques, we tracked the entire TGF-β1-induced process of Smad2/Smad4 complexes formation as well as their transport through nuclear pore complexes (NPCs) in live cells with a high spatiotemporal resolution of 2 ms and < 20 nm. Our single-molecule FRET data have revealed that in TGF- β1 treated cells Smad2/Smad4 complexes formed in cytoplasm, imported through the NPCs as entireties, and finally dissociated into monomers in the nucleus. Moreover, we found that monomeric Smad2 or Smad4 cannot accumulate in the nucleus without the presence of TGF-β1, mainly because both of them have an approximately two-fold higher nuclear export efficiency compared to their nuclear import. Remarkably and reversely, complexes of Smad2/Smad4 induced by TGF-β1 can rapidly concentrate in the nucleus due to its almost four-fold higher nuclear import rate in comparison with its nuclear export. Thus, we believe that the determined TGF- β1 depended transport configurations and efficiencies for monomers or complexes of Smad2 and Smad4 elucidate the basic molecular mechanism to understand their nuclear transport and accumulation.

P3318
Board Number: B607
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Differential activation of ERK, JNK, and p38 mitogen-activated protein kinases (MAPKs) is central to cellular decisions ranging from cell cycle progression to apoptosis. In this study, we use an optogenetic and live-cell imaging approach to selectively and acutely activate individual kinases and dissect cross-regulation of MAPKs and its determination of cell responses. Focusing on the MAPK kinase, MKK6, photo-uncaging constitutively active MKK6 activates isoforms of the stress-activated MAPK p38.
subfamily but not JNKs, consistent with prior literature. Uncaging MKK6 strongly inhibits basal and growth factor-stimulated ERK activity, but surprisingly this negative crosstalk is not affected by pharmacological inhibition of p38 nor shRNA depletion of p38alpha, both of which ablate MKK6-mediated p38 kinase activity in our cells. We also show that photo-activation of MKK6 is sufficient to induce caspase-3 activity and release of cytochrome C from mitochondria, hallmarks of apoptosis. The course of MKK6-induced apoptosis is accelerated when type IA phosphoinositide 3-kinases are inhibited; notably, MKK6-induced apoptosis still occurs when p38 kinase activity is inhibited, but in a smaller fraction of cells.

Post-Translational Modifications in Signaling

P3319
Board Number: B608
Integration of Post-Translational Modification Spaces in Lung Cancer Signaling Pathways.
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To elucidate patterns in post-translational protein modifications (PTMs) that may point to characteristic disease signatures and etiology in lung cancer, we coupled immunoprecipitation using modification-specific antibodies with Tandem Mass Tag (TMT) mass spectrometry to compare 45 lung cancer cell lines to normal lung tissue, and cell lines treated with anti-cancer drugs. These data reveal more than 4000 proteins modified by phosphorylation, methylation, and acetylation, and represent the first large-scale integrative analysis of these PTMs together. A cluster-filtered network approach was used to interrogate the data. We posit that clustering of protein modifications under different conditions reveals patterns specific to the system, and that PTM clusters that contain proteins known to interact with one another are likely to represent functional signaling pathways. We optimized clustering methods based on a machine learning algorithm (t-SNE) using a combination of statistical relationships. PTM clusters were used to filter protein-protein interaction (PPI) relationships to retain only interactions between proteins whose modifications co-clustered. The resulting cluster-filtered network (CFN) removed bias in protein interaction databases, and outlined signaling pathways specific to lung cancer cell lines. We found that a number of proteins modified by more than one type of PTM had negative correlations between different PTMs, suggesting that molecular “OR” switches govern a surprising number of signal transduction events. We also showed that the acetyltransferase EP300 appears to be a hub in the network of pathways involving phosphorylation, acetylation, and methylation. In addition, the data shed light on the mechanism of action for geldanamycin, the HSP90 inhibitor, and indicate that acetylation and methylation, together with phosphorylation, are involved in regulation of the cytoskeleton, endocytosis, and control of gene expression through RNA binding proteins.
In contrast to non-transformed cells, cancer cells are able to survive and proliferate in stressful conditions often characterized by hypoxia, lack of nutrients, and exposure to chemotherapeutic drugs. Cancer cells accomplish this, in part, by rewiring or activating specific signal transduction cascades. A primary signaling pathway induced by a variety of stresses in cancer cells is the p38 MAP kinase (MAPK) pathway. Upon activation, p38 phosphorylates multiple downstream substrates including the serine/threonine kinase MAPKAP kinase 2 (MK2). However, the precise roles that MK2 plays in breast cancer progression are poorly understood. Here we demonstrate that p38-MK2 signaling pathway is constitutively active in both breast cancer cell lines and in primary human tumor samples. Moreover, we show that shRNA-mediated knockdown of MK2 in SUM149 breast cancer cells reduces cell proliferation. To gain additional insight into the biological functions of this kinase in breast cancer, we performed a proteomics-based screen to identify novel substrates of MK2. Using this approach, we identified and subsequently validated the DNA helicase RECQ1 as a novel substrate of MK2. As a member of the RecQ family of helicases, RECQ1 plays an important role in relieving replicative stress and has recently been implicated as a susceptibility gene in familial forms of breast cancer. We show that MK2 phosphorylates RECQ1 solely at S392, a residues located within the helicase domain of the enzyme, and that MK2-mediated phosphorylation of RECQ1 enhances its catalytic activity as determined by a branch migration assay. We further demonstrate that overexpression of a non-phosphorylatable RECQ1 mutant in triple-negative breast cancer cells sensitizes these cells to DNA damage-induced apoptosis, while expression of a phosphomimetic mutant promotes cell proliferation and enhances resistance to cell death in response to treatment with adriamycin and hydroxyurea. Collectively, these results implicate RECQ1 as a critical substrate of MK2 in breast cancer cells and suggest that MK2-mediated phosphorylation of RECQ1 may foster the development of genomic instability and resistance to chemotherapy.

Constitutively activating mutations in Gaq and Gα11 (Gαq/11) have been reported in up to 93% of uveal melanomas. Although constitutively active Gaq/11 promote uveal melanoma tumorigenesis through activation of multiple downstream pathways, no therapies inhibit constitutively active Gaq/11. Studies suggest that upon activation of some Ga subunits increased palmitate turnover and increased cytoplasmic localization occur. The purpose of our studies is to understand the role of palmitoylation in trafficking and signaling of constitutively active Gaq/11 in uveal melanoma. Using live cell imaging and cellular fractionation of HEK 293 cells and uveal melanoma cells, constitutively active GaαG209L and GaαQ209P show decreased localization at membranes compared to wild type Gaα, suggesting increased turnover of attached palmitate in constitutively active mutants. Moreover, immunofluorescence
microscopy revealed that a palmitoylation-deficient constitutively active mutant displays a complete loss of plasma membrane localization and an inability to signal as measured by Rho- and Rac-dependent YAP translocation into the nucleus, TEAD-dependent luciferase activity, and ERK phosphorylation. These studies demonstrate that palmitoylation of mutationally activated Gαq/11 is required for its signaling functions. Although live cell imaging and cellular fractionation experiments reveal decreased membrane localization of constitutively active Gαq compared to wild-typeGαq, our studies suggest that complete loss of membrane localization abolishes constitutively active Gαq-dependent signaling. Disruption of constitutively active Gαq/11 palmitoylation and localization may be an effective strategy for inhibiting constitutively active Gαq/11 oncogenic signaling in uveal melanoma.

P3322
Board Number: B611
O-GlcNAc Transferase Regulates Glioblastoma Acetate Metabolism via Regulation of CDK5-dependent ACSS2 phosphorylation.
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Cancer cells preferentially utilize glycolysis to supply the necessary precursors for macromolecular biogenesis. A subset of the glucose taken up is shunted into the hexosamine biosynthetic pathway where it is used to synthesize UDP-GlcNAc, a substrate of O-GlcNAc transferase (OGT), which serves to modify cytoplasmic and nuclear proteins with O-linked sugar moieties. The post-translational addition of an O-GlcNAc moiety to serine or threonine residues can modulate the activity of nuclear and cytoplasmic proteins. Here, we show that both OGT and O-GlcNAcylation are elevated in glioblastoma cell lines compared to normal human astrocytes. Moreover, immunohistological analyses of human glioblastoma show increased OGT staining correlated with disease progression. Interestingly, when OGT expression is reduced in glioblastoma cells, we detect a significant reduction in anchorage-independent growth, cellular acetyl-CoA concentrations, and decrease in free fatty acids. Importantly, reducing OGT expression in an orthotopic intracranial mouse model resulted in significant reduction in tumor growth and extension of survival. Conversely, overexpressing OGT was sufficient to increase anchorage-independent growth, acetyl-CoA concentrations, and intracellular lipids. Mechanistically we show that OGT overexpression regulates carbon-flux of acetate to acetyl-CoA, a reaction that requires the enzymatic activity of acetyl-CoA synthetase 2 (ACSS2). ACSS2 is critical for OGT-mediated regulation of glioblastoma cell growth, as overexpression of ACSS2 partially rescue cell growth defects. Proteomics analysis identified Ser267 on ACSS2 as being uniquely phosphorylated when O-GlcNAc levels were elevated. This residue was predicted to be a substrate for Cdk5-dependent phosphorylation, which we confirmed with site-directed mutagenesis, immunoprecipitation, and in vitro kinase assay experiments. Thus, we show that OGT and O-GlcNAcylation are required for glioblastoma growth, via a novel regulatory mechanism of acetate and lipid metabolism via regulation of CDK5/ACSS2, and suggests that OGT and CDK5 may be a novel therapeutic targets for treatment of glioblastomas.
P3323

Board Number: B612

N-terminal protein modifications regulate the activity of Myosin Regulatory Light Chain 9 in colorectal carcinoma cells.
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Dysregulation of N-terminal post-translational modifications (Nt-PTMs) is found in multiple cancers and developmental disorders. However, the exact roles Nt-PTMs play in regulating protein function remain poorly understood. Our objective is to clarify the role of N-terminal methylation (Nt-Me) and acetylation (Nt-Ac) on the function of Myosin Regulatory Light Chain 9 (MYL9). MYL9 is a key cytoskeletal regulator and transcription factor and is the first protein confirmed to undergo both Nt-Me and Nt-Ac. Nt-PTM restricted mutants of MYL9 were generated by modifying the consensus sequence targeted by Nt-methyltransferases and Nt-acetyltransferases. These mutants were assayed alongside wildtype (WT) protein for effects on MYL9 stability and activity in HCT116 human colorectal carcinoma cells. MYL9 half-life was tracked using the photoswitchable fluorescent protein Dendra2. Dual loss of Nt-Ac and Nt-Me resulted in an increase in MYL9 half-life, while individual loss of either Nt-Ac or Nt-Me produced no change, suggesting that these modifications redundantly promote protein turnover. We also found that Nt-PTMs regulate the transcriptional activity of MYL9. Upon TNFα stimulation, MYL9 promotes transcription of Intracellular Adhesion Molecule 1 (ICAM-1). HCT116 cells were transduced with WT or mutant MYL9, and ICAM-1 transcript levels were measured by qPCR before and after TNFα stimulation. TNFα treatment resulted in an increase in ICAM-1 transcription when WT MYL9 was present, but this effect was significantly greater in cells expressing an Nt-Ac deficient form of MYL9. No change from WT was seen with an Nt-Me deficient MYL9 variant. This suggests that Nt-Ac reduces the ability of MYL9 to promote ICAM-1 transcription, and identifies a role for this modification in diminishing inflammatory signaling. We are currently testing whether Nt-Ac reduces the transcriptional activity of MYL9 by limiting its nuclear translocation or its ability to bind the ICAM-1 promoter. Our preliminary data thus far indicates TNFα treatment induces nuclear translocation equally for all forms of MYL9. Finally, we are investigating the role of MYL9 Nt-PTMs in its ability to promote cell migration. As Nt-acetylation occurs in the cytoplasm and seems to block MYL9 transcriptional activity, we predict it will be a positive regulator of cell motility. Together, these findings begin to delineate the regulatory role of Nt-PTMs on MYL9 function, which has important implications for multiple disease states, including cancer metastasis and inflammation.

P3324

Board Number: B613

BCAR3, a cell migration-associated protein, is regulated by the E3 ligase Cullin 5-SOCS6.
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Cell migration is important for embryogenesis, tissue repair, and other important biological processes. Migrating cells form protrusions in the direction of motion and attach to their surroundings through multi-protein structures called focal adhesions (FAs), which are assembled at the leading edge and disassembled behind. Adaptor proteins breast cancer antiestrogen resistance 3 (BCAR3) and p130Cas (Cas) form a complex in FAs. BCAR3 is elevated in more aggressive, metastatic cancers. Additionally,
BCAR3 regulates pathways that contribute to membrane protrusion, cytoskeletal remodeling, migration, and antiestrogen resistance. These pathways, if aberrant, could contribute to a cancer phenotype. However, very little is known about how BCAR3 is regulated. We found that BCAR3 expression is regulated by the E3 ubiquitin ligase, Cullin 5 (Cul5) in breast epithelial MCF10A cells. In the absence of Cul5 (knockdown or CRISPR knockout), BCAR3 protein, but not RNA, is increased. Cul5 substrate specificity is conferred through different adaptors that bring the substrate to the E3. We found that BCAR3 is bound by SOCS6, a member of a family of Cul5 adaptor proteins called suppressors of cytokine signaling (SOCS). Knockdown of SOCS6 increases BCAR3 protein levels, suggesting that SOCS6-Cul5 complexes mediate BCAR3 turnover. SOCS6 contains an SH2 domain, which binds phosphotyrosine. Mutating the SH2 domain inhibits BCAR3 binding, suggesting that phosphorylation is required. Deleting the C-terminus of BCAR3 is sufficient to inhibit binding to SOCS6. This suggests that the phosphorylation of a tyrosine residue in the C-terminus of BCAR3 mediates its association with SOCS6.

BCAR3 is stabilized and bound by Cas, which also binds Cul5-SOCS6, suggesting that Cas may be required for Cul5-SOCS6 interaction with BCAR3. However, a BCAR3 mutant that cannot bind to Cas still associates with SOCS6, suggesting that Cas is not required for BCAR3-SOCS6 association. Additionally, knocking down Cas does not disrupt BCAR3-SOCS6 association. While more experiments are needed to establish mechanism, the results suggest a model in which BCAR3-Cas interaction stabilizes BCAR3 by inhibiting SOCS6 binding. By investigating how BCAR3 turnover is regulated, we hope to identify factors that increase BCAR3 turnover in cancers where it is overexpressed.

**P3325**

**Board Number: B614**

Inhibition of deacetylases attenuates lipid accumulation and caspase 3 activation in pancreatic beta cells under the duress of glucolipotoxicity: Potential roles for CD36 activity.

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The cluster of differentiation 36 (CD36), a membrane glycoprotein, facilitates uptake of long-chain fatty acids in various cell types including the pancreatic beta cell. It is widely felt that CD36 promotes lipid accumulation and storage, thus contributing to the pathogenesis of metabolic stress and diabetes. Recent evidence also indicates that CD36 undergoes posttranslational acetylation and deacetylation. However, putative roles of such modifications in the onset of beta cell dysregulation under pathological conditions (glucolipotoxicity; GLT) remain largely unknown. Using pharmacological approaches, we tested the hypothesis that acetylation/deacetylation signaling steps are involved in CD36-mediated lipid accumulation and downstream apoptotic signaling steps in pancreatic beta (INS-1 832/13) cells under GLT conditions.

INS-1 832/13 cells were exposed to GLT conditions (20 mM glucose plus 0.5 mM palmitate) in the absence or presence of known inhibitors of deacetylase, including valproic acid (VAP; 2.5-5.0 mM) or trichostatin A (TSA; 0.25-0.5 μM) or sulfo-n-succinimidy oleate (SSO; 200 μM), an irreversible inhibitor of CD36. Lipid accumulation was determined by oil-red-o staining and quantified by image analyses (Image J software). Western blotting assessed CD36 expression and caspase 3 activation. Exposure of INS-1 832/13 cells to GLT resulted in significant lipid accumulation without affecting the CD36 expression. SSO markedly suppressed GLT-mediated lipid accumulation. Treatment of pancreatic beta cells with TSA or VPA significantly reduced GLT-associated lipid accumulation with no discernable effects.
on CD36 expression. Furthermore, TSA or SSO treatment significantly reduced GLT-induced caspase 3 activation. Based on these findings, we propose that a deacetylation signaling step might regulate CD36-mediated lipid accumulation and caspase 3 activation in pancreatic beta cells exposed to GLT conditions. Identification of specific deacetylases that control CD36 functional activity should provide novel clues for the prevention of islet dysfunction under conditions of metabolic stress such as GLT.

P3326
Board Number: B615
The function of Metabolic Syndrome Complex 1 (MSC1) in myogenesis by modulating YY1 transcriptional activity.
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The development of skeletal muscle requires the progression of a highly ordered cascade of events comprising myogenic lineage commitment, myoblast proliferation, and terminal differentiation. Metabolic syndrome Complex 1 (MSC1) is a multidomain protein and subunit of a lysine acetyltransferase complex that acetylates histone H4 and p53 but the function of MSC1 is unclear. MSC1 Knockout (KO) mice die shortly after birth and display a wide variety of phenotypes within the skeletal and hematopoietic systems. YY1 binds DNA through four C-terminal zinc finger domains and can function as an activator or repressor of gene expression. In the present study, we found that protein and mRNA expression level of MSC1 is decreased during myoblast differentiation in C2C12 cells and YY1 is also decreased during myogenesis. And MSC1 overexpression results in inhibition of myogenesis. Therefore, overexpression of MSC1 does not result in the formation of myotubes. On the other hand MSC1 knockdown was showed induction of myoblast differentiation. In addition, MSC1 directly regulates YY1 expression level how was bind YY1 promoter. The promoter activity of YY1 decreases with the progress of differentiation but its promoter activity is maintained without decrease when MSC1 is overexpressed. Also MSC1 transgenic mouse had an unequal shape and length of muscle fibers. The muscle development also seemed to have occurred abnormally. However, the leg muscles of the MSC1 TG mouse showed no significant difference in size from the wile type mouse when viewed visually. Taken together, these results demonstrate that MSC1 has a negative effect on muscle differentiation both in vitro and in vivo. Based on these finding, MSC1 may have an important role in myogenesis.

P3327
Board Number: B616
The SMYD methyltransferase Set6 interacts with the GimC/prefoldin complex to regulate cell growth and stress response pathways.
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Protein lysine methylation has emerged as a critical regulator of signaling pathways that promote proper growth, development, and differentiation in response to environmental cues. While lysine methylation has been detected on a large fraction of the proteome, we have a limited understanding of its potential roles in signaling and its contribution to cell growth and development. Specifically, the SMYD protein lysine methyltransferases (KMTs) have been identified as key regulators of skeletal and cardiac muscle development, with clear roles in myogenic differentiation and links to cardiac disease. Furthermore, the
aberrant regulation of SMYD proteins is associated with both solid tumor progression and blood cancer. Despite their critical roles in diverse developmental and pathological pathways, our knowledge of SMYD protein substrates and their potential functions in signaling is severely lacking. The *Saccharomyces cerevisiae* protein Set6 is a conserved member of the SMYD family of enzymes, although little is known about its biochemical or biological function. Here, we performed a proteomic screen to identify interacting partners of Set6 and uncovered an interaction with the GimC/prefoldin complex, a chaperone complex for tubulin, actin and other proteins that also plays a role in transcriptional elongation. Consistent with these findings, loss of Set6 confers mild benomyl sensitivity and cell cycle defects on cells and strongly suppresses phenotypes associated with GimC/prefoldin dysfunction, suggesting that Set6 also genetically functions in the same pathway as GimC/prefoldin. The preliminary biochemical analysis also demonstrates that Set6 is likely an active methyltransferase on multiple non-histone proteins, and we will present data investigating the hypothesis that methylation by Set6 promotes proper functioning of the GimC/prefoldin complex. Overall, this work sheds new light on conserved pathways targeted by SMYD family methyltransferases that will be important to understanding their function in development and disease in metazoan systems.

**P3328**

**Board Number: B617**

Poldip2 is an oxygen-sensitive mitochondrial protein that controls Oxidative/glycolytic metabolism balance and Proteasome activity.

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The polymerase delta interacting protein 2 (Poldip2) is a nuclear-encoded mitochondrial protein of unknown function. We recently reported that Poldip2 is repressed under hypoxia and that its deficiency results in repressed mitochondrial function and increased glycolytic activity. However, the mechanisms responsible for this metabolic reprogramming and its consequences are unknown. In this study, we show that Poldip2 controls the lipoylation and consequently the activity of the pyruvate and α-ketoglutarate dehydrogenase complexes by a mechanism that involves Clp-protease complex-mediated degradation of Acyl-CoA Synthetase Medium-Chain Family Member 1 (ACSM1), a lipoate-activating enzyme required for the utilization of lipoic acid derived from the salvage pathway. With regards to the increased glycolytic activity, we found that Poldip2 deficiency inhibits PHDs inducing HIF-1-dependent upregulation of Hexokinase 2. As a result, we observed an increase in the flux through the Hexosamine biosynthetic pathway (HBP), a glucose metabolic pathway that produces uridine diphosphate N-acetylglucosamine, the sugar donor for the O-GlcNAc transferase (OGT). The OGT-catalyzed transfer of O-GlcNAc is a post-translational modification implicated in cell signaling. Interestingly we found that Poldip2 deficiency induces the inhibition of the ubiquitin proteasome system (UPS) by OGT-dependent O-GlcNAcylation of the 19S regulatory unit of the 26S proteasome regulatory subunit 4 (PRS4).

Additionally, we demonstrated that Poldip2-mediated inhibition of the UPS is required for the hypoxia-induced expression of connective tissue growth factor (CTGF), a cytokine that participates in multiple pathologies including fibrotic diseases and cancer. In all, we demonstrate that Poldip2 is an oxygen sensitive protein that regulates the activity of key enzymes of the Krebs cycle by coupling proteasome activity to mitochondrial function and fibrotic responses under hypoxia.
P3329
Board Number: B618
Prolyl dihydroxylation of extra-ribosomal Rps23/uS12 regulates hypoxic adaptation in fission yeast.
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Low-oxygen adaptation is transcriptionally controlled by the Sterol regulatory element-binding protein Sre1N in fission yeast. Sre1N is inactive in the presence of oxygen through direct binding to the prolyl-3,4-dihydroxylase Ofd1. We show that under hypoxic conditions, Ofd1 is sequestered from Sre1N by the nuclear import adaptor Nro1 and the Ofd1 substrate Rps23/uS12. Ofd1, Nro1, and Rps23 form a complex in the cytosol that traffics to the nucleus to deliver dihydroxylated Rps23 for assembly into the small ribosomal subunit. In cells lacking Nro1, we find that Rps23 is predominantly unmodified or monohydroxylated using SILAC and targeted mass spectrometry. Low oxygen stabilizes the Ofd1-Nro1-Rps23 complex by inhibiting Ofd1-dependent dihydroxylation of Rps23. This sequestration model for Sre1N regulation by Ofd1 is further supported by our discovery of a shared Ofd1-binding sequence in Sre1N, Nro1, and Rps23, and by our ability to control Sre1N activation by reducing or elevating Rps23 levels. Since ribosomal protein expression is tightly correlated to the growth state of the cell, this allows nutrients to control Ofd1 availability and thus Sre1N activity. Ofd1 is one of several hydroxylases that modify ribosomal proteins. Our work provides a paradigm for how ribosomal oxidases use their substrates to regulate extra-ribosomal signaling networks to coordinate pro-growth pathways in the cell.

P3330
Board Number: B619
Antiproliferative Activity of Natural Compounds Isolated from Artemisia species.
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Throughout human history, natural products have been used in traditional medicines. Today, plants continue to retain their historical significance as important sources of natural and novel compounds useful directly as medicinal agents and as sources for generating derived-natural compounds. Artemisia is a large and diverse genus of plants with approximately 400 species. In this study, we examined the effect of plant extracts prepared from two different species of Artemisia (Artemisia douglasiana and Artemisia tridentata tridentata) on survival and melanogenesis of B10F16 murine cell lines.

Several extracts from aerial parts of Artemisia species were prepared with selective organic solvents (e.g., hexane, ethyl acetate). Plant extracts were dried, dissolved in DMSO to a concentration of 100 mg/ml, and then added to media containing B10F16 murine cell lines in a concentration dependent manner. After incubation, the effect of these plant extracts on cell proliferation and melanin production were evaluated by the MTT proliferation assay and by measuring the absorbance of the collected sample at a wavelength of 405 nm, respectively.

Both species of Artemisia inhibited proliferation of B10F16 murine cell lines in concentration depended manner. However, Artemisia tridentata tridentata showed the strongest inhibitory effect on melanogenesis even in the presence of IBMX. Furthermore, GC/MS analysis showed a unique chemical composition for each species of Artemisia.
Therefore, these observations indicate that both species of Artemisia may contain several potential natural compounds, which are able to block cell proliferation as well as melanin production in murine cell lines.

**P3331**  
**Board Number: B620**  
Trim13 E3 ligase regulates stability of orphan nuclear receptor Nur77 via casein kinase 2.  
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Nur77, also known as NGFI-B, NR3, or NR4A1, was the first member of the NR4A family to be identified as a gene induced by NGF. Nur77 is an immediate early response gene and plays a critical role in cellular processes in response to diverse stimuli including cytokines, stress, and apoptotic signals. Nur77 has been implicated in a many pathological processes, including cancer, immune alterations and metabolic or neurological diseases. Here we show that Nur77 protein is degraded by ubiquitin-proteasome mechanism and determined that the ubiquitination by E3 ligase is important for an efficient targeting of Nur77 to degradation. We found that the ubiquitination of Nur77 is mediated by the tripartite motif 13 (Trim13), a RING-type E3 ubiquitin ligase. An interaction between Nur77 and Trim13 was confirmed by co-immunoprecipitation assay. We determined ubiquitination site in Nur77 that is important for Trim13-mediated degradation. Moreover, motif scan analysis provided potential that specific sites are able to be phosphorylated by CK2. Treatment of TBB or CX-4945, CK2 inhibitors, increased Nur77 protein levels. Nur77 levels were significantly decreased in cells overexpression of CK2a, but not in cells expressing inactive mutant K68M form. CK2a increased Nur77 ubiquitination, but had less effect on inactive mutant. We also found the phosphorylation site by CK2 that is important for Nur77 stability. Importantly, the introduction of special serine mutant abrogated Trim13-induced Nur77 degradation and ubiquitination. Taken together, these results suggest that CK2a-mediated Nur77 phosphorylation is target for its degradation by Trim13.

**P3332**  
**Board Number: B621**  
Phosphorylation of the HPV E6 oncoprotein by DNA damage response kinases links the E6 interaction with 14-3-3 proteins and p53.  
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Human Papillomavirus (HPV) is a major cause of human cancers, with cervical cancer being the most prevalent. The virus encodes two oncoproteins, E6 and E7, together which are responsible for the initiation and maintenance of malignancy. The cancer-causing E6 oncoproteins are characterized by the presence of a PDZ binding motif (PBM) on the C-terminus. This motif confers interaction with a variety of cellular proteins involved in the regulation of cell polarity, and phosphorylation by PKA switches E6 substrate specificity to members of the 14-3-3 protein family. In this study we have been interested in analyzing the conditions under which E6 is subject to phosphorylation within the PBM. Using a variety of approaches, we demonstrate a striking increase in the levels of E6 phosphorylation by a number of DNA damage response kinases, including those that are induced following treatment with chemotherapeutic agents. In order to understand the biological relevance of these phospho-modifications of E6, we have analyzed their effects upon the ability of E6 to inhibit p53 transcriptional activity. We show that
phospho-E6 has increased interaction with 14-3-3 proteins, a direct consequence of which is an inhibition of p53 transcriptional activity. These studies demonstrate that, under conditions that induce the DNA damage response, E6 has acquired an alternative means of disrupting p53 activity, through its PBM phosphorylation and subsequent sequestration of 14-3-3 from p53. This has important implications for how E6 contributes towards the development of malignancy: it provides an alternative means of inhibiting p53 activity, explains why the PBM is specifically conserved in cancer causing E6 oncoproteins, and explains why the residual levels of p53 in cervical cancer are nonetheless subject to inactivation by E6.

P3333
Board Number: B622
Investigating the function and mechanism of Gpa2 phosphorylation.
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G proteins are important molecular switches that are involved in transmitting a variety of signals from outside to the inside of the cells. G proteins are highly conserved, which allows studying their mechanisms of regulation in model organisms such as the budding yeast Saccharomyces cerevisiae. Gpa2 is a yeast G alpha protein that functions in the glucose signaling and pseudohyphal growth pathway. Through the usage of phos-tag SDS-PAGE, we found that in normally growing cells a substantial amount of Gpa2 protein exists as phosphorylated species. Phosphorylation of Gpa2 requires its N-terminal region, a region that is important in mediating the interaction of Gpa2 with GPCR as well as the plasma membrane. We tested possible kinase(s) that could regulate Gpa2 phosphorylation and found that protein kinase A and casein kinases are involved in this process. In the future study, we will identify Gpa2 phosphorylation sites and then investigate the potential role of Gpa2 phosphorylation.

P3334
Board Number: B623
Investigating Gpa2 Phosphorylation in Saccharomyces cerevisiae.
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Gpa2 is a G alpha protein in a nutrient signaling pathway in yeast. Recently, we found that Gpa2 undergoes elevated levels of phosphorylation as well as accumulation in the mitochondria in response to the treatment of nitrogen starvation. We hypothesize that phosphorylation of Gpa2 could regulate its localization. Typically, the N-terminal region of G alpha proteins functions to interact with the cell membrane. If phosphorylation does control the observed localization of Gpa2 inside of the cell, it is very likely the modification occurs at the N-terminal region of the protein. To test this, we constructed a truncated mutant of Gpa2 and demonstrated that the N-terminal region of the protein is indeed required for this phosphorylation. Simultaneous mutating the 10 serine residues within the N-terminal region of Gpa2 to alanine also blocked Gpa2 phosphorylation. This further supported phosphorylation sites are located on the N-terminal region of Gpa2. In future studies, we will work to identify the kinase(s) responsible for phosphorylating Gpa2, which is a critical step in understanding the signaling roles of this modification; furthermore, we will investigate the precise role of phosphorylated Gpa2.
Mechanotransduction 2

P3335
Board Number: B625
Visualizing Direct Interactions in the Mechanobiome.
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Every biological process, ranging from cell migration to embryogenesis, relies on a cell’s ability to adapt to changing mechanical environments. By studying the model shape change process cytokinesis in Dictyostelium, we find that the cell is a finely tuned control system, with proteins that modulate their behavior in response to mechanical and biochemical signals. We have uncovered the integrated control system that endows the cell with the ability to drive contractility and furrow ingress in the presence of various mechanical and environmental stresses. Although we know many of the players involved in this control system, their biochemical interactions that allow force propagation through the cortical network are still poorly defined. To identify the direct interactions that govern a cell’s mechanical response, we performed immunoprecipitation followed by mass spectrometry on two key nodes of the control system, the scaffolding protein IQGAP2 and the actin crosslinker cortexillin I. This approach led to the discovery of potential binding partners of these nodes. Using fluorescence cross-correlation spectroscopy (FCCS), we have confirmed many of these interactions in live cells, and by measuring concentrations and apparent binding coefficients, we are elucidating the network of biochemical interactions.

From the mass spec analysis, myosin II was identified as a biochemical interactor of both cortexillin I and IQGAP2. By FCCS, myosin II has a measured apparent in vivo KD of 0.6 μM with cortexillin I, and 1.5 μM with IQGAP2. Interestingly, the myosin II-IQGAP2 and cortexillin I-IQGAP2 interaction in vivo KD’s decrease by approximately 5-fold in an iqgap1 null. This, in combination with stoichiometry information from Single Molecule Pulldown, suggests a potential mechanism for the role of IQGAP1 as a negative regulator on the myosin II-cortexitillin I mechanoresponsiveness. In addition, we identified a few unusual interactors by mass spec analysis, such as RNP1, discoidins, and methylmalonyl semialdehyde dehydrogenase (mmsdh), which we previously identified in genetic suppressor screens, providing greater evidence for their function in the network. Using the cytokinesis control system as a model, we are identifying a quantitative interaction map, complete with new interactions, that is uncovering new biochemistry associated with the mechanobiome.
P3336
Board Number: B626
Force generation via β-muscle myosin, titin, and α-actinin drives cardiac sarcomere assembly from focal adhesions.
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Truncating mutations in the sarcomere protein titin cause dilated cardiomyopathy due to sarcomere insufficiency. However, it remains mechanistically unclear how these mutations decrease sarcomere content in cardiomyocytes. Using human induced pluripotent stem cell derived cardiomyocytes and live microscopy, we characterized the fundamental mechanisms of cardiac sarcomere formation. We identify that titin truncating mutations lead to an impairment of sarcomerogenesis and subsequent myofibrillar assembly. We observe that sarcomerogenesis initiates at focal adhesions, where nucleation and radial assembly of α-actinin-2 containing fibers provide a template for z-body fusion and subsequent striation. Using CRISPR/Cas9-mediated engineering, we mechanistically identify that titin provides an essential mechanical connection that propagates diastolic traction stresses from β-cardiac myosin, but not nonmuscle myosin II, to focal adhesions during sarcomerogenesis. Ablating focal adhesions or decoupling titin binding from focal adhesions abolishes sarcomere assembly. The β-cardiac myosin-titin-focal adhesion mechanical connection transmits the forces required to direct α-actinin-2 radial fiber assembly and sarcomere formation. Together these results identify the mechanical and molecular components critical for human cardiac sarcomerogenesis and provide insights into the molecular and environmental origins of cardiomyopathies.

P3337
Board Number: B627
Development and implementation of a Förster Resonance Energy Transfer based biosensor for measuring intracellular tension and force.
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During cellular processes such as cell adhesion, migration and cell division, the actin cytoskeleton exhibits varying changes in tension to generate mechanical force. Nonmuscle myosin 2 (NM2) is an actin binding motor protein that generates the contractile force to facilitate these cellular processes. We have developed a Förster Resonance Energy Transfer (FRET) based biosensor probe of Nonmuscle Myosin 2 (NM2); this probe enabled us to measure the dynamic changes in intracellular tension during various cellular processes. We utilized the recently published tension sensor module (TSMod) in which a 40 amino acid long elastic domain from a spider silk protein was inserted between fluorophores mTFP1 (Donor probe) and venusA206K (acceptor probe) that undergoes low or high FRET depending on the state of the protein (tensed or relaxed) attached to it. We strategically designed and cloned the tension sensor module into the coiled-coil domain of NM2A and NM2B cDNA. The biosensor was then introduced into osteosarcoma (U2OS) and kidney epithelial cell lines (HEK293 and MDCK) by stable and transient transfections. Live cell fluorescence lifetime imaging microscopy (FLIM) was used to quantify
the molecular forces by measuring the FRET efficiency, and cells expressing NM2A-TSMod or NM2B-TSMod tension sensors indicated higher and varying FRET efficiency (11.3±4.5%) compared to the donor only control probe (mTFP1-NM2A or mTFP1-NM2B) (2.79±1.4%). Our NM2 biosensor has identified dynamic changes in tension and force within the cell during various activities. First, molecular forces in the range of 3-8 pN were identified in different regions of the cell, as well as along the individual actin filaments in both adherent and migrating cells. The mechanotransduction on a single actin filament was characterized by the fluorescence correlation spectroscopy (FCS), and indicates a fast force transmission (~500 ms) on a single actin filament in U2OS cells. In addition, we measured the mechanical force map of the MDCK cells during cellular migration, molecular forces of actin on the focal adhesion sites exhibit an alternative pattern varying from 2.5 pN to 7.2 pN within 7.2±3 mins. Our NM2 TSMod biosensors can be applied to visualize and measure dynamic tension changes in cells during various physiological processes.

P3338
Board Number: B628
Improved and tunable molecular tension sensors reveal extension-based control of vinculin loading.
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Despite the importance of applied forces and stiffness of the cellular microenvironment in many developmental, physiological, and pathophysiological processes, the molecular mechanisms enabling cells to sense and respond to mechanical stimuli are poorly understood. Forster resonance energy transfer (FRET)-based molecular tension sensors, which enable the visualization of the forces experienced by specific proteins in living cells, are uniquely suited to study these mechanisms. However, the limited dynamic range in existing sensors has hindered their widespread use. The most common design of these sensors involves two fluorescent proteins (FPs) linked by an unstructured, extensible domain. In response to applied load, the domain extends, reducing FRET, and producing a readily detectable optical signal. To enable the rational design of novel tension sensors with improved force sensitivities, we developed a first principles model based on standard descriptions of polymer mechanics to describe the behavior of these sensors under load. Guided by this model, we altered aspects of the tension sensors, such as the optical properties of the FPs as well as the length and composition of the extensible domain, to improve sensor sensitivity and create a collection of sensors for use in living cells. By utilizing a sensor optimized for studying vinculin mechanobiology, we readily observe that loads supported by vinculin are not uniformly distributed within individual adhesion structures. Also, comparison of multiple sensors with distinct mechanical properties reveals the existence of an extension-based, instead of the traditionally assumed force-based, mechanism regulating vinculin loading. Simple simulations of the mechanical characteristics of FAs suggest that the extension-based control is most likely due to extension-controlled mechanical inputs, such as the step size of a molecular motor or the discrete increases in length associated with a growing actin filament, regulating the loading of vinculin. The model also can predict the behavior of readily achievable FRET-based tension sensor designs. Based on the optical properties of commonly used FPs, the length of springs typically used in FRET-based tension sensors, and estimates of the persistence length of unstructured polypeptides, we also calculate the mechanical behavior of an additional 1020 possible designs. The various sensors are predicted to have sensitivities covering the range of 1-25 pN (1-14 nm) with different sensors exhibiting wide varieties of characteristics, including extremely sharp transitions or broad responsiveness. These
rationally-designed sensors should uniquely enable a wide variety of novel studies and be very useful in elucidating key mechanisms of mechanotransduction

P3339
Board Number: B629
Detecting Vinculin Load-Dependent Protein Recruitment to Focal Adhesions.
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Cells are sensitive to alterations in the mechanical nature of the extracellular microenvironment. In order to interact with the extracellular matrix (ECM), cells assemble integrin-mediated adhesion structures termed focal adhesions (FAs). To detect the mechanical nature of the ECM, cells apply forces generated by the actomyosin cytoskeleton to FAs, altering the activities of key proteins to enable complex cell functions such as cell migration and differentiation. However, the fundamental mechanisms underlying FA-based mechanosensitive signaling are unclear. To study these processes, we use a FRET-based molecular tension sensor that reports the loads across the key mechanical linker protein vinculin. Specifically, we seek to investigate the relationships between these loads, actomyosin structures, and the signaling state (composition) of FAs. Initial experiments combining FRET imaging with phalloidin labeling of actin structures showed high loads across vinculin in the cell periphery where FAs are associated with either stress fibers or lamellar protrusions. Strikingly, the distribution of the loads supported by vinculin within individual FAs associated with these two distinct actin structures are different. Within FAs loaded by stress fibers, we observed a spatial gradient in the loading of vinculin with the highest tension at the edge of the FA proximal to the cell membrane, while loading of vinculin associated with lamellar protrusions appears uniform. Consistent with these observations, FAs exhibit uniformly distributed loads during the early stages of cell spreading, when protrusion is enhanced, and the observation of gradients in vinculin loading coincides with the formation of stress fibers during the later stages of spreading. Also, the expression of constitutively active forms of Rac1 and RhoA lead to enhancements of FAs exhibiting uniform or gradient loading of vinculin, respectively. Furthermore, the mechanical state of vinculin within an individual FA is a strong predictor of the signaling state (composition) of that FA. By combining FRET-imaging with immunofluorescent staining, we screened 22 proteins for vinculin tension sensitivity and associated actomyosin structure preference and observed evidence of distinct mechanosensitive signaling nodes within specific actomyosin structures. For example, VASP, a direct binding partner of vinculin preferentially localizes to loaded FAs associated with stress fibers and not similarly loaded FAs associated with protrusions. In total, this work shows that both the magnitude and the molecular basis of vinculin loading impacts mechanosensitive signaling, and suggests that these distinct relationships may mediate the diverse mechanosensitive aspects of FAs.

P3340
Board Number: B630
Tissue geometry directs patterns of bioelectricity and growth.
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Epithelial cells form intact tissues wherein cells communicate with their neighbors by juxtacrine signaling, diffusible morphogens, and mechanical stresses. Mechanical stress gradients can transmit information about the geometry of the tissue to its constituent cells and lead to the emergence of
patterns of proliferation within the population, with highest proliferation at sites of maximum stress. Here, we found that tissue geometry also controls the pattern of cellular membrane voltage (Vm), defined as the electric potential difference between the cytoplasm and external medium. Manipulation of Vm has been demonstrated to impact cellular behaviors including proliferation and apoptosis. Using our microfabricated epithelial tissues in combination with the voltage-reporter dye DiBac4(3), we observed Vm depolarization in cells located along the convex edges of epithelial tissues. These are the same high-stress regions where cells show high proliferation. Surprisingly, we also observed an increase in apoptotic markers in cells within these regions. These observations suggest a relationship between Vm, proliferation, and apoptosis during the cellular response to tissue-generated mechanical stress, and a combined role of these factors during the orchestration of global patterning events.

P3341
Board Number: B631
Traction force microscopy using embedded marker arrays with an implied zero-displacement state.
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Cytoskeletal tension generated by cells directly governs initiation of intracellular signaling cascades that mediate cell fate in a tension-dependent manner. A better understanding of how tension-dependent signaling cascades are initiated and maintained may provide new strategies for preempting the onset of pathological processes but this is difficult to achieve with existing approaches. One major limitation in current approaches to measure cell-generated traction forces, a direct indicator of cytoskeletal tension, is the need to chemically relax or completely remove the cells being investigated to obtain a material zero displacement state for force calculations. This limitation inhibits molecular analysis of signaling processes in contractile cells. To address this limitation, we developed a new traction force microscopy platform which utilizes patterned arrays of fluorescent fiducial markers within a PEGDA hydrogel to measure material deformation induced by contractile cells. Using an image-guided, pulsed (140fs; 740nm) laser, the marker arrays are composed of Gaussian-like features with full-width, half-maximum dimensions of 0.86μm in XY and 3.45μm in Z, with center-to-center spacing of 2.18μm in XY and 3.5μm in Z. Precise vertical alignment of the fiducial markers allows for facile measurement of shear displacement, within 66±40nm, through comparisons of displaced markers near the cells with non-displaced markers sufficiently below the surface. Normal displacements at the surface are measured from a surface approximated by local regression of non-deformed regions to provide simultaneous measures of shear and normal forces. We demonstrate the utility of this platform in measuring cellular tractions without interrupting cellular function using human umbilical vein endothelial cells (HUVECs) imaged at timepoints ranging from 4hrs to 72hrs post-seeding. Time-lapse cellular traction measures confirm the elasticity of the substrate, even in cases where markers were displaced greater than 4μm from their original locations and subsequently released. Consistent with previous findings, the total output of individual HUVECs, measured as the sum of surface shear displacements, increased with cell spread area. Additionally, HUVECs sharing a border with another cell generate less traction per unit area compared to isolated cells during early spreading (p=0.0136). During migration, HUVECs utilizing blebbing generate larger normal forces compared to those utilizing filipodia and/or lamellipodia (p=0.0006). We are currently optimizing this platform to couple it with standard immunofluorescence labeling to investigate the role of tension on activation of adhesion site signaling proteins (FAK and paxillin) and present preliminary findings towards this goal.
Many cell types polarize and migrate directionally in response to spatially organized ECM cues and other organized cues from the extracellular matrix (ECM) in the form of contact guidance. Collagen and other ECM components in vivo are often organized into wavy, sinusoidal bundles rather than linear fibers. For example, in tumors bearing ovarian stroma, sinusoidal collagen bundles are observed with a lateral curvature radii that can vary between 2 and ~30 microns, and are predictive of tumor grade. To investigate how cells respond to curved ECM, we used nanofabrication to create solid ridges with the approximate cross-section of large collagen bundles (300-600 nm), with lateral curvatures of varying wavelength or amplitude, which were then coated with ECM as a proxy for curved ECM bundles. On either linear or short wavelength curvature substrates, cell shape was polarized such that they were elongated and aligned with the pattern propagation, and their Golgi were likewise oriented in front of the nucleus with respect to this axis. However, as the wavelength of curvature approaches the average diameter of the cell spread area, cells are depolarized with respect to the axis of ridge wave propagation. Focal adhesions, Arp2/3, free barbed ends, and PIP3 accumulate at sites of substrate curvature, mimicking leading edge machinery, even within the cell center. Integrin engagement, PI3K activity and myosin II (myo2) activity are all required for the polarization response. On cell-scale curvatures, myo2 assembles and flows centripetal to the curvature, like arcs observed in two-dimensional lamella. On shorter wavelength curvatures, while actin polymerization sites still occur along curved portions, myo2 flow is altered such that myosin and actin assemble onto long linear bundles spanning the curvature, enforcing an elongated cell shape. When contractility is inhibited, this no longer occurs, and cells flow along the ridge pattern and lose polarization. Conversely, highly contractile cells such as fibroblasts continue to polarization along the long axis of the ridges even at cell-diameter curvatures, extending stress fibers across the waveforms. We propose a model in which cells “detect” curvature of ECM by actomyosin arc and bundle formation which are constrained by adhesion sites. When curvature is low enough for actomyosin bundle deformation, compliance with the curvature occurs thus depolarizing cells, while on shorter wavelength, higher curvature substrates, contractile bundles prevent cell compliance to the curvature, allowing for robust polarization of cells along ECM bundles despite local curvatures. This model would predict that tumor cells may overcome curved bundle induced depolarization by increasing their contractility to migrate along curved bundles found in vivo.
Mechanotransduction properties of the PECAM-1 cytoplasmic tail.

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Vascular endothelial cells (ECs) are a well-known cell system used in the study of mechanobiology. We previously showed that platelet EC adhesion molecule 1 (PECAM-1, CD31), a cell adhesion protein which localizes to regions of EC-EC contact, was rapidly tyrosine phosphorylated in cultured ECs exposed to flow or cyclic stretch. Src-homology 2 domain-containing protein tyrosine phosphatase 2 (SHP2) was found to then bind phosphorylated PECAM-1 and activate the extracellular signal-regulated kinase1/2 (ERK1/2) signaling cascade, a known flow-activated signaling pathway. Although PECAM-1 tyrosine phosphorylation is well characterized in ECs exposed to fluid shear stress, it is less so in cyclically stretched cells. Here, using a two-dimensional cell stretch apparatus, we show that the stretch-induced change in EC shape is a dynamic and reversible response. We also show that the EC-EC contact site is a major region for stretch-induced mechanosignaling. We hypothesized that external forces applied to a monolayer of ECs are directly transmitted to PECAM-1, causing the molecule to be stretched and in turn, allowing phosphorylation sites in its cytoplasmic domain to be exposed and phosphorylated. This hypothesis requires the presence of a stretchable structure within the PECAM-1 cytoplasmic domain.

Force spectroscopy measurements were performed with a construct containing cytoplasmic PECAM-1 domains inserted between I27 motifs, a recombinant string of the structural elements from titin. This strategy allowed us to identify the events in which a single molecule is being pulled and to detect the unravelling of the cytoplasmic domain of PECAM-1 by force. The response by PECAM-1 to mechanical loading was heterogeneous but with magnitudes as high as or higher than the naturally force bearing I27 domains. In conclusion, the PECAM-1 cytoplasmic domain has a structure that can be unfolded by externally applied force and this unfolding of PECAM-1 may be necessary for its phosphorylation, the first step in the PECAM-1 mechanosignaling cascade. *E.M. and J.L.S. contributed equally to this work.
under in vivo conditions. Vascular smooth muscle cells (VSMCs) are constantly subjected to mechanical stimulation in physiological and pathological conditions. Phenotype and functional changes of VSMCs, including production of reactive oxygen species, proliferation and migration are often associated with mechanical stimulation. Since mechanical forces are known to induce rearrangement of cytoskeleton that also participates in endocytosis process, we asked whether cyclic mechanical strain may reduce MNP uptake by VSMCs. Primary culture of VSMCs were obtained from spontaneously hypertensive rats with an explant technique and used between passages 3 to 8. VSMCs were cultured in Flexcell plate to near confluence prior to be subjected to cyclic strain (1 Hz, 10% elongation) for 2 to 8 hrs, followed by exposure to polyacrylic acid-coated magnetic nanoparticles (MNPs; 200 nm, 25 μg/cm²) for additional 4 hrs. VSMC-associated MNPs (MNPcell) was analyzed with a KSCN method, which revealed high capacity of MNP internalization by VSMC in a concentration-dependent manner. Application of cyclic strain for 4-12 hrs significantly reduced MNPcell by up to 65% (P <0.05). Confocal microscopy demonstrated that both cyclic strain and fibronectin coating of the culture plate reduced VSMC uptake of MNPs by 71% and 40%, respectively. Incubation of MNPs with VSMCs for 4 hrs induced microvilli formation associated with MNP internalization observed with transmission electron microscopy; whereas cyclic strain stimulated formation of jigsaw-like protrusion from cell surface in the presence of MNP. However, pretreatment of N-acetylcysteine (0.1 mM), an antioxidant, did not alter the effects of cyclic strain on MNPcell. In conclusion, cyclic strain reduced MNP internalization by VSMCs, which may be due to cytoskeleton rearrangement; such effects of cyclic strain may preserve nanocomposites to the extravascular target.

P3345
Board Number: B635
The SMuSh pathway is essential for survival during growth-induced compressive mechanical stress.
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Cells that proliferate in a confined environment eventually build up mechanical compressive stress. For example, mechanical pressure can emerge from the growth of cancer cells or microbes in their natural settings. However, while the biological effects of tensile stress have been extensively studied, little is known about how cells sense and respond to mechanical compression. By combining genetic analysis with microfluidic approaches, we discovered that compressive stress is sensed through a module consisting of the mucin Msb2 and Sho1, which is one of the two osmosensing pathways in budding yeast. This signal is transmitted via the MAP kinase Ste11 to the cell wall integrity pathway. We term this mechanosensitive pathway the SMuSh pathway, for Ste11 through Mucin / Sho1 pathway. The SMuSh pathway is necessary for G1 arrest and cell survival in response to growth-induced pressure. Our study demonstrates the ability of budding yeast to specifically respond to mechanical compressive stress raising the question of whether homologous pathways confer mechano-sensitivity in higher eukaryotes.
**P3346**

**Board Number: B636**

A novel role for the small heat shock protein, HspB1, in the response of cells to mechanical stress.

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The mechanical environment exerts a tremendous influence on cells, but the molecular mechanisms involved in mechanical stimulation, sensing and response are not entirely clear. We hypothesized that the activation of MAPK pathways in mechanically stimulated fibroblasts may directly impact the resulting actin reorganization through post-translational modifications, subcellular redistributions, and actin polymerization and reinforcement. Mechanical stimulation (via uniaxial cyclic stretch) activates p38 MAPK and MK2 kinase which increases phosphorylation of the downstream target HspB1 (Hsp25/27), a small heat shock protein implicated in actin regulation. We eliminated HspB1 expression using a CRISPR/Cas9-nuclease targeting strategy and determined that the normal stretch-stimulated actin thickening was disrupted without HspB1. Using fluorescence microscopy we found that the phosphorylated HspB1 protein localizes to the ends of actin stress fibers in stretch-stimulated cells. These cytoskeletal structures extending from focal adhesions towards the cell center are known as “comet tails” and include retrograde actin flux away from focal adhesions. Actin comet tails and cell migration are inversely correlated, so we next examined the effect of HspB1 on migration using time lapse microscopy. Compared to parental wild-type (WT) cells, cells lacking HspB1 protein exhibited increased migration. The HspB1-null cell migration returned to WT levels with expression of an HspB1-rescue construct. These HspB1-null phenotypes (decreased actin stress fiber reinforcement and increased cell migration) were reminiscent of those previously described for cells lacking the mechanosensitive cytoskeletal regulator and comet tail constituent zyxin. To determine if HspB1 and zyxin might function cooperatively or in parallel pathways of actin remodeling, we used the CRISPR/Cas9-nuclease targeting strategy to generate double-mutant HspB1/zyxin-null cells and compared them to single-null cells. The HspB1/zyxin double-null cells displayed an enhanced failure of the actin reinforcement response to mechanical stress, suggesting that HspB1 and zyxin may act through parallel pathways. This work identifies HspB1 as a new player in the field of mechanotransduction.

**P3347**

**Board Number: B637**

Effects of Mechanical Stress on Remodeling of Periodontal Ligament.

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Periodontal ligament (PDL), which connects the teeth to the alveolar bone, is always exposed to mechanical stress such as occlusal force. The response of PDL cells to mechanical stress has been studied by many dentists and researchers. However, it was difficult to obtain the quantitative data related to mechanical stress effect on PDL in vivo. Here we study the effects of stretch and pressure on PDL fibroblasts. First, we cultured PDL fibroblasts under uniaxial cyclic stretch for 16 hours. After 4 hours, PDL cells and actin stress fibers were aligned in the vertical direction to the stretch axis. These results suggest that mechanical stress regulate the orientation of PDL cells to support the teeth. Second, we used high hydrostatic pressure microscope to observe PDL fibroblasts under high pressure in real time. Our systems control the level of hydrostatic pressure and stimulate cells equally. They also apply...
various patterns of occlusion to cells by controlling the hydrostatic pressure. Furthermore, high hydrostatic pressure microscope observes cells and molecules under high pressure in real time. As a result, high hydrostatic pressure (> 20 MPa) contracts PDL fibroblasts and actin stress fibers. After the release of pressure, contracted cells restart spreading on the surface after high pressure releasing process. Our data suggest excessive occlusal force induces the collapse of PDL and occlusal force at biological range affects homeostasis of PDL. Our systems directly show mechanical stress effect on PDL and give us new clinical knowledge about the treatment of periodontal diseases.

P3348  
Board Number: B638  
Direct observation of cell mechanics under high hydrostatic pressure.  
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Activities of daily living such as walking and sitting pressurize articular cartilage. Chondrocytes, which constitute articular cartilage, synthesize collagen and aggrecan to maintain the function of articular cartilage. Aggrecan is a type of proteoglycan, and collagen type II is the main articular cartilage. However, the effect of pressurization on chondrocytes at a molecular level is poorly understood due to the lack of methods that directly observe cells under high pressure condition. Here we used hydrostatic pressure to apply higher pressure to chondrocytes in vitro and investigated cell morphology, molecules, and gene expression levels under high pressure condition. We observed higher pressure (dozens of MPa) decreased the size of chondrocytes and collapsed actin actin stress fibers. We also used high pressure microscope to directly observe chondrocytes under high pressure. The chondrocytes adhesion area was decreased to ~80% at 20 MPa for 1 h. However, after pressure releasing process, cells restarted to spread on the surface. We also found that gene expression levels of collagen and aggrecan were increased during the incubation after pressurizing process. Our results suggest that pressurization to chondrocytes is related to maintenance of articular cartilage function.

P3349  
Board Number: B639  
Water Pumping performance of human polycystic kidney disease epithelial cells.  
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Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary disorder caused by mutations in the genes PKD1 and PKD2, which encode for the proteins polycystin 1 (PC 1) and polycystin 2 (PC 2). A major characteristic of the disease is formation and progressive growth of numerous fluid-filled cysts in the renal tubules. Previous studies have shown that cysts are formed due to rapid cell proliferation and are sustained by hydrostatic pressure generated by the mural epithelial cells by pumping ions and water into the lumen. However, previously no work has been done to quantify the water pumping performance of normal and diseased cells. Also the influence of external cues like fluid shear stress, osmolarity and hormonal perturbation on the pump performance of renal epithelial cells in a physiologically relevant setup is not yet studied.

We have developed a Kidney-an-a-Chip microfluidic set-up, that can measure the water flux across renal epithelium with a volumetric resolution ~0.785 uL. The device is capable of applying a range of mechanical, chemical and osmotic perturbations, both on the apical and basal domains of the
epithelium. By seeding the device with primary ADPKD cells derived from human renal cysts, the water flux as a function of the hydrostatic pressure difference between the apical and basal domain for different values of shear stresses was measured. The apical to basal water flux increased from 3.33 to 6.67 μL/min/cm² with change in shear stress from 0.125 to 0.25 dyn/cm², but it decreased to 3.33 μL/min/cm² with further increase in shear stress to 0.5 dyn/cm². The water flux was also found to change as a function the hydrostatic pressure on the apical side. Our observations reveal that renal epithelial cells can be modelled as mechano-biological pumps whose performance can be mapped under both normal and diseased conditions.

P3350
Board Number: B640
Forces and dynamics in three-dimensional epithelia of controlled size and shape.
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Mechanobiology of epithelial tissues plays a central role in morphogenesis, tissue compartmentalization, protection against pathogens, wound healing, and tumor progression. Current understanding of epithelial mechanobiology relies on measurements of forces and deformations obtained in flat epithelial layers. However, most epithelia in vivo exhibit a curved three-dimensional shape that encloses a pressurized lumen. Here we provide direct measurements of epithelial mechanics in three-dimensional cell sheets. Using soft micropatterned substrates we produce massive parallel arrays of epithelial domes with controlled size and basal shape. By measuring 3D deformations of the substrate we obtain a direct measurement of epithelial tractions and luminal pressure. The local tension in the freestanding epithelium is then mapped by combining measured luminal pressure and local curvature. We show that tension and dome curvature remain isotropic and uniform during perturbations of the contractile machinery, osmotic shocks, and spontaneous fluctuations of dome volume. By examining dome tension over time-scales of hours and for nominal strains reaching 300%, we establish that epithelial sheets exhibit superelastic behavior. Despite the fact that the dome is subjected to uniform tension, the areal strain of each individual cell can differ by more than one order of magnitude. This remarkable heterogeneity is suggestive of a mechanical instability caused by limited availability of structural components of the actin cortex. We develop a 3D vertex model that captures both the global tension/strain relationship in the dome and the strain heterogeneity. Our study provides the first direct measurement of pressure, tension and strain in three-dimensional epithelia and shows that epithelial cells can sustain extreme deformations under constant tension.
P3351
Board Number: B641
Ca2+-calmodulin dependent kinase kinase II regulates mechanosensitive actin stress fiber assembly.
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Ventral stress fibers are contractile actomysin bundles, which guide adhesion, migration, and morphogenesis of non-muscle cells. Their assembly and alignment are under precise mechanosensitive control, and thus stress fiber networks undergo rapid alterations upon changes in the biophysical properties of cell’s surroundings. In migrating cells, stress fiber maturation requires mechanosensitive activation of the 5’AMP-activated protein kinase (AMPK), which subsequently inhibits actin filament assembly at focal adhesions through phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Here we identify Ca2+-calmodulin-dependent kinase kinase II (CaMKK2) as a critical upstream factor controlling mechanosensitive activation of AMPK during stress fiber maturation. CaMKK2 accumulated to focal adhesions located at the ends of ventral stress fibers, and its inhibition, or depletion of Ca2+, led to defects in mechanosensitive regulation of AMPK and VASP. Consequently, inactivation of CaMKK2 resulted in the loss of mature contractile stress fibers, accompanied by diminished cell-exerted forces. Collectively, these data provide evidence that Ca2+, CaMKK2, and AMPK form a locally-activated, mechanosensitive signaling cascade in focal adhesions that is critical for maturation of contractile stress fibers in migrating cells.

P3352
Board Number: B642
Spatiotemporally varying wall shear stress modulates lymphatic endothelial cell alignment and transcriptional regulation.
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The lymphatic system is an essential though sometimes overlooked part of the cardiovascular system. One-way valves within lymphatic vessels, which are formed from lymphatic endothelial cells during embryonic development, are required to maintain unidirectional fluid flow. At present relatively little is known about how these valves form during lymphatic development, and how they regenerate in the adult. Fluid flow is proposed to be a key cue in determining the location and timing of valve formation. We built a novel in vitro cell culture device that reproduces key aspects of the flow environment at sites of valve formation. Using this device, we observed that human lymphatic microvascular endothelial cells (HLMVECs) reoriented perpendicular to the flow direction at the region of maximum wall shear stress (WSS), i.e. at the site of maximum constriction, and exhibited enhanced nuclear localization of FOXC2, a transcription factor required for valve formation. In addition, we found that HLMVECs exposed to oscillating flow, such as occurs in vivo, showed a marked increase in their responsiveness to WSS. Further experiments revealed that the cell surface protein E-selectin was required for HLMVECs to orient perpendicular to the flow at sites of flow constriction. Our observations support the hypothesis that spatial and temporal variations in WSS provide synergistic physical cues that may help to define the timing and location of valve formation, and implicate E-selectin as a possible component of a mechanosensory mechanism for sensing localized gradients in WSS.
P3353
Board Number: B643
Investigating calcium dynamics in lymphatic endothelial cells subjected to flow-induced wall shear stress.
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Spatial variations in fluid flow are thought to help trigger the formation of valves within the lymphatic circulatory system during embryonic development. However, how the physical stimulus provided by fluid flow may direct the changes in gene expression required for valve formation is not well understood. Previous studies in developing mouse embryos have shown that lymphatic valves preferentially form near sites of vessel constriction and branching, where the lymphatic endothelial cells (LECs) which form the valve are exposed to gradients in wall shear stress (WSS). Previous work also identified the Ca\(^{2+}\) and Calcineurin-dependent nuclear localization of the transcription factor NFATc1 as a critical cue in driving valve formation. However, how the physical stimulus provided by fluid flow may influence Ca\(^{2+}\) signaling in LECs remains poorly understood. We have subjected LECs treated with the calcium indicator Fluo4 to spatially varying WSS such as occurs at sites of valve formation in vivo. We found that LECs displayed oscillatory pulses of cytoplasmic Ca\(^{2+}\) release in response to spatial gradients in WSS, and that Ca\(^{2+}\) pulses were more frequent in the areas of highest WSS. However, the Ca\(^{2+}\) duty cycle showed the opposite trend, with shorter individual pulses in the regions of highest WSS. Ongoing work aims to identify the molecular mechanisms that couple the physical cue provided by fluid flow to the activation of Ca\(^{2+}\) pulses. In summary, our data indicate that Ca\(^{2+}\) dynamics in LECs are exquisitely sensitive to local variations in WSS. These data support the hypothesis that the dynamics of stored Ca\(^{2+}\) release may play a key role in coupling the physical cue provided by fluid flow to the changes in transcriptional regulation required for valve formation.

P3354
Board Number: B644
Functional analysis of larval chordotonal organ mechanics in *Drosophila*.
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In *Drosophila* larvae and adults, chordotonal organs (ChOs) are ubiquitous mechanoreceptors, converting a diverse range of physical forces such as sound, vibration and stretch into biological responses. The mechanoelectrical transduction, operated by an active, force-generating process, has been linked to adaptation motors. The underlying force generator, however, is poorly known, and the functional dissection of ChO mechanics *in vivo* has been challenging. We combine electrophysiological analyses with mechanical stimulation, and correlate mechanical properties and active manipulation with neuronal activity. We find that myosin II motors power contraction of the cap cells of ChO and regulate mechanosensation. Our *in vivo* model reveals larval ChOs as complex, but accessible organs to study the molecular machinery involved in the regulation and encoding of mechanical forces by primary mechanoreceptor neurons.
Signaling Networks Governing Cell Migration

P3355
Board Number: B645
Regulation of RhoA by STAT3 Coordinates Glial Scar Formation.
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Understanding how the transcription factor signal transducer and activator of transcription 3 (STAT3) controls glial scar formation may have important clinical implications. We show that astrocytic STAT3 is associated with higher amounts of secreted MMP2, a crucial protease in scar formation. Moreover, we report that STAT3 inhibits the small GTPase RhoA, and thereby controls acto-myosin tonus, adhesion turnover, and migration of reactive astrocytes, as well as corralling of leukocytes in vitro. The inhibition of RhoA by STAT3 involves ezrin, the phosphorylation of which is reduced in STAT3-CKO astrocytes. Reduction of PTEN levels in STAT3-CKO rescues reactive astrocytes dynamics in vitro. By specific targeting of lesion-proximal reactive astrocytes in Nestin-Cre mice, we show that reduction of PTEN rescues glial scar formation in Nestin-Stat3+/− mice. These findings reveal novel intracellular signalling mechanisms underlying the contribution of reactive astrocyte dynamics to glial scar formation.

P3356
Board Number: B646
Novel biosensor reveals the regulation and coordination of GEF-H1 with protrusion machinery.
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Rho family GTPases are molecular switches that control multiple cellular functions including cellular movement. Their activity is regulated with precise spatio-temporal dynamics by guanine exchange factors (GEFs). While biosensors have revealed the dynamics of Rho GTPases in living cells, little is known about the subcellular distribution and timing of GEF activation. Guanine exchange factor H1 (GEF-H1) activates RhoA at the leading edge of moving cells and plays a critical role in focal adhesion turnover and mechanosensing. GEF-H1 is unique among GEFs because it associates with microtubules (MTs). We identified a new mode of GEF-H1 regulation by Src kinase and used it to design a fluorescent biosensor that reports GEF-H1 activation in living cells. The biosensor was designed using general structure-based principles that could be extended to the other 68 members of the Dbl family of GEFs. Live cell imaging experiments revealed the spatio-temporal relationship between GEF-H1 activity and RhoA activity at the cell edge. GEF-H1 is activated during protrusion with precise timing and position relative to RhoA activity and edge dynamics. The biosensor also showed that GEF-H1 is localized at the MTs in its inactive state, and that GEF-H1 is active away from the MTs. Using a new quantitative image analysis method and imaging both MT and GEF-H1 activity in the same cell, we saw that MT tip dynamics control GEF-H1 activity in specific regions relative to cell protrusions. These results show that GEF-H1 regulates cell migration by mediating crosstalk between the MT and actin networks. Current studies exploring proteins that regulate GEF-H1, and the role of GEF-H1 in linking MT dynamics and focal adhesion turnover will be described.
P3357
Board Number: B647
*Ganoderma lucidum* extract reduces cell migration and invasion in triple-negative metastatic breast cancer.

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Triple-negative metastatic breast cancer (TNMBC) is characterized by the absence of estrogen and progesterone receptors and the absence of HER2 overexpression. According to statistics, breast cancer (BC) is known as the leading cause of cancer among women in the United States and Puerto Rico. Approximately, 15% of all BCs and women typically display shorter overall survival and an early peak of distant recurrences at 3y after diagnosis. TNMBC is known to have an aggressive clinical behavior with a high risk of both local and distant relapses that frequently present as visceral and/or brain metastases. The treatments for TNMBC are limited besides the use of chemotherapy and currently there are no approved targeted therapies. The mortality of TNMBC could be a result of the lack of an effective treatment to decrease the migration of the cancer cells to other tissues. Hence, it is highly important to understand migration process of cancer cells and find effective therapies for TNMBC. Therefore, we aimed to test the effects of the medicinal mushroom *Ganoderma lucidum* extract (GLE) on TNMBC cell migration and the regulation of proteins involved in this process. MDA-MB-231 cells were treated with vehicle or 0.97 mg/mL (IC50) of GLE for 24hrs. The effect of GLE on cell migration was assessed by wound healing assay and fluorescence staining of F-actin. Additionally, cell invasion assay was performed using the Transwell Invasion Assay. The protein expression of Rac1/2/3, Cdc42, Lamellipodin and Rho was tested by immunoblotting. The activity of Rac1 was tested by a pulldown activity assay and immunoblotting. GLE decreased MDA-MB-231 cell migration as shown by reduced wound closure. Moreover, there was a significant reduction in the expression of Rac1/2/3, Cdc42, and Lamellipodin proteins. GLE also significantly decreased the activity of Rac1, and significantly decreased invasion of MDA-MB-231 cells after 24h treatment. Thus, we can conclude that our results suggest the potential of GLE as a natural anti-TNMBC migration agent. This work was supported by NIGMS SC3GM111171 (MMM), NIMHD G12MD007583 (MMM), P20GM103475 (UPR-pilot MMM), NIMHD G12MD008149 (MMM), NIMHD G12MD007587 (MMM), NIGMS R25GM110513 (TRF), NIMHD G12MD007579, and Title-V-PPOHA P031M105050 and Title-V-Cooperative P031S130068 from the U.S. Dept of Education, and PRSTRT SGRP 2017-00143 (MMM). The content is solely the responsibility of the authors and does not necessarily represent the official views of the supporting agencies.

P3358
Board Number: B648
Long-range intercellular communication in collective cell migration.

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Collective cell migration of cohesive groups involves intercellular mechanical communication transmitted between adjacent cells through cell-cell contacts to eventually drive long-range
communication. We discovered how local mechanical fluctuations induce long-range communication and identified some potential molecular players driving this mechanism. By designing and applying new analytical methods to migrating monolayers of epithelial cells, we find that cells at the front transmit mechanical cues by inducing normal and shear strains on neighboring follower cells. Accumulation and propagation of these mechanical cues over time and space create groups of cells that migrate and exert forces in a coordinated manner. Such motion patterns direct cells from within the monolayer toward the sites of shear-strain-induced motion at the monolayer front. These results provide a model of long-range mechanical communication between cells, in which local alignment of velocity and stress translates local mechanical fluctuations into globally collective migration.

Efficient collective migration depends on a balance between contractility, cytoskeletal rearrangements, and adhesions, all controlled by GTPases of the RHO family. Spatiotemporal analysis revealed a surprising role of the RHO GTPase RhoA in regulating long-range communication that was mirrored upon slight down-regulation of myosin-II contractility. A comprehensive screen uncovered a group of RhoA-activator guanine nucleotide exchange factors (GEFs) that are implicated in intercellular communication. Downregulation of these GEFs differentially enhanced propagation of guidance cues through the group, defining two functional clusters: RHOA-ARHGEF18 and ARHGEF3-ARHGEF28-ARHGEF11, with RHOC as an intermediate between them. We conclude that for effective collective migration the RhoA-GEFs/RhoA/C/actomyosin pathways must be optimally tuned to compromise between generation of motility forces and restriction of intercellular communication.

P3359
Board Number: B649
The ArfGAP Drongo regulates Myosin-II mediated contractility during the migration of Drosophila border cells.
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Cell migration is implicated in various important biological processes, notably it is central for the dissemination of cancer cells. Previously, our lab has showed that endocytosis regulates cell guidance and cell-cell coordination during collective cell migration. Our hypothesis is that other events of vesicular trafficking might be implicated in collective cell migration. Mainly, we propose that small GTPases of the Arf family, important for the formation of vesicles and sorting of cargo in these vesicles, and their regulators might regulate the localization of determinants of collective cell migration. During oogenesis in Drosophila melanogaster, a group of cells named border cells, migrate collectively towards the oocyte. We performed a candidate RNAi screen, to deplete Arf GTPases and their regulators specifically in borders cells. We found that every Arfs, two ArfGAPs and one ArfGEFs are necessary for normal border cell migration. However, we found that depletion of Arfs affects the integrity of the border cell cluster and cause a disruption the Golgi apparatus. Interestingly, depletion of the ArfGAP drongo did not disrupt the golgi apparatus or the formation of the border cell cluster, suggesting a specific role in border cell migration. Indeed, live-imaging showed that drongo depleted clusters are able to form protrusions at the front of migration but are unable to detach and migrate towards the oocyte. We later found that Myosin-II activity, which is normally localised at the front and back of the cluster, and is necessary for detachment was mislocalised in drongo depleted clusters. Active Myosin-II was dispersed around the cluster and significantly reduced at its back. Accordingly, we found an accumulation of the myosin phosphatase regulatory subunit Mbs at the back of the clusters depleted for drongo. Finally, we found in complementation assays that the function of Drongo during border cell
migration requires its ArfGAP activity. We are currently investigating a mechanism where *drongo* depletion leads to the overactivation of an Arf and promotes the recruitment of Mbs at the back of the cluster.

Overall, our work demonstrates that the ArfGAP Drongo is necessary for the collective cell migration of border cells by promoting the activity of Myosin-II at the back of the cluster through restriction of the spatio-temporal activity of the myosin phosphatase regulatory subunit Mbs.

**P3360**

**Board Number: B650**

**Alternative Polyadenylation of RECK Regulates Cell Migration and Invasion.**

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Cell migration is a highly conserved process involving cytoskeletal reorganization and restructuring of the surrounding extracellular matrix. We developed a model in which fibroblast migration and invasion are regulated by alternative polyadenylation (APA) of REversion-inducing-Cysteine-rich protein with Kazal motifs (RECK). RECK encodes a GPI-anchored protein that inhibits extracellular matrix metalloproteinases (MMPs) through its Kazal motifs. Previous studies found that RECK-/- MEFs show slower migration than wild type as a result of defective focal adhesion formation, increased levels of GTP-bound Rac1 and Cdc42 activity, and lower levels of detyrosinated tubulin that is associated with more stable tubulin in migratory cells. We found that a short RECK isoform generated by coding sequence APA plays an opposing role to the long RECK isoform, and promotes cell migration and invasion. We discovered that mRNA encoding the short RECK isoform is expressed at higher levels in proliferating cells (which are also more migratory), TGF-β treated fibroblasts, and cancer tissue. Knockdown of the shorter isoform of RECK resulted in reduced fibroblast migration and invasion, while knockdown of the canonical longer isoform of RECK lead to rapid fibroblast migration and invasion, implicating the short RECK isoform as a new player in fibroblast migration and invasion. We hypothesize that the short RECK isoform suppresses the activity of the long RECK isoform through protein-protein interactions. Our biochemical analysis revealed the interaction between short RECK and long RECK isoforms. We also found that the Kazal motif domain, that is important for suppressing MMP activity, was critical for interaction with the short RECK isoform and the short RECK isoform was able to compete with MMP9, but not MMP2, for binding to the Kazal motif region of the long RECK isoform. Furthermore, we discovered that both short and long RECK isoforms can affect tubulin post translational modification (PTM) levels, and that altering tubulin PTMs represents a functionally important mechanism by which RECK isoforms can affect cell migration. Finally, we show that tubulin PTM levels can be regulated by short RECK and this requires the presence of the long RECK isoform. Our studies provide a new paradigm for the mechanisms through which APA can affect cell migration and invasion using the example of RECK protein.
P3361  
**Board Number: B651**  
Gio2 protein modulates the migratory capability of prostate cancer cell lines downstream or independently of PI3K and RAC1 activation.  
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Growth factors, cytokines, and hormones regulate migration and invasion in different cancer cell types, including prostate cancer cells. Previously we have shown that the migratory capability of prostate cancer cells depends on the activation of both G-protein coupled receptors (GPCRs) and PI3K/AKT/mTOR pathways. We have also shown that Gio2 protein has an essential role in migration of prostate cancer cell lines. The current study investigated the interaction among Gio2 and PI3K/AKT/RAC1 activation in the induction of migration in prostate cancer cells, in response to both GPCR and tyrosine kinase receptor (RTK) activation. Conditional knockdown of Gio2 by siRNA in PC3 and DU145 prostate cancer cell lines, generation of Gio2-deficient PC3 prostate cancer cell lines by CRISPR/Cas9 gene editing approach, and overexpression of constitutively active RAC1 protein in PC3 cells (PC3-RAC1Q61L), were performed, followed by migration and invasion assays. Western blots were used to validate the knockdown and knock out of Gio2 protein and to determine the activation of PI3-kinase pathway. G-Lisa assay for RAC1 and fluorescent staining were also performed. Pretreatment of PC3 cells with pertussis toxin (PTX) resulted in attenuation of transforming growth factor beta1 (TGFβ1) and oxytocin (OXT) induced migration and PI3K activation, without affecting EGF-induced PI3K activation and cell migration. Knockdown and knock out of endogenous Gio2 resulted in attenuation of cell migration and invasion in response to all ligands, but did not influence EGF-induced activation of PI3K pathway. These results suggest a role of Gio2 downstream or independent of PI3K/AKT/mTOR activation, in EGF-induced cell migration. Basal and EGF-induced activation of RAC1 in PC3 and DU145 cells were not affected after Gio2 knockdown. On the other hand knockdown of endogenous Gio2 in PC3-RAC1Q61L caused a significant reduction in the migratory behavior in EGF treated cells, indicating that Gio2 acts downstream or independent of RAC1. We also observed that knockdown of Gio2 in both parental PC3 and PC3-RAC1Q61L and in the Gio2-deficient PC3 prostate cancer cell lines resulted in reduced cell size, F-actin rearrangement and inhibition of lamellipodia formation, and these effects were not reversed by the ligands used in the present study. These results suggest that Gio2 protein acts at two different levels which are both dependent and independent of GPCR and PI3K signaling pathways, affecting cell migration and morphology of prostate cancer cells.

P3362  
**Board Number: B652**  
Differential effect of Mammalian Target of Rapamycin Complexes 1 (mTORC1) and 2 (mTORC2) in the migration of Prostate Cancer cells.  
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PI3K/AKT/mTOR pathway plays an essential role in the regulation of cell migration and invasion. Mammalian Target of Rapamycin (mTOR), a major component of PI3K/AKT pathway, exists in two distinct complexes-mTORC1 and mTORC2. Major regulators of mTORC1 and mTORC2 are two distinct proteins Regulatory-Associated Protein of mTOR (RAPTOR) and Rapamycin-Insensitive Companion of Tuesday-407
mTOR (RICTOR) respectively. Recent studies have shown that phosphorylation of Serine2488 on mTOR activates mTORC1 whereas phosphorylation of Serine2481 on mTOR activates mTORC2. The relative contribution of each complex towards cell migration is not well known. We observed that inhibition of RAPTOR and RICTOR individually using siRNA significantly decreased migration in PC-3 cells during a 5 hr Transwell migration assay. We also observed that inhibition of RAC1 (Ras-related C3 Botulinum Toxin Substrate 1, essential for cell migration) using RAC1siRNA or active RAC1 inhibitor NSC23677 had two effects. The first is a decrease in migration of PC-3 cells with/without EGF stimulation and the second a decrease in phosphorylation of mTORC2 but no effect on the phosphorylation of mTORC1 in the presences of EGF stimulation. Next, we developed PC-3 cell lines stably overexpressing EGFP-tagged Wild-Type RAC1 (RAC1WT) or constitutively active RAC1 (RAC1Q61L) by plasmid isolation, transfection, cell sorting and antibiotic selection. In PC-3 cell lines stably overexpressing RAC1WT or RAC1Q61L, we observed that the phosphorylation of mTORC2 is inhibited by NSC23677 in the presences of EGF whereas phosphorylation of mTORC1 is unaffected under similar treatment conditions. We also observed by immunoprecipitation and western blot analysis that RAC1 forms a complex with mTOR in PC-3 cells stably overexpressing RAC1WT or RAC1Q61L after stimulated with EGF. In conclusion, we propose that the interaction of RAC1 with mTOR lead to phosphorylation/activation of mTORC2 and in turn might affect cell migration in prostate cancer cells.

P3363

Board Number: B653

Phosphoinositides regulate cytoskeletal reorganization and extracellular matrix adhesion.

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Angiogenesis is the process when new blood vessels are formed from existing blood vessels. During angiogenesis, endothelial cells (ECs) generate a polarized leading edge through a process that involves the coordinated reorganization of the actin and microtubule cytoskeleton. Cytoskeletal reorganization and EC branching are necessary to generate physical contacts between the cell and the extracellular matrix (ECM). Together these processes help drive directional migration, a critical component that aids in the formation of new blood vessels. The phosphatidylinositol lipids have been identified as key mediators for directed motility. Upon stimulation, phosphatidylinositol 3,4,5-triphosphate (PI3P) is synthesized from phosphatidylinositol 4,5-bisphosphate (PI(2)) by a family of phosphoinositide-3-kinases (PI3Ks) at the plasma membrane (PM). PM PI3P is important for actin polymerization at the leading edge of migrating cells through the activation of the small GTPase Rac1. PM PI3P is much more abundant when compared to PI(2). PI3P is thought to be generated at the PM at sites of focal adhesions (FA) and has been implicated in promoting activation of various FA-associated proteins. While PM PI3P restricts cortical actin polymerization, it is unclear how PM PI3P is organized in the cell or how it regulates cell polarity and migration. We hypothesized that the regulation of PM PI3P levels functions to locally modulate cytoskeletal organization and EC-ECM adhesion. To test this hypothesis, we used an inositol polyphosphate 5-phosphatase fused rapamycin-triggered heterodimerization strategy (iRAP) to deplete PM PI3P levels in ECs. Cells co-expressing GFP-actin/GFP-Paxillin, and iRAP with PI3P readouts were collected and analyzed by live cell imaging. Experimental data reveal that PM PI3P depletion disrupted acto-myosin bundles, inhibited the assembly of nascent FA, increased the number of mature FAs, and promoted oscillatory protrusions and retractions. Independent of PM PI3P localization, the PI3P biosensor localized to actin stress fibers associated with mature FA, and this localization was reduced by treatment with blebbistatin, supporting a role for myosin II contractility in the localization of PI3P near FAs. Thus, spatial and temporal dynamics of phosphoinositide levels modify EC morphology and polarity.
through actin reorganization and cell-ECM adhesion. Future investigations will focus on the role of both PIP2 and PIP3 in controlling EC polarization and FA assembly.

P3364
Board Number: B654
Plasma Membrane PI(4,5)P2 Threshold Regulates Cell Migration Speed and Morphology.
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During gradient sensing, many signaling and cytoskeletal markers become asymmetrically distributed, and cells establish a polarity circuit. A similar excitable network was shown in cells during cytokinesis. Phosphoinositide-linked signals at the poles of dividing cells were activated in a manner equivalent to those found at the front of migrating cells. Signals at the furrow between two daughter cells were similar in nature to those found at the rear of migrating cells. To further demonstrate the importance of plasma membrane (PM) PI(4,5)P2 in maintaining cell morphology and breaking cell symmetry during polarized morphologies, we have used the inducible rapamycin (iRAP) system. iRAP was used to recruit various enzymes to the PM that regulate PM PI(4,5)P2 levels. Cell migration and the localization of various signaling and cytoskeletal biosensors were then monitored and recorded when PI(4,5)P2 levels were depleted or elevated. We find that lowering PM (PI4,5)P2 levels inhibits cell excitability, contributes to cell rounding, and reduced rates of cell migration. Depletion of PM PI(4,5)P2 is excitatory and increases Ras and PI3 Kinase activity. Cells show a variety of phenotypes when PI(4,5)P2 levels are depleted, including oscillatory behavior and dramatic changes in cell morphology. Similar effects were observed in Dictyostelium cells lacking the PI5 Kinase (pik1), whose loss leads to dramatic decreases in PM PI(4,5)P2 levels. We also investigated the localization of ForminA, which has a putative PI(4,5)P2 motif. ForminA localizes in a reciprocal manner with that of a marker for Rac activity during cell oscillations, with ForminA moving to the cytosol and Rac moving to the PM during cell spreading. Interestingly, this ForminA also localizes to the rear of cells undergoing chemotaxis and is found at the furrow of dividing cells. Our data suggests that the PM levels of PI(4,5)P2 are critical for altering cell speed and morphology, with lower levels contributing to excitable networks controlling cell protrusions. PM PI(4,5)P2 levels above a threshold support regulators that contribute to quiescent membrane activity and actomyosin contraction. Interestingly, we find that lowering PIP2 levels alone does not appear sufficient for triggering these excitable networks, and another component is likely activated. We propose that this unknown molecule becomes positively charged during the formation of protrusions, and plays a major role in the transient binding affinity of proteins that are lipid anchored and have positively charged binding motifs.

P3365
Board Number: B655
Location-Dependent Responses to Epithelial Cell-Cell Contact – Tail Following Complements Contact Inhibition to Facilitate Collective Migration.
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Contact inhibition of locomotion (CIL), largely drawn from observations of head-on collisions between individual migrating cells, has been the predominant paradigm to describe contact-mediated interactions of migrating cells. However, it poorly accounts for the behaviors observed in collective migration of epithelial cells. Using micropatterned substrates to facilitate contacts between different
regions of cells, we found that CIL applies specifically to interactions with neighboring heads. Contacts with other regions elicited migratory responses towards the tails of other migrating cells, including the induction of a new migrating front from stationary cells, causing one cell to follow another. This novel behavior, termed contact promotion of locomotion (CPL), complements CIL to allow a more complete understanding of the migration of both individual cells within a population and cell collectives. We further deployed a single-file, cell train model based on micropatterned substrates to facilitate the investigation of the effects of these behaviors on collective migration. Cells in migrating trains showed a preference to follow predecessor cells over free space on branched micropatterns. Moreover, treatment with a Frizzled-Dishevelled inhibitor NSC 668036 ablates both the tail-following behavior of individual cells and the collective migration of cell trains, which implicates the Wnt signaling pathways in both CPL and collective migration and suggests that CPL is responsible for the tail following behavior of collective migration. Our findings extend the paradigm of CIL to provide a more complete picture of the effect of cell-cell contact on cell migration, and uncover a new mechanistic understanding of collective migration.

P3366
Board Number: B656
The *Drosophila* TNFα ortholog, Eiger, decreases tension and facilitates deformation in a neighboring tissue to expedite macrophage invasion.
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How changes in the tension and stiffness of the environment alter cell migration has been frequently studied *in vitro*, yet much less is known about such parameters affect migration in a living organism. We study the invasive movement of *Drosophila* macrophages (plasmatocytes) into the germband during development, a process that displays molecular and cellular similarities with vertebrate immune cell transmigration. Here we find that an intricate interplay between two neighboring tissues occurs during development to facilitate this tissue invasion by macrophages through the regulation of tissue tension. Eiger, the *Drosophila* ortholog of TNFα, is required in the amnioserosa, a tissue neighboring the germband, to facilitate macrophage invasion between the germband ectoderm and the mesoderm. Once the macrophages migrate up to the amnioserosa, Eiger is released and binds on the ectoderm to Grindlewald, which is also required for macrophage invasion. Eiger and Grindelwald act in the ectoderm to enhance the apical localization of a Crumbs complex component known to bind Myosin phosphatase, called Patj. Eiger and Grindelwald limit the apical localization of active Myosin and thus decrease apical tension, as assessed by laser cuts. The importance of this pathway is indicated by our ability to rescue the defect in macrophage germband invasion in *eiger* mutants through over-expression of Patj or RNAi-based downregulation of Myosin. 2-photon movies in *eiger* mutant embryos show no alteration in the speed of macrophage migration up to the germband but do reveal an increase in the time the first macrophage takes to enter into the germband and to move along the region where Myosin is enriched. We show through segmentation analysis of movies visualizing ectodermal cell outlines that *eiger* mutants display less and slower ectodermal cell deformation as the macrophage enters. As we see no evidence for a change in the force exerted by macrophages in the *eiger* mutant, our work supports the conclusion that higher stiffness in surrounding cells decreases cell migration in an *in vivo* context, and that Eiger acts to decrease tension and stiffness to expedite tissue invasion. Our findings contrast to prior results from other labs showing that increased stiffness of the ECM facilitates cell migration *in vivo*. We have evidence that in zebrafish TNFα also down-regulates Myosin activity to enhance the entry of macrophages into the developing brain to form microglia, arguing that this regulatory effect is not limited to the fly. Thus we demonstrate for the first time *in vivo* that the tension of surrounding cells can
limit tissue invasion and show that a TNFα family member acts to modulate this effect through a novel signaling pathway.

P3367
Board Number: B657
Regulation of traction force mediated by the calpain small subunit and Galectin-3.
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During cell migration, mechanical forces are generated by the acto-myosin cytoskeleton and transmitted to the extracellular matrix producing traction to move the cell forward. These forces are known as traction forces and are critical for cell migration. The Calpain family of proteases have been implicated in numerous aspects of cell migration, including the regulation of cell spreading, contraction and the formation and disassembly of adhesion sites. Calpain 1 and Calpain 2, the most well-characterized holoenzymes within the Calpain family, are proteolytically active only in the presence of the small subunit Calpain 4. Previous studies from our lab have shown that Calpain 4 regulates the production of traction force in migrating mouse embryonic fibroblasts (MEF), independent of the large subunit’s catalytic activity. Furthermore, we found that Calpain 4 is crucial for the tyrosine phosphorylation and secretion of Galectin-3, a galactoside-binding protein known to cluster and activate the integrin adhesion receptors. Additionally, secreted Galectin-3 can rescue the magnitude of traction forces, which is defective in Calpain 4-deficient cells. We recently determined that the inhibition of c-Abl kinase, which is known to phosphorylate Galectin-3 on tyrosine residues, decreased Galectin-3 secretion leading to lower traction forces. We also found that the phosphorylation of Galectin-3 on tyrosine 107 is essential for the signal transduction and the generation of traction forces. This study provides insight into the signaling mechanism through which Calpain 4 and secreted Galectin-3 regulates the production of traction forces, thus regulating cell migration.

P3368
Board Number: B658
Serine Threonine Kinase-40 (STK40) and Cell Migration: Interaction with Mitogen-Activated Protein Kinase (MAPK).
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Cell migration is important for embryonic development, tissue regeneration and cancer metastasis. Recent research identified cell migration-related genes, but how they crosstalk with each other remain unclear. To resolve this mystery, we conducted a “two-hit” cell migration screen using short hairpin RNA (shRNA) and small molecules inhibitors. Among our candidates, we are particularly interested in STK40, a putative serine/threonine kinase. Previous literature reports showed that STK40 knockdown increased cell motility. However, our two-hit screen revealed STK40 knockdown plus PD98059 (MEK inhibitor) resulted in a dramatic suppression of cell motility. We therefore hypothesize that STK40 interacts with MAPK signaling to regulate cell migration.
To validate this hypothesis, we first conducted various cell migration assays by analyzing different parameters to investigate how STK40 regulates cell migration in epithelial and endothelial cells. We discovered that STK40 improved speed in both cell lines and improved coordination in SAS. Our immunofluorescent assays also showed that altering STK40 level altered E-cadherin and phalloidin, which may explain STK40 improvement in coordination. Further exploration will aim at how STK40 and...
MAPK regulate cell migration, hoping to disclose the mechanism of the regulation of STK40 in cell migration.

P3369
Board Number: B659
Cellular externalization control of actin-binding protein profilin-1 is a novel mechanism for MKL-dependent regulation of cell migration.
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Megakaryoblastic leukemia (MKL)/serum-response factor (SRF)-mediated gene transcription orchestrates the dynamic remodeling of the actin cytoskeleton which includes control of a wide range of genes including SRF itself and others related to structural and regulatory components of the actin cytoskeleton. In this study, we demonstrate that MKL impacts the cellular level of profilin-1 (Pfn1), a key regulator of actin cytoskeletal dynamics and cell migration. Modulation of Pfn1 by MKL occurs through change of expression of signal transducer and activator of transcription 1 (STAT1) that is dependent on its SAP-domain function rather than its conventional SRF-mediated action. Unexpectedly, we found that MKL modulates Pfn1 through controlling its cellular externalization. Finally, we demonstrated that MKL can affect cell migration through controlling Pfn1 thus establishing a functional link between MKL and Pfn1 in the context of cell migration. Collectively, these findings reveal a novel regulatory mechanism of cell migration downstream of MKL that extends beyond and above its SRF-mediated action and involves cellular retention control of actin-binding proteins.

P3370
Board Number: B660
A polymorphism of BLT2 leads to an enhanced ligand sensitivity.
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Recently, single-nucleotide polymorphisms (SNPs) in G-protein coupled receptors (GPCRs) have been suggested to contribute to physiopathology and therapeutic effects. Leukotriene B4 receptor 2 (BLT2), a member of the GPCR family, plays a critical role in the pathogenesis of several inflammatory diseases, such as cancer and asthma. However, no studies on BLT2 SNP effects have been reported to date. In this study, we demonstrate that BLT2 SNP (rs1950504, Asp196Gly), a Gly-196 variant of BLT2 (BLT2 D196G), causes enhanced cell motility under low-dose stimulation of its ligands. In addition, we demonstrated that Akt activation and subsequent production of reactive oxygen species (ROS), both of which act downstream of BLT2, are also increased by BLT2 D196G in response to low-dose ligand stimulation. Furthermore, we observed that ligand binding affinity of BLT2 D196G was enhanced compared to BLT2. Through homology modeling analysis, it was predicted that BLT2 D196G loses ionic interaction with R197, thereby potentially resulting in increased agonist-receptor interaction. This report is the first SNP study on BLT2 and shows that BLT2 D196G enhances ligand sensitivity and thereby increases cell motility in response to low-dose ligand stimulation.
**P3371**

**Board Number: B661**

The NDR kinase Tricornered regulates membrane handling and collective cell migration in the Drosophila egg chamber epithelium.

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Development and wound healing depend on tissue rearrangements mediated by collective cell migration. We study a highly coordinated collective cell migration that occurs in the follicular epithelial layer of the Drosophila egg chamber. The follicular epithelial cells migrate along a basement membrane extracellular matrix (ECM) surrounding the egg chamber. This migration is essential for the elongated shape of the mature egg. We take advantage of this model system to identify proteins and processes required for coordinated cell migration. Two proteins essential for egg elongation are the NDR kinase Tricornered (Trc) and its activator Furry (Fry). Loss of these proteins results in round eggs and loss of planar actin polarization at the basal surface of follicle cells, two hallmarks of migration defects. Here, we show using live imaging that Trc and Fry are required for collective follicle cell migration and maintenance of the structure of the basal plasma membrane adjacent to the ECM. Trc localizes to the plasma membrane and to membrane-adjacent vesicle-like structures. Loss of Trc or Fry by RNAi or in mutant clones causes excess membrane shedding from the rear of each migrating cell. We also observe this phenotype when endocytosis is disrupted, suggesting that Trc may regulate endomembrane trafficking. We have previously shown that the atypical cadherin Fat2 is localized to the trailing edge of follicle cells and signaling through Fat2 is essential for cell-cell interaction during migration. Loss of Trc causes Fat2 at the trailing edge to become more dispersed, likely through disruption of overall trailing edge membrane structure. This poor organization may affect the ability of Fat2 to mediate cell-cell interaction, thereby blocking migration. In future work, I aim to determine 1) the mechanism by which Trc regulates endomembrane trafficking and 2) if Trc regulates follicle cell migration through Fat2.

**P3372**

**Board Number: B662**

Effect of PI3 Kinase Inhibition on Human Dermal Fibroblast and Human Epidermal Keratinocyte Wound Healing.

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Cell proliferation and cell migration are two essential parts of normal wound healing. Both of these cellular functions are regulated by phosphoinositide 3-kinases (PI3-kinases). PI3 Kinases regulate cellular proliferation through their activation of the G-protein, Rac GTPase. Rac GTPase activation activates AKT causing a series of interactions whose downstream affect activates mTOR, downregulating apoptosis and upregulating the cell cycle resulting in increased proliferation (Rafalski and Brunet, 2011; Peltier et al, 2007). PI3 Kinase’s role in cell motility is not as clearly defined. What is known is that RAS binds directly to PI3 Kinase to increase cell motility. Preventing RAS from binding to PI3 Kinase results in upregulation of E-cadherin expression due to an increase in expression of the protein Reelin. This increase in focal adhesion protein decreases cell motility and migration (Castellano et al, 2016). Additional data has shown that PI3 Kinase regulates actin through regulation of the protein Cofilin (Kolsh et al, 2008). We used cell tracking reagents and live cell kinetic assays for a quantitative analysis of the effects of PI3 kinase inhibition on dermal fibroblast and epidermal keratinocyte wound healing.
**P3373**

**Board Number: B663**

**Immunohistochemical Analysis of Singed Protein in Wild-Type and CASK Knockout Fly Lines.**

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_Drosophila_ border cell migration is a process whereby a cluster of 8-10 specialized follicle cells known as border cells transport two non-motile follicle cells, known as polar cells, to the oocyte during oogenesis in the fly. _Drosophila_ egg chambers are comprised of a single oocyte and 15 supportive nurse cells. In turn, the oocyte and nurse cells are surrounded by a single layer of follicular epithelial cells. Arrival of the border cell cluster to the oocyte occurs during stage 10 _Drosophila_ egg chamber development. The transported polar cells will ultimately form the micropyle, the structure which permits sperm entry during fertilization events. Egg chambers in which the cluster fails to reach the oocyte by stage 10 are said to exhibit defects in border cell migration. Although this process has been investigated from a developmental biology perspective, it has also recently been recognized that border cell migration provides an attractive system to study coordinated cell movement. Recent RNA interference studies suggest that knockdown of the CASK (Ca2+/-CaM-dependent serine protein kinase) protein results in incomplete border cell migration. However, the molecular mechanisms by which CASK influences this critical cellular process have yet to be elucidated. In order to gain further insight into the function of CASK in _Drosophila_ border cell migration, immunohistochemical analysis using the border cell specific protein Singed was performed in four wild-type fly lines (Oregon R, yw, w1118, p33) and the p18 (CASK knockout) fly line. Results of the stained protein staining demonstrate successful labeling of border cell clusters in >200 egg chambers in each of the aforementioned fly lines. Future work will focus on quantitative analysis of stage 10 ovaries to determine whether CASK knockout flies demonstrate defects in border cell migration as suggested by previous RNA interference studies.

**P3374**

**Board Number: B664**

**Validation of CASK gene expression in Drosophila ovaries.**

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CASK is a Ca2+/-CaM-dependent serine protein kinase belonging to the Membrane Associated Guanylate Kinases (MAGUK) protein family. All MAGUK family members contain PDZ, SH3, and GK domains. However CASK is unique in that it possesses an additional N-terminal CaMK-like domain. This distinct structure allows CASK to function as a scaffolding molecule for channels and receptors at cell-cell junctions. Recent work in _Drosophila_ has shown that CASK is also involved in regulating coordinated cell movement. Specifically, an RNAi screen demonstrated that knockdown of CASK results in defective border cell migration. Border cell migration is a process by which two non-motile follicular epithelial cells known as polar cells are transported from the anterior end of the developing egg chamber to the developing oocyte by specialized cells known as border cells. This process serves as an important model of collective cell movement _in vivo_. Despite this potential role for CASK in border cell migration, little work has been done to characterize CASK gene or protein expression in _Drosophila_ ovary. Gene prediction models and ModENCODE data suggest that there are seven CASK isoforms expressed in _Drosophila_ ovaries (CASK-A, -B, -D, -E, -F, -G, -H). To verify the expression of each of these isoforms in _Drosophila_ ovaries, primer sets were designed to detect each of the seven CASK isoforms. Previous work
demonstrated that each isoform is expressed in w1118 (wild-type) pooled Drosophila ovary samples, but expression was variable when analyzed across three independently pooled w1118 samples. Current work focused on isolation of new RNA samples from the w1118 wild-type fly line, as well as two additional wild-type fly lines - yw (yellow white) and OreR (Oregon R). RNA was isolated from three pooled samples of w1118, yw and OreR total fly and ovary homogenates and cDNA was synthesized using the Superscript IV first-strand cDNA synthesis kit. As variability between samples has been a limiting factor in the verification of CASK isoform expression, each cDNA sample was validated using primer sets to the Rp49 housekeeping gene and the MAGUK family gene, Dlg (Discs Large). PCR with the control primers on the independently pooled samples demonstrated equivalent expression levels across samples for both primer sets. Future work will involve standard reverse transcriptase-PCR on the validated cDNA with each of the seven CASK isoform primer sets to authenticate CASK gene expression across pooled samples in three separate wild-type fly lines. Characterizing CASK isoform expression within Drosophila ovary tissue will provide insight into which isoform may be mediating cell movement during border cell migration.

P3375
Board Number: B665
Generation of UAS-CASKRA-GFP and UAS-CASKB-GFP Transgenic Constructs.
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Border cell migration is a developmentally regulated process in Drosophila oogenesis in which two nonmotile polar cells are transported from the anterior of the egg chamber to the oocyte by a group of 4-6 motile cells known as border cells. Given the plasticity of the Drosophila model system, border cell migration has served as a noteworthy model of collective cell migration. Calcium/calmodulin-dependent serine protein kinase (CASK) belongs to the membrane-associated guanylate kinases (MAGUK) protein family and recent RNA interference screens in Drosophila ovary have suggested that knockdown of this protein results in defective border cell migration. Drosophila express two major classes of CASK isoforms, known as CASK alpha and CASK beta isoforms. The CASK alpha isoforms encode a truncated CASK protein possessing the canonical MAGUK domains, PDZ (PSD-95-Dlg-Zo1), SH3 (Src Homology 3) and GK (Guanylate Kinase) domains. The CASK beta isoforms encode the full-length protein, which possesses each of the above domains as well as an additional N-terminal CamK-like domain and two L27 (Lin-2/Lin-7) domains. Previous work has not determined which class of isoforms is responsible for the cell migration defects observed in during Drosophila oogenesis. Moreover, no work has been done to characterize the molecular means by which CASK may contribute to cell movement en masse. In order to begin to delineate the function of CASK in border cell migration, the current work focuses on the generation of both pUAS-CASKRA-GFP and pUAS-CASKB-GFP constructs for production of transgenic fly lines. The CASK_RB open reading frame was previously PCR amplified and the results presented here demonstrate successful subcloning into the pENTR-D-TOPO entry vector to yield the pENTR-D-TOPO-CASKRB. The CASKRA open reading frame has also been obtained in a pFlc vector backbone. Future work on this project will involve performing a Gateway cloning reaction to yield the desired pUAS-CASKB-GFP construct and subcloning of CASKRA into the pENTR-D-TOPO entry vector. As there is no commercially available antibody to Drosophila CASK, generation of these transgenic fly lines will allow for characterization of CASK’s subcellular localization within the Drosophila egg chamber, thereby providing valuable insight into CASK’s potential role in border cell migration. Moreover, these fly lines will allow for rescue experiments with CASK knockout fly lines to determine which CASK isoforms are necessary for successful coordinated cell movement.
P3376
Board Number: B666
PI(3,4)P2-Directed Negative Feedback Control of Ras Activity in Dictyostelium.
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Ras proteins serve as key mediators of the downstream signaling response evoked by guidance cue receptor activation and are therefore important regulators of cell migration. In Dictyostelium, the landscape of Ras regulation by guanine nucleotide exchange factors, GEFs, which promote signaling and GTPase-accelerating proteins, GAPs, which inhibit Ras signaling, is very unclear. Our recent work implicates phosphatidylinositol 3,4-bisphosphate, PI(3,4)P2, in the regulation of Ras activity at the leading edge of migrating cells. PI(3,4)P2 like many phosphoinositides, regulates membrane localization of proteins via an interaction with lipid binding motifs such as pleckstrin homology, PH, domains. A genome-wide search for PH domains with high similarity to the PI(3,4)P2-binding PH domain of polarity regulator CynA, identified several PH domain containing GAP proteins. Live cell microscopy revealed a transient localization of these GAPs to the base of cup-shaped macropinosome crowns in Dictyostelium. Depleting cellular PI(3,4)P2 by knocking out enzyme Dd5p4 which converts PI(3,4,5)P3 to PI(3,4)P2, blocked the localization of GAPs to the cup shaped protrusions. The size, duration and frequency of initiation of Ras patches were all increased in Dd5p4− cells. Chemotaxis to cAMP was also impaired in Dd5p4− cells. These findings point us to the hypothesis that Ras activity at cellular protrusions is regulated by the PI(3,4)P2-dependent localization of GAP proteins.

Chemotaxis and Directed Cell Migration

P3377
Board Number: B667
Proper actin network architecture enforces polarization during cell migration.
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Efficient cell migration requires that actin assembly is spatially restricted to form a single leading edge in response to external stimuli. In neutrophils, cells of the innate immune system that rely on chemotaxis to track down pathogens, the protrusive force of actin polymerization leads to increases in membrane tension. These tension increases confine subsequent actin polymerization to a single site at the leading edge, producing a well-defined polarity axis. A multitude of actin assembly pathways function alongside one another to drive neutrophil migration, but it remains unclear whether different types of actin networks can generate similar membrane tension increases in response to polarity cues. The WAVE regulatory complex (WRC), an actin nucleation-promoting factor, plays a central role in directed migration by organizing the assembly of highly-branched actin networks at the leading edge of cells. Here, we assess the role that actin network organization plays in generating plasma membrane tension by using CRISPR-Cas9 to disrupt WRC in neutrophil-like HL-60 cells. WRC-null cells failed to maintain a single axis of polarity and instead simultaneously formed multiple sites of protrusion initiation in a non-polarized fashion. Consistent with their inability to restrict polarization, WRC-null cells also showed impaired membrane tension generation. The polarity defect was rescued by driving increases in membrane tension via hypo-osmotic treatment, demonstrating that membrane tension generation...
relies on WRC-dependent actin assembly. Surprisingly, WRC-null cells produced significantly higher levels of actin polymer, in contrast to previous observations that cellular actin polymer levels and the amount of tension experienced by the plasma membrane are highly correlated. Our results show that actin assembly via WRC efficiently drives increases in membrane tension to provide long-range negative feedback to protrusion formation. WRC-dependent actin polymerization ensures that migrating neutrophils maintain a single ‘front-to-back’ polarity axis.

**P3378**

**Board Number: B668**

**ARF1 recruits RAC1 with mutually dependent regulatory circuits in neutrophil chemotaxis.**

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**Background:** The small GTPase ARF1 mediates membrane trafficking mostly from the Golgi, and is essential for the G protein-coupled receptor (GPCR)-mediated chemotaxis of neutrophils. In this process, ARF1 is activated by the guanine nucleotide exchanger GBF1, and is inactivated by the GTPase-activating protein GIT2. Neutrophils generate the Gβγ-PAK1-αPIX-GIT2 linear complex during GPCR-induced chemotaxis, in which αPIX activates RAC1/CDC42, which then employs PAK1. However, it has remained unclear as to why GIT2 is included in this complex.

**Results:** We investigated the association between ARF1 and RAC1/CDC42 during the f MLP-stimulated chemotaxis of HL60 cells. We found that the silencing of GBF1 significantly impaired the recruitment of RAC1 to the leading edges, but not PAK1, αPIX, RAC2, or CDC42. A significant population of RAC1 colocalized with ARF1 at the leading edges in stimulated cells, whereas f MLP activated both ARF1 and ARF5. Consistently, the silencing of ARF1, but not ARF5, impaired the recruitment of RAC1, whereas the silencing of RAC1 did not affect the recruitment of ARF1 to the leading edges.

**Conclusions:** Our results indicated that the activation of ARF1 triggers the plasma membrane recruitment of RAC1 in GPCR-mediated chemotaxis, which is essential for cortical actin remodeling. Thus, membrane remodeling at the leading edges appears to precede actin remodeling in chemotaxis. Together with the fact that GIT2, which inactivates ARF1, is an integral component of the machinery activating RAC1, we proposed a model in which the ARF1-RAC1 linkage enables the regulation of ARF1 by repetitive on/off cycles during GPCR-mediated neutrophil chemotaxis.

**P3379**

**Board Number: B669**

**CCR7 homo-dimerization regulates CCR7 ligand-dependent cell migration and signaling.**

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Chemokine receptors contribute to various cellular processes including cell migration, proliferation and survival. The CCR7 plays pivotal roles in T cell migration from the blood and dendritic cell migration from the peripheral tissues into secondary lymphoid tissues, and tumor cell metastasis to lymph nodes.
Although a previous study suggested that the efficacy of CCR7 ligand-dependent T cell migration correlates with CCR7 homo- and heterodimer formation, the exact extent of contribution of the CCR7 dimerization remains unclear. In this study, we demonstrated a direct contribution of CCR7 homo-dimerization to CCR7-dependent cell migration and signaling. Induction of stable dimerization by the inducible dimerization system (iDimerize) showed that CCR7 homo-dimerization enhanced CCR7-dependent cell migration and ligand binding, whereas CXCR4/CCR7 hetero-dimerization showed little effect. When CCR7 homo-dimerization was inhibited by a synthetic peptide derived from the CCR7 fourth transmembrane domain, CCR7-dependent cell migration, ligand-dependent CCR7 internalization, ligand-induced actin rearrangement, and Akt and Erk signaling were all significantly decreased. The CCR7-derived peptide impaired ligand-induced cell migration in CCR7-expressing breast cancer cells and human peripheral blood T cells as well. Our study indicates that CCR7 homo-dimerization critically regulates CCR7 ligand-dependent cell migration and intracellular signaling in multiple cell types.

P3380

**Board Number: B670**

**The involvement of SAMSN1 in mast cell dynamics.**

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Mast cells (MCs) are multifunctional cells derived from bone marrow, that reside in connective tissue at the interface between the host and the outside environment. Upon activation, MCs produce and release a large range of biologically active mediators. SAMSN1 is a member of a novel gene family of putative adaptor and scaffold proteins containing SH3 and SAM (sterile alpha motif) domains. In the present study, for the first time, SAMSN1 was detected in MCs, both by Western blots and by immunofluorescence. In order to investigate the role of SAMSN1 in MCs, RBL-2H3 MCs were knocked-down (KD) for SAMSN1 using shRNA. SAMSN1 was reduced about 60% in the KD cells when compared to non-transduced RBL-2H3 MCs. Since SAMSN1 is known to be involved in reorganization of the actin cytoskeleton in other cell types, the function of SAMSN1 in mast cell migration was characterized using CCL3 as a chemoattractant in a Transwell system. After 3 hours of incubation, the migration rate of KD RBL-2H3 MCs was about 40% less when compared to non-transduced RBL-2H3 MCs. MC migration was then investigated in Bone Marrow derived MCs (BMMCs) from SAMSN1 deficient mice (C57BL/KaLwRijHsd) and wild type (WT) mice using Stem Cell Factor (SCF) as a chemoattractant in a Transwell system. After 3 hours of incubation, the migration rate of BMMCs from SAMSN1 deficient mice was 40% less than when compared to BMMCs from WT mice. Since migration rates were reduced in SAMSN1 deficient cells, in vitro, and MCs can be recruited to peripheral sites by various stimuli, MC recruitment in vivo was investigated. MC recruitment to the peritoneal cavity was characterized after iP injection of the chemoattractants SCF or Interleukine-3 (IL-3). After injection of SCF, SAMSN1 deficient mice had approximately 40% fewer MCs recruited to the peritoneal cavity when compared to WT mice. While, after injection of IL-3, the number of MCs recruited to the peritoneal cavity of SAMSN1 deficient mice was 50% less than when compared to WT mice. The number of MCs at peripheral sites was also lower in the SAMSN1 deficient mice. Metachromatic MCs were 50% and 30% lower respectively in the skin and tongue of SAMSN1 deficient mice in comparison to WT mice. These findings indicate that SAMSN1 plays a role in MC migration, recruitment and distribution. The results from this study contribute to a better understanding of the role of SAMSN1 in MCs and may provide additional therapeutic approaches to the treatment of allergic and inflammatory processes.
P3381

Board Number: B671
MAL2 overexpression induces cell membrane protrusion formation, and decreases cell migration via its FPAP motif in liver-derived cancer cells.
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Over 80% of all human carcinomas are derived from polarized epithelial cells that are characterized by the loss of or failure to achieve polarity. Thus, it is important to consider how loss or dysregulation of polarized protein trafficking contributes to malignant transformation. This is of particular interest to us because MAL2 (myelin and lymphocyte protein 2) - a protein that we have been investigating in studies of polarized hepatic trafficking for several years - is highly up-regulated in a host of human cancers including renal carcinoma, cholangiocarcinoma, and cancers of the stomach, breast, ovary and pancreas. High MAL2 expression has also been linked to a bad prognosis in patients with ovarian or pancreatic cancer, yet how MAL2 overexpression leads to cancer progression is not known. To identify possible mechanisms, we overexpressed WT MAL2 in hepatoma-derived Clone9 cells that lack endogenous MAL2. We determined that MAL2 overexpression led to actin-based protrusion formation with MAL2 localized to their tips. Because the MAL2 cytosolic N-terminus encodes V PPPP and FPAP sequences that resemble the F/L/W/YPPP recognition sites for EVH1 (enabled, VASP, homology1) motifs present in the actin-associated Ena/VASP proteins, we engineered recombinant adenoviruses with either or both of these proline-rich motifs mutated to alanines. In cells expressing the FPAP or double mutant, actin protrusion were not observed, implicating the FPAP as the responsible motif. Surprisingly, Boyden chamber assays indicated that migration was not enhanced in MAL2 overexpressing cells which we confirmed with scratch assays imaged with the CytoSmart live cell imaging platform, and MTT cell proliferation assays. Similarly, human-derived hepatocellular carcinomas Hep3B cells with endogenous MAL2 displayed abundant protrusions formation and decreased migration. MAL2 knockdown reversed these phenotypes. Because invasive cells display altered polarity with directional membrane delivery and recycling to the invasive front, we predict that MAL2-induced actin protrusions may explain its role in regulating vesicle delivery to the migrating front. We further predict that this is mediated by MAL2 dual specificity in associations with rafts and actin binding proteins, a possibility we continue to explore.

P3382

Board Number: B672
Cxcl12a/Cxcr4b and the transcription factor Myca orchestrate cell migration during zebrafish pronephros repair.
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Acute kidney injury represents a frequent complication of severe human disease characterized by a rapid loss of renal function. Despite improvements in supportive care, the overall morbidity and mortality rates have remained unchanged. To study the immediate repair mechanisms after acute
In kidney injury, the zebrafish pronephros was ablated with a high-energy laser during the first two days post fertilization, and the repair response was monitored by high-resolution video-microscopy. In one-day-old embryos pronephric tubules were repaired by an actomyosin-based mechanism that irreversibly occluded the injured pronephric tubules. In contrast, pronephric injuries in two-day-old embryos were repaired by a rapid migratory response that re-established the patency of the tubules. This repair response was characterized by accelerated migration, occurred independently of fluid flow, cilia, Wnt signaling and in the absence of epithelial-to-mesenchymal de-differentiation, and reversed the direction of the posterior-to-anterior collective cell migration that shapes the proximal pronephros during organogenesis. Gene expression analysis of the two developmental stages uncovered the chemokine cxcl12a and the transcription factor myca as putative candidates involved in the migratory repair response. Indeed, two-day-old embryos deficient for cxcl12a or its receptor cxcr4b exhibited striking repair abnormalities. Similar repair defects were observed after pronephric injury of myca-deficient embryos. The defective repair after myca knockdown was partially rescued by cxcr4b overexpression, suggesting that myca acts in part through upregulation of cxcr4b. In contrast to their essential role in collective cell migration of the posterior lateral line primordium, both cxcl12a and cxcr4b were dispensable for pronephric collective cell migration. Confirming the zebrafish results, kidney-specific knockout of Cxcl12 and Myc in mice delayed the recovery after ischemic/reperfusion injury. Transcriptional analysis of Cxcl12 and Myc deficient kidneys revealed increased Cxcr4 expression as well as suppressed mitochondrial functions and reduced retinoic acid metabolism. All-trans retinoic acid, which is known to stimulate Cxcr4 expression and mitochondrial metabolism, improved renal recovery after ischemic/reperfusion (I/R) injury. Our findings suggest that cxcl12a and myca are up-regulated in two-day-old embryos to facilitate rapid cell migration in response to injury, and to overcome the actomyosin-based repair mechanism utilized by one-day-old zebrafish embryos. Cxcl12 and Myc contribute to the early recovery after I/R injury of the kidney, establishing the pronephros injury model as a tool to identify molecules involved in kidney repair and to design novel therapeutic approaches.

**P3383**

**Board Number: B673**

Collective dynamics over long time scales and large length scales reveals distinct cell migration phenotypes.

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Migrating cells play a role in diverse biological processes which can incorporate both individual cell motion and collective behavior. As the role of collective motion becomes clearer, especially in diseases such as metastatic cancer, there is growing interest in the emergent properties of collective migration. In this study, we analyze the collective migration of cell sheets using particle image velocimetry (PIV), an approach which is being increasingly used to study migration due to its ease of application. In our analysis, we show that PIV can be used to study multiple length and time scales and can yield insight into biological mechanisms that play a role in collective behavior.

The properties of each cell in a migrating cell sheet interact to lead to experimentally observed collective motion. By comparing experimental PIV flow fields from migrating MCF10A cells to the behavior of simulated cell groups, we connect individual cell properties to collective behavior over the

**Tuesday-420**
millimeter-scale of the cell monolayer. We find that cell migration at the boundary can affect migration within the monolayer without the need to introduce leader cells at the boundary or specify large-scale features in the simulations. Our model suggests that collective motion can be enhanced by increasing the cells’ sense of polarity or by increasing the cells’ overall activity.a

In addition to tissue-scale trends in collective behavior, migrating cell groups include localized dynamic features which can change over multiple time scales. We developed a set of measurement techniques to extract multiple features of collective motion, including the chaotic character of the cell sheet. By applying these techniques to both non-malignant (MCF10A) and tumorigenic (MCF10CA1a) breast epithelial cells, we find differences in collective behavior that are not found from measuring speed alone. We use these techniques to show that these cell lines vary in their response to a perturbation to E-cadherin, a cell-cell adhesion molecule. MCF10A cells use E-cadherin for short time coordination of collective motion yet remain coordinated over hours even with decreased E-cadherin expression. In contrast, the migration behavior of MCF10CA1a cells shows decreased collective dynamics and is insensitive to the change in E-cadherin expression.b

Our analysis provides further evidence of the applicability of PIV for understanding collective migration. These analysis techniques—which can be applied directly to phase contrast, time lapse images—allow for the measurement of collective behavior over large length and time scales. These measurements can be used to understand collective migration on scales which are relevant to processes such as cancer metastasis.

a10.1088/2057-1739/2/2/025001

b10.1088/2057-1739/2/2/025001

P3384

Board Number: B674

Cytoskeletal dynamics during wound reepithelialization in vivo.

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A critical step in wound healing is reepithelialization, or migration of epithelial cells into a wounded region. Several recent studies in vivo have revealed the tissue-scale patterns of growth and migration during epidermal reepithelialization, but a detailed understanding of the cytoskeletal dynamics in individual cells is lacking. We have observed the migration of the basal epidermal cells of embryonic zebrafish during reepithelialization at high spatial and temporal resolution, using spinning disk confocal microscopy. These basal epidermal cells are also known as keratocytes, and while isolated keratocytes have been studied extensively in culture for their rapid and persistent migration, much less is known about their physiological function in vivo.

We have found that keratocytes in vivo initiate rapid and dramatic migration en masse within minutes of tissue wounding. This study has revealed several novel insights: First, keratocytes adopt an elongated “squid-like” morphology as they migrate, which differs dramatically from their characteristic shape in culture; this suggests that the physical environment of the tissue significantly impacts keratocyte migration. Second, during reepithelialization keratocytes separate from each other, unlike in collective migration in cultured monolayers, where cells often remain attached even as they migrate. Third, cell stopping at the end of reepithelialization is accompanied by a burst of myosin localization at the back of the lamellipodium. Together, these results suggest that collective migratory responses differ markedly in vivo and ex vivo, particularly in the nature of the physical contact between cells. Furthermore, these results provide insight into the dynamics of cell collectives when they stop migrating, which has been difficult to observe in tissue culture models of reepithelialization. Future work promises to clarify...
molecular details about the dynamics of adhesions and myosin localization during reepithelialization, shedding light on this classic example of cell migration in an in vivo context.

P3385
Board Number: B675
Electric Fields Coalesce Lipid Raft and Caveolin to Direct Cell Migration.
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In development and wound healing, endogenous electric fields (EFs) promote and direct cell proliferation and migration. Surface proteins have been proposed as sensors of the electrophoretic and electroosmotic forces that polarize cell membranes and intracellular signaling events. Yet modification of surface protein charges or alternating field directions offer conflicting evidences. We hypothesize that dynamic membrane nanodomains, rich in highly charged glycolipids, are the primary sensors of EFs. Theoretical modeling reveals EF-induced lipid raft polarization and aggregation. Indeed, super-resolution microscopy demonstrates that applied EF polarizes lipid raft distribution and increases their sizes. Disruption of lipid rafts abolishes directional migration and intracellular signaling polarization, while inhibition of signaling molecules or actin polymerization does not change raft distribution. Furthermore, EF induces caveolin oligomerization and preferential distribution. Caveolin knockdown suppresses EF-induced directional migration and intracellular signaling polarization. Interestingly, although inhibition of signaling molecules does not suppress caveolin or raft polarization in EF, functional blocking of integrin reduces caveolin polarization. Inhibition of rafts and integrin polarization from caveolin knockdown suggests reciprocal interactions amongst raft, integrin and caveolin, and the integrity of all three components are necessary for EF-directed migration. In summary, lipid rafts respond to EFs as mobile complexes that polarize, coalesce, and partition membrane proteins, and in turn activate intracellular signaling events to orient cell migration. Our results establish a fundamental mechanism for cell electrosensing and provide a new role in lipid raft mechanotransduction.

P3386
Board Number: B676
Directed Schwann cell migration can be guided by extracellular mechanical gradients.
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Peripheral nerve injuries often result in significant disability and greatly reduced quality of life. Successful nerve regeneration depends upon directed migration of specialized repair-state Schwann cells (SC) across a nerve defect. Following migration, these repair SCs provide directional guidance for severed axons to regrow and restore function back to their innervation target. During this regenerative process, the critical features displayed by these SCs are directed migration and elongated cellular morphologies. Although several groups have studied directed migration of SCs in response to chemical or topographic cues, the current understanding of how the mechanical environment influences migration remains largely understudied and incomplete. Therefore, the focus of this study was to evaluate SC migration and morphodynamics in the presence of stiffness gradients, which revealed that SCs can follow extracellular gradients of increasing stiffness, in a form of directed migration termed durotaxis. On laminin-coated polyacrylamide substrates embedded with either a shallow (0.04 kPa/mm)
or steep (0.97 kPa/mm) stiffness gradient, SCs displayed durotaxis, increasing both their speed and directionality along the gradient materials, fabricated with elastic moduli in the range found in peripheral nerve tissue (0.05-0.54 kPa or 0.87-12.99 kPa). Uniquely and unlike cell behavior reported in other cell types, the durotactic response of SCs was not dependent upon the slope of the gradient. When we examined whether durotaxis behavior was accompanied by a pro-regenerative SC phenotype, we observed altered cell morphology, including increases in spread area and the number, elongation, and branching of the cellular processes, on the steep but not the shallow gradient materials. This phenotype emerged within hours of the cells adhering to the materials and was sustained throughout the 24 hour duration of the experiment. Control experiments also showed that unlike most adherent cells, SCs did not alter their morphology or cytoskeletal architecture in response to uniform materials of different stiffnesses (0.05, 0.54, 0.87, and 12.99 kPa). This study is notable in its report of durotaxis in response to a stiffness gradient slope, which is two orders of magnitude less than reported elsewhere in the literature, suggesting SCs are highly sensitive detectors of mechanical heterogeneity. All together, this work identifies durotaxis as a new migratory modality in SCs, and further shows that the presence of a stiffness gradient of 0.97 kPa/mm can support a pro-regenerative cell morphology, which mimics the specialized in vivo phenotype of repair-state SCs.

P3387
Board Number: B677
Plasma membrane PI(4,5)P2 levels regulate chemotactic signaling pathways and actin networks in Dictyostelium.
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Phosphatidylinositol 4,5-bisphosphate (PIP2) has been shown to be involved in the chemotactic signaling pathways. However, PIP2 has been largely underappreciated as a regulatory component in this signaling network. In a polarized Dictyostelium, the PI3’Kinase (PI3K) translocates to the plasma membrane (PM) of the leading edge while the tumor suppressor PTEN distributes to the rear of the cell. Higher PIP2 levels at the back are maintained through the PIP2 binding and activity of PTEN, in a positive feedback loop. PI5 kinase (PI5K) which is activated downstream of protein kinase B also contributes to PIP2 synthesis across the entire cell PM. However, PI3K and phospholipase C (PLC) use PIP2 as a substrate, so it is locally depleted at the leading edge of a migrating cell. We have found that the activity of the GTPase Ras is reciprocally regulated with PTEN localization, and our work suggests that PIP2 levels set up a threshold preventing the activity of signaling molecules normally active at the leading edge of a cell. To test this, we used Dictyostelium mutant strain lacking the type I PI5K, pikl, (pikl-). These cells have highly reduced PIP2 levels and higher Ras activity compared to wild-type cells. We found that pikl- resemble other phenotypes observed when we use an inducible system to deplete PIP2. We observed that typical leading edge biosensors diffuse to the cytosol when the pikl- round-up and translocate back to the PM when pikl- cells spread. These observations suggest that PIP2 levels go down as cells spread, and elevate as cells round up and contract. F-actin polymerization also occurs as the cell spread. Remarkably, Ras and PI3K biosensors were still activated on the PM when actin polymerization was inhibited with Latrunculin A (LatA). Interestingly, the crescent signaling responses in LatA-treated pikl- cells were reminiscent of responses seen in pten- cells. The lower levels of PM PIP2 in pikl- cells is likely below the minimal level of PIP2 needed to block Ras activity contributing to the excitable cell phenotype observed.

Tuesday-423
P3388
Board Number: B678
Inter-species repression of light-dependent photoresponses in diatoms.
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Diatoms, the single-celled siliceous golden algae ubiquitous to aquatic communities, are crucial primary food sources to numerous marine and freshwater ecosystems. In order to understand the structure and maintenance of healthy aquatic ecosystems it is important to understand the ecological factors regulating the ability of motile diatoms to successfully migrate through their local assemblages in order to successfully exploit resources such as light and nutrients. Previously, our lab has shown how several large single-celled diatom species display characteristic responses to local conditions such as temperature and light. We determined that several of these conditions such as adhesion and directional responses can be modified by the presence of other diatom species, and that this effect can be dependent on the relative abundance of diatom species present. For example, previous studies on light-stimulated direction change indicated that Stauroneis (Stauroneis phoenicenteron) cells had a 2-3 fold longer response time (i.e. slower, repressed response) when in the presence of increasing densities of Craticula (Craticula cuspidata) cells, while Cratiula showed no such density dependent change (Diatom Research, 2016, 31:173–184). In our current study, we measured the time-dependence of this effect, generating a mixed assemblage of Stauroneis and Craticula cells, and measuring the light-stimulated direction change response time of both Craticula and Stauroneis as a function of the duration of their co-incubation. Our results confirm that Stauroneis cells are repressed in the presence of Craticula, showing a response time that increases within 20-30 minutes. Stauroneis cells show an average response time of 29 ± 2 s within the first 0-10 minutes after incubation with Craticula, which increases significantly to 50 ± 5 s for cells 20-30 minutes after incubation (P < .006). The Craticula in the mixed assemblage shows no significant repression in responsiveness over this same period of time, with an average response time of 13 ± 1 s (P = 0.51 for differences over time). Stauroneis in the absence of any Craticula also show no significant change in response time over a period of about an hour, with an average response time of 37 ± 2 s (P = 0.65 for differences over time). These studies suggest that there is some type of inter-species effect between Stauroneis and Craticula that represses the light-stimulated response time of Stauroneis, but not Craticula, and may aid in the cells’ ability to have niche partitioning and greater ecological success in algal communities. This work was supported by grants through the DePaul College of Science and Health, the DePaul University Research Council, and equipment purchased previously through NSF Grant IBN-9982897.

P3389
Board Number: B679
Stability on the edge: probing the biophysical mechanisms of polarity maintenance in motile cells.
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Tuesday-424
Neutrophil chemotaxis to sites of inflammation and infection is one of the first steps in a healthy immune response. In their surveillance of the human body, neutrophils must undergo polarized migration over long distances through a host of different physical environments - in the bloodstream, along blood vessel walls, and within many mechanically distinct tissues. The long-term goal of this research is to understand how neutrophils regulate their actin cytoskeleton in order to maintain polarized migration through dynamic physical settings. Using high-speed microscopy of neutrophil-like HL-60 cells, we have shown that cell leading edge shape undergoes micron-sized fluctuations that dynamically appear and equilibrate on the order of seconds. We have developed image analysis tools to quantify properties of these fluctuations, which we use as a read-out of the biochemical and physical feedback mechanisms used to maintain polarization. In addition, we have developed a minimal physical model of actin growth against a membrane to interpret these results, predict how changes in properties of actin growth affect leading edge dynamics, and identify candidate actin binding proteins that are likely to regulate polarity maintenance. This minimal model recapitulates many aspects of lamellipodial behavior in vivo, such as treadmilling and decay of actin intensity away from the leading edge. In the future we will iteratively test and update this model to determine the minimal set of feedback interactions that can reproduce polarized cell migration behavior. We will then use the model to predict how neutrophils employ these feedback mechanisms to respond to forces in their environment - informing how neutrophil migration, and thus immune function, might vary between tissues with different mechanical properties.

P3390
Board Number: B680
Cellular decision-making in symmetric directional dilemmas.
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Neutrophils are the most abundant circulating leukocytes in humans, comprising the first line of innate immune defense. As neutrophils migrate towards sites of infection and inflammation they encounter a highly heterogeneous environment. Tasked to navigate through microscale obstacles, neutrophils continuously engage in decision-making processes. Through genetic and pharmacological loss-of-function experiments, we know many of the key components required for neutrophil chemotaxis. However, there are still fundamental gaps in our understanding of how these components interact to generate directional decisions in symmetry-breaking situations.
We investigate how neutrophil-like cells make directional decisions when presented with symmetric directional dilemmas. To this end, we are challenging single HL-60 cells with microfluidic devices that harbor symmetrically bifurcating migration channels. We found that cells that encounter symmetric bifurcations exhibit two different decision-making modes. In the first, upon reaching the bifurcation the cell immediately begins to migrate along one direction. In the second, the cell extends a leading edge along both directions of the bifurcation and subsequently retracts the leading edge from one side, continuing to migrate along the other direction. In the later case, we have seen no measurable difference in the leading edge actin intensity until very late in the decision process. Additionally, the positioning of the nucleus and that of the microtubule organizing center do not have a predictive value. Lastly, under conditions of extreme confinement, cell motility becomes bleb-driven and a third decision-making mode arises, where a cell seemingly selects a direction right away only to change it later.
By precisely controlling the cellular environment and measuring the intrinsic biochemical and mechanical interactions between regulatory molecules and structural components we aspire to build a model for cellular decision-making that accounts for cytoskeletal dynamics and signaling activity.

**P3391**  
**Board Number: B681**  
**Regulation of Haptotaxis via Dynamic Lamellipodia.**  
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Cells in vivo migrate in response to directional cues such as differing concentrations of extracellular matrix (haptotaxis). However, a complete mechanistic understanding of how cells sense and respond to haptotactic cues is lacking. In order to generate the forces required to drive cell migration, actin filaments must be nucleated and organized in a precise manner. One primary mechanism by which new actin filaments are nucleated is the Arp2/3 complex. In order to dissect the mechanism of haptotaxis on fibronectin, an agnostic approach using microfluidic chambers that allow direct imaging of fibroblast haptotaxis on gradients of fibronectin was utilized. The requirement for the Arp2/3 complex for haptotaxis was confirmed via knockdown, knockout and inhibition in a range of cells, indicating that this result is not cell type specific. We utilized knockdown and inhibitors to elucidate a pathway for haptotaxis, as well as kymography and FRAP to study cellular and actin dynamics during haptotaxis.

From this we recently elucidated the haptotaxis pathway (King SJ et al., JCS 2016) whereby differential engagement of integrins triggers a Src/Rac/WAVE pathway that leads to Arp2/3 activation. Resulting in differential actin dynamics and protrusion dynamics, ultimately leading to directed migration towards higher concentrations of extracellular matrices. Interestingly, merely the presence of a lamellipodium has been shown to be insufficient to regulate haptotaxis; fascin knockdown cells display lamellipodia but cannot haptotax on fibronectin (Johnson HE et al., JCB 2015). In this paper we hypothesized that fascin may direct the formation of the lamellipodium via filopodia to regulate haptotaxis. Utilizing fascin phosphorylation mutants that disrupt fascin/actin binding (Zanet J et al., JCB 2012; Villari G and Jayo A et al., JCS 2015), in a knockdown rescue model, we are exploring the role of fascin in haptotaxis and the dynamics of the cytoskeleton. Interestingly, we have discovered that fascin’s ability to bundle actin is dispensable for haptotaxis. Fascin is regularly misregulated during cancer as are many of the proteins involved in haptotaxis; therefore, a full understanding of its role in haptotaxis will likely contribute to a greater understanding of cancer progression with therapeutic benefits.

**P3392**  
**Board Number: B682**  
**PI(3,4)P2, a new player in chemotaxis?**  
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Motile cells can detect shallow gradients of specific chemical signals in their environments and migrate accordingly. This directed cell movement is defined as chemotaxis and is essential for various cell types to carry out their biological functions. During chemotaxis front proteins are recruited to extending pseudopods, which themselves are concentrated at the anterior of the cell. Conversely, back proteins dissociate from nascent pseudopods. I am interested in studying the events occurring at the trailing edge of migrating cells, what back proteins bind to, and how back components regulate polarity. We
identified a Pleckstrin Homology domain (PH domain) containing protein, CynA, which localizes strongly to the lagging edge of cells and is involved in symmetry breaking and promotes polarity. Furthermore, we found CynA preferentially binds to P(3,4)P2 over PIP3 on lipid strips and on reconstituted lipid vesicles. P(3,4)P2 is an important player in signal transduction in chemotaxis. However, the dynamics and mechanisms of P(3,4)P2 mediated signal transduction in this process remain largely unknown. We used this novel P(3,4)P2 biosensor to investigate the role of CynA in chemotaxis. Our data indicated that in uniformly stimulated cells, CynA-GFP-CynA falls off the membrane and translocates to the cytosol, which suggests that P(3,4)P2 levels decrease upon cAMP stimulation. In addition, we studied the dynamics of P(3,4)P2 localization during chemotaxis using CynA PH dimer- KIKGR, a photoconvertible green-to-red fluorescent protein. We found CynA PH dimer- KIKGR localizes in the lagging edge of the cells, as well as in vesicles coming from the protrusions at the leading edge. Our observations suggest that vesicle trafficking might be the source of P(3,4)P2 gradient from the trailing to the leading edge of the migrating cells.

**Integrins and Cell-ECM Interactions 2**

**P3393**

**Board Number: B684**

The Role of Integrin-dependent Paracrine Signaling from Keratinocytes in Regulating Myofibroblast Differentiation.

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During wound healing, keratinocytes promptly restore the epidermal barrier and secrete paracrine factors that regulate wound cell functions. Dysregulation of this paracrine signaling can potentially contribute to a spectrum of pathologies: from wounds that fail to heal to those that are fibrotic, such as hypertrophic scars. Our labs have recently established that keratinocyte integrins α3β1 and α9β1 play critical roles in the regulation of wound paracrine signaling by demonstrating that epidermal α3β1 promotes angiogenesis, but α9β1 negatively regulates these α3β1-dependent paracrine effects through paracrine stimulation of endothelial cells (Longmate et al., J Cell Bio, 2017). We suspect that integrin-dependent paracrine signaling from keratinocytes may also play a role in regulating differentiation of myofibroblasts, the cell type that deposits and contracts the ECM during scarring and fibrosis. Recently we determined that conditioned medium (CM) derived from α3+ expressing mouse keratinocytes (α3+ MK) induces expression of cyclooxygenase 2 (COX-2) in fibroblasts. Exogenous prostaglandin E2 (PGE2), one product of the COX-2 pathway, represses markers for myofibroblasts such as TGF-β-induced expression of alpha smooth muscle actin (α-SMA) and formation of stress fibers. In addition, PGE2 suppresses TGF-β-mediated nuclear translocation of myocardin related transcription factor A (MRTF-A). Furthermore, COX-2 knockdown in fibroblasts reduces PGE2 levels and relieves the suppression of α-SMA expression. These in vitro data support a role for fibroblast COX-2-dependent, PGE2 signaling in modulating TGF-β-induced myofibroblast differentiation. Building on our earlier finding that α3β1 induces matrix metalloproteinase 9 (MMP-9) expression in MK cells (Iyer et al., J Cell Sci. 2005; Missan et al., 2015; Longmate et al., J Cell Bio. 2017), we now find that GM6001 (a broad-spectrum inhibitor of MMPs) and a MMP-2/9 specific inhibitor, each suppresses the ability of CM from α3+ MK cells to induce fibroblast COX-2 expression. Furthermore, either gelatin-agarose-mediated depletion of MMP-2/9 in α3+ MK CM, or dicer-substrate siRNA-mediated knockdown of MMP-9 in α3+ MK cells, attenuates α3β1-dependent induction of fibroblast COX-2. Importantly, CM from MK cells that express both integrins α3β1 and α9β1 (α3+/α9+ MK) induces COX-2 in fibroblasts to a lesser degree than CM from α3+ MK cells.
alone, consistent with our hypothesis that α9β1 suppresses the paracrine signaling functions of α3β1. Our findings support a novel role for coordinated functions of keratinocyte integrins α3β1 and α9β1 in controlling the secretion of paracrine factors such as MMPs that regulate fibroblast gene expression and differentiation during wound healing.

P3394
Board Number: B685
Interplay between cell-cell and cell-extracellular matrix forces regulate myocardial proliferation in the heart.
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Cells sense and transduce mechanical signals through cell-extracellular matrix (ECM) adhesions and cell-cell adhesions. After birth, contractile force generation and tissue stiffness increase as cardiac output increases to support the needs of the newborn organism and its growing heart. During this postnatal period, the N-cadherin complex assembles at the intercalated disc (ICD) as integrin-ECM adhesions decrease, accompanying the switch from hyperplastic to hypertrophic growth. However, little is known regarding the reciprocity between integrin and N-cadherin adhesions in the regulation of cell cycle withdrawal that occurs in cardiomyocytes after birth. Alpha-catenin function as mechanosensors and transduce the intercellular force from N-cadherin to the actin cytoskeleton. To investigate mechanotransduction in the heart, cardiac-specific αE- and αT-catenins double knockout (DKO) mice were generated. The reorganization of N-cadherin and integrin-ECM complexes was examined at postnatal day (P) 4, P7, P14, and P60. DKO hearts exhibited aberrant N-cadherin localization accompanied by alteration of α5/β1 integrin distribution. Normally found at the lateral membrane, α5 and β1 accumulated at the ICD with disrupted N-cadherin adhesion. FN matrix assembly, as monitored by immunofluorescent staining and its insolubility in the detergent deoxycholate (DOC), was increased in DKO hearts. Meanwhile, focal adhesion kinase (FAK) was activated in early postnatal DKO hearts. Complementary experiments performed with deformable substrata demonstrated that stiffness-mediated proliferation was dependent on FAK activity. Finally, treatment of DKO pups with the lysyl oxidase (LOX) inhibitor, β-aminopropionitrile (BAPN), was sufficient to reduce cardiomyocyte proliferation to wild-type levels. This data demonstrates that α-catenins regulate the balance between cell-cell and cell-matrix adhesions, which, in turn, controls cardiomyocyte proliferation, thus providing a molecular explanation for loss of regenerative potential shortly after birth.

P3395
Board Number: B686
Laminin 521 Enhances the Expansion and Engraftment of Mouse Satellite Cell-Derived Myoblasts.
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Large-scale expansion of myogenic progenitors is necessary to support the development of high throughput, bioengineered cellular assays in vitro and to develop cellular therapies for rare muscle diseases. A significant challenge exists to significantly expand myogenic progenitors since they progressively lose their ability to differentiate when cultured and passaged long term in vitro. In order to
overcome this challenge, we evaluated the consequence of propagating mouse and human myogenic stem cell progenitors on various extracellular matrices to determine if they could enhance long-term myogenic potential. We comprehensively examine the effect of physiologically relevant laminins, laminin 211 and laminin 521, compared to traditionally utilized ECMs (e.g. laminin 111, FN, and matrigel) to assess their capacity to preserve myogenic stem cell potential. Laminin 521 supported the increase of myogenic proliferation in early phases of expansion and was the only substrate facilitating high-level fusion following more than 8 passages in mouse cultures. In human cultures, laminin 521 also supported increased proliferation during expansion and superior differentiation with myotube hypertrophy and increased nuclear spacing. In addition we performed engraftment studies in NOD/SCID mice using these ECMs and established that laminin-521 was superior to LN-111 and matrigel expanded cells. These results suggest that laminin-521 could be employed to facilitate the development of future cellular therapy approaches that employ satellite cells.

P3396
Board Number: B687
The role of αvβ5 integrin in axon specification of cerebellar granule cell precursors.
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Vitronectin (VN), which is an extracellular matrix protein, is known to be involved in the regulation of proliferation and differentiation of cerebellar granule cell precursors (CGCPs) in mice. We previously revealed that VN promotes the progress of the initial differentiation stage of CGCPs. However, it has not been identified which receptor for VN is involved in the function of VN in CGCPs. A prime receptor candidate for VN is integrin (Itg). It is known that αvβ3 Itg and αvβ5 Itg are expressed in CGCPs. Then, we examined which Itg serves as a receptor for VN in CGCPs and what phenomenon the receptor contributes to in the initial differentiation stage of CGCPs. First, the effects of the knockdown (KD) of αv, β3, β5 Itg by the siRNAs on the proliferation and differentiation of CGCPs were analyzed by immunohistochemistry of the CGCPs. The KD of αv Itg and β3 Itg up-regulated the number of proliferation marker-positive cells, but the KD of β5 Itg up-regulated the number of TAG1, a marker of initial differentiation, -positive cells in CGCPs. These results of β5 Itg, not β3 Itg, is consistent with our previous results of VN. This finding motivated us to check the role of β5 Itg as a receptor for VN. The KD of β5 Itg using shRNA-expression lentivirus abrogated the promotion of initial differentiation stage progression in CGCPs by VN, indicating that αvβ5 Itg as a receptor of VN contributes to the progress of initial differentiation stage in CGCPs. Next, we focused axon specification, which has been reported to be observed in the initial differentiation stage of CGCPs and speculated that VN and αvβ5 Itg contribute to axon specification in the initial differentiation stage of CGCPs. To check this speculation, we examined the effect of VN and β5 Itg on the axon specification of CGCPs. The knockout of VN and the KD of β5 Itg up-regulated the ratio of CGCPs with no axon, and the addition of VN and the over-expression of β5 Itg up-regulated the ratio of CGCPs with 2 or more axons. These results indicated that αvβ5 Itg and VN contributes to the axon specification in CGCPs. The axon specification is known to be an essential process in the initial differentiation stage. It is suggested that the progress of the initial differentiation stage is suppressed by the inhibition of the axon specification by the knockout of VN and the KD of β5 Itg. Moreover, the effects of addition of VN were abrogated by KD of β5 Itg. Taken together, αvβ5 Itg is served as a receptor for VN in the initial differentiation stage in CGCPs and has the critical role to specify axon in CGCP.
P3397
Board Number: B688
The planar cell polarity protein Vangl2 regulates cell-extracellular matrix interactions underlying proper membrane protrusive activity.
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Planar cell polarity proteins are implicated in a variety of morphogenetic processes including embryonic cell migration. The transmembrane protein Vang-like 2 (Vangl2) is specifically required for planar cell polarity and directed migration during zebrafish gastrulation. These cell behaviors occur in the context of a fibrillar fibronectin-containing extracellular matrix (ECM). While cell-ECM interactions are thought to regulate cell migration, it is unclear how Vangl2 influences these events. Using a cell culture model system, we previously showed that human VANGL2 negatively regulates membrane type-1 matrix metalloproteinase (MMP14) and activation of secreted matrix metalloproteinase 2 (MMP2). We have now investigated the functional relationship between VANGL2, integrin αβ3, and MMP2 activation. We provide evidence that VANGL2 regulates cell adhesion to the ECM proteins fibronectin, laminin, and vitronectin. Inhibition of MMP14 activity suppressed the cell adhesion defect in VANGL2 siRNA transfected cells. Furthermore, our data show that MMP14 and integrin αv are both required for increased proteolysis by VANGL2 knockout cells. Lastly, our data suggest that integrin αvβ3 is a novel VANGL2 binding partner. These findings begin to dissect the molecular underpinnings of how VANGL2 regulates MMP activity and cell adhesion to the ECM. Previous data established that, compared to wild type, vangl2/trilobite mutant zebrafish gastrula cells lack directionality and meander during their migration towards the dorsal body axis. Notably, we have reported that vangl2/trilobite mutant embryos also have increased Mmp activity and decreased fibronectin ECM assembly. Using time-lapse confocal imaging we now show that loss of zebrafish Vangl2 function increases the number of both filopodia-like and larger membrane protrusions in migrating gastrula cells. This phenotype is accompanied by decreases in polarized protrusive activity and cell migration directness. To determine the role of the ECM in membrane protrusion formation, we performed protrusion analysis on wild-type cells injected with fibronectin morpholinos. Our data show that embryos with reduced fibronectin protein expression exhibit membrane protrusion formation similar to vangl2/trilobite mutant cells, suggesting that defective cell-ECM interactions underlie at least a portion of the mutant phenotype. Together, our in vitro and in vivo data suggest a model whereby Vangl2-dependent regulation of cell-ECM interactions is required for proper membrane protrusive activity and directed cell migration.

P3398
Board Number: B689
Integrin αvβ3 and α6β1 cross-talk of dermal fibroblasts on integrin specific peptide polysaccharide matrix.
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A limited number of cross-talks between different integrin subtypes had been identified. Here, we focused on various cross-talks of two different subtypes of integrins and investigated the mechanisms using the unique integrin binding peptides conjugated chitosan matrices. Five different integrin binding peptides, FIB1 (integrin αvβ3), EF1zz (integrin α2β1), S31 (integrin α3β1), CS1D (integrin α4β1), and A2G10 (integrin α6β1), were individually mixed, and ten different two-peptides mixed chitosan matrices
were prepared. Human dermal fibroblast (HDF) attachment activities to the ten different mixed peptide-chitosan matrices were different depending on the combination of peptides and their mixture ratios. Six different pair of mixed peptides-chitosan matrices did not show any specific variation by mixture of peptides, and three mixtures (FIB1/A2G10−, FIB1/CS1D−, and EF1zz/CS1D-chitosan matrices) promoted HDF attachment. Of the three HDF attachment promoting combinations, FIB1/A2G10-chitosan matrix significantly promoted HDF attachment and spreading. FIB1 is fibronectin derived RGD containing peptide that binds integrin αvβ3, and A2G10 is derived from laminin α2 chain that binds integrin α6β1. The promotion of HDF attachment was conspicuous when A2G10 and FIB1 was mixed with 3:7 molar ratio. Since FIB1 include RGD in its sequence, laminin derived RGD containing peptide of A99 was examined to clarify this promotion, and A99/A2G10-chitosan matrix was prepared. A99/A2G10-chitosan matrix promoted HDF attachment as same as FIB1/A2G10-chitosan matrix. Further, the promotion of cell attachment was also observed laminin-111 and fibronectin mixture. From these results, we could identify the specific combination of integrin-integrin cross-talks that promotes the cell attachment using the mixed peptides chitosan matrix method.

P3399  
Board Number: B690  
DDesmoplastic-ECM induces aberrant active α5β1 integrin endocytosis to sustain a pro-pancreatic cancer desmoplastic stroma phenotype.  
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Pancreatic ductal adenocarcinoma (PDAC) presents one of the highest mortality rates amongst all neoplasias. The desmoplastic (Desmo) microenvironment, characteristic of this malignancy, plays a pivotal role in its aggressive progression. This pro-tumor niche is produced and sustained by activated (i.e., Desmo) cancer-associated fibroblasts (CAFs). We have previously demonstrated the occurrence of a CAF Desmo phenotype correlated to bad patient outcomes. This phenotype is characterized by an aberrant intracellular accumulation of active α5β1 integrin. Using our in vivo-mimetic 3D human stroma system, we were able to induce this phenotype on human naïve pancreatic stellate cells by culturing these into Desmo CAF-derived extracellular matrices (ECMs). We found that the intracellular accumulation of active α5β1 integrin depends on αvβ5 activity, and that this integrin crosstalk is needed to maintain the CAF-like phenotype. The hypothesis being tested in this study postulates that Desmo-ECM-induced alterations to α5β1 integrin trafficking dynamics, induced by outside-in αvβ5 integrin signaling, are responsible for the intracellular sequestering of active α5β1, which in turn fuels a forward loop to sustain and propagate the CAF Desmo phenotype.

Our data suggests that in response to Desmo-ECM, CAFs display changes in the expression and regulation of key vesicle transport molecules, such as Cav1, Rab27, CLIC3 and CD81 and that these changes are lost in CRISPR edited αvβ5 integrin-null CAFs. These results prompted the notion that alterations in endocytic routes of active α5β1 integrin-containing vesicles are triggered by CAFs binding to Desmo-ECM. Moreover, knockdown of CD81 expression prevented the intracellular accumulation of active α5β1 integrin and resulted in the reversion of the pro-PDAC CAF phenotype back to an anti-tumor, akin to normal stroma, phenotype. These findings suggest a potential clinical relevance for gauging PDAC’s stromal phenotypes in patients. Moreover, our results could facilitate the potential development of new therapeutic targets aimed to reprogram Desmo stroma and harvest its natural tumor-restrictive benefits.

Funding provided by National Cancer Institute (NCI) R01 CA113451; U.S. Department of Defense (DOD) Idea Award with Special Focus in Military Personnel-Affected Pancreatic Cancer W81XH-15-1-0170.

Tuesday-431
Fibrinogen is a major blood plasma protein which is converted into fibrin by thrombin to form fibrin clot. Fibrinogen is involved in a variety of biological functions, such as thrombus formation, inflammation, wound healing and leukocyte adhesion. Fibrinogen consists of six polypeptide chains in two sets of three polypeptides (α, β, γ) and of three domains with two distal and one central domains. It was shown that the central and distal domain of fibrin bind to β2 integrin on leukocyte via I domain of α subunit of β2 integrin. In this study, we further characterized fine structure of central domain of fibrin responsible to β integrin binding, by using ELISA type binding analysis with fibrin derived proteins. As results, the β chain of fibrin central domain bound to αX-I domain at a high level and α chain bound a less extent level, suggesting that the β chain of fibrin fragment E and a major binding site for αX-I domain. To further characterize the major site in β chain of fibrin fragment E, peptide inhibition experiment was carried out. As a result, peptide corresponding to G14~A25 region of the β chain has an inhibition effect on the αX-I domain binding to β chain of fibrin fragment E. Therefore it is likely that G14~A25 region of the β chain is a major binding site. In addition G29~D40 region of α chain in recognized as a binding site and G17~C28 region of α chain as a minor binding site for αX-I domain. Besides there two regions, other sites of fibrin fragment E still has a possibility as binding regions but in less significant manner.

The nervous system can mitigate an exacerbated pro-inflammatory immune response in an immunosuppressive setting. Studies also suggest that neurotransmitters can trigger immune responses such as cytokine secretion, integrin expression, and chemotaxis. We observed a basal expression of receptors for glutamate on mouse T lymphocytes. Both CD4+ and CD8+ T cells significantly upregulated expression of glutamate receptors (GluRs) following TCR stimulation, with CD8+ T cells maintaining a higher and prolonged receptor expression than CD4+ T cells. Concomitant with the upregulation of T cell activation molecules CD69, CD25 and CD44, proliferating CD8+ T cells presented higher levels of GluR3 and GluR1 when compared with non-proliferating cells, as well as a higher production of IFN-γ and granzyme B. We also evaluated the role of neurotransmitters in modulating and/or activating T cells in mammary (4T1HA) tumor model that express viral hemagglutinin (HA) as a defined immunodominant antigen of low avidity. We observed that HA-reactive CD8+ T cells in the tumor-infiltrate showed an upregulated expression of GluR3 and GluR1 receptors when compared with control group. Although the dynamics of glutamate receptor signaling on T cell function is not completely clear, the blockade of GluR signaling by using specific antagonists down regulates expression of activation markers as CD69 and
CD25, impairs T cell proliferation, however, it does not interfere with their cytolytic activity neither their ability to express effector molecules. Early TCR mediated molecular pathways are also affected by GluR signaling, suggesting an interaction between them. Overall, data suggest that glutamate might have a costimulatory effect on T cell activation and could be a promising strategy for enhancing anti-tumor T-cell immunity, most likely by increasing T cell survival.

P3402

Board Number: B693

Genetic dissection of Cell-ECM adhesion in vivo using CRISPR/Cas9-mediated genome engineering of the Talin locus.

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Cell adhesion to the extracellular matrix is of fundamental importance to the development of multicellular organisms. This process entails the coordinated assembly of the integrin family of adhesion receptors with cytoplasmic proteins complexes required to regulate integrins and transduce their signal. An essential component of these complexes is the cytoskeletal protein talin, which is required for integrin function and regulation. Talin has essentially two functions orchestrating the assembly of the intracellular adhesion complex, which associates with integrin, and regulating the affinity of integrins for the ECM. Talin is a large 270-kDa protein comprised of multiple domains capable of a wide variety functions such as scaffolding, adaptor, regulatory and mechanotransduction. These are believed to enable talin to precisely regulate integrins in wide variety of developmental processes. To understand the role of these different domains, our group and others have performed extensive structure-function analysis through the expression of specific Talin mutants in Drosophila Melanogaster. Although instrumental, an important caveat of these studies was the resulting ectopic expression of the Talin constructs. To circumvent this problem, we have established a CRISPR/Cas9 strategy to efficiently engineer the endogenous Talin locus. Through this method, we successfully generated mutant flies by specifically mutating key residues and allowing us to directly assay these modifications in vivo in the context of Drosophila development. We present here our findings on 3 different talin mutants. The first one, K17E, targets a site in the FO subdomain of the talin head previously shown to be able to bind rap-1, a small GTPase known as a master regulator of cytoskeletal remodeling. Surprisingly, phenotypic studies of this mutant revealed that this site is essential for talin function. Indeed, K17E embryos were unable to localize talin to integrins leading to severe adhesion defects. This indicates that direct Rap1 binding to talin is required for its proper localization. This novel model of Talin recruitment to the integrins redefines the previous canonical view where Rap1 was thought to not interact directly with talin, but through the adaptor protein RIAM. The remaining mutants target the two principal talin actin binding sites, ABS2 and ABS3. Here, we separately investigated the roles of each to precisely dissect their contribution to talin function. Our results suggest that each actin binding site has distinct function in the establishment and maintenance of Cell-ECM adhesion. Together these results shed new insight on talin function and opens new interesting prospects with the future analysis of additional talin mutants.
P3403
Board Number: B694
Mechanical and Signaling Roles for Keratin Intermediate Filaments in the Assembly and Morphogenesis of Mesendoderm Tissue at Gastrulation.
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Coordination of individual cell movements is critical for the assembly and morphogenesis of tissues at gastrulation. These movements generate forces but how cells respond to and transmit these forces remains unclear. Xenopus mesendoderm cells employee integrin based adhesions to migrate collectively along a fibronectin (FN) substrate assembled by blastocoel roof (BCR) cells. One question still to be addressed is the distribution of individual cell traction stresses that are involved in propelling this tissue across the BCR. Traction force microscopy (TFM) was used to detect and quantify traction stresses generated by cells within the mesendoderm tissue. We demonstrate that leading edge cells are primarily responsible for generating traction stresses necessary for migration and that these stresses are balanced by intercellular stresses in following row cells. This arrangement of traction stresses is further reflected in the morphology of these cells. Leading edge cells extend broad stable lamellipodia while following row cell protrusions are smaller, more dynamic and include long filopodial-like structures. TIRF microscopy of cells expressing GFP-tagged paexillin reveals mature focal adhesions (FAs) formed by cells at the leading edge with rapid turnover of smaller FAs evident in following row cells. A gradient of Rac1 activity was detected in leading edge cells expressing a FRET-based biosensor, with highest Rac1 activity noted in forward protrusions. Increased Rac1 activity coincides spatially with highest traction stresses generated by the leader cells. Following row cells lack a Rac1 activity gradient. Keratin(8) intermediate filaments (IFs) are known to localize at cell adhesions in mesendoderm cells in response to anisotropic tension on C-cadherin adhesions (Weber et al. 2012. Dev Cell 22, 104-115). Depletion of keratin(8) IFs with antisense morpholinos results in higher traction stresses in following row cells, misdirected protrusions, and the formation of actin stress fibers anchored in streak-like focal adhesions. A connection between Rac1 and keratin(8) IFs was explored using a photoinducible Rac1 construct to increase Rac1 activity in targeted regions of the cell. Local activation of Rac1 activity resulted in reduced keratin(8) filaments and increased lamellipodial protrusions. Morpholino knockdown of keratin(8) IFs also resulted in disruption of the Rac1 activity gradient. Together, these results suggest a functional antagonism between Rac1 and keratin (8) IFs. We propose that maintenance of mechanical integrity in the mesendoderm by keratin intermediate filaments is required to balance forces within the tissue and to coordinate collective cell movements. This work was supported by USPHS grant GM094793.

Cadherins and Cell-Cell Interactions

P3404
Board Number: B695
Mechanosensitive cadherin adhesion and its regulation.
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Mechanical signals are essential for the formation, differentiation, and remodeling of tissue. Classical cadherin cell-cell adhesion proteins play key roles in sensing mechanical forces and transducing them
into biochemical signals. While the functional consequences of cadherin adhesion have been extensively studied, little is known about the molecular mechanisms by which cadherins sense extracellular mechanical stimuli and regulate adhesion. To address this critical gap, we used an integrated approach that tightly couples highly predictive computer simulations, ultrasensitive single molecule Atomic Force Microscopy, and precise manipulation of cadherin interactions with the cytoskeleton. Our results show that: (i) cadherin ectodomains adhere in two conformations that respond to mechanical stimuli in distinctly different manners, by either strengthening or weakening adhesion [1, 2]; (ii) cadherins interconvert between these alternate conformations [3]; and (iii) cadherin ectodomain conformation is regulated by the highly dynamic actin cytoskeleton. Our results provide a mechanistic understanding of how the cytoskeleton regulates cadherin adhesion and cadherin response to extracellular mechanical stimuli.


P3405
Board Number: B696
Mutations disrupting planar cell polarity alter Celsr1-mediated cell adhesion and dynamics.
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Planar cell polarity (PCP), the collective and organized polarization of cells along a tissue plane, is fundamental to early embryonic development and tissue organization in complex, multicellular organisms. Compromised PCP results in severe developmental disorders including neural tube defects, ciliopathies and cardiomyopathies. A hallmark feature of PCP is the asymmetric localization of core PCP components at intercellular junctions. This junctional PCP complex is thought to be organized via homophilic interactions of the principle PCP protein, Celsr, a large atypical cadherin. While important developmental roles for Celsr at the tissue-level have been established, an understanding of Celsr extracellular cell-cell interactions at the individual cell and molecular level is lacking. The overall goal of this work is to elucidate how Celsr1 mediates adhesive interactions to coordinate asymmetrical PCP protein localization and function. Using a combination of junctional recruitment and cell aggregation assays in cadherin-free K562 cells, we confirmed that mouse Celsr1 mediates calcium-dependent, homophilic cell adhesion through its cadherin repeat-containing extracellular domain. Interestingly, disease-associated PCP variants Celsr1Crsh and Celsr1Scy, which harbor mutations in the extracellular cadherin domains, mediated cell aggregation comparable to wildtype (WT). However, these mutants lack the ability to enrich at epithelial cell junctions, suggesting that intercellular adhesion alone cannot fully explain Celsr1 functions. Surprisingly, Celsr1Crsh aggregates segregated from Celsr1WT clusters when mixed, demonstrating this single point mutation generates a novel Celsr variant with altered homophilic binding properties. Further observations suggest Celsr1Crsh is defective in lateral clustering, which may function to stabilize and reinforce the adhesive complex. Celsr1Crsh displayed broader cell surface distribution and reduced junction localization relative to WT in K562 cells. Further, the stable association of Celsr1 at the membrane, which depends on Celsr1’s extracellular domain, was substantially reduced.

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in the Celsr$^{1\text{Crsh}}$ mutant. Together, our data indicate that Celsr1 mediates homophilic adhesion and that perturbing its adhesive properties compromises functional PCP. Based on the altered distribution, stability and adhesive properties of Celsr$^{1\text{Crsh}}$ protein, we hypothesize that this mutation may lie within a cis-interacting region that functions to stabilize and reinforce the adhesive complex. Domain mapping, biochemical approaches and advanced imaging techniques are ongoing to detail Celsr’s function as an intercellular bridge to mediate PCP complex assembly, maturation and functional asymmetry.

P3406
Board Number: B697
Organization of E-cadherin on the cell surface of A431 cells.
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Adherens junctions (AJs) are major intercellular adhesion structures in vertebrates. Despite the critical role of AJs in tissue integrity and morphogenesis, the detailed organization of their key protein, E-cadherin inside and outside of AJs remains controversial. According to the traditional point of view, AJs are formed by E-cadherin clustering in cell-cell contacts. This model was recently challenged by Single Molecule Localization Microscopy (SMLM) experiments, which detected structurally identical E-cadherin clusters all over the cell surface of A431D cells (Wu et al., 2015, Dev Cell, 32:139-54). This observation implied that E-cadherin clustering is not a key process in AJ assembly. Since A431D cells are highly abnormal, we re-investigated the E-cadherin organization using SMLM on the surface of A431D parental A431 cells. Using E-cadherin tagged with photoswitchable fluorescent protein Dendra2, we showed that AJs in these cells can reach more than one µm in length and consist of tightly packed cadherin clusters with crystal-like density interspersed within sparser cadherin regions. No E-cadherin clusters were found outside of AJs. To exclude the possibility that such extrajunctional clusters were overlooked because of their small size, the weakness of Dendra2 fluorescence, and/or the presence of endogenous E-cadherin, we analyzed A431 cells in which the latter was knocked-out. The extracellular region of the recombinant E-cadherin in these cells was tagged by GFP and was located in SMLM by monovalent Alexa Fluor 647-conjugated anti-GFP nanobody. This approach confirmed that E-cadherin is a monomer outside of AJs. These results strongly support the idea that cadherin clustering is an essential mechanism of AJ assembly and is triggered by adhesive cadherin-cadherin interactions.

P3407
Board Number: B698
The role of cadherin compensation during adherens junction assembly and maintenance in MDCK cells.
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The process of cadherin switching is one of the most notable indicators of Epithelial to Mesenchymal Transition (EMT). A classic example of this type of switch is during cancer metastasis where there is often a shift in the expression of Epithelial cadherin (E-cad) to Neural cadherin (N-cad) with in a tumor. Depending on the cell type, numerous cadherins are expressed at any given time. In this study we investigated two of the most abundant cadherins in the Madin Darby canine kidney (MDCK) cell line, E-cadherin and Placental cadherin (P-cad). While E-cadherin is well known for its ability to initiate epithelial junction assembly as well as maintaining proper tissue permeability, P-cadherin has primarily been studied within the context of collective cell migration and tissue tension. We looked at P-cadherin

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expression and localization during adherens junction assembly when E-cadherin expression and function were perturbed. Using the calcium switch method to synchronize junction assembly, we discovered P-cadherin’s expression and localization to be similar to E-cadherin’s. We also looked at both the localization and expression levels of P-cadherin within E-cadherin overexpressing cells and noted significant changes in its localization. These findings support the cadherin compensation hypothesis, whereby one cadherin could be forced into multiple roles depending on a change in another cadherin’s expression. Lastly, we investigated the affect of the cadherin compensation process on downstream signaling pathways by looking at a common signal component, the small Rho GTPase Cdc42. Given Cdc42’s known roles in actin cytoskeletal remodeling, necessity for junction assembly, and its role in establishing polarity, and necessity for directed cell migration, we investigated the localization and activity of Cdc42 following E-cadherin perturbation. Finally, we investigated the changes in cadherin expression and contact localization following Cdc42 inhibition. We expect this research will be used to better understand one of the more critical mechanisms during cancer metastasis and provide novel insight to prevent cancer progression.

P3408
Board Number: B699
Branched actin networks at endothelial adherens junctions push against each other to maintain cadherin transinteraction.
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Adherens junctions (AJs) are mechanosensitive cadherin-based intercellular adhesions that extensively interact with the actin cytoskeleton and carry most of the mechanical load at cell-cell junctions. Both Arp2/3 complex-dependent actin polymerization, which generates pushing force, and nonmuscle myosin II (NMII)-dependent contraction, which produces pulling force, are necessary for AJ morphogenesis. Which of these actin systems interacts with AJs is unknown. Their specific contribution to AJs is also not fully understood with the role of Arp2/3 complex-dependent nucleation in mature linear AJs being especially obscure. By platinum replica electron microscopy of endothelial cells, we show that VE-cadherin immunogold colocalizes with Arp2/3 complex-positive branched actin network in different types of AJs, while actin-NMII bundles are located more distally. The branched networks at AJs consist of two oppositely oriented subsets with VE-cadherin in the middle. After inhibition of the Arp2/3 complex, mature linear AJs split with detergent-insoluble VE-cadherin remaining at the gap edges. In contrast, VE-cadherin is lost from gap edges following detergent extraction in cells with inhibited actin-myosin contractility. We propose that Arp2/3 complex-dependent branched actin networks push against each other to maintain trans-interaction between VE-cadherins of contacting cells, whereas NMII activity stabilizes adhesion complexes at the cytoplasmic faces of the AJ. Additionally, tangential actin bundles serve as a launch pad for assembly of the junctional branched networks and as a structural base that restrains pushing force generated by the branched network and its retrograde flow within a narrow space between contacting cells. Our data provide an important conceptual framework to explain results of functional experiments that target various components and regulators of the actin cytoskeleton AJs.
P3409
Board Number: B700
Essential Role of Obscurin Kinase Domain-1 in Cardiac Cell Adhesion and Communication by Regulating the Phosphorylation of N-Cadherin.
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Obscurin-B is a giant protein composed of adhesion and signaling motifs. Our earlier work demonstrated that the serine/threonine kinase domains (Kin1 & Kin2) of obscurin-B are enzymatically active [1]. Particularly, Kin1 undergoes autophosphorylation, and phosphorylates the cytoplasmic domain of N-cadherin (N-cad) in vitro [1]. We now show that endogenous obscurin-B containing Kin1, co-localizes with N-cad at cell junctions in neonatal rat ventricular myocytes (NRVM), and that their distribution is coordinated and regulated by Ca2+. Consistent with this finding, ectopically expressed, constitutively active Kin1 (Kin1-CA) preferentially targets to the cell membrane where it forms a complex with N-cad. Overexpression of Kin1-CA significantly increases cardiomyocyte aggregation, adhesion, intracellular communication, and stiffness, as shown by aggregation, dispase, dye transfer, and atomic force microscopy assays, respectively. Given that N-cad is a substrate of Kin1 in vitro and has a well-documented role in modulating cell adhesion, gap junction assembly, and cell stiffness, we postulated that Kin1-CA might exert these effects, at least in part, via phosphorylation of N-cad. To assess this hypothesis, we first used 2D electrophoresis to examine the phosphorylation profile of N-cad in situ. Overexpression of Kin1-CA results in a major shift of N-cad spots towards a lower pH, which is indicative of the generation of less positively charged or more phosphorylated species, thus suggesting that N-cad is a substrate of Kin1 in vivo, too. This observation was supported by mass spectrometry, which revealed that Kin1 phosphorylates N-cad at Ser-788, and the use of phospho-specific antibodies, which confirmed the presence of phosphorylation at Ser788 in cardiac lysates. Importantly, phosphorylation of N-cad at Ser788 was significantly enhanced (~3-fold) following insulin stimulation, implying its physiological relevance. Overexpression of phospho-mimetic N-cad (Ser788Glu), but not phospho-ablated N-cad (Ser788Ala), in NRVM significantly increased the rate of dye transfer, suggesting that Kin1 enhances gap junction communication through phosphorylation of N-cad. We are currently examining the effects of phospho-mimetic N-cad on cell aggregation, adhesion and stiffness using our established approaches. In summary, our studies are the first to show that Kin1 plays key roles in cardiomyocyte adhesion, communication and stiffness, at least in part via phosphorylation of N-cad at Ser788. While the regulation of E-cad via phosphorylation has been extensively studied, little is known about the regulation of N-cad. Our studies are therefore highly novel with important functional implications for cardiac structure and function for both N-cad and obscurin-B.

P3410
Board Number: B701
Barrier enhancing function of Src and VE cadherin phosphorylation in endothelial cells revealed by synthetic biology approach.
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Permeability of the endothelial monolayer is regulated at the level of adherens junctions (AJs), multiprotein structures mediating cell-cell interactions. Src tyrosine kinase can stimulate AJ disassembly, but its activity is also required for AJ formation, suggesting a dual function for Src in
regulation of the endothelial barrier. However, defining a barrier protective role for Src has been difficult due to limitations of current methods. To overcome existing limitations we employed novel protein engineering technology that provides tight temporal regulation of Src kinase activity in living cells. This allowed us to identify a previously undescribed role for Src in the regulation of endothelial barrier function. Src activation resulted in a distinct biphasic temporal effect on endothelial permeability. Initially, Src induction led to strengthening of endothelial barrier and accumulation of VE cadherin at the AJs. Only prolonged activation of Src increased barrier permeability. Importantly, activation of another Src Family Kinase (SFK) member, Lyn, only promoted barrier permeability, suggesting that the barrier enhancing function is specific to Src. Src-mediated transient barrier strengthening was accompanied by formation of morphologically distinct reticular AJs. Reticular AJs were found in areas of overlap between contiguous endothelial cells, and exhibited reduced localized permeability. Organization of reticular AJs suggests that they form in membrane protrusions. In agreement with this hypothesis, we observed increased formation of lamellipodia that occurred concurrently with Src-mediated formation of reticular AJs. Interestingly, Src-induced barrier enhancement was also accompanied by robust phosphorylation of VE cadherin. However, VE cadherin phosphorylation did not affect its interaction with binding partners, p120- and β-catenin, and it did not disrupt VE cadherin localization to AJs. Furthermore, VE cadherin phosphorylation at Tyr731 was required for Src-mediated barrier enhancement. Therefore, our results reveal an unexpected protective role for Src and VE cadherin phosphorylation in the regulation of endothelial permeability. To further support these observations, we also show that activity of SFKs and VE cadherin phosphorylation on Tyr731 are required for barrier enhancing effect of S1P treatment. Our studies also demonstrate that Src activity mediates recovery of the endothelial barrier after thrombin treatment. These data support a novel role for Src uncovered by a synthetic biology approach, and indicate that Src signaling mediates barrier enhancement in endothelial cells downstream of physiological stimuli.

P3411
Board Number: B702
E-cadherin is recruited by the pathogen Enteropathogenic Escherichia coli and binds the Tir:intimin complex.
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Enteropathogenic Escherichia coli (EPEC) is a major cause of childhood diarrhea and causes significant morbidity and mortality in developing countries. To design intervention strategies towards EPEC infections, molecular understanding of the infection strategy is essential. Upon infection, EPEC attaches to the surface of the small intestinal epithelial cells, where it secretes several virulence factors, modifying the cell surface, strengthening EPEC attachment, and promoting growth of microcolonies on the epithelium. EPEC attachment disrupts many structural and functional components within the host cell, and recruits several basolateral proteins to the apical microcolonies. EPEC produces its own receptor, Tir (Translocated intimin receptor), which is translocated into the host cell plasma membrane by the type III secretion system (T3SS). Here, Tir serves as a receptor for the EPEC adhesin intimin, which enables tight attachment of EPEC to the host cell. We discovered that E-cadherin is one of the basolateral proteins recruited to EPEC microcolonies on the apical surface, and explored the significance of this observation. Using fluorescently tagged E-cadherin, we confirmed that E-cadherin is indeed
recruited to the EPEC infection site. Time-lapse imaging revealed that recruitment occurs at later stages of infection. To test the role of E-cadherin in attachment and microcolony formation, we pre-incubated the bacteria with the soluble extracellular domain of E-cadherin before infection. This significantly decreased EPEC adhesion to the host cells, indicating that the extracellular domain of E-cadherin can bind EPEC and block attachment to host cells. We found that Tir/Intimin facilitated this binding, since addition of purified Tir to a non-pathogenic E. coli strain BI21 expressing intimin was sufficient to promote E-cadherin binding. Direct interaction between the soluble domains of intimin and E-cadherin was also detected in vitro, using a far-western interaction assay. Altogether, our data indicate that the Tir-intimin complex formation promotes recruitment of E-cadherin, which stabilizes EPEC attachment to host cells at later stages of infection. This molecular complex may be an attractive target to block colonization of the cell surface by pathogenic bacteria.

**P3412**
**Board Number: B703**
**Anillin dependent stabilization of Rho signaling at adherens junctions influences cell extrusion and collective cell migration.**
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Adherens junctions sense and propagate mechanical forces by coupling the acto-myosin contractile apparatus of adjacent cells. Zonula Adherens (ZA) in simple epithelial polarized cells exemplifies this property of adherens junctions. It is a specialized zone where apically concentrated E-cadherin is coupled to perijunctional actomyosin bundles to generate tension. RhoA signaling and actin regulators play an essential role in generating tension and maintaining the integrity of the ZA. We have now identified a novel mechanism in which RhoA signaling is stabilized at the ZA by anillin and myosin II. This is achieved by recruitment and stabilization of a pool of anillin by myosin II at the ZA in the interphase MCF-7 cells. The junctional anillin, then imparts cortical stability to active RhoA by binding to RhoA-GTP through its Anillin Homology (AH) domain. Anillin stabilized GTP-RhoA is then accessed by effectors like ROCK1 and mDia1 to generate junctional tension. Depletion of Anillin resulted in reduced RhoA signaling at ZA and resulted in reduced junctional tension. Interestingly, anillin’s ability to potentiate RhoA signaling is co-opted by the transcription factor Snail for expulsion of cells from monolayer and also to maintain epithelial cohesivity during collective migration of cells. These results highlight a novel extramitotic role for anillin in regulating RhoA signaling at the zonula adherens.
P3413
Board Number: B704
A non-canonical Notch signaling complex regulates adherens junctions and endothelial barrier function.
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The vascular barrier that separates blood from tissues is actively regulated by the endothelium and is essential for transport, inflammation, and hemostasis. Hemodynamic shear stress plays a critical role in maintaining endothelial barrier function, but how this occurs remains unknown. Here, using an engineered organotypic model of perfused microvessels and confirming in mouse models, we identify that activation of the Notch1 transmembrane receptor directly regulates vascular barrier function through a non-canonical, transcription independent signaling mechanism that drives adherens junction assembly. Shear stress triggers proteolytic activation of Notch1 and the release of the Notch1 transmembrane domain, which is the key domain that mediates barrier establishment. Expression of the Notch1 transmembrane domain is sufficient to rescue Notch1 knockout-induced defects in barrier function, and does so by catalyzing the formation of a novel receptor complex in the plasma membrane consisting of VE-cadherin, the transmembrane protein tyrosine phosphatase LAR, and the Rac1 GEF Trio. This complex locally activates Rac1 at cell-cell contacts to drive the formation of cortical actin fibers, strengthen adherens junctions, and establish barrier function. Canonical Notch transcriptional signaling is highly conserved throughout metazoans and is required for many processes in vascular development, including arterial-venous differentiation, angiogenesis, and remodeling; here, we establish the existence of a previously unappreciated non-canonical cortical signaling pathway for Notch1 that regulates vascular barrier function, and thus provide a mechanism by which a single receptor might link transcriptional programs with adhesive and cytoskeletal remodeling.

P3414
Board Number: B705
Dissecting the function of classical cadherins in stratified epithelial morphogenesis.
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Establishment and maintenance of the epidermis is coordinated by progenitor cells residing in the deepest basal layer that are tightly adherent to each other and to cells in the spinous layer above them. Our lab and others have shown that basal cells promote differentiation by two discrete mechanisms: 1) “delamination,” or detachment of basal cells from the underlying basement membrane; and 2) “asymmetric cell divisions,” perpendicular orientation of the mitotic spindle to specify daughter cells of different fates. Interestingly, both of these processes require remodeling of adhesions in a coordinated manner.

In the developing epidermis, it is well established that during oriented cell divisions, positioning of mitotic spindle is directed by LGN (\textit{Gpsm2}), an intracellular scaffolding protein that localizes to the cell cortex during mitosis. It remains poorly understood how LGN is differentially localized in basal progenitors during either planar (generally thought to be symmetric) or perpendicular (asymmetric) orientation. Our lab has previously shown that LGN is localized to the apical cell cortex during
perpendicular divisions in epidermis, where it can promote perpendicular divisions which promote stratification. However, in oral epithelia and developing hair placodes, LGN can display different patterns of cortical localization where it promotes distinct division orientations. While distinct patterns of cortical LGN distribution are likely to underlie the choice between symmetric and asymmetric divisions, little is known about how LGN localization is regulated either by intrinsic or extrinsic cues. Cadherins are an essential class of cell-cell adhesions that maintain skin integrity at apical junctions through the formation of adherens junctions (AJ). Prototypical E-cadherin (Cdh1) has been proposed to influence the cortical localization of LGN by a direct interaction between its juxtamembrane-domain (JMD) and tetra-tricopeptide (TRP) motifs of LGN. A highly-conserved aspartic acid residue (D758) in the JMD is required for this interaction. While this residue is conserved in most other cadherins, P-cadherin (Cdh3) has a glycine substitution which should render it incapable of binding LGN. Indeed, we have found that Pcad and LGN are localized to mutually exclusive cortical domains in basal cells, while Ecad shows significant colocalization with LGN at the apical cell cortex. This suggests that both Ecad and Pcad may have different functions in regulating the localization of LGN. We find that epidermal Ecad loss increases the frequency of oblique divisions at the expense of perpendicular divisions, but interestingly, LGN expression appears to be unaffected. This suggests the effect of Ecad on spindle orientation may not be entirely mediated by LGN.

P3415
Board Number: B706
Adherens Junction Components Regulate Mitotic Spindle Orientation in Embryonic Epidermis.
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Adherens junctions (AJs) are cell-cell adhesion complexes consisting of transmembrane cadherins which link to the actin cytoskeleton via the catenins and associated scaffolding molecules. Cadherins form homodimers in trans and bind directly to the catenins via their cytoplasmic tail. In turn, α-catenin binds to tensile F-actin via its C-terminus, producing an open "loaded" conformation allowing for vinculin binding. In turn, vinculin binds additional actin filaments, theoretically increasing potential load. Previous studies have demonstrated that afadin, also an F-acting binding molecule, can bind α-catenin via a proximal region theoretically only exposed in the loaded conformation. Other studies have shown that afadin is essential for normal orientation of the mitotic spindle and capable of binding directly to the spindle orientation protein LGN. Interestingly, previous results have shown that α-catenin null mutants displayed abnormal spindle orientation in the epidermis. Taken together, these data suggest a role for components of the AJ in regulating proper orientation of the mitotic spindle either via a direct link to the spindle orientation machinery, or via maintenance of cortical, actin-dependent tension. The goal of this study is to investigate a link between numerous AJ components and mitotic spindle orientation and to elucidate the mechanism(s) governing previously observed mitotic phenotypes. Our data demonstrate that afadin, α-catenin, vinculin, and E-cadherin are all essential for normal spindle orientation in the embryonic mammalian epidermis. Surprisingly, loss of afadin or α-catenin does not perturb early apical recruitment or accumulation of LGN despite subtle effects on the upstream polarity cue, Par3. However, both afadin and α-catenin mutants demonstrate a novel mitotic phenotype where the spindle is unlinked from apical orientation cues specifically at the metaphase-anaphase transition. Furthermore, we were able to detect errors in the maturation of nascent adhesions in afadin mutant cell lines, in vitro. Of particular interest, it appears afadin is required for normal recruitment of vinculin to Tuesday-442
the AJ, loss of which reduces staining of the tension-dependent α18 epitope of α-catenin. Taken together, our data suggest adherens junctions regulate spindle orientaiton, in vivo, potentially by maintaining cortical tension or rigidity during the metaphase-anaphase transition.

P3416
Board Number: B707
Cardiomyocytes Assemble Force-Resilient Adherens Junctions through Vinculin and Afadin.
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With every heartbeat, the junctional complexes that couple cardiomyocytes must transmit the mechanical forces of actomyosin contraction while maintaining adhesive homeostasis. Cardiomyocytes are linked end-to-end by intercalated discs (ICDs), specialized junctions that couple cell signaling, electrical and mechanical operations. The adherens junction (AJ) couples the actomyosin network to the ICD to provide structural integrity and mechanical continuity. However, little is known about how the actin cytoskeleton is linked to the ICD or how ICD AJs resist the mechanical forces of contraction to preserve tissue integrity.

The core of the AJ is the cadherin-catenin complex, and alpha-catenin is the primary link between the AJ and actin. In epithelia, the AJ senses and responds to mechanical force through tension-dependent changes in alpha-catenin conformation that reveal binding sites for actin-binding proteins like vinculin and afadin. To understand the AJ-actin interface at the ICD, we combined proteomics with confocal and electron microscopy to define the composition and architecture of AJs in mouse neonatal cardiomyocytes. We first used proximity biotinylation and mass spectrometry to identify proteins associated with the N-cadherin cytoplasmic tail along the ICD membrane. A number of alpha-catenin ligands were identified, including vinculin and afadin. Immunostaining revealed that both were recruited to myofibril integration sites at AJs in cardiomyocytes. Transmission electron microscopy revealed that myofibrils were coupled at chevron-shaped AJs, similar to junctions between mature cardiomyocytes in the heart and reminiscent of tension-bearing tricellular AJs in epithelia.

We postulated that vinculin and afadin were recruited to alpha-catenin to strengthen the AJ-actin interface at the ICD. To delineate roles of alpha-catenin, vinculin and afadin ligand recruitment and AJ stability, we built a series of EGFP-tagged N-cadherin:alpha-catenin fusion constructs. We found that vinculin and afadin were recruited independently to the N-cadherin:alpha-catenin fusions in a tension-dependent manner. Vinculin recruitment was sufficient to localize and stabilize N-cadherin at the ICD membrane in cardiomyocytes, suggesting that vinculin stabilizes the AJ at sites of myofibril integration. In contrast, afadin recruitment was sufficient to localize N-cadherin but did not promote N-cadherin stability, indicating that afadin alone cannot replace vinculin in linking AJs to myofibrils. We propose a model in which alpha-catenin, vinculin and afadin cooperate to form a force-resilient AJ that anchors myofibrils at the ICD membrane and establishes mechanical continuity between cardiomyocytes.
P3417
Board Number: B708
Talin is related to cadherin-mediated cell-cell adhesions through its direct-binding domains to catenins.
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Talin was molecularly considered to be exclusively involved in integrin-mediated adhesions between cells and matrix/cells. Our previous study found that a C-terminal region of talin resultant from a calpain proteolytic cleavage, called the VAD fragment, is located within cadherin-mediated cell-cell contacts and enhances this type of adhesion complex. However, it still remains unknown how talin, or its fragment, relates to the cadherin adhesion complex. In this study, by using immuno-coprecipitation and Mass Spectrometry, we found that the VAD fragment of talin has previously unknown binding capacities for beta- and alpha-catenins, which are well-established components within the cadherin complex. By using purified recombinant proteins, we confirmed that the VAD fragment of talin has direct binding affinities for both catenins. Furthermore, the presence of the VAD fragment appears to drastically enhance the interaction between alpha- and beta-catenins, which would explain the functional role of the VAD fragment in enhancing cadherin-based adhesions. Finally, by expressing smaller truncations of the VAD fragments fused with fluorescence proteins in mammalian cells, we found that several discreet domains are required for the localization of the fragment into the cadherin mediated adhesion sites. Our study is among the first to suggest that talin contains direct binding capacities to catenins, which may constitute a novel mechanism mediating the interaction between integrin- and cadherin-based adhesions.

P3418
Board Number: B709
E-cadherin mediated homotypic cell–cell interaction confers cytokine independence in human erythroleukemia.
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Pure erythroid leukemia (PEL) is a rare and aggressive cancer (~3 months of average survival from diagnosis) with unknown etiology. Since cytokine signals control hematopoietic stem cell functions, we hypothesized that cytokine-independent survival and proliferation of erythroid progenitor cells is crucial for PEL development. To test this hypothesis, a novel cytokine-independent subline, UT-7/EI, was developed from erythropoietin (Epo)-dependent erythroleukemic progenitor cell line UT-7/Epo. The UT-7/EI cells survived without Epo and formed aggregates as they proliferated, suggesting augmented cell-cell adhesion. In agreement with this, differential gene expression profiling and network analysis uncovered the enrichment of E-cadherin (CDH1)-mediated homotypic cell-cell adhesion pathway in UT-7/EI cells. The upregulation of CDH1 mRNA and protein was further validated using qRT-PCR (6.5-fold relative to UT-7/Epo, p < 0.01) and immunoblot analysis. In confocal imaging studies, E-cadherin-β-catenin adhesion complexes were concentrated at adherence junctions of UT-7/EI cells. Moreover, calcium chelation abrogated cell-cell adhesion and induced the death of UT-7/EI cells. To further understand the role of CDH1 in cytokine-independent survival of UT-7/EI, its expression by Epo was investigated. Relative to untreated controls, Epo suppressed the expression of CDH1 in UT-7/EI cells at 24 hrs (4.3-fold), 48 hrs (16.7-fold), and 72 hrs (12.8-fold) with a corresponding increase in SNAIL2 expression (4.9-fold at 48 hrs), a repressor of CDH1 transcription (p < 0.01). Consequently, Epo abrogated the cell-cell adhesion of UT-7/EI cells without affecting their viability. These results suggest
that CDH1 and Epo-dependent survival pathways are mutually exclusive. In CFU-GEM assay, UT-7/E1 cells formed erythroblast-like colonies with positive heme staining and high CD36 expression (3.22-fold relative to UT-7/Epo, p ≤ 0.05), a marker of immature erythroblasts in PEL. In summary, our study identified a role for CDH1-mediated cell-cell adhesion pathway in developing cytokine-independence in PEL. Therefore, suppression of CDH1-mediated homotypic cell-cell adhesion may improve therapeutic outcomes in cytokine-independent PEL.

P3419
Board Number: B710
Analysis of biological networks with biomimetic microsystem platforms.
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Post-translational modifications and nonsynonymous single nucleotide polymorphisms can significantly alter protein biological function and influence responses of individuals to pathogens, medications, and diseases. Understanding how small changes in protein composition influence specific communication with other proteins is of greatest importance for personalized medicine. Here, we describe a novel system approach for analysis of complex interactions within biological systems. Using His-tagged proteins covalently-linked to Ni-NTA beads as a biomimetic microsystem platform in combination with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) followed by our enrichment pathway, we analyzed how phosphorylation of End Binding protein 3 (EB3) influences the protein complex formation and signaling networks in primary human endothelial cells. We found that this EB3 “phospho-switch” resulted in increased activation of the cell-cell adhesion pathways and decreased activation of the cell proliferative pathway. We propose this microsystem platform may be a valuable tool in identifying activation of different biological networks upon changes in protein structure, which can be found in disease states.

P3420
Board Number: B711
Differential Recruitment of Afadin and EPLIN to E-cadherin Adhesions.
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E-cadherin adhesions are essential for the dynamic events of morphogenesis as well as for the maintenance of the architecture of adult epithelial tissues. In epithelial tissues, E-cadherin from one cell binds to E-cadherin from the neighboring cell, couples to the actin cytoskeleton in each cell and transmits cell-generated actomyosin forces across the tissue. However, it does so at cell-cell interfaces that have multiple types of cell-cell adhesions molecules which may or may not influence E-cadherin function, depending on the context. To clearly delineate the function of E-cadherin adhesions alone in organizing the actin cytoskeleton, we created a biomimetic interface with oriented, immobilized E-cadherin ectodomains that supports the formation E-cadherin adhesions alone, with C2bb epithelial cells. In contrast to glass surfaces coated with randomly oriented E-cadherin ectodomains, this interface avoids the formation of other adhesions such as focal adhesions. We wanted to determine how certain key proteins implicated in linking E-cadherin to the actin cytoskeleton are localized at E-cadherin adhesions in the absence of other cell-cell adhesions. We found that afadin, which is a cytoplasmic partner of the nectin adhesion molecule, can be recruited to E-cadherin adhesions in the absence of
nectin based adhesions. In contrast, we found that EPLIN, which has been shown to link the E-cadherin-catenin complex to F-actin, did not concentrate at E-cadherin adhesions. Rather, it localized to actin filaments proximal to E-cadherin adhesions. These results show how different proteins that can constitute the E-cadherin-actin link can be differentially localized in the presence of just E-cadherin adhesions.

P3421
Board Number: B712
Septins regulate VE-cadherin – mediated junctional integrity of human endothelial monolayers.
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The junctional integrity of endothelial monolayers plays crucial roles in controlling transmigration of molecules and immune cells. Both scanning electron microscope (SEM) and transmission electron microscope (TEM) images of the junctions of human microvascular endothelial monolayers showed dynamic features of membrane, which suggested that membrane-associated proteins regulated junctional integrity. In this study, we demonstrated that Septin protein regulated VE-Cadherin-mediated junctional integrity. Immunofluorescence staining showed that septin2 was localized at the junctions of endothelial cell monolayers, yet was not co-localized with VE-Cadherin and actin filaments. Structured illumination microscope (SIM) super-resolution images showed curve and scallop-shaped polymer at the junctions, especially, at the regions where membrane dynamically protruded. Here, we have found that shRNA technique-mediated septin 2 suppression altered the arrangement of VE-Cadherin at the junctions and up-regulated expression levels of VE-Cadherin, which contributed to the reduction of transendothelial electric resistance (TEER) and transmigration event of immune cells. All together, we suggested that cytoskeletal protein, septin2 played an important role in junctional integrity of endothelial monolayers.

P3422
Board Number: B713
The interplay of WAVE-dependent branched actin and Cadherin junction components promotes Cadherin trafficking and junctional maturation.
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Our lab studies embryonic morphogenesis in C. elegans. Our earlier work showed that branched actin, powered by the WAVE/SCAR and Arp2/3 complexes, promotes polarized events during epithelial tissue development. These events include epidermal cell migration during ventral enclosure, and the polarized formation of the embryonic intestine. The principal indicators of a fully polarized epithelial cell are apical junctions, which promote adhesion and communication between the cells. Actin is essential for cell polarity and is an integral component of the epithelial apical junction, yet the interactions of branched actin regulators with apical junction components are not completely clear. Conflicting findings have further raised questions on its role. It is widely accepted that branched actin contributes to the initial formation of apical junctions. However, only recently has the role of branched actin and its regulators in junctional maturation been established. The C.elegans Apical Junction (Ce AJ) contains independent complexes, the cadherin/catenin complex (CCC), and the DLG-1/AJM-1 complex (DAC).The C. elegans CCC is comprised of homologs of classical E-cadherin (HMR-1), α-catenin (HMP-1), β-catenin (HMP-2). In this study we address how branched actin regulators contribute to apical junction maturation. We have
used live imaging of C. elegans embryos and newly available CRISPR-tagged strains (from our lab and the Goldstein lab) to define the developmental time course of accumulation of apical junction components relative to the branched actin regulators WVE-1 and ARP-2/ARX-2 as the embryonic intestinal epithelium becomes polarized. Live imaging of WVE-1 and Cadherin components allow us to address how the WAVE complex and Cadherin components regulate each other to promote the development of the apical junction. Newly developed transgenic and endogenously tagged strains allow us to image intestinal apical actin enrichment in live embryos undergoing junction formation to clarify in a better way how the two main C. elegans apical junction complexes contribute to apical actin during development. Our FRAP studies suggest that WAVE contributes to Cadherin trafficking by regulating the dynamics of Cadherin turnover at the developing apical junction. Comparison of the WAVE effects on Cadherin dynamics to known trafficking components identifies a new role for WAVE dependent branched actin in Cadherin trafficking at the apical junction. Thus, in this study we propose a developmental model for junction formation where branched actin regulators are tightly interconnected with Cadherin junctions through their previously unappreciated role in Cadherin transport.

P3423
Board Number: B714
VE-PTP Scaffold Function in Adherens Junction Stabilization.
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Vascular Endothelial Protein Tyrosine Phosphatase (VE-PTP) is an endothelial-specific phosphatase that dephosphorylates multiple proteins including Vascular Endothelial (VE)-cadherin, the main adhesion protein of the adherens junctions (AJ). However, the basal role of VE-PTP in stabilization of VE-cadherin adhesion complexes remains unclear. VE-cadherin establishes adhesion events through trans-interaction with opposing VE-cadherin molecules at AJs. In resting endothelial monolayers, it undergoes continues exchange between junctional and cytosolic pools. Using VE-cadherin tagged with the photoconvertable protein Dendra2, we have demonstrated that VE-PTP regulates basal VE-cadherin steady-state kinetics at AJs in a phosphatase-independent manner. Overexpression of wild-type (WT) or phosphatase “dead” VE-PTP mutant similarly decreased the VE-cadherin dissociation rate from AJs as compared to control cells expressing empty vector. In agreement with these results, VE-PTP depletion increased VE-cadherin dissociation rate. Because Rac1 stabilizes AJs by reducing mechanical tension across VE-cadherin adhesion, we analyzed junctional activity of Rac1 as well as the tension applied to VE-cadherin using respective FRET biosensors. We observed that overexpression of VE-PTP caused both Rac1 activation at AJs and reduction of tension across VE-cadherin adhesion, accompanied by an inhibition of RhoA activity. VE-PTP knockdown decreased Rac1 activity, increased RhoA activity, and increased tension/stress at AJs. Interestingly, binding partner studies indicate that VE-PTP binds to and inhibits GEF-H1, a RhoA activator. Further studies will elucidate the role of VE-PTP in endothelial barrier stabilization through GEF-H1 interaction.
P3424

Board Number: B715
TRIP6 inhibits the Hippo signaling pathway in response to tension at adherens junctions.
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The Hippo pathway regulates cell proliferation, contact inhibition, apoptosis, and stem cell maintenance and differentiation. The pathway responds to changes in the mechanical environment of the cell including tension across the tissue. Although tension is an important regulator of various cellular behaviors, how tension is sensed and the signal transduced to control Hippo pathway activity is unclear. The Hippo pathway kinases LATS1/2 regulate cellular responses to mechanical stimuli through the transcriptional co-activator YAP. Here, we show that the human LIM domain protein TRIP6 acts through LATS1/2 to regulate YAP activity and localization in response to tension at cell-cell junctions. TRIP6 binds directly to LATS1/2 and inhibits its activity. TRIP6 can inhibit LATS1/2 activation by competing with the LATS1/2 activator MOB1 for binding to LATS1/2. We show that TRIP6 recruits LATS1/2 to cell-cell junctions in response to tension. Conditions that reduce tension at cell junctions by inhibition of actin stress fibers (by treatment with actin or myosin disrupting drugs, serum starvation, or high cell density) block both TRIP6-LATS1/2 binding and localization to cell-cell junctions. In contrast, stretching cells stimulates recruitment of TRIP6 and LATS1/2 to adherens junctions. TRIP6 binds to the known tension responsive adherens junctions protein vinculin, and binding of TRIP6 to vinculin is also tension sensitive. Tension-dependent recruitment of TRIP6 to adherens junctions, TRIP6-LATS1/2 binding, and YAP activity all depend on vinculin. Together these results indicate that TRIP6 acts as part of a mechanical sensor at adherens junctions to mediate cellular responses to tension across tissues through control of the Hippo signaling pathway.

P3425

Board Number: B716
Alpha-T-catenin N-terminus functions with the M-region to regulate vinculin binding.
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Cardiomyocyte contractions place unique physical and regulatory demands on the protein complexes that join these cells together to form the heart. Cardiomyocytes are connected by intercalated discs (ICDs), specialized junctions that couple signaling and mechanical operations. The ICD comprises adherens junctions (AJs) and desmosomes that connect the actin and intermediate filament cytoskeletons, respectively, to the plasma membrane. The core of the AJ is the cadherin-beta-catenin complex, and alpha-catenin serves as the primary link between the complex and actin. Two alpha-catenin proteins are expressed in the mammalian heart, alpha-E(Epithelial)-catenin and alpha-T(Testes)-catenin. Mechanical tension regulates alpha-E-catenin conformation: actomyosin-generated force stretches the M(middle)-region to reveal binding sites for cytoskeletal proteins like vinculin. Intramolecular interactions maintain the alpha-E-catenin M-region in an autoinhibited state to limit vinculin binding in the absence of force. However, little is known about the molecular properties of alpha-T-catenin. Here we show that the alpha-T-catenin N-terminus cooperates with the M-region to regulate vinculin binding. Limited proteolysis experiments revealed that the alpha-T-catenin M-region adopts a more open conformation than alpha-E-catenin. Furthermore, isothermal titration calorimetry
(ITC) experiments showed that the alpha-T-catenin M-region binds vinculin D1 (N-terminus) with low nanomolar affinity, demonstrating that the alpha-T-catenin M-region is not autoinhibited. However, the alpha-T-catenin head domain (N-terminus plus M-region) binds vinculin approximately 1000-fold more weakly, suggesting that the N-terminus influences M-region binding to vinculin. The beta-catenin/N-cadherin complex binds alpha-T-catenin with a similar affinity to that of alpha-E-catenin but, notably, beta-catenin binding does not affect the interaction between alpha-T-catenin and vinculin. Finally, experiments in epithelial cells show that alpha-T-catenin can recruit vinculin to cell-cell contacts. Together, our results suggest that the N-terminus functions with the M-region of alpha-T-catenin to regulate vinculin binding. We postulate that the distinct molecular properties of the alpha-T-catenin M-region are optimized to meet the structural and mechanical demands of adhesion along the ICD membrane in cardiomyocytes.

**P3426**

**Board Number: B717**

**Evolutionary Rate Covariance (ERC) analysis identifies regulators of intercellular adhesion in Drosophila.**

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Adherens junctions (AJs) are cadherin-based complexes that are required for cell-cell adhesion. It is critical to understand the genetic basis of these structures since their disruption is the primary cause of many developmental abnormalities and deadly diseases. Cadherins recruit the cytosolic catenin proteins p120-catenin, beta-catenin and alpha-catenin to form the core cadherin:catenin complex. Many secondary binding proteins, such as vinculin, afadin and ZO-1 have been identified in recent years as important catenin binding partners that regulate and stabilize AJs. Nevertheless, additional proteins may influence AJ biology. To identify genes that regulate AJ biology, we used a computational Evolutionary Rate Covariation (ERC) analysis screen to identify genetic components of the cadherin regulatory machinery. ERC relies on the principle that genes that function in common pathways and complexes have evolutionary rates that covary between species. ERC analysis has been used to identify novel gene functions in yeast, flies and mammals. We applied ERC analysis to generate a list of >100 genes with high ERC values relative to Drosophila shotgun (DE-cadherin). To test whether the identified genes function in cadherin-based adhesion, we used an in vivo RNAi-based knockdown approach in the Drosophila egg chamber to examine follicular epithelium (FE) organization and border cell (BC) migration. In the FE, we generated clones of RNAi expressing cells by using FRT/FLP recombination system in conjunction with GAL4-UAS expression system. Interestingly, knockdown of the candidate genes did not disrupt FE organization. It is possible that the AJs in this tissue are resilient to minor genetic perturbations induced by RNAi expression. Next, we used a silo-GAL4 construct to drive expression of RNAi specifically in the BCs. In contrast to FE, knockdown of multiple (18/35 tested) candidate genes caused either a delay in BC migration or disrupted BC cluster adhesion. BC migration and adhesion are cadherin-dependent, suggesting that these genes play a direct or indirect role in regulating AJs. Notably, mammalian orthologs of several identified genes – including CG16952 (Btbd7), CG42684 (Rasal2) and PDZ-GEF (Rapgef6) – have potential roles in AJ formation. For example, Btbd7 is a known nuclear repressor, which regulates E-Cadherin transcription and epithelial cell motility, and Rasal2 is a tumour suppressor with roles in epithelial to mesenchymal transition. Our results suggest that ERC analysis is an effective tool to identify components of the AJ complex in Drosophila. Our study also highlights the number of genes that converge together to regulate AJ-mediated intercellular adhesion.
P3427

Board Number: B718

Identification of cardiomyocyte adhesion complexes by proximity proteomics.

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Cell-cell adhesion is essential for tissue function and integrity. Cardiomyocytes possess a specialized adhesion architecture, the intercalated disc (ICD), adapted to withstand the forces of contraction. The ICD is composed of adherens junctions, desmosomes and gap junctions. Adherens junctions, through their direct linkage to actin cytoskeleton, transduce contractile force across neighboring cardiomyocytes. The core of the adherens junction is formed by the cadherin-catenin complex. N-cadherin is the classical cadherin expressed in cardiomyocytes; however, the proteins associated with the N-cadherin complex along the ICD membrane are largely unknown. We used biotin ligase-mediated proximity labeling and mass spectrometry to identify proteins in close proximity to the C-terminus of N-cadherin in mouse primary neonatal cardiomyocytes. We identified over 250 proteins. Approximately 120 are recognized components of the E-cadherin interactome in epithelial cells and 30 are known ICD proteins. Notably, more than 100 of the identified proteins are neither associated with the adherens junction in epithelia nor recognized at ICD proteins. Enrichment analysis suggested that these novel proteins are highly enriched in cardiovascular disease related proteins. Protein interaction networks constructed using known protein interactions provide a holistic view of cytoplasmic complex organization along the ICD. Our study is among the first to identify and resolve the proteins associated with N-cadherin at cardiomyocyte cell-cell contacts. We identified more than one hundred novel protein components of the adhesion complex, providing a proteomics-based reference for the adherens junction complex in cardiomyocytes.

Bioengineering of Cell-Matrix Interactions

P3428

Board Number: B719

Three-dimensional modeling of metastatic breast cancer dormancy using tunable PEG-based hydrogels.

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Metastasis is the leading cause of breast cancer-related mortality, with only a few palliative treatment options available for patients. One of the major challenges of targeting metastatic disease is the presence of disseminated tumor cells (DTCs), that lie dormant in secondary organotropic niches within the body for extended periods of time. Specifically targeting these DTCs as an approach towards reducing metastatic outbreaks is difficult owing to their quiescent behavior, poorly understood cellular characteristics, and limits of clinical detection. Tumor dormancy is manifested in one of two ways: cellular dormancy (G0-G1 arrest) or dormant micrometastasis (angiogenic dormancy). Targeting the dominant mechanism determining metastatic breast tumor dormancy is of prime interest toward suppressing metastatic outbreaks and improving patient survival rates.

To establish an in vitro system for improved understanding, detection, and treatment of dormant breast cancer, we have developed a three-dimensional (3D) hydrogel culture platform for the recapitulation of key microenvironmental features of the dormant niche. In this study, we encapsulated MDA-MB-231
metastatic breast cancer cells within cell-responsive peptide-conjugated poly(ethylene glycol diacrylate) hydrogels and maintained them in 3D culture for 15 days. The hydrogel stiffness and cell-adhesiveness were modulated through the introduction of additional crosslinking polymer networks and cell-adhesive ligands to cover a wide range of extra-cellular matrix (ECM) characteristics. MDA-MB-231s encapsulated within these hydrogel matrices were investigated for viability (via Live/Dead staining), early-stage apoptosis (via Annexin V staining), and proliferation (via EdU staining) every 5 days post-encapsulation for a total of 15 days.

Our results indicate that cancer cell dormancy is dually dependent on cell-adhesiveness and matrix stiffness. Increasing stiffness leads to reduced proliferation but also increased apoptosis while increasing matrix adhesiveness leads to higher proliferation. The cell proliferation and viability were quantified to vary between 5-10% and 80-90% respectively under the tested conditions, thereby demonstrating that an optimum matrix formulation has been devised that restricts proliferation of cancer cells through cellular dormancy while still maintaining high viability in 3D culture. This platform is being further implemented to provide insight into the cellular and molecular characteristics of dormant breast cancer cells and to facilitate investigation of organotropite dormancy behavior that could aid in future cancer drug discovery initiatives.

P3429
Board Number: B720
Matrix malleability regulates cancer cell migration through confining microenvironments.
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Breast cancer cells must invade and migrate through basement membranes (BM) to metastasize during ductal carcinoma progression. Studies of cancer cell invasion and migration have shown that there are two modes: one that is protease-independent, involving cells squeezing through micron-sized pores, and one that is protease-dependent, involving cells biochemically degrading matrix with invadopodia protrusions. Invasion of the BM is thought to require protease degradation, due to its confining, nanometer-sized pores. However, many studies investigating how pore size limits invasion have utilized rigid or elastic pores. In contrast, many physiological tissues and extracellular matrices (ECM) are viscoelastic, with matrix able to flow in response to applied forces. Matrix flow can be plastic or irreversible, making the matrix “malleable”. Malleability can be related to matrix viscosity, but is distinct from matrix stiffness. Here, we show that matrix malleability enables invasion and migration of breast cancer cells through confining microenvironments. We developed a set of interpenetrating network (IPN) hydrogels for 3D culture in which malleability could be modulated independent of stiffness, and which were nanoporous, minimally degradable, and presented BM ligands. Strikingly, cells in high malleability IPNs exhibited spread morphologies and carried out protease-independent migration through the nanoporous IPNs, while cells in low malleability IPNs mostly stayed rounded and did not migrate. Mechanistically, cells in high malleability IPNs first extended invadopodia protrusions, identified by localization of cortactin and Tks5. Invadopodia exerted both protrusive and contractile forces to initiate invasion. Then, cells generated protrusive forces at the leading edge during migration, plastically opening channels in the matrix to migrate through. While the function of invadopodia was thought to be exclusively for ECM degradation, these results demonstrate an additional function of invadopodia is in generating force. These findings reveal a new mode of protease-independent migration, in which cells generate force to migrate through confining matrix if the matrix is sufficiently malleable.
P3430

Board Number: B721

Synergistic regulation of skeletal muscle maturation with sphingosine-1-phosphate and biomimetic matrix nanotopography.

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Study Objective: We have developed an approach in which the benefits of biomimetic matrix nanotopography and sustained sphingosine-1-phosphate (S1P) signaling could be harnessed synergistically to induce the formation of structurally organized skeletal muscle tissues that are more mature.

Methods: Substrate Fabrication and Functionalization with S1P: Substrates were generated using a capillary force lithography (CFL) technique in which PLGA is nanopatterned with a mold applied with constant pressure and heat. For this study, substrates with 800 x 800 x 600 nm (groove width x ridge width x groove depth) feature sizes were used. Fabricated substrates were functionalized with S1P using 3,4-dihydroxy-L-phenylalanine (DOPA).

Cell Isolation and Culture: Mice were sacrificed 3 days after cardiotoxin-induced injury. Injured muscles were harvested under sterile conditions and digested tissue was passed through strainers to remove debris, muscle fibers, and multinucleated cells. Resulting mononuclear cells were then seeded onto each respective substrate and cultured for 10 days.

Results: Nanopatterned substrates induced a greater degree of structural organization in the form of aligned myotubes. These substrates also appeared to enhance the myogenic development of cultured progenitor cells, as a greater number of MHC+ myotubes were observed in the patterned environment. When coupled with S1P signaling, myogenesis and subsequent maturation was further enhanced. The expression of \textit{MyoD}, \textit{MyoG} and \textit{Myh15}, which are expressed in late-stage or terminally differentiated muscle cells, was greater in cells not only on patterned substrates, but also significantly in cells on substrates with 175 \(\mu\)M S1P. Experiments were conducted to determine whether the maturation observed as a result of the synergistic signaling from S1P and nanopatterning translated to improvements in myotube function. Myotube contraction displacement and velocity, as well as Ca\(^{2+}\) flux, was significantly increased in tissues cultured on nanopatterned substrates with 175 \(\mu\)M S1P. Flox-out of S1P receptor 1 in cultured cells resulted in a lack of S1P-mediated maturation, while nanotopographical guidance cues were still observed to exert an effect.

Conclusions: We have demonstrated that by harnessing signaling cues from both biomimetic nanotopography and S1P, it is possible to enhance the maturation and overall function of cultured skeletal muscle progenitors all without the need for incorporating multiple growth factors.
P3431
Board Number: B722
A contractile hoop stress aids in balancing sudden hydrostatic pressure perturbation in a tubular epithelium.
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Epithelium lines major organs where functional tubular structures consist of polarized and differentiated epithelial cells, such as endothelium lining the arterial walls and kidney proximal tubule epithelium. These epithelia are constantly subjected to hydrostatic pressure stresses which can result in ruptures. As seen in aging and inflammation, epithelium rupture imposes detrimental effects. Therefore it is important to understand how epithelium maintain homeostasis upon rapid changes in hydrostatic pressure that might result in ruptures.

We have developed a microfluidic device to investigate the response of epithelium to hydrostatic pressure changes. A mono-layered MDCK\textsubscript{II} epithelium lines a tubular channel in collagen (7mg/ml rat-tail Type-I) of diameters ranging from 75um to 150um. Via time-lapse imaging, the dynamics of the collagen channel with and without an epithelium were measured in response to varied hydrostatic pressure perturbations (~1kPa). A rapid increase in channel diameter was first detected because of the elasticity of the tissue, followed by the drop from the maximum to a constant value for a long period, dictated both by elasticity and permeability of the material. The dynamics can be modeled in terms of poro-elastic responses. Our hypothesis was tested in collagen gels formed at different gelation temperatures and cross-linking conditions. While the system without cells deformed by ~5\%, the epithelium deformed by ~2\% upon sudden increase in hydrostatic pressure. It was found that epithelium balances the effect of sudden perturbation by a contractile hoop stress. Theoretical modelling revealed that this constant hoop stress acts in a way that the outcome is an increment in the effective stiffness of the epithelial layer (previously reported to be ~10-20kPa) and that it exists in a pre-stressed condition. This suggests that the epithelium behaves in a non-linear regime even at low levels of perturbation as opposed to previous findings.

Quite intriguingly, upon perturbation by higher hydrostatic pressures(~2-5kPa), the tubular epithelium manifested identical responses as the collagen, suggesting the contractile hoop stress by epithelium is disrupted under such condition. In summary, we have devised a platform to simulate the tissue responses to hydrostatic perturbations and characterized the differential dynamic behaviors upon variable parameters.

P3432
Board Number: B723
T is for Tension: Micropatterns, Machine Learning, and the Role of the Microenvironment in Mesoderm Induction.
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Rapid advancements in stem cell biology over the past two decades have motivated exciting new directions in tissue engineering. As the possibility of tissue and complete organ culture in vitro moves closer to reality, the need for an efficient production of fully specified cell populations is moving to the fore. In contrast to the large body of literature uncovering a myriad of chemical cues which can be used to achieve efficient directed differentiation, there has been relatively little investigation into the role of
the mechanical microenvironment. In this work, we discuss the interplay between cytoskeletal tension and cell-cell contact during mesoderm induction. Decreasing cell density increases tension and we show that at early stages of mesoderm commitment, increasing tension increases Brachyury expression and thus differentiation efficiency. On the other hand, we show that without sufficient cell-cell contact, at late stages of mesoderm commitment there appears to be an absence of endothelial committed populations. Only in the unique microenvironment at the edge of small patterns do we find the balance of high cell-cell contact and high tension to generate both high differentiation efficiency and spatial organization. We go on to develop a learning algorithm used to predict which environments are likely to give rise to differentiated populations and those which may remain stem. Hopefully these predictions may be used to aide the design of new tools for directed differentiation.

P3433
Board Number: B724
Plasticity in cell migratory modes on orthotropic fiber architectures.
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Cell migration is crucial in wound healing, organ development, tissue regeneration as well as pathological conditions such as cancer metastasis. It is now well appreciated that biophysical cues imposed by the fibrous extracellular matrix (ECM) immediately surrounding the cell regulate its migratory behavior. Cells migrate efficiently (high persistence over long distances) through the ECM by adopting a variety of shapes and motility mechanisms \((1, 2 \text{ and } 3D)\) as they squeeze, push or tug through the dense or sparse and aligned or random ECM in a process commonly referred to as plasticity. Cell migration \textit{in vitro} has been traditionally studied on 2D flat surfaces, and recently in 3D gels. These studies have highlighted the responsiveness and plasticity of cell migration to material stiffness, but lack the ability to capture the role of ECM fiber curvature, orientation, and organization on migration in a repeatable and controlled manner. Here, we demonstrate that crosshatch fiber networks of controlled inter-fiber unit cell spacing \((3,6,18,36 \text{ and } 54 \mu\text{m})\) regulates plasticity of the migratory phenotype \((1, 2 \text{ and } 3D)\) of metastatic thyroid cancer (Hras1) cells. Contrary to intuition, cells on dense networks \((3 \text{ and } 6 \mu\text{m})\) exhibit polarized migration in shapes as observed in 3D gels. At medium spacing \((18 \text{ and } 36 \mu\text{m})\), cells attain traditional 2D shapes of a broad leading edge and a narrow trailing edge, with the migration described by a diffusive random walk model. Finally, at large spacing \((54 \mu\text{m})\), cells attain stable non-migratory kite shapes at intersections or migratory spindle 1D shapes on suspended fibers in between the fiber intersections. Cells in dense networks have elongated morphologies (aspect ratio: \(5.5\pm0.2\)) and short focal adhesion cluster (FAC) lengths resulting in fastest migration. The migration speed decreases with increasing scaffold porosity due to longer FAC lengths (FAC length: \(5\pm0.4, 3.8\pm0.2, 7.7\pm0.3, 12.9\pm1.4, 15\pm0.8 \mu\text{m}\) and Speed: \(46.9\pm4.6, 41.7\pm2.8, 33\pm2.7, 31\pm2.3, 25.3\pm1.5 \mu\text{m}/h\) on 3, 6, 18, 36 and 54 \(\mu\text{m}\) respectively). This consequently affects the dynamics of migration, as wider spacing networks favor a distinctive \textit{recoil} of the trailing edge, which causes the nucleus to undergo rapid deformation followed by a gradual relaxation. Thus, faster relaxation times of the nucleus are recorded in cells moving on dense networks, thereby indicating potentially softer cell bodies, which is generally considered a biophysical marker for enhanced metastatic potential. Taken altogether, our approach of precisely controlling fiber diameter and spacing provides new means to study plasticity in cell migration and notably for the first time shows that anisotropic substrates are not the ‘only’ requirement for achieving persistent cell migration.
P3434
Board Number: B725
Penetrability of Prostate Tumor Cells Grown in 3D Culture by Prostate Targeting Agents.
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Properly modeling tumor anatomy remains an issue in understanding aspects of cancer, such as metastatic potential, and in designing agents which effectively penetrate the tumor mass. Objective: The purpose of this project is to develop tissue culture methods which allow tumor cells to grow as a 3D tumor mass rather than as a flat 2D culture. Methods: Culturing via cellular matrices such as Matrigel allows the ability to culture cancer cells into spheroids, which model better a tumor’s anatomy. Results: Such culture methods allow for the testing of Targeted Molecular Imaging Agents (TMIAs) which bind to specific biomarkers on the cancer cells allowing measurement of the number of biomarker molecules on a tumor cell and testing the TMIAs' penetrability. The localization of our TMIAs, A1 and B1, which are bound to the dye Cy5.5, was tested on the prostate cancer line C4-2 which overexpresses the marker prostate specific membrane antigen (PSMA). 3D culturing and spheroid formation of the line was achieved by changing the Matrigel concentration. The cell line PC-3 (PSMA-) was used for the negative control to demonstrate specificity of the TMIA. Conclusion: It was demonstrated that the TMIAs were able to penetrate the internal cells of the spheroids and bind to the PSMA molecules on the surface of the cells. This may allow the TMIAs to be effective in various applications including treatment and in-vivo tumor imaging. Determining the binding of TMIAs to target cancer cells should facilitate the development of TMIAs and result in molecules which target to metastatic tumors illuminating their presence, size, and structure thus allowing better clinical treatment and hopefully enhanced cancer survival.

P3435
Board Number: B726
Cell-cell adhesion and myosin activity controls the curvature-dependent cortical actin assembly in mammary gland epithelium.
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It has been demonstrated in recent studies that geometry might play an important role in cell behaviors, ranging from mucus production, cell migration and the origination and prorogation of cancer stem cells. Despite the studies on potentially curvature-sensitive proteins at the nanoscale, it is not clear how cells sense curvature and other geometric parameters at cellular and multi-cellular-level. To systematically study whether and how curvature determines cell behaviors, we developed a technique combining 3D printing and soft lithography to fabricate open channels with radii ranging from 40µm to 90µm. We examined various protein expression levels of mouse mammary epithelial cells EPH4-EV cells at different curvatures. EPH4-EV cells are used to simulate the formation of mammary gland epithelium, which has been observed previously to exhibit differential phenotypes depending on the local geometry of the mammary ducts. We found that cortical actin is 1.3-fold more enriched in cells grown on curvature of 1/60µm and 1.5 fold more enriched in 1/40µm when compared to curvature of 1/90µm. Inhibiting myosin phosphorylation via blebbstatin restored the cortical actin formation in cells cultured in channels of high curvatures, implying myosin is involved in curvature-dependent cortical actin assembly. Furthermore, it was also observed that cell-cell contact is required in the curvature-dependent cortical
actin assembly. The prominent cortical actin assembly usually seen at 1/40μm and 1/60μm was not
detected when the cell seeding density resulting in sparse cell-cell contact. Our finding suggests myosin-
mediated cortical actin assembly might be a length scale-appropriate machinery for curvature sensing at
the multi-cellular level.

P3436

Board Number: B727

Maskless Quantitative Multi-protein Photopatterning to orchestrate cellular microenvironment.
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Cell biology is faced with significant challenges when attempting to create complex microenvironments
to unravel intricate mechanisms involved in cell adhesion, cell polarity, cell migration etc... These
challenges can be overcome by molecular printing which involves the controlled deposition of molecules
on a substrate at the micrometer scale. These approaches have developed tremendously in the past few
years and micropatterned substrates are now routinely used for biological research. To yield biologically
relevant data, printed biomolecules should mimic the complexity of the in vivo microenvironment.
Micrometer-scale gradients of multiple proteins are thus highly desirable. Here we present a maskless
quantitative multi-protein photopatterning solution which is based on the light-induced molecular
adsorption of proteins (LIMAP) technology. This system combines a UV illumination module and a
specific photoactivatable reagent (PLPP). The combined action of UV-light and PLPP locally degrades
antifouling polymer brushes allowing for the adsorption of proteins in a well-defined area. PRIMO relies
on a wide-field DMD-based projection system coupled to an epifluorescence microscope to project
custom-defined patterns of UV light onto a cell culture surface. As a result, micrometer scale patterns
are generated within seconds. The remaining background allows for the sequential patterning of
multiple proteins. Controlled protein gradients of custom-defined shape can also be patterned. In
addition, PRIMO technology allows micro-manufacturing by photopolymerization of UV-sensitive
materials and protein patterning onto pre-existing 3D surfaces.
This technology empowers biomedical research in neurobiology, immunology, stem cell biology,
oncology and tissue engineering.

P3437

Board Number: B728

New approach to forming embryoid bodies of ES cells by using Low Adhesive Scaffold Collagen.
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Japan

[Objective] Embryonic stem (ES) cells are having an ability to differentiate and proliferate. That is, it has
the feature of propagating (self-replicating) while retaining the ability (pluripotency) to differentiate into
any cell constituting the tissue of the body. Generally, ES cells form planar colonies in maintenance
culture with or without feeder cells. On the other hand, when ES cells are differentiated into tissue cells,
cell aggregates called as embryoid bodies (EB) are formed. These days, we succeeded in developing low
adhesive scaffold collagen (LASCol, patent pending). On LASCol-coated dish, cells form spheroid. In this
study, we report that ES cells spontaneously form EB weakly adherent to LASCol. [Methods] Two types
of culture dish, coated with LASCol or gelatin, were used. Mouse ES cells were cultured on each coat
dish in which feeder cells were previously cultured. GFP fluorescence observation was used to
distinguish ES cells from feeder cells. To evaluate undifferentiated potential, we examined the alkaline phosphatase (ALP) activity of the ES cells and the mRNA expression levels of undifferentiation-related genes (Oct-4 and Nanog). The motility of EB was monitored by time-lapse observation with a microscope. [Results] Feeder cells and ES cells independently adhered to LASCol, and ES cells collided each other one after another and got to form EB with well maintaining ALP activity. No feeder cells existed in EB, and besides, ES cells on LASCol formed EB even without feeder cells. We confirmed that the undifferentiation potency of ES cells on LASCol was kept as similarly as gelatin-coated dish culture. [Summary and Conclusions] We demonstrated that LASCol has the ability to form embryoid bodies of ES cells and to maintain undifferentiated potential. [Funding] This work was supported by the Adaptable and Seamless Technology Transfer Program through target-driven R&D, AMED (AS2414037P to K.M.) and JST (AS2715177U to K.M.).

P3438
Board Number: B729
Active wetting of epithelial tissues.
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Development, regeneration and cancer involve changes in cell mechanical properties that lead to drastic transitions in tissue geometry and dimensionality. Given the fluid nature of living tissues, these transitions have been experimentally studied and theoretically modelled in terms of the physics of wetting phenomena, which describes how a fluid droplet spreads on a solid surface. However, physical forces, the effective determinants of tissue spreading, have never been measured in the context of tissue wetting. Here we perform a systematic study of tissue mechanics during epithelial wetting/dewetting. We induce a progressive expression of E-cadherin in a confined monolayer of MDA-MB-231 cells and, simultaneously, we measure tissue forces using Traction Force Microscopy and Monolayer Stress Microscopy. The gradual formation of intercellular junctions produces a continuous increase in tissue contractility (pMLC), triggering a two-fold increase in tissue forces that ends up in a spontaneous wetting-dewetting transition. To understand how this transition arises from tissue active properties, we develop a wetting model based on active gels theory. Combining theory and experiments, we find that wetting-dewetting transition results from a competition between contractility and traction forces, which introduces a new length scale, defining a critical size for tissue wetting. Strikingly, this implies that the critical tissue contractility driving the transition is dependent on tissue size, a phenomenon that has no counterpart in passive wetting/dewetting physics. Furthermore, we find that the critical tractions, which depend linearly on substrate ligand density, are the mechanical threshold for tissue spreading. Finally, we show that long-wavelength morphological instabilities in our fluid interface, together with active fluctuations, explain tissue shape dynamics during dewetting. We conclude that tissue spreading can be understood as an active wetting transition of a viscous polar fluid.
P3439

Board Number: B730

Developing Tunable Bioink for Versatile 3D Bioprinting.
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With the advances in instrumentation, 3D bioprinting technology has matured over the years for potential applications that require high precision in cell placement and geometry. For instance, in order to study cell migration, tissue repair, and tumor progression at physiological conditions, it is desirable to print organoids in mimicry of the in vivo tissue conditions. However, currently available bioinks lack the tenability in adjusting mechanical properties such as stiffness and porosity to represent in vivo conditions. We have developed biocompatible and tunable alginate-based bioink for in vitro tissue printing. By adjusting the concentration, gelling, and crosslinking conditions of the alginate/gelatin composite bioink, it can be used for direct ink writing to produce printed tissues at a 500 micron scale. The bioink is extruded from the printing head to form 3D geometries as supportive scaffolds for 3D cell culture. Gelatin is used as a rheological modifier to enhance the printability of the alginate ink. Complex, tissue-like 3D architectures can be achieved by embedding multiple cell types in our bioink. We also verified that fibroblasts were able to stay viable and replicate during a 7-day incubation period. In summary, our bioink is biocompatible, mechanically tunable and can form stable structures for 3D bioprinting. It is suitable to be used in organoid fabrication where physiological biophysical microenvironments can be recreated faithfully.

P3440

Board Number: B731

Extracellular matrix dimensionality reduces cellular cortical tension to stimulate Arf6/Rac/p38 pro-survival signaling in mammary epithelial cells.
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Tumor metastasis is limited by the ability of cells to survive varying microenvironmental stresses. Metastatic cancer cells encounter heterogeneous two dimensional (2D) or three dimensional (3D) extracellular matrix (ECM) landscapes with variable biophysical properties. Here, we interrogated the role of ECM dimensionality in cell behavior. We employed compliant polyacrylamide gels conjugated with defined ECMs to recapitulate ECM landscapes and interrogated the cellular biophysical and biochemical response to these parameters. We found that non-spread premalignant mammary epithelial cells (MECs) plated on compliant 2D polyacrylamide gels underwent apoptosis, whereas non-spread cells survive when overlaid with laminin. Traction force microscopy, atomic force microscopy
indentation, and laser ablation studies revealed that MECs encountering a 3D ECM have reduced cortical tension. Computational modeling predicted that such drop in cortical tension should lead to an increase in the number and/or residence time of actin protrusions. To test the prediction, we ectopically expressed F-actin and plasma membrane markers (GFP-tagged F-tractin and farnesylated mcherry, respectively) in MECs to examine the plasma membrane topography and actin protrusion dynamics. Indeed, MECs in 3D ECM had longer and more stable actin protrusions. The computational model also predicted that these protrusions should increase the area of negative curvature, which recruit proteins that induce or sense negative membrane curvature. Because non-spread MECs survive in 3D ECM and have more negative membrane curvature, we speculated that negative curvature associating proteins may lead to the activation of pro-survival pathways. Consistent with the prediction, MECs interacting with a 3D ECM had higher Arf6 activity and increased its downstream pro-survival Rac/p38 signaling. Loss and gain of function studies also revealed that Rac/p38 signaling promotes MEC survival in a 3D ECM. Moreover, knockdown of key Arf6 GEFs or reducing levels of negative curvature-inducing protein Exo70 compromised MEC survival in a 3D ECM, while loss of Arf6 GAPs enhanced the survival of non-spread MECs in 2D. Importantly, reducing cortical tension of non-spread MECs on a 2D ECM through myosin inhibition promoted negative curvature in MECs as well as cell survival. Our results provide the first evidence demonstrating that ECM dimensionality alters the biophysical properties of cells to modulate an Arf6/Rac/p38 pro-survival signaling axis.

P3441
Board Number: B732
PC3 cells deterministic immobilization on biochemically-patterned slides by micro-contact printing - Effect of the patterns shapes, size and pitch
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Microstructured cell immobilization has been shown to be a useful tool in different areas of cell biology such as neuron motility, cell signaling responses facing physical stress, cell migration and the study of cell behavior under different extracellular matrix protein composition, etc. The micro-contact printing of extracellular matrix proteins on conventional glass slides or coverslips turned out to be an efficient method to fabricate micropatterned cell culture substrates. Automated printing by the InnoStamp40 is a powerful tool to control the stamping parameters and obtain reproducible patterns of different shapes, sizes and pitches. Here we present a dedicated study in which different pattern sizes and gaps for the deterministic immobilization of PC3 prostate cancer cells were screened. Automated microcontact printing was used to create microstructured slides with four different feature types with different sizes and gaps. The spreading of the immobilized cells on the different patterns and the selectivity of the immobilization on the adhesive patterns were monitored in comparison to the antifouling background. We found that the number of immobilized cells per pattern decreased in direct correlation with its size and independently of its shape. Patterns with a pitch larger than 50µm did not present any junction between them, while for smaller pitches, some cells immobilized between the patterns creating a junction. The optimal parameters to create microstructured cell arrays with different pattern types are presented.
P3442
Board Number: B733
Programmable viscoelastic matrices from artificial proteins.
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Extracellular matrix compliance influences cellular adhesion and migration, proliferation and apoptosis, and differentiation. Much of our current knowledge of the effects of substrate stiffness on cellular behavior is based on elastic substrates, in particular cross-linked polyacrylamide hydrogels. Biological tissues, however, are viscoelastic and exhibit stress relaxation and energy dissipation on physiologically relevant timescales. While emerging evidence suggests that these physical properties also influence cellular behavior, materials in which viscoelasticity can be precisely engineered are currently lacking. Here, we describe programmable hydrogel matrices assembled from artificial recombinant proteins designed to be cross-linked by covalent bonds involving cysteine residues, by association of helical domains as coiled coils, or by both mechanisms. Using these proteins, we construct chemical, physical, and chemical-physical hydrogel networks that deform elastically or viscoelastically depending on the type of cross-linking (Dooling et al., Adv. Mater., 2016, 28, 4651–4657). In viscoelastic networks, the amount of stress relaxation is tuned by controlling the ratio of physical cross-linking to chemical cross-linking, and the timescale for stress relaxation is tuned over five orders of magnitude by single point mutations to the coiled-coil physical cross-linking domain (Dooling and Tirrell, ACS Cent. Sci., 2016, 2, 812–819). The genetic engineering approach also allows biological activity to be encoded directly within the protein sequence in the form of cell-adhesive domains and proteolytic cleavage sites. The capacity to program the viscoelasticity and biological activity of hydrogel matrices is anticipated to have applications in studying and engineering cell-matrix interactions.

Chaperones, Protein Folding, and Quality Control 2

P3443
Board Number: B735
Subcellular localization, uptake and dynamics of inorganic polyphosphate in mammalian cells.
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Inorganic polyphosphate (polyP), a linear chain of 3 to over 100 orthophosphates, is a highly conserved and universal molecule found in all species tested. Yet, unlike in bacteria where its biosynthetic pathway has been well characterized, little is known about polyP metabolism in mammals. This is largely due to a lack of feasible tools for detection and manipulation. Here we utilize a specific probe called MBP- EcPPXc (a fusion protein of maltose binding protein and the polyP binding domain of E. coli exopolyphosphatase) to monitor subcellular localization and dynamics of polyP by immunofluorescence. In several cell culture models including HeLa cells and primary human fibroblasts, we observed endogenous polyP in the nucleus (with enrichment in the nucleolus), plasma membrane and distinct foci in the cytoplasm. By using exogenously applied polyP300 chains labeled with Alexa Fluor 647 (polyP300 - AF647), we furthermore discovered that cells are able to rapidly take up polyP from the environment through endocytosis. This finding enables us to increase cellular polyP level under normal culture condition, and offers the unique opportunity to reveal the physiological function of polyP in mammalian cells. Finally, we observed a massive increase of polyP in response to hypochlorous acid (HOCl) treatment, the first known induction of polyP synthesis in mammalian cells. Taken together, we have
now established the tools that will finally allow us to uncover the physiological roles of this enigmatic polymer in mammalian systems.

**P3444**
**Board Number: B736**
**Exploring compartment-specific protein disaggregases to combat neurodegenerative diseases.**
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Protein aggregates accumulate in several neurodegenerative diseases. Protein disaggregases have great therapeutic potential as they can reverse toxicity caused by protein aggregates and soluble oligomers by disassembling these structures and recovering natively-folded proteins. The yeast protein disaggregate, Hsp104, disassembles disease-associated protein aggregates and soluble oligomers to suppress toxicity. Hsp104 has a yeast mitochondrial homologue, namely Hsp78. I targeted mitochondrial (mt) Hsp78 (mtHsp78) to the cytoplasm (cHsp78) and potentiated its activity via homologous mutations that nonselectively potentiated Hsp104. Three cHsp78 variants selectively rescue α-synuclein (αSyn), FUS, or TDP-43 toxicity in yeast. Three different mtHsp78 variants rescue αSyn toxicity in yeast without affecting cytoplasmic αSyn aggregation. We are exploring the mechanism of rescue for each of these variants. The results presented here will help to further our understanding of the demands of compartment- and substrate-specific protein disaggregate activity.

**P3445**
**Board Number: B737**
**Small HSP αB-Crystallin is a hydrolyzed eggshell membrane-response chaperone and keeps hydration of epidermis keratinocyte through the interaction of lipid membrane regulator-acid sphingomyelinase.**
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Small Heat Shock Protein alphaB-crystallin (αB) has various essential biological roles not only to protect cells from apoptosis but also improve function of differentiated tissues that constitutively receive stresses like mechanical stress on slow-twitch muscle (Atomi et al., 1991) and heart (Pereira et al., 2014), and light on lens. Present study is intended to address interesting and important relation of molecular chaperone αB and solubilized eggshell membrane (ESM) and was succeeded in adding ESM beneficial effect of increased hydration on epidermal keratinocytes as well as dermal fibroblasts previously reported (Ohoto-Fujita et al., 2011). Recently we show that αB, chaperone for tubulin/microtubule controls shape and adhesion of glioma and myoblast cells in the absence of stress (Shimizu et al., 2016) through its chaperone-like activity (Ohoto-Fujita 2007). Both significant increased hydration of the arm skin and decreased wrinkles of the face after ESM-2-weeks-topical applications for women were associated with significant increased SMPD-1 gene expression encoding acid sphingomyelinase that produces ceramide by hydrolyzing sphingomyelin after 10 days-application on hairless mice skin. To know the mechanism of epidermis hydration by ESM, 1 to 10 (w/v) % of ESM was applied to a dispase II separated epidermis from dermis and human embryonic keratinocyte, and fixed after various time points, and the localization of αB, ceramide, and acidic SMase (aSMase) were analyzed. αB colocalized with membrane associated aSMase that produces ceramide. Co-

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immunoprecipitation of αB and aSMase was observed in mouse epidermis homogenate. αB highly expresses in muscle and lens, whereas the expression level is different depending on activity properties like long-lasting contraction and reception of stresses. Cells in such tissues are required “endurance”, which results in long-lasting higher calcium level, which needs special endoplasmic/sarcoplasmic reticulum calcium channel (shown in slow skeletal and heart muscles), and also long-lasting suppliable energy source lipid, of which metabolism needs mitochondria. Although exact reason is not known, recently sHSPs was found to be the most important protein for keeping proteostasis linked to longevity. αB has been thought to be a cytosolic chaperone supporting structural regulation of cytoskeleton, however now considered as chaperone for ER and also mitochondrial stresses, both are the matter of lipid membrane. The present study offers another role of αB, regulating membrane dynamics probably related to tubulin/microtubule cytoskeleton dynamics, contributing to anti-apoptosis effect against constitutively receiving stresses like gravity, light and dehydration and convert them to beneficial stimuli for adaptation.

P3446
Board Number: B738
Endoplasmic reticulum morphology serves as predictor for stress severity.
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The Unfolded Protein Response (UPR) serves to counteract the accumulation of proteins in the Endoplasmic Reticulum (ER) during stress. The initial phases of the UPR adapt cells to ER stress, but chronic UPR activation can lead to cell dysfunction and pathology. Our work using gene expression analysis uncovered a distinct UPR, activated in the liver of zebrafish and humans, that correlates with the accumulation of lipid in hepatocytes (fatty liver). We reasoned that ER morphology alterations would occur during an adaptive phase of the UPR, prior to gene expression changes in response to disease, and asked whether it could indicate an early stress response. We tested this utilizing intravital confocal imaging of 5 days post fertilization (dpf) old transgenic zebrafish expressing an ER-marker in hepatocytes (Tg(fabp10:ER-tdTomato)). Tunicamycin, a well-established ER-stressor causing fatty liver, induced dramatic changes in the ER marker from a reticular pattern to motile punctae first observable by 12 hours of tunicamycin treatment. At 24 hours exposure 80% of the livers contained punctae. To identify the nature and origin of these structures, we tested their co-localization with the trans Golgi apparatus using a transgenic marker (Tg(bact2:Galt-GFP)) and with acidic compartments using Lysotracker. We found no co-localization with the Golgi-marker, but Lysotracker co-stained and was present in 63% of the larvae by 12 hours of tunicamycin treatment compared to 6% of livers in untreated controls. We hypothesize that this represents the activation of ER-phagy to mitigate severe ER stress and suggests that markers of ER-phagy indicate the organisms most prone to disease. Our preliminary data shows that UPR target genes are upregulated in livers from tunicamycin treated larvae with multiple punctae per hepatocyte, compared to those with milder phenotypes. These findings suggest that ER morphology may be prognostic for identifying stressed and disease prone cells.
P3447

Board Number: B739

Suppressing Aβ toxicity with potentiated Hsp104 variants in a yeast model of Alzheimer’s disease.
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As the sixth leading cause of death, Alzheimer’s disease (AD) continues to be an intractable public health issue. 5.3 million Americans suffer from the declines in memory, cognition, and behavior characteristic of the disease. AD is currently incurable and unpreventable. Research has shown AD is linked to the formation of cytoplasmic neurofibrillary tangles (NFTs) and extracellular senile plaques (SPs) in the brain1. NFTs are comprised of tau protein2 and SPs are comprised predominantly of amyloid beta peptides (Aβ) of amino-acid length 40 (Aβ40), 42 (Aβ42) and 43 (Aβ43)3. The amyloid cascade hypothesis proposes that AD is fundamentally caused by the accumulation of misfolded Aβ oligomers, which can form highly ordered plaques in brain regions with severe neurodegeneration. One therapeutic strategy is to target amyloid fibrils and toxic oligomers for disaggregation. Hsp104, a protein disaggregase found in yeast, has been previously engineered to display potentiated activity against other proteins such as α-synuclein, TDP-43, and FUS4, aggregates of which underpin Parkinson’s disease, amyotrophic lateral sclerosis, and frontotemporal dementia. Here, we show that some of these engineered Hsp104 variants rescue the toxicity of Aβ42 and Aβ43 in a powerful yeast model of AD5, which has revealed important risk factors for AD in humans. Remarkably, several enhanced Hsp104 variants also rescue Aβ42 toxicity in the context of the metazoan nervous system in C. elegans6. We further assessed the activity of these Hsp104 variants in yeast when they are targeted through the secretory pathway alongside Aβ42. Curiously, we found that the enhanced Hsp104 variants exhibit off-target toxicity when they are targeted to the secretory pathway and fail to rescue Aβ42 toxicity.

References:

P3448

Board Number: B740

Engineering Potentiated Hsp70 Variants to Combat Neurodegeneration.
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When proteins become misfolded, they can form prions, amyloids, amorphous aggregates, and toxic oligomers, which have been implicated in aging and neurodegenerative diseases such as Parkinson’s
disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS). Protein-misfolding processes are remediated by the metazoan disaggregase system (MDS), which may become overwhelmed under these disease states. Here, we present our work on engineering Heat Shock Protein 70 (Hsp70), a component of the MDS, to counter toxic protein-misfolding events. We utilize yeast as a high-throughput and facile proteinopathy model to study the mechanism of these engineered Hsp70 variants against the PD-linked protein, alpha synuclein, and the ALS-linked proteins, TDP-43 and FUS. Through this model, several Hsp70 variants have been identified with the capacity to selectively rescue PD- and ALS-linked proteotoxicity. This work will contribute to our understanding of how we can therapeutically engineer human Hsp70s to combat protein misfolding implicated in neurodegenerative diseases.

P3449

Board Number: B741

The Ribosome-Associated Complex Suppresses [PSI+] Prion Formation in Yeast.
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Prions are self-propagating infectious protein conformations. In mammals, prions underlie the transmissible spongiform encephalopathies, a set of invariably fatal neurodegenerative disorders associated with misfolding of the mammalian prion protein, PrP. In recent years, increasing numbers of fungal proteins capable of infectiously propagating altered conformations have been discovered. Multiple yeast prions can produce beneficial phenotypes resulting in increased fitness in the presence of stressors, possibly by serving as epigenetic switches that can rapidly confer beneficial heritable phenotypes. To gain critical insight into the mechanisms by which cells regulate prion formation, propagation and toxicity, we examined the role of the ribosome-associated complex (RAC) chaperones in the formation and toxicity of the [PSI+] prion in yeast. The [PSI+] prion results from a self-propagating amyloid form of the essential translation termination factor Sup35, and is manifested phenotypically as elevated levels of nonsense suppression. The RAC consists of the Hsp70 chaperone Ssz1 and the Hsp40 chaperone Zuo1, which anchors the complex onto ribosomes and stimulates the ATPase activity of the Hsp70 chaperone Ssb. Previous work in the lab has shown that yeast cells lacking Zuo1, and thus lacking RAC function on ribosomes, exhibit higher frequencies of spontaneous and induced [PSI+] formation. Cells expressing variants of Zuo1 that are unable to associate with ribosomes, or that are unable to stimulate Ssb ATPase activity, exhibit similarly high levels of prion formation. These findings are consistent with a role for the RAC in chaperoning nascent Sup35 to prevent misfolding of the N-terminal prion domain as it emerges from the ribosome. An alternative role for Zuo1 is its ability to function as a transcriptional co-activator for the pleiotropic drug resistance pathway, which enhances resistance to various drugs and environmental toxins. Its role in this pathway requires unfolding of the C-terminal domain of Zuo1, which prevents its association with ribosomes. Since a variety of environmental stress conditions are known to induce prion formation, RAC dissociation from ribosomes under these conditions may facilitate prion formation. Our current work aims to understand the mechanism by which Zuo1 dissociation from ribosomes is triggered in response to environmental stress.
The nucleus of a eukaryotic cell is enclosed by a double-membrane structure, the nuclear envelope, which separates the nucleoplasm from the cytoplasm. The outer nuclear membrane is continuous with the endoplasmic reticulum (ER), whereas the inner nuclear membrane (INM) acts as the intermediate connecting the outer nuclear membrane to the nucleoplasm. As with most cellular compartments, the proteome of the INM is highly specific. Proteins targeted to the INM can be associated with the membrane through peripheral interactions or integrally embedded within the INM. The sorting of integral INM proteins after synthesis at the ER is achieved through either an energy dependent transport or by simple diffusion-retention. Due to the extremely high rate of protein synthesis and subsequent misfolding present at the ER, a mechanism for protein quality control is present to ensure dysfunctional or unwanted proteins are removed. A large majority of membrane associated proteins are degraded by the ubiquitin proteasome through the endoplasmic reticulum associated protein degradation (ERAD) pathway. ERAD functions in a step wise manner which involves the target protein being ubiquitylated by the joint actions of a ubiquitin conjugating enzyme and ubiquitin ligase. There are two redundant ubiquitin conjugating enzymes, Ubc6 and Ubc7, known to function with one of the three independent ubiquitin ligases (Doa10, Hrd1 and the Asi complex) in budding yeast, creating three separate branches for ERAD mediated degradation. The Asi pathway is thought to be a major ERAD pathway specific for degrading INM associated proteins. The conserved SUN-domain protein, Mps3, which resembles a type II single-pass transmembrane protein, is localized to the inner nuclear membrane. Its N-terminus is in the nucleoplasm, whereas the characteristic SUN-domain of Mps3 is in the lumen of the nuclear envelope. We have found that Mps3 has a half life of 45 minutes, and its degradation is regulated by Ubc7. However, there is no discernable change in Mps3 stability when any of the known ERAD ubiquitin ligases, including the Asi complex, are inactivated. Our findings suggest another ubiquitin ligase that is specific for the degradation of Mps3 is present at the INM, providing further evidence for an inner nuclear membrane associated degradation pathway (INMAD) that functions independently of ERAD.

The Golgi apparatus, a complex multi-layered endomembrane compartment, is the major site of protein modification and sorting within a cell. Additionally, it serves as an important platform for spatially-regulated signaling. Golgi homeostasis mechanisms that ensure precise processing and proper localization of protein substrates and signaling complexes remain poorly understood, due in large part to the lack of strategies capable of specifically inducing Golgi stress. Current tools used to perturb the Golgi, such as the ionophore nigericin or the glycosylation inhibitor xyloside, induce numerous off-target effects that confound studies on Golgi homeostasis. To overcome this limitation, we developed a novel
methodology to establish a Golgi-specific stress model that involves the expression of an artificial Golgi-localized protein and its targeted destabilization by small molecule hydrophobic tagging. The present study establishes for the first time that protein unfolding perturbs the Golgi, leading to a specific Golgi stress response that is distinct from the ER stress response. Using RNA sequencing, we transcriptionally profile our stress inducer and compare it to the current state-of-the-art Golgi stressors nigericin and xyloside. We find that stress induced by protein unfolding in the Golgi is specific to the Golgi apparatus, while nigericin and xyloside affect a broad range of cellular processes. Furthermore, from the obtained dataset, we uncovered a group of previously uncharacterized proteins involved in Golgi structural maintenance, knockdown of which leads to Golgi fragmentation. Together, these findings establish important new aspects of Golgi auto-regulation.

P3452
Board Number: B744
Unfolding of a Golgi-localized Protein Identifies Important Protein Quality Control Mechanisms in the Early Secretory Pathway.
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Eukaryotic cells have evolved to utilize distinct reaction environments by employing a complex endomembrane system enclosing specialized organelles. Each of these organelles needs to maintain a quality control system to ensure the fidelity of its specific proteome and prevent toxic protein aggregation. The endoplasmic reticulum (ER) employs chaperones and the ER-associated degradation (ERAD) pathway to ensure the proper folding of proteins and to mark misfolded proteins for degradation. Proteins evading the ER quality control system or becoming damaged at later stages of their life cycle encounter downstream surveillance mechanisms. The Golgi apparatus is a major component of the secretory pathway which sorts and modifies incoming proteins from the ER. How the Golgi maintains proteostasis and exerts quality control measures is very poorly understood. To enable studying post-ER proteostasis mechanisms we have developed a tool that involves the expression of a Golgi-resident HaloTag2 fusion protein and its subsequent induced unfolding by covalent attachment of a small molecule hydrophobic tag. A similar approach in the ER has established that unfolded HaloTag2 is recognized and degraded by the ERAD machinery, validating the construct as a quality control substrate. Conversely, HaloTag2 unfolding in the Golgi does not lead to its degradation. Confocal imaging studies show that distinct carriers selectively export the unfolded protein from the Golgi. Three hours after inducing protein destabilization, the unfolded protein is localized to the ER, where it associates with molecular chaperones of the ER quality control machinery. The small-molecule induced protein unfolding approach serves as an important tool for identifying key features of organelle-specific proteostasis mechanisms. Together, our data demonstrate that the Golgi apparatus maintains a quality control system that is able to recognize unfolded proteins and induce their selective export, thus demonstrating the existence of a novel quality control mechanism in the early secretory pathway.
P3453

Board Number: B745

Modulation of calreticulin expression reveals a novel mechanism of Z variant alpha-1 Antitrypsin disposal.
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Introduction

Alpha-1-antitrypsin deficiency (AATD) is an inherited disease characterized by emphysema and liver disease. AATD is most often caused by a single amino acid substitution at amino acid 342 in the mature protein, results in Z mutation of the AAT gene (ZAAT). This substitution is associated with misfolding and the accumulation of ZAAT in the Endoplasmic reticulum (ER) of hepatocytes and monocytes, causing a toxic gain of function. It has been shown that retained ZAAT is eliminated by ER-associated degradation (ERAD). ERdj3, one of the soluble ER luminal DnaJ homologue along with calreticulin directly interact with misfolded ZAAT. We hypothesize that depletion of each of these chaperones will change the fate of ZAAT polymers. Our study demonstrates that calreticulin modulation reveals a novel ZAAT degradation mechanism mediated by exosomes.

Methods

Human PiZZ hepatocytes and K42 mouse fibroblast cell line were grown in DMEM/F12 containing 10% FBS and transfected with or without calreticulin siRNA. To confirm interaction between EC ERdj3 and ZAAT and calreticulin regulatory role in ZAAT-ERdj3 secretion, quantitative RT-PCR, immune blotting, immunofluorescence, co-immunoprecipitation and flow cytometry were performed in PiZZ cell line.

Results

ERdj3 and calreticulin directly interact with ZAAT in PiZZ hepatocytes. Silencing calreticulin or preventing its interaction with ZAAT, induces calcium independent ZAAT-ERdj3 secretion through exosome pathway. This co-secretion decreases ZAAT aggregates within the ER of hepatocytes.

Conclusion

We demonstrate that calreticulin has an inhibitory effect on exosome mediated ZAAT-ERdj3 secretion. This is a novel ZAAT degradation process that involves DnaJ homologue chaperone bound to ZAAT. In this context, calreticulin modulation may eliminate the toxic gain of function associated with aggregation of ZAAT in lung and liver, thus providing a potential new therapeutic approach to the treatment of AATD related liver disease.

P3454

Board Number: B746

Redox modification of the Hsp70 nucleotide exchange factor Fes1.
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Elevated levels of reactive oxygen species (ROS) are associated with many degenerative disorders. Increased cellular ROS levels correlate with accumulation of irreversibly oxidized proteins, which are prone to misfolding and aggregation. 70-kDa heat shock proteins (Hsp70s) are a conserved family of ATPases that serve as molecular chaperones to refold misfolded and aggregated proteins, such as those damaged by ROS. As such, Hsp70s play a critical role in protein quality control under oxidative stress. To examine the role of cytosolic Hsp70s in coping with oxidative stress, we screened S. cerevisiae strains mutated for each of the chaperones and their cofactors for peroxide sensitivity. Notably, we found that the nucleotide exchange factor Fes1 is required for cell survival during oxidative stress, and that when
cells are grown in medium containing oxidant, Fes1 is modified directly by ROS at three methionine residues. Preliminary data demonstrate that this oxidative modification of Fes1 reversibly alters its nucleotide exchange activity. We hypothesize this modification confers a protective role during stress conditions allowing cells to sense and respond to elevated ROS levels. Altered Hsp70 activity, as a consequence of Fes1 modification, may help cells cope with elevated ROS by limiting protein aggregation, targeting misfolded polypeptides for degradation, and/or activating the heat shock response. We are currently focused on characterizing these events to determine how Fes1 modification affects cell physiology and Fes1’s protein quality control activities in the cell.

P3455
Board Number: B747
The ASNA-1/ENPL-1 redox sensitive complex modulates C.elegans insulin secretion.
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ASNA-1 is a positive regulator of insulin secretion in C.elegans and mice. To understand its biochemical role, we are studying candidate interacting proteins that may promote its activity. We have analyzed the function of C.elegans ASNA-1 candidate gene GRP94/ENPL-1. enpl-1 mutants have growth and reproductive defect. ASNA-1 gene positively regulates the secretion of the C.elegans insulin DAF-28. Phenotypes of enpl-1 mutants overlap with asna-1 mutants. We have identified a role for the chaperone ENPL-1 as a new positive regulator of insulin signaling that acts at the level of insulin availability similar to ASNA-1. We hypothesized that they might physically interact. Using Y2H and GST pulldown analysis we showed that ENPL-1 binds to ASNA-1 through defined subdomains and in vivo interactions were revealed by IHC in embryos. In worms these two proteins interact in neurons which are also one of the sites for insulin secretion. Moreover, in DAF-28/insulin mutants the level of binding decreases significantly. This finding suggest that an ASNA-1/ENPL-1/DAF-28 complex is needed for proper processing of insulins in C.elegans. ASNA-1 and ENPL-1 show increased binding under oxidative stress conditions. The S.cerevisiae homolog of ASNA-1 acts as a dimer to promote targeting of tail-anchored proteins to the ER membrane and upon oxidation, converts to a tetrameric state in which it acts as a general chaperone. Worm ASNA-1 as well is present in both states: oxidized and non-oxidized and conversion is controlled via two cysteines. Under oxidative stress conditions ASNA-1 changes its shape which allows increased binding to ENPL-1, without changing its total level. Our aim is to further investigate conditions which favor or inhibit structural changes of ASNA-1 to be able to specifically control these processes. We hypothesize that insulin secretion in C.elegans needs the interaction between ASNA-1 and ENPL-1 in order to happen. In the ER, ENPL-1 promotes the proper cysteine bond formation in insulin molecules via a molecular complex. Only then, the complex of the properly folded insulin together with ENPL-1 leaves the ER and translocates to Golgi apparatus where can meet the tetrameric ASNA-1 chaperone. ASNA-1 and ENPL-1 creates a complex, which allows ENPL-1 to release insulins which can be secreted through dense core vesicles outside the cell. Discovering the mechanism of insulin folding and secretion and the relation to two chaperones will lead to a better understanding of insulin maturation/secretion and better design of diabetes targeting drugs.
P3456

Board Number: B748

Amyloids as the natural protein storage reservoirs.
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Amyloids represent protein fibrils with highly ordered spatial structure that makes them one of the most stable biogenic particles. Amyloids are resistant to treatment with ionic detergents, proteinases and other protein denaturants, and can survive in the environment for years. For a long time, amyloids were mainly perceived as the lethal pathogens causing about 40 incurable disorders in humans and animals. During last decades, amyloid became clear to be not only pathogenic but also functionally important variant of the quaternary protein structure. A wide variety of functional amyloids was identified in different species of prokaryotes and eukaryotes. Among various functions of amyloids, protein storage takes an important place. Different toxins in bacteria and hormones in humans are stored in the amyloid state. Plants are poorly studied group in the field of amyloid biology. Several plant proteins or peptides were shown to have amyloid properties only in vitro or in heterological systems in vivo. The main functions of these plant proteins are defense from the pathogens as well as regulation of flowering [for a review, see 1]. We performed large-scale bioinformatic analysis of the amyloidogenic properties of 2.9 million proteins in 75 plant species using SARP and WALTZ algorithms. We identified protein families and functional groups, in which potentially amyloidogenic proteins are overrepresented. We found that seed storage proteins are highly enriched with amyloidogenic regions, and this feature is evolutionary conservative among land plants [2]. Experimental analysis performed with recently developed PSIA approach [3] revealed several candidates for novel amyloid-forming proteins among storage proteins. Taking together, our data suggest that amyloid formation is likely to represent an important mechanism of protein storage in seeds. This work was supported by the Russian Science Foundation, Grant №17-16-01100. References: [1] Antonets K.S., Nizhnikov A.A. Amyloids and prions in plants: facts and perspectives. // Prion., 2017, In Press. [2] Antonets K.S., Nizhnikov A.A. Predicting amyloidogenic properties of the plant proteomes. // Int. J. Mol. Sci., 2017, In Press. [3] Nizhnikov A.A., Nizhnikov A.A., Ryzhova T.A., Volkov K.V., Zadorsky S.P., Sopova J.V., Inge-Vechtomov S.G., Galkin A.P. Interaction of prions causes heritable traits in Saccharomyces cerevisiae. // PLOS Genetics, 2016, V.12, e1006504.

P3457

Board Number: B749

Broader perspectives of secretory pathway quality control revealed through the study of misfolded GPI-anchored proteins.
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Secretion of misfolded proteins can result in disruptive and toxic molecular interactions, such as those associated with prion and Alzheimer’s diseases. Accordingly, cells have evolved multiple coordinated protein quality control (QC) pathways to target and retain misfolded secretory pathway proteins in the endoplasmic reticulum (ER) for refolding or degradation via ER associated degradation (ERAD) or autophagy. Yet, we now appreciate that some misfolded secretory pathway proteins escape degradation at the ER and traverse the secretory pathway before being secreted and/or destroyed in
lysosomes. For example, misfolding mutants of a prevalent class of membrane proteins called glycosylphosphatidylinositol-anchored proteins (GPI-APs), which includes prion disease-associated mutants of PrP, are selectively cleared out of the ER by a stress-enhanced, Tmp21-dependent pathway. We named this ER export pathway RESET (for Rapid ER Stress-induced Export). Upon release from the ER, the misfolded GPI-APs move through the Golgi and transiently reside on the plasma membrane before being internalized for lysosomal degradation. Recently, we discovered that a subset of misfolded transmembrane proteins, including human disease mutants of LDLR, also associate with Tmp21 and are cleared from the ER by RESET. We are now dissecting the precise role of Tmp21 and associated factors in the ER export of misfolded GPI-APs and transmembrane proteins. Additionally, we are characterizing the currently unknown QC system(s) that target and send misfolded GPI-APs on the cell surface to lysosomes for degradation.

P3458
Board Number: B750
Prions may act as the gain-of-function mutations.
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Prions are proteins that can form in the same conditions two or more structurally distinct states, at least one of which is infectious. Prions were initially described as the proteinaceous infectious agents that cause neurodegenerative pathologies in humans and animals. However, it soon became clear that prions are also implicated in different biological processes in a wide range of species. The most common point of view postulates that prion formation leads to functional inactivation of prion-forming protein similarly to the deletion of corresponding structural gene or loss-of-function mutation. Certainly, in contrast to mutations, prion formation is not caused by changes in genetic material, but is determined by conformational change of the prion-forming protein. Here we used [SWI\textsuperscript{1}] prion of yeast \textit{Saccharomyces cerevisiae} as a model to compare the effects of prion formation and deletion of prion-encoding gene at the transcriptome-wide level. [SWI\textsuperscript{1}] is a prion of Swi1 protein, which is a component of evolutionary conservative chromatin remodelling complex SWI/SNF. We analyzed transcriptomes of the [SWI\textsuperscript{1}], [swi\textsuperscript{1}], and swi1Δ strains. We found that both, prion formation and deletion of Swi1, lead to changes in the expression of hundreds of genes. Nevertheless, 20 genes that are downregulated in the swi1Δ strain are upregulated in the [SWI\textsuperscript{1}] strain. Thus, formation of [SWI\textsuperscript{1}] prion affects expression of several genes in a manner similar to the gain-of-function mutation. Taking together, our data show that the effect of prion formation are not equal and may be even opposite to the effects of the deletion of prion-encoding gene. This work was supported by the grants of the Russian Foundation for Basic Research (17-04-00816) and the President of the Russian Federation (MK-3240.2017.4). The authors acknowledge Research Park of SPbSU for the opportunity to use equipment of “Biobank” and “RRCMCT”.
During heat shock, single-celled organisms must be able to dynamically sense and respond to changes in the protein-folding environment, such as temperature, to ensure proteostasis and survival. The budding yeast S. cerevisiae grows best in acidic (low-pH) conditions, yet during logarithmic growth maintains a resting cytosolic pH just above neutral, expending a considerable portion of cellular ATP resources to do so. When cells experience acute stresses such as high temperatures or energy depletion, intracellular pH drops. Although changes in intracellular pH have been shown to influence the cellular response to starvation, in the case of heat shock little is known about the interplay between changes in pH and other, more well-studied processes such as the transcription and translation of the heat shock genes, and the behavior of the protein homeostasis machinery. In order to understand how changes in intracellular pH might influence the heat shock response, we dictate intracellular pH with an ionophore, use a pH-sensitive GFP derivative to measure intracellular pH, and simultaneously measure the production of heat shock proteins using quantitative, multicolor flow cytometry.

We find that cells that are held at the pre-shock pH during heat shock almost completely fail to synthesize certain heat shock proteins in an interval where acidified cells do so robustly. We measure relative growth rates and demonstrate that the intracellular pH profile during stress also alters cellular fitness during recovery. Finally, we correlate production of molecular chaperones to intracellular pH during recovery at the single-cell level. These findings implicate pH regulation as a key aspect of the cellular stress response.

Translation of heat shock proteins is regulated by poly(A)-binding protein assembly. How do cells sense and respond to changes in the environment? During heat stress, numerous eukaryotic RNA and protein species assemble into macromolecular bodies - stress granules - the function of which is largely unknown. Simultaneously, cells increase transcription of genes encoding heat shock proteins, many of which are molecular chaperones. In addition to increased transcription, the mRNA for these genes is also translated more efficiently. Thus, heat shock protein production is regulated by both transcription and translation. These heat-induced mRNAs have exceptionally A-rich 5′ untranslated regions (UTRs), which have long been known to mediate cap-independent translation. We observe that poly(A)-binding protein (PABP, Pab1 in S. cerevisiae) is bound to these A-rich UTRs during exponential growth. Pab1 is able to repress translation of its own mRNA by binding a similar A-rich UTR. We find that Pab1 represses translation of mRNA by binding A-rich UTRs of heat shock proteins. Further, we propose that during heat-shock, as Pab1 assembles into heat-induced RNA-protein granules, Pab1 releases bound 5′ UTRs, and relieves repression. We have recapitulated Pab1-mediated repression and heat-induced relief with an in vitro translation system. Moreover, yeast expressing assembly-impaired Pab1 are not able to grow at elevated temperature; this result is consistent with disruption of an
assembly-mediated regulatory mechanism. Viewed through this lens, formation of RNA-protein granules is a regulatory response, whose functions include translational regulation.

P3461  
**Board Number: B753**  
**Differential impact of several PD-associated genes on the toxicity associated with wild-type and familial mutant forms of α-synuclein in a yeast model.**  
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Parkinson’s disease (PD) is a neurodegenerative disease characterized by motor impairment. The aggregation of the α-synuclein in the midbrain dopaminergic neurons and the corresponding death of these cells underlie these symptoms. PD can be both genetic and sporadic. Familial PD is directly caused by a mutation in one of at least 10 genes, including SNCA, DJ1, VPS35, and ATP13A2. SNCA encodes α-synuclein and has six identified missense mutations (A30P, E46K, H50Q, G51D, A53E, and A53T) that each cause early-onset PD. Sporadic PD is linked with several risk genes, including VPS13, Sac1, and Swa2. In our budding yeast model system, we first find wild-type, E46K, A53T, H50Q, and A53E α-synuclein to be toxic to yeast and they show varying degrees of membrane binding and aggregation, while A30P and G51D α-synuclein are relatively non-toxic and show cytoplasmic diffuse localization. What is still not well understood is whether the PD-causing and PD-risk genes mentioned above can influence the toxicity properties of this six familial mutants of α-synuclein. To explore this connection, wild-type and familial forms α-synuclein were studied in yeast strains that were singly deleted for these six PD-linked genes. Results indicate that some gene deletions increased α-synuclein toxicity (DJ1, VPS13), others reduced toxicity (ATP13A2, VPS35), while some had no effect (Swa2, Sac1). In each case, those genes that altered α-synuclein toxicity did so in a familial mutation-specific way. Our findings suggest that each familial mutant creates cellular toxicity in a unique way regulated by different subsets of genes/cellular processes, offering doors for additional mechanistic insight into the mode of toxicity with each mutant.

P3462  
**Board Number: B754**  
**The Saccharomyces cerevisiae SUMO-targeted ubiquitin ligase subunit Slx5 reduces the toxicity and transcriptional activity of poly-Q expanded huntingtin.**  
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Cell viability and gene expression profiles are altered in cellular models of neurodegenerative disorders such as Huntington’s Disease (HD). Using the yeast model system, we show that the SUMO-targeted ubiquitin ligase (STUbl) Slx5 reduces the toxicity and abnormal transcriptional activity associated with a mutant, aggregation-prone fragment of huntingtin (htt), the causative agent of HD. We demonstrate that expression of an aggregation-prone htt construct with 103 glutamine residues (103Q), but not the non-expanded form (25Q), results in severe growth defects in slx5Δ and slx8Δ cells. Concomitantly, an extra copy of SLX5 reduces the accumulation of 103Q aggregates in the cytosol of wild type cells while overexpression of SUMO led to diffuse nuclear staining of htt. This prompted us to assess the effect of STUbls on the transcriptional properties of aggregation-prone htt. Expression of 25Q, 55Q and 97Q fused to the Gal4 activation domain (AD) resulted in reporter gene auto-activation. Remarkably, the

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auto-activation of htt constructs was abolished by expression of Slx5 fused to the Gal4 DNA-binding domain (BD-Slx5) but not an Slx5 mutant (BD-Slx5ΔSIM) that fails to interact non-covalently with SUMO. In support of this observation we find that chromatin-associated poly-Q expanded htt is reduced when Slx5 levels are elevated. Finally, we show that RNF4, the human orthologue of Slx5, curbs the aberrant transcriptional activity of aggregation-prone htt in a tissue culture model of Huntington’s disease. This research indicates that STUB1s are functionally required to counteract the reduced cell viability and abnormal transcriptional profiles observed in protein aggregation disorders such as HD.

P3463
Board Number: B755
Functions of TPR Containing Proteins in the Endoplasmic Reticulum.
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The endoplasmic reticulum (ER) is a complex, multi-functional organelle, comprised of a continuous membrane and lumen that is organized into several functional regions with various roles including protein translocation, folding, quality control, secretion and the unfolded protein stress response. Total cellular protein homeostasis is maintained by a complicated chaperone network and when the various factors are categorized into functional families, the largest family consists of proteins containing tetra-trico peptide repeats (TPR). TPRs are well-studied structural motifs that support intermolecular protein-protein interactions in various organelles and organisms, consisting of 34 amino acids with a degenerate consensus sequence, making them highly acceptable and capable of interacting with a wide range of ligands. Since TPR motifs are ubiquitous and adaptable scaffolds, we hypothesized that additional TPR domain-containing proteins could contribute to complex formation and ER compartmentalization. Putative TPR containing proteins, identified from database searches and an in silico library (Regan, Yale University), were subjected to SignalP 4.0 and TargetP1.1 to identify those targeted to the secretory pathway. This analysis identified 10 TPR containing proteins. While some of these proteins have been confirmed to be ER localized and currently determined to be involved in chaperone function/modification and ERAD, a clear understanding of the remaining 7 is currently absent. Confocal microscopy and carbohydrate analysis revealed that TMTC1-4 reside in the ER. TMTC1-4 were determined to be integral membrane proteins by alkaline extraction and trypsin protection assays on isolated ER microsomes showed that their C-terminal ends are in the ER lumen. Recent data has implicated TMTC1-4 in O-mannosylation. We seek to determine the role they may play in O-mannosylation and resulting disease states characterizing previously determined mutations.
1Larsen et. al (2017) Proc Natl Acad Sci USA 10.1073/pnas.1708319114

Regulation of Aging

P3464
Board Number: B756
Molecular insights into life span through changes in tRNA export and transcription activation.
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Aging is significant risk factor for a variety of serious diseases, and is regulated by several cellular and molecular signaling pathways. One such pathway involves tRNA nucleocytoplasmic transport. tRNAs are transcribed in the nucleus and must be exported through nuclear pore complexes (NPCs) to mediate...
protein translation in the cytoplasm. We and others discovered that inhibition of tRNA export in *S. cerevisiae* increases replicative life span (RLS), which is defined as the number of daughters an individual yeast cell produces prior to senescence or death. RLS is a robust system to model the aging process due to the conservation of several longevity factors in metazoans and the short time frames required for analysis. Our prior studies showed that deletion of the nucleoporin Nup100 or its GLFG domain, an intrinsically disordered region consisting of glycine-leucine-phenylalanine-glycine repeats that regulates passive diffusion and active transport through NPCs, is sufficient to increase RLS. Since *nup100Δ* and tRNA export mutants both display increased life spans, we tested whether tRNA transport is affected by Nup100. Fluorescence in situ hybridization experiments reveal isoleucine, tyrosine, and tryptophan tRNAs, but not serine or methionine tRNAs, accumulate in the nuclei of *nup100Δ* cells. In addition, protein levels of the transcription factor Gcn4 are elevated in *nup100Δ* mutants, and *GCN4* is necessary for the increased life spans of *nup100Δ* cells. In *S. cerevisiae* cells, intron-containing and mature tRNAs are exported from nuclei; however, the majority of tRNAs present in *nup100Δ* cells are spliced, suggesting re-export of mature tRNAs is inhibited. Functional assays indicate that transport of the tRNA-binding nuclear transport receptors Los1 and Msn5 are unaffected in *nup100Δ* cells. Together, these results suggest a model whereby Nup100 is required for the re-export of specific mature tRNAs. Nuclear accumulation of these tRNAs in *nup100Δ* cells then extends life span through a Gcn4-dependent signaling pathway. Since the GLFG domain of Nup100 is critical for regulating longevity, we speculate Nup100 is required for an unidentified nuclear transport event, independent of Los1 or Msn5, that is necessary for tRNA export. We are currently investigating how Nup100 influences tRNA export, and whether Gcn4 overexpression is sufficient to increase RLS. Through these studies, we mechanistically define several important signaling events that modulate longevity.

**P3465**

**Board Number: B757**

**IRBIT links nucleotide metabolism to aging in the gut.**

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Within the gut, there are age-related changes in the microbiota, immune signaling and inflammation response. At the cellular level, aging in the gut is coupled with decreased epithelial integrity. Using the genetically tractable model organism Drosophila melanogaster, we have identified IRBIT as a key protein in maintaining gut homeostasis and epithelial integrity in aging.

We recently reported a conserved role for IRBIT in inhibiting ribonucleotide reductase (RNR), an enzyme that produces dNTPs within the cell for DNA synthesis. During embryogenesis, in situ hybridization reveals localization of IRBIT to regions that will become the midgut, and it is expressed highly in the adult midgut. WT flies show a marked decrease in IRBIT protein level as they age, hinting at a function for IRBIT in aging. Therefore, we probed IRBIT’s potential role in regulating gut aging by generating an IRBIT null fly (IRBIT/-). One day old WT and IRBIT/- flies are indistinguishable at the tissue architecture level. However, rapid progressive aging occurs in the IRBIT/- flies, whereby one week old flies resemble dramatically older WT flies. IRBIT/- flies show an increase in undifferentiated enteroblast progenitor cells, that are RNR positive. This increase in RNR positive progenitor cells contributes to the tissue dysplasia. IRBIT/- flies also show decreased cell-cell contacts when stained for junctional proteins in the posterior midgut epithelium. These phenotypes are fully rescued with full length IRBIT; suppressing RNR by hydroxyurea treatment also rescues the IRBIT/- phenotype, which reveals IRBIT’s role in aging is
through RNR inhibition. Finally, artificial elevation of dNTP levels extensively phenocopies the IRBIT deletion. Altogether, these data suggest that IRBIT plays a key role in aging and tissue homeostasis in the fly gut via its role antagonizing RNR in nucleotide metabolism. We are currently examining the interplay of IRBIT with inflammatory pathways and microbiota changes that are characteristic features of aging in flies.

P3466
Board Number: B758
Jaboticaba extract prevents inflammation and hormonal imbalance in the prostatic microenvironment of high-fat-fed aging mice.
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Jaboticaba is a Brazilian fruit rich in phenolic compounds, such as anthocyanin, which display antioxidants and anti-inflammatory effects. Aging and obesity are involved in prostatic lesions, due to their association with cellular proliferation, inflammation, and hormonal imbalance. Thus, the aim of this study was to evaluate the effect of jaboticaba extract (JE) in molecular pathways related to inflammation and hormonal metabolism, during aging and/or high-fat diet ingestion in mice prostate. Seventy FVB mice was distributed in the following experimental groups: YG(young-3 months old), SE(senile-11 months old), HfSE(senile+high-fat diet), JSEI(senile+0.003mL/gJE), JSEII(senile+0.006mL/gJE), HfJSEI(senile+high-fat diet+0.003mL/gJE) and HfJSEII(senile+high-fat diet+0.006mL/gJE). After 60 days, animals were anesthetized, ventral prostates were collected and submitted to morphological analysis and AR, ERα, COX-2, NFkB immunohistochemical and/or Western Blotting evaluation. Aging itself increased prostate cellular proliferation, well-differentiated adenocarcinoma, microacini and inflammatory infiltrate frequency as well as the COX-2, NFkB and ERα levels, and reduced AR level in relation to JV group. The high-fat diet intensified the senescence alterations. HfSE group presented significantly higher cellular proliferation, well-differentiated adenocarcinoma, microacini and fibromuscular layer frequency, besides increasing AR, ERα, COX-2 and NFkB levels compared to SE group. The treatment with JE at both doses reversed most of the alterations seen during aging by lessening the cellular proliferation, the well-differentiated adenocarcinoma and inflammatory infiltrates frequency as well as the COX-2 level in senile mice. Only JSEII group showed lower fibromuscular layer frequency and reduced AR, ERα and NFkB levels compared to SE group. Moreover, both JE doses in HfJSEI and HfJSEII groups reduced cellular proliferation, microacini, well-differentiated adenocarcinoma, inflammatory infiltrates and fibromuscular layer frequency as well as AR, ERα, COX-2 and NFkB levels in relation to HfSE group. HfJSEII group showed even lower frequency of cellular proliferation and atrophy in relation to HfJSEI group. Thus, JE exerted a dose-dependent positive effect, probably acting on the ERα pathway, which led to prostate microenvironment recovery and hormonal and inflammation balance, as well as to the maintenance of prostate typical morphology, impaired by aging process and/or high-fat diet ingestion. Therefore, JE can be indicated as an alternative therapy for the prevention of prostatic damage during aging and/or overweight.
Proteopathic proteins have characteristic phase behaviors.
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Protein self-assembly regulates the function of many proteins. Under certain conditions or stimuli, the same protein can form different assemblies that correspond to different cellular activities. For example, ASC, an inflammasome adaptor protein, is an inactive monomer in mammalian cells, however, in response to a pathogen or cellular danger signal, ASC undergoes prion-like polymerization which then leads to caspase-1 activation and subsequent programmed cell death. In contrast, for many neurodegenerative disease-related proteins, the formation of a high-order assembly underlies pathology. For example, oligomeric forms of Aβ42 are believed to be toxic and a precursor of the amyloid aggregates that characterize Alzheimer’s disease. However, the mechanistic differences between functional and dysfunctional assembly are unclear. Thus, distinguishing distinct assembled forms of the proteins is a pivotal step to reveal their inter-relationships and roles in disease. Here, I applied Distributed Amphifluoric FRET (DAmFRET), a FRET- and imaging flow cytometry-based approach developed in our laboratory to study protein self-assembly, to show that ectopically expressed disease-associated proteins, including Aβ42, -synuclein (Parkinson’s Disease), and amylin (type II diabetes), each accumulate in yeast cells as diffuse oligomers that are metastable with respect to higher order assemblies. We also found that cell size was negatively affected only in cells that harbored these oligomeric forms of -synuclein or Aβ42, but not in cells that contained -synuclein in an exclusively monomeric form, or Aβ42 in high order assemblies. The oligomers of -synuclein or Aβ42 also inhibited the proliferation of their host cells. Moreover, among several -synuclein mutants examined, only the A30P mutant disrupted oligomerization. It likewise alleviated toxicity. Our results suggest that a propensity to form soluble oligomers (that are metastable with respect to higher order assemblies) commonly underlies proteotoxicity. This ongoing study is aimed to explore the connection between the different assemblies and the underlying pathology of the disease-associated proteins.

Increasing glucose uptake suppresses age-dependent reductions in ATP levels in brain neurons and behavioral deficits in Drosophila.
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Aging has been associated with changes in metabolism at system and cellular levels. Brain neurons are highly energy demanding cells and require local energy supply due to their polarized structures. Disruption in energy metabolism in neurons is thought to cause age-related functional declines as well as neurological and neurodegenerative diseases. However, temporal and special information regarding age-dependent changes in ATP levels in brain neurons is limited. Using Drosophila as a model system,
here we analyzed the age-dependent changes in ATP levels in the cell bodies and axons in brain neurons using FRET-based ATP biosensor. We focused on the mushroom body structure, in which the cell body region and the axonal region are easily distinguished. ATP levels in the axon were similar between young and aged flies. In contrast, we found that ATP levels in the cell body were significantly reduced during aging. To gain insight into the mechanisms underlying age-dependent reduction in ATP levels in the cell bodies, we first compared the numbers and quality of mitochondria between young and aged flies via ultrastructural analyses. While the number of mitochondria was similar between young and aged fly brains, mitochondria with abnormal cristae were more often observed in aged brain neurons, suggesting that ratio of damaged mitochondria was increased during aging. Thus, increased ratio of damaged mitochondria was correlated with age-dependent reductions in ATP levels in the cell bodies. We next compared mRNA levels of glycolytic enzymes in young and aged fly brains. We found that mRNA levels of several rate-limiting enzymes in the glycolytic pathway were reduced during aging. Interestingly, flies with knockdown of pfk, one of the key glycolytic enzymes, did not show age-dependent reductions in ATP levels in the cell body, suggesting that reduced glycolysis also contributed to age-dependent reduction in ATP levels. Interestingly, we also found that enhancement of glucose uptake by neuronal overexpression of glucose transporter (GLUT) can suppress age-dependent declines in ATP levels in the cell body. Moreover, neuronal overexpression of GLUT suppressed the age-dependent decline in locomotor functions in flies. Taken together, these results suggest that age-dependent reductions in ATP levels are due to mitochondrial damage and reduced glycolysis, while reduction in ATP levels as well as decline in neuronal functions can be suppressed by increasing GLUT into neurons. Further study of regulatory mechanisms underlying glucose uptake in brain neurons may lead to novel therapeutics against normal aging and age-related neurodegenerative diseases.

P3469

Board Number: B761

Role of sphingolipid enzymes in the regulation of stress response and aging through detoxification protein pathways.

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Sphingolipids play an important role in different regulatory processes including those for structural support and cell signaling. Sphingosine is a sphingolipid involved in regulating cell death, whereas sphingosine-1-phosphate (S1P) is a sphingolipid that induces cell proliferation and inhibits apoptosis. Thus, the enzymes that control cellular levels of sphingolipids, such as sphingosine kinases ceramide synthases, and acid sphingomyelinases, are critical to cell survival decisions. When cells face stress, such elevations of reactive oxidative species (ROS) that damage proteins and lipids, they must respond to counteract stress. Cellular responses to ROS include the upregulation of detoxifying proteins such as glutathione S-transferases (GSTs) and superoxide-dismutases (SODs). Recently, it has also been shown that sphingolipids are important for stress responses in animals. We show here that C. elegans mutants lacking sphingosine kinase (sphk-1) have poor heat and oxidative stress responses, whereas animals lacking certain ceramide synthases exhibit greater stress response. In particular, the stress resistance phenotype of ceramide synthase mutants is dependent on sphk-1, suggesting that conversion of sphingosine to S1P may be critical to maintaining stress responses. However, it is not known whether sphingolipids directly influence stress response through detoxification protein pathways. To examine this, we further analyzed a panel of C. elegans sphingolipid metabolism mutants; sphingosine kinase (sphk-1), ceramide synthase (hyl-1, hyl-2), and acid sphingomyelinase (asm-3) in oxidative stress assays. We found that H2O2 treatment induced the the expression of sod-3/SOD, using GFP transcriptional

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Sphingolipids are cellular signaling molecules known to play vital roles in locomotion and neuromuscular function in organisms. In particular, sphingosine-1-phosphate (S1P), promotes neurotransmitter and neuropeptide release, is produced by the activity of the enzyme sphingosine kinase. Sphingolipids are interconverted through enzymatic activity that mediates lipid content within a cell. However, S1P levels change with age, but it is not known how loss of S1P and changes in sphingosine kinase activity mediate changes in neuronal aging. Using the model organism C. elegans, we aimed to determine the effects of loss of sphingolipid metabolism enzymes on the maintenance of locomotion and neuromuscular function in age. Using movement, we aim to measure how sphingolipid metabolism affects health span, defined as the amount of time an organism lives until the onset of morbidity, or serious or chronic illness. To do this, we examined thrashing and body bending behavior in aging animals at multiple time points; we used C. elegans knockouts of the sphingolipid pathway enzymes including sphingosine kinase (sphk-1), ceramide synthases (hyl-1, hyl-2), and an acid sphingomyelinase (asm-3). We found that the loss of sphk-1 leads to decreased thrashing behavior and declined neuromuscular function with age in comparison to wildtype animals. However, loss of hyl-1 results in increased thrashing behavior in age. Furthermore, we wanted to determine the role of sphingolipids during development and adulthood. To do this, we assessed the role of sphingolipid metabolism enzymes during different windows of age through the use of RNAi-mediated knockdown, which also allowed us to assess combinations of enzymes.

P3471
Board Number: B763
Poly(ADP-ribose) (PAR) promotes TDP-43 liquid droplet formation but reduces TDP-43 aggregation.
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TAR DNA-binding protein 43 (TDP-43) is a nuclear RNA-binding protein (RBP) that contains a C-terminal prion-like domain (PrLD). Approximately 97% of cases of amyotrophic lateral sclerosis (ALS) show mislocalization and cytoplasmic aggregation of TDP-43. Interestingly, several other RBPs with PrLD, including Fused in Sarcoma (FUS) and heterogeneous nuclear ribonucleoprotein A1 and A2 (hnRNP A1 and hnRNP A2), also mislocalize and aggregate in ALS. These RBPs are components of stress granules,
which are membrane-less organelles that form via liquid-liquid phase separation in cells experiencing stress. Research has been done on FUS and hnRNP A1 and A2 showing that these proteins are capable of liquid-liquid phase separation in vitro, so we thus hypothesized that purified TDP-43 will similarly undergo liquid-liquid phase separation. Furthermore, it is thought that aberrant phase transition of these RBPs leads to misregulation of stress granule dynamics, which then induce toxicity and disease. Therefore, understanding the basis of TDP-43 recruitment to stress granules may provide potential avenues for ALS therapies. Previous research indicated that Poly (ADP-ribose) (PAR) is required for stress granule assembly. Interestingly, TDP-43 contains a putative PAR binding motif and localizes to stress granules that are Poly (ADP-ribosylated) (PARylated). This suggested that poly (ADP-ribose) (PAR) may recruit TDP-43 into stress granules, and so we further hypothesized that PAR may promote TDP-43 phase separation by binding TDP-43. We purified SUMO tagged TDP43 and here show that SUMO-TDP-43 is indeed capable of phase separating into liquid droplets in vitro. Moreover, the addition of PAR promotes TDP-43 liquid droplet formation while mutating the putative PAR-binding motif dramatically reduces TDP-43 phase separation. Additionally, TDP-43 is an inherently aggregation prone protein that also forms solid aggregates upon cleavage of the SUMO tag. We used turbidity measurements to monitor this aggregation kinetics and found that addition of PAR reduced the formation of solid aggregates. Together our results suggest that poly (ADP-ribose) promotes liquid-liquid phase separation of TDP-43 while also preventing TDP-43 from forming solid aggregates.

P3472

Board Number: B764

Young blood serum increased cellular response associated to wound healing in old rats.
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Aging is a natural process which is associated with the appearance of a number of diseases that contribute to progressive deterioration in the quality of life. Aging affect negatively wound healing. However, the mechanisms underlying this phenomenon remain unclear. Here, we studied the in vivo wound healing process during aging using a rat skin repair model in young and old rats and an in vitro model using primary skin fibroblast from young and old rats and human gingival fibroblast. To do that, we evaluated histologically using Masson Trichrome the in vivo effect of young and old rat blood serum in skin wound healing in young (2 months old) and old rats (18 months old). We next evaluated in vivo and in vitro myofibroblast differentiation. To do that we used rat blood serum applied topically in skin wound form young and old rats, rat skin fibroblast obtained from young and old rats and human blood serum classified into 3 groups: “young” from 18 to 22 years old, “middle-aged” from 30 to 48 years old and “aged” over 50 years old. Myofibroblastic differentiation, measured through alpha-smooth muscle actin (α-SMA) was decreased in the presence of middle-aged and aged serum compared to young serum. Moreover, the topical use of young blood serum in skin wound repair improves wound healing and myofibroblast differentiation in old rats. The present study suggests that serum factors present in middle-aged and aged individuals may be responsible, at least in part, for the altered responses observed during wound healing in aging.
Funding Fondecyt 11140064 (MC).
P3473
Board Number: B765
Alcohol causes a defect in telomere homeostasis.
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Telomeres are repeated DNA sequences at the distal tips of eukaryotic chromosomes. The linear nature of chromosomes causes a dual problem for telomeres; the end replication problem and the end-protection problem. The end-replication problem is solved by telomerase, which is a ribonucleoprotein composed of telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT). Telomerase adds the TTAGGG repeat to chromosome ends using TERC as a template. Shortened telomeres are observed in chronic drinkers and shorten telomeres are a factor in pathology of many diseases. Despite all that is known about the adverse effect of alcohol, the molecular mechanism of alcohol-induced telomere shortening is still unclear. To address this question, we establish a zebrafish model of alcohol consumption to ascertain how alcohol impacts telomere homeostasis. We find that TERRA, a long noncoding RNA that originates from telomeres and is needed for telomere heterochromatin, was phase-shift relative to the normal TERRA rhythm. In addition, it also appears that alcohol intake disrupts the diurnal rhythm of TERT and TERC. Our data suggest that alcohol intake alters normal telomere homeostasis on multiple levels, including TERRA expression, heterochromatin formation, TERT and TERC expression. These findings reveal multifarious mechanisms involved in alcohol-induced telomere shortening.

P3474
Board Number: B766
Functions of CLIC proteins in heat stress in C. elegans.
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Chloride intracellular channel proteins (CLIC) are multifunctional proteins. Mammalian CLIC family, which has seven members, varies in subcellular localization and cellular functions. Cellular stress molecules induce endogenous CLIC4 to translocate from the cytoplasm into the nucleus. Mammalian Schnurri-2 is required for CLIC4 nuclear translocation in response to Transforming Growth Factor – beta (TGF-beta) but not required for CLIC4 nuclear function. Schnurri-2 is a transcription cofactor in the BMP pathway. However, the physiological functions of CLIC in whole animals level are not well understood, in particular how the genes regulate thermostolerance is largely unknown. To address these issues, we took advantage of viable CLIC mutants in C. elegans and characterized its functions in heat stress and aging. Schnurri-2 is homologous to C. elegans SMA-9 which functions in the DBL-1/TGF-beta pathway. There are two CLIC homologs in C. elegans: EXL-1 and EXC-4. exc-4 mutants develop cysts in the excretory canal, while abnormal phenotypes of exl-1 mutants have not been identified. We analyzed integrated EXL-1::GFP lines in wild type background and observed strong fluorescence in intestinal cells, which is consistent with previous study. EXL-1::GFP indeed is translocated into the nucleus under various heat shock conditions. Supporting functional importance of this, exl-1 loss-of-function mutants are thermosensitive, in compare with wild type animals. Meanwhile, we found that EXL-1, not EXC-4, bears a non-classic Nuclear Localization Signal (NLS). This may explain why exl-1 translocates into the nucleus upon heat stress, while exc-4 still remains in the cytoplasm. Our phylogenetic analysis across evolutionary species shows that duplication of CLICs in protostomes and deuterostomes occurred independently. We also observe that EXL-1 nuclear translocation occurs in a timely ordered manner in the intestine, from

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posterior to anterior regions. Furthermore, we also generated double mutants of exl-1 and DBL-1/TGF-beta pathway components and will dissect the genetic interactions between exl-1 and the DBL-1 pathway in the future.

P3475
**Board Number: B767**
**Human Dermal Stem/Progenitor Cell-derived Conditioned Medium Ameliorates Ultraviolet A-induced Damage of Normal Human Epidermal Keratinocytes.**
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Purpose: The present study aimed to define the epidermal moisturizing effects of human dermal stem/progenitor cell-derived conditioned medium (hDSPC-CM) on normal human epidermal keratinocytes (NHEKs). Methods: To investigate the epidermal moisturizing effects of hDSPC-CM on NHEKs, I measured 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity and gene expression and performed cell viability assay and hyaluronic acid (HA)—enzyme-linked immunosorbent assay (ELISA). Results: In the present study, I elucidated the effects of hDSPC-CM on the mRNA expression of hyaluronan synthase 2 (HAS2), aquaporin 3 (AQP3), keratin 1 (KRT1), and keratin 10 (KRT10) and HA production. Real-time polymerase chain reaction (real-time PCR) revealed that hDSPC-CM increased HAS2 and AQP3 expression in NHEKs, and HA-ELISA revealed that hDSPC-CM increased HA production in NHEKs. On the other hand, hDSPC-CM decreased KRT1 and KRT10 expression in NHEKs. Conclusions: The results revealed that hDSPC-CM is a potent moisturizing cosmetic ingredient for epidermal moisturization. Based on this study, I anticipate further studies on mechanisms underlying the effects of hDSPC-CM on epidermal keratinocytes to develop not only cosmetics but also healthcare medicines.

P3476
**Board Number: B768**
**Crataegus pinnatifida extract increases lifespan of Drosophila melanogaster.**
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*Crataegus pinnatifida* is known to possess various medicinal properties. In this study, we investigated the lifespan-prolonging effects of *Crataegus pinnatifida* extract (CPE) on in vivo using *drosophila melanogaster* and explored its underlying mechanism. We found that CPE significantly extended the lifespan of drosophila melanogaster in both male and female. To elucidate the efficacy of CPE to the longevity, we evaluated the expression of aging related genes, such as atg5, atg8, TRF, and Hep. In addition, CPE improved the antioxidant ability in drosophila by regulating the antioxidant enzymes of superoxide dismutase (SOD), catalase, Thioredoxin (Trx) and hemeoxygenase (HO) in RNA level as well as in protein level. The progress of antioxidant ability was confirmed by prolonged survival time of drosophila melanogaster treated with CPE in H2O2 challenge test. Furthermore, we found that increased lifespan by CPE was associated with increased lipid level and locomotion activity. Therefore, our data suggest that CPE extended the lifespan in *drosophila melanogaster* attributing to exerts upregulating endogenous antioxidant response and at least partially associated with its interaction with increasing lipid level.
Chemical Cell Biology

**P3477**  
**Board Number: B770**  
Probing mitochondrial dynamics and heterogeneity during cell state switching using multiplexed, environment-sensitive fluorescent dyes.  
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Mitochondria are known as power house of the cell as they efficiently produce ATP through Oxidative Phosphorylation (OxPhos). Apart from this, mitochondria also play significant role in regulating cellular metabolism, calcium and ROS signaling as well as in programmed cell death. Despite decades on research, precise and real-time information on mitochondrial dynamics and functionality, is still limiting. For example, recently it has been shown that cellular migration during metastasis relies, in part, on mitochondrial motility and precise positioning within the cell. Similarly, morphology and activity of mitochondria are linked with maintenance of stemness as well as triggering of differentiation event. Therefore, unraveling the spatio-temporal localization as well as functional heterogeneity of mitochondria during various cellular states appears to be crucial for understanding the coherent behavior of cell. To better visualize the functional dynamics of mitochondrion, we have developed red-emitting, multi-functional, novel mitochondrial probes that are sensitive to local environment, specifically parameters like viscosity, pH, ROS, etc. The developed dyes have low toxicity and very high photo-stability, allowing their use in long term imaging. In this presentation, we will show these dyes have yielded new insights into mitochondrial dynamics in embryonic stem cells as well as onset of differentiation. In a different example, we have also used our new dyes to probe mitochondrial heterogeneity within primary ‘activated’ cells during cell migration. These results would be placed in the context of our larger efforts to build new ways of probing ‘cellular dynamics’ with a focus on physico-chemical changes in the cell.

**P3478**  
**Board Number: B771**  
Discovery and development of novel antifungal compounds from marine endophytic fungal sources.  
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Discovery of new drugs have shifted from plants to microorganisms due to the enormous diversity. Marine endophytic fungi (MEF) have not been explored as compared to their terrestrial counterparts in the treatment of diseases. We set out to identify and develop novel antifungal compounds from marine endophytic fungi sources using yeast genomics guided approach. To achieve this, isolated marine endophytic fungi (Top 6) were fermented (1-2 litres) and extracted with ethyl acetate after 4 weeks. Crude extracts were tested against S. cerevisiae and C. albicans with majority of the extracts showing activity above 10mm zone of inhibition. Characterization of the crude extracts and fractions were performed against C. albicans, S. cerevisiae and mutant S. cerevisiae. The activities of the crude extracts
were tested under various chemical conditions against S. cerevisiae and C. albicans with most of the extracts maintaining their activities across the chemical conditions. Crude extracts from the selected 6 MEF were fractionated by Kupchan’s solvent partitioning and preparative thin layer chromatography (TLC). The data obtained from this study showed that the extracts possessed distinct activities and were able to maintain their activities even when the cells were under different chemical conditions. A total of 59 active fractions were obtained from the 3 selected MEF after the preparative TLC. It is estimated that 148 active fractions will be obtained after the analysis of all 6 selected MEF. Two of the fractions from MEF 134 were highly active. An HPLC-HRMS full scan was performed on fractions 134FDV5V7 and 134FDV5V9. Both fractions (V7 and V9) had 2 peaks that were similar, all others were different. Preliminary structural elucidation of the compounds identified based on ms/ms showed all structures to be novel. Hence 100 Litre cultures of MEF 134 have been prepared for large scale product isolation.

P3479
Board Number: B772
Remodeling and Visualizing Bacterial Peptidoglycan to Understand Crohn’s Disease.
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Bacterial cells surround themselves with a peptidoglycan cell wall, an essential structure that resists changes in osmotic pressure and other environmental insults. To a certain degree, the peptidoglycan is also essential to humans as antibiotics target its destruction and fragments activate immune responses. Crohn's disease is believed to arise from a misrecognition of the commensal bacteria that inhabit the human body. Normally, the body recognizes fragments of bacterial cell wall; however in CD this pathway is disabled. Chemical tools to investigate the problem of misrecognition are needed to understand why the innate immune system fails in CD. We have developed novel syntheses of bacterial cell wall fragments, expression systems for mutated innate immune receptors and a series of assays to measure and map binding events. Here we present two independent methods to metabolically label the glycan backbone of the bacterial peptidoglycan and track its breakdown. We have developed the methodology to label and visualize the glycan backbone. As chemists, we are passionate about working with other cell biologists to incorporate this methodology into their projects.

P3480
Board Number: B773
Exploration of a novel cyclic peptide that senses lipid-packing defects.
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Regulation of dynamics of cellular membrane is vital for cell functions such as membrane trafficking. Development of the tools to observe or artificially regulate membrane dynamics including membrane curvature and tension will gain novel insights into cellular events. In this study, we have explored a new cyclic peptide to sense membrane curvature, or lipid-packing defects, using a random non-standard peptide integrated discovery (RaPID) system in which cyclic peptides (\(~ 10^{13}\)) can be produced by in vitro translation of mRNA libraries. We used 0.03 μm-filtered DOPC liposomes containing biotin-labeled DOPE as high curvature membrane immobilized on Streptavidin magnetic beads. We selected the peptides that bound to the small liposomes by excluding the peptides that strongly bound to the beads. After several selection cycles, the obtained peptide sequences were analyzed and then the 4 peptides
labeled with nitrobenzoxadiazole (NBD) were chemically synthesized as candidates to sense lipid-packing defects. We carried out NBD fluorescence assay to examine the binding affinity of each peptide to various liposomes with different compositions and sizes. We have found one of the peptides can sense lipid-packing defects. This peptide was consisted of cyclic peptide and tail peptide. The linear peptide without cyclization was less sensitive to lipid-packing defects than the original one. This suggests that cyclic structure could enhance the ability to sense lipid-packing defects. We expect that our findings lead to the development of a novel peptide sensor for membrane curvature in living cells or for small vesicles such as exosomes.

P3481
Board Number: B774
Study of the purinosome in HeLa cells and Caenorhabditis elegans model systems.
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Objectives: Defects of ADSL and ATIC enzymes of de novo purine synthesis (DNPS) cause two known metabolic disorders – ADSL deficiency and AICAr ibosiduria. This malfunction leads to accumulations of substrates of the affected enzyme and afterwards to unspecific neurological symptoms. Moreover, there is clear disruption of multienzyme complex, purinosome. We decided to prepare human cellular model (HeLa cells) and multicellular model (Caenorhabditis elegans) to study intracellular compartmentalization and formation of purinosome.

Methods: CRISPR-Cas9 method was used to prepare HeLa cells deficient for specific steps of DNPS. The DNPS proteins in HeLa cells were immunofluorescently labeled. The DNPS proteins in C. elegans were fused with fluorescent protein GFP or mCherry. The intracellular compartmentalization of purinosome was observed by fluorescence confocal microscopy.

Results: Deficient HeLa cells for GART, ADSL and ATIC enzymes showed no signal overlap, which suggest the disruption of purinosome formation. HeLa cells deficient for PFAS and PAICS enzymes showed fine granular staining and reduced formation of purinosome compared with control HeLa cells grown in the same conditions. These findings correspond with previous studies, that the structurally intact proteins are important for purinosome assembly. The organism C. elegans with translational reporters of human homologues PPAT::GFP and GART::mCherry appears to be valuable model for study of purinosome formation in vivo. It showed us that the purinosome complex is dynamic structure. Its localization differs in different developmental stages and it is represented in different types of tissues.

Conclusions: We prepared CRISPR-Cas9 genome-edited model HeLa cells and multicellular model organism C. elegans with translational reporters. Both models gave us interesting insights into purinosome assembly and offer us opportunity to furthermore investigate the physiological and pathogenic mechanisms of purinosome formation and regulation.

This work was supported by grant GAUK 1102217; grant AZV 15-28979A from the Ministry of Health, CZ; and programs: PROGRES-p24/LF1/3, SVV UK 260367 from the Charles University in Prague, CZ, BIOCEV (CZ.1.05/1.1.00/02.0109) and NPU II MSMT LQ1604.
P3482
Board Number: B775
Bioorthogonal fluorescent probe for molecular imaging of cancer aggressiveness.
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While early stage cancers that were hard to detect can now be analyzed by high content imaging screening and many of these early detected cancers are not life threatening. Although it is possible to detect early stage cancers with appropriate screening methods, some typical lesions are cancer and still cannot always distinguish from the cancers which are not life threatening. Some certain biomarkers are frequently used to detect early stage of some cancer types. However, we still do need better specific markers such as small fluorescent molecules that can tag some of the certain proteins in cancer cells to monitor aggressiveness of the cancer at a molecular level. Likewise, fluorescence imaging is referred to as a “tool” used to detect spatially or temporally resolved cancer cells, tissues and their surroundings. We therefore aim to develop new bioorthogonal imaging agents (fluorophores) using fluorescence spectroscopy for monitoring aggressive cancers and the less aggressive ones. The design of suitable imaging agents for biological labeling requires consideration of both the reactivity of the nucleophilic functional group and good spectroscopic features of the fluorophore. Although newly designed fluorophores do react with carbonyls at neutral pH to form a hydrazone product, the reaction is very challenging. In this work, confocal microscopy was here used to be an effective approach to distinguish two cancer cell lines with different metastatic ability, based on the changes in their fluorophore compositions due to their oxidative stress levels. A moderately metastatic (A498), and an advanced metastatic (ACHN) prostate cancer cell lines were monitored with the newly synthesized fluorophore using confocal microscopy after treatment with certain amount of hydrogen peroxide. The experimental and the analyzed results indicate that confocal microscopy can be used to monitor the changes of the key fluorophores response to carbonylation of various cell lines with different cancer risk levels. The method introduced here may also form the basis of a synthetic small molecule based diagnostics approach to give a new insight in molecular cancer staging.

P3483
Board Number: B776
Structure-activity relationship study for α-dystroglycan binding peptide A2G80 derived from mouse laminin α2 chain sequence.
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Dystroglycan (DG) is a component of the dystrophin-glycoprotein complex (DGC) in skeletal muscle. DGC is a molecular complex composed of dystrophin and membrane glycoprotein group and forms a structure linking the basement membrane and the actin skeleton. The association of basement membrane-cytoskeleton mediated by DGC is thought to be important for maintaining the strength of the muscle cell membrane which can withstand mechanical stress such as elongation and contraction of the muscle. DG binds with dystrophin directly under the cell membrane, it is bound extracellularly to basement membrane molecules such as laminin and play a role as a molecular axis linking the cytoskeleton and basement membrane. DG consists of two subunits α and β, αDG, which is an extracellular subunit, binds ligands. βDG is a transmembrane subunit, which binds αDG to the cell surface extracellularly, and binds dystrophin within the cell. Laminin is known as a ligand molecule of αDG. Laminins are major components of basement membrane, consists of three different subunits, α, β,
and γ chains, and so far, five α, three β, and three γ chains have been identified. We have constructed the synthetic peptide library derived from the laminin sequences, and identified functional peptides. We have reported that A2G78 (GLLFYMARINHA, mouse laminin α2 chain, 2796-2807) and A2G80 (VQLRNGFPYFSY, mouse laminin α2 chain, 2812-2823) peptides bind αDG [1]. In this study, we investigated the structure-activity relationship of A2G80 to identify the specific residues for binding to αDG. The αDG was rough purified from mouse muscle myoblast cell line C2C12. The A2G80 and its derivative peptides substituted each amino acid residue to alanine were synthesized by Fmoc-solid phase methods, furthermore, the biotinylated peptides of A2G80 and its Ala substituted peptides were prepared. The circular dichroism (CD) spectra of the peptides were measured. The peptides substituted Asn5, Phe7, Pro8, Tyr9, Phe10, and Ser11 for Ala were different spectra to A2G80 peptide. The binding activity was evaluated by enzyme immunoassay using biotinylated peptides and crude αDG. The A2G80 peptide bound crude αDG with dose-dependent manner. The peptides substituted Val1, Gln2, Arg4, Gly6, Phe8, Tyr9, and Tyr12 to Ala lost the αDG-binding activity. From the result of X-ray crystallographic analysis, it is reported that the Gln2 and Arg4 were critical residue for binding to αDG. [1] Suzuki N, Hozumi K, Urushibata S, Yoshimura T, Kikkawa Y, Gumerson JD, Michele DE, Hoffman MP, Yamada Y, Nomizu M. Identification of alpha-dystroglycan binding sequences in the laminin alpha2 chain LG4-5 module. Matrix Biol. 2010; 29: 143-151.

**P3484**

**Board Number: B777**

**Inhibition of osteoclast formation and function by EW33.**

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Many bone diseases such as osteoporosis and rheumatoid arthritis are due to an increase in osteoclast number or activity, of which control thus has significant clinical implications. Here, we show that EW33, a natural compound having anti-inflammatory activity, inhibited osteoclast differentiation and function. It inhibited osteoclast differentiation with decreased activation of NFATc1, mitogen-activated protein kinases (MAPKs), Src and CREB. Such effects of EW33 were reversed by co-treatment of hydrogen peroxide, and it also reduced reactive oxygen species by scavenging them or activating Nrf2. In addition, it significantly attenuated the survival, migration and bone resorption of differentiated osteoclasts with decreased expression of RANK, c-Fms, caveolin-1 and p130Cas. Furthermore, it inhibited inflammation-induced bone erosion in the calvaria of mice. These results suggest that EW33 may inhibit osteoclast formation through the redox-dependent regulation of MAPKs, Src and CREB during RANKL-induced osteoclastogenesis, and attenuate osteoclast function by regulating caveolin-1 and p130Cas expression.

**P3485**

**Board Number: B778**

**EW17 inhibits osteoclastogenesis and attenuates the migration and resorption of osteoclasts.**

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Excessive number and activity of osteoclast cause many bone disease such as osteoporosis and rheumatoid arthritis. Thus, the control of osteoclast formation and function has significant clinical
implications. EW17 was identified from the screening of phytochemicals inhibiting RANKL-induced osteoclast differentiation. EW17 inhibited the activation of NFATc1 with decreased activation of NF-kB, JNK and Src, and c-Fos expression during osteoclastogenesis. It decreased intracellular reactive oxygen species by scavenging them or activating Nrf2. In addition, it inhibited the migration and bone resorption of osteoclasts with decreased expression of Src, p130Cas and caveolin-1. Moreover, it inhibited inflammation-induced bone erosion in mouse calvariae and ovariectomy-induced bone loss in mice. These results suggest that EW17 may inhibit osteoclast differentiation by regulating NF-kB, JNK and Src through the control of redox status and attenuate osteoclast function by regulating Src, p130Cas and caveolin-1 expression, and support its therapeutic potential for treating bone diseases caused by moderate osteoclast activity.

P3486

Board Number: B779

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The study reports biological derived synthesis of gold nanoparticles using marine bacterial isolates of Arabian Sea, Karachi. The isolates were identified as Vibrio alginolyticus, V. natrigens, V. campbelli and Bacillus aquimaris using 16S rRNA gene sequence similarity. The synthesized nanoparticles showed a prominent peak at 550 nm; with SEM image result analysis showing particles within the range of 85-200 nm. Particles of diverse shapes were visible from the SEM image analysis. Most of the particles generated were elliptical or oval with abundance of spherical shaped nanostructures however, a few triangular shaped particles were also visible from SEM images of B.aquimaris with maximum size of 118 nm and smallest particle of 85 nm. The EDX further confirms the particles to be of gold with one prominent peak visible at 2 KeV while three to four smaller peaks are also visible around 8 and 9 KeV. The in vitro cytotoxicity studies of biosynthesized gold nanoparticles against leukemia cell line show the particles to be of non toxic nature with no effect on cell morphology or cell viability making them ideal targets for delivery of genes or drugs as well as bioimaging tools.

P3487

Board Number: B780

Purification of an exceptionally resilient and long-lived orange fluorescent protein from the Gulf anemone Calliactis tricolor.
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Green fluorescent protein (GFP) was one of the most useful discoveries of the 20th Century, due to its subsequent use in cell and molecular tagging applications, resulting in three Nobel Prizes in 2008 (Noble Media AB 2014). GFP and similar proteins, including anthozoan fluorescent proteins, have been used in numerous research efforts to probe cellular structure and function since its introduction. We have discovered a similar protein in a variant of the common anemone, Calliactis tricolor, from the Alabama Gulf Coast, which, unlike other anthozoan fluorescent proteins (Curr. Opin. Chem. Biol. 20:92; Curr. Opin. Biol. 12:505), is extraordinarily long-lived and resilient. In February 2014 and 2015, anemones were collected by trawling at a depth of 8 to 20 m south of the Mobile Bay Entrance. The anemone’s mouth and mesenteries produce intense orange fluorescence, peak emission at 510 nm and 570 nm when illuminated by blue light at 500 nm and 540 nm. Illumination at 490 nm gives the perception of
brilliant orange fluorescence. The protein appears to be freely associated with the cytoplasmic space of mesentery cells; purification of the protein from a cleared cytosolic homogenate by 5-20% sucrose gradient rate-zonal centrifugation yields a ~10S trimeric quaternary complex with three bands at 31, 25 and 10 kDa as revealed by SDS-PAGE. LC-MS/MS reveals that the fluorescence moiety is novel and has effectively no homology to any previously known fluorescent protein; moreover the fluorescent group is found only in the largest subunit. Ongoing whole genome analysis and differential transcriptomic analyses seek to reveal genome organization and appropriate regulatory elements and should provide data for future incorporation of this group into cellular probes. Our work reveals that this is a very easy to purify, resilient and easily handled protein, albeit limited currently by availability of the host, which has been impacted by heavy rainfall in the collection area. Funded by the Office of the Provost and the Office of the Vice President for Research and Economic Development, Auburn University.

P3488
Board Number: B781
A chemical-genetic toggle switch for phosphorylation events applied to Plk1 signaling.
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Protein kinases are a large family of enzymes that regulate pathways through phosphorylation of threonine, serine, or tyrosine phosphoacceptors. Yet, untangling these signaling networks proves challenging as most kinases are pleiotropic, with large numbers of molecular targets and functions. It is not currently possible to chemically control a single phosphorylation with tight temporal control. Here we describe a chemical-genetic toggle switch for serine/threonine kinases that allows for control of a particular phosphorylation event. This is accomplished by regulating phosphoacceptor preference, allowing threonines to be chemically controlled while serines remain phosphorylated. We demonstrate this system in RPE1 cells using polo-like kinase 1, a pleiotropic and essential mitotic kinase. We first generated Plk1S and Plk1T alleles, selective for serine or threonine phosphoacceptors, respectively, by mutating sites in the phosphopeptide interface that engage the substrate. We verified Plk1S (L197F) and Plk1T (L197S/L211A) as phosphoacceptor-specific mutants through in vitro kinase assays with purified substrates containing S, T, or both S/T as phosphoacceptors. To evaluate phenotypic effects, these constructs were used to complement a cell line driven by Plk1as, which can be chemically controlled by 3-MBPP1 without affecting Plk1S or Plk1T. Neither Plk1S nor Plk1T were sufficient to restore all essential mitotic functions of Plk1, as cells failed to proliferate by comparison with either phosphospecific allele. Surprisingly, however, Plk1T is sufficient to restore mitotic progression to anaphase, but is unable to restore essential roles of Plk1 in cytokinesis. By contrast, Plk1S was unable to restore any kinase functions surveyed, suggesting that threonine phosphorylations have less functional significance. To demonstrate the ability to toggle phosphorylations from always on (S) to chemically controlled (T), we expressed the motor protein Kif2b47, in which 4 serine phosphoacceptors are mutated to threonines. As predicted, expression of Kif2b47 with Plk1S yields a chemical-genetic toggle switch where Kif2b can now be chemically controlled. When switched off, there is a sharp increase in the frequency of misaligned chromosomes, attributable solely to this particular phosphorylation event. These findings demonstrate a method whereby specific phosphorylations are designated to specific functions in a temporally controlled manner, eliminating confounding factors associated with overexpression of non-phosphorylatable mutants. While we have applied this technique to Plk1, it can in principle be used to chemically control any desired S/T phosphorylation event to more clearly define kinase-signaling networks.
Development of protein tools to study receptor clustering on cell membrane.

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Receptor proteins freely move membrane and convert extracellular stimuli into intracellular responses by spatial arrangement that brings receptor clusters. Receptor clustering on cell membrane is one of the key mechanisms underlying cellular signal transduction, so understanding it is crucial to illuminating the function of biological systems. Despite of the significance, visualizing receptor clusters in detail have been obscure because it requires the use of methods that reveal both molecular and supramolecular in sub-nanometer level. We engineered protein tools, ‘Membrane (M) -BiFC’, based on BiFC (Bimolecular Fluorescence complementation) to visualize and control the receptor clustering. BiFC is one of the most widely used tools for direct visualization of protein-protein interactions in living cells. The strategy of BiFC is to split a fluorescent protein into two non-fluorescent fragments at the proper site. A fluorescent complex is formed when two proteins (that are fused to fragments of a fluorescent protein) interact with each other. The interaction between the fusion proteins leads the association of the fluorescent protein fragments. However, the main limitation of current BiFC assays is a low signal to noise ratio caused by self-assembly between the two non-fluorescent fragments. It contributes to false-positive fluorescence, which makes data interpretation difficult. Here, we developed new BiFC systems, ‘M-BiFC’, which have low self-assembled properties for studying receptor clustering on cell membrane. Charged superfolder GFP and Venus (mutation form of YFP) proteins were engineered to form M-BiFC fragment pairs. SpyCatcher-Spytag and Calmodulin-M13 binding pairs were used for specific protein-protein interactions of fused proteins. Various Split pairs of charged sGFP as well as Venus proteins were tested for reduced false-positive fluorescence during overexpression in bacterial cytosols. After then we applied M-BiFC to membrane system of mammalian cell. We demonstrate M-BiFC offers new scaffold to enable visualization of the clustering of diverse transmembrane receptors including Death receptor, GPCR(G protein coupled receptor) and integrins in multiple cell types.
ions are observed, which indicate a bidentate NO3- coordination [2]. The molar conductivity (65 S-cm2-mol-1) of the cobalt chloride complex with IQ-1 gives an evidence for the presence of one chloride ion in the outer sphere. The other complexes showed low values of molar conductivity, indicating the absence of an outer sphere. Cu(II) complexes demonstrated the highest superoxide dismutase (SOD)-like activity in a murine bone marrow leukocytes, generated superoxide anion, with IC(50) values in the nanomolar range.


Acknowledgements: Research has been partly supported by the Russian Foundation for Basic Research, Grant No. 17-43-220969 and the state project «Science», № 4.8192.2017/8.9. Study of SOD-like biological activity was supported by the Russian Science Foundation, Grant No. 17-15-01111.

P3491
Board Number: B784
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Oxidative stress is the result of an imbalance between pro-oxidant and anti-oxidant species inside the body. An excess of reactive oxygen species (ROS) introduces covalent modifications in proteins, lipids and DNA altering their structure and function. These covalent modifications are a result of oxidation and introduce an aldehyde or ketone functional group on the biomolecule, which is referred to as carbonylation. We have shown previously that a coumarin fluorophore, containing a hydrazine functional group, may be used to probe these carbonylated biomolecules in live cells. This fluorophore, however, has limited versatility such as high excitation energy, photobleaching, and low brightness. In order to improve upon these, a novel hydrazide containing fluorophore has been synthesized that is able to conjugate to the carbonylated biomolecules in live mammalian cells. This probe can be excited in the lower energy green fluorescent protein (GFP) channel, which is common to many fluorescence microscopes. Furthermore, the quantum yield of the molecule undergoes a large increase upon covalent conjugation with aldehydes and ketones. The fluorophore is resistant to photobleaching and is therefore a promising probe for observing the fate of carbonylated biomolecules in live cells and for use in high-throughput and high-content cellular assays for the detection of protein and lipid carbonylation.
P3492
Board Number: B785
Polymeric Particles for the Control Release of Bioactive Molecules to PC12 Cells.
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The demonstration of a molecular delivery system that consistently releases a protein to mammalian cells is of special interest for the elimination of experimental errors. Researchers could use protein loaded microspheres instead of retreating the cells with solubilized protein solutions every or every other day. PC12 cells, derived from rat pheochromocytoma, are a common cell type used to model neuronal differentiation [1] and we specifically tested our microspheres using PC12 cells. This cell line will grow neurite-like outgrowths when exposed to NGF (nerve growth factor) [2]. Prior to protein encapsulation, we tested the encapsulation methodology on two model small molecules. Ibuprofen, a household anti-inflammatory drug, and retinoic acid, a bioactive lipid, were encapsulated in polylactic acid (PLA) microspheres. The release profiles of these molecules and their biocompatibility details were acquired and quantified. Next, we prepared PLA microspheres incorporating a dye-labeled protein via the double emulsion synthesis method to demonstrate the encapsulation proteins in PLA microspheres. We envision expanding our work to further include other non-fluorescent but bioactive proteins such as NGF. This presentation will focus on microsphere synthesis and characterization with and without PC12 cells and with and without encapsulated molecules. Quantitative and statistical analysis results will include live-cell microscopy data.


P3493
Board Number: B786
Cholic Acid Conjugation as a Tool for Enhancing Intracellular Delivery of Linker-Extended Constructs.
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Chemical tagging of biologically active entities is instrumental to the research at the interface of chemistry, biology and biomedicine. Such tools deliver reliable ways to study existing, discover novel and molecularly engineer new variants of mechanisms of action for a plethora of chemical probes, including small molecules, gene products and conjugates thereof. A major challenge when designing probes that bear additional functionality is their decreased cellular uptake, which is essential for probes aimed at intracellular targets. The extension of a given probe by a polyethyleneglycol (PEG) or other linker often results in strongly diminished or no intrinsic biological activity. To address this challenge, we report that direct conjugation to cholic acid derivatives serves as a useful tool in overcoming decreased biological activity of the linker-extended constructs. Moreover, we systematically explore structure–activity relationships of cholic acid conjugation using cell biological assessment of the constructs targeting three unrelated targets to demonstrate the potential utility of this approach.
P3494

Board Number: B787

Identification of natural compounds that alter myoblast cell cycle kinetics.
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It is common in the elderly to experience sarcopenia, a loss in muscle mass. There are many different possibilities of treatment; many of which are currently on the market, but none of the pharmaceuticals are completely efficient. Increasing muscle mass is perceived as the main therapeutic route to remediate this disorder. To increase muscle mass, there is a requirement for proliferation, differentiation, and fusion of muscle stem cells, also known as satellite cells, allowing for the formation of new muscle. The objective of this study was to identify nutraceuticals that can promote proliferation in satellite cells. We used a mouse satellite cell line, C2C12, to screen 133 natural compounds to observe any potential increase in proliferation. A thymidine analog, EdU, was used to monitor progression into S phase of the cell cycle and MTT, a tetrazolium dye, was used to report cell number. The usage of both EdU and MTT allows for an overall view of cell proliferation in high-serum media (growth media; HSM) or low-serum media (arrest media; LSM). Andrographisolide is one of a few compounds that decreased (P = 0.02) C2C12 proliferation when cultured in HSM. Morphological assessment coupled with EdU incorporation and MTT mitochondrial activity suggest the cells suffered from growth arrest at either G1 or S phase. By contrast, indole-3-carbinol inhibited (P = 0.01) proliferation of the C2C12 cells in LSM but had no effect on cells cultured in HSM. Additional compounds, such as berberine chloride, were identified to have the ability to alter mitochondrial activity independent of the cell cycle indicating a metabolic effect. Overall, our data demonstrate that natural compounds can inhibit or stimulate proliferation of C2C12 cells and affect metabolic function. Ongoing studies are examining the direct effect of the compounds on satellite cell bioactivity in vivo with the goal of increasing muscle in mice suffering sarcopenia or muscular dystrophy.

P3495

Board Number: B788

Research in the molecular mechanism of coagulation activity of sulfur-containing monoterpenoids of pinane series.
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Currently we have synthesized a series of sulfur-containing terpenoids of varied structure with the large set of the functional groups. Previously we have already revealed antifungal, anti-inflammatory, anti-helicobacter, antimicrobial and other types of activity of sulfur-containing monoterpenoids. Sulfoxide synthesized on the basis of naturally occurring monoterpene (-)-β-pinene was investigated for its coagulation properties. The possibility of using sulfoxide for correction of hemostasis was determined in vitro on human blood plasma obtained from healthy donors not taking antiplatelet and anticoagulant medications. The anticoagulant activity of sulfoxide was measured by activated partial thrombosis time, thrombin time and prothrombin time. As a result of a study it was shown that the sulfoxide of pinane series in contrast to acetylsalicylic acid and clopidogrel completely inhibited activation of platelets induced by adrenaline, collagen and arachidonic acid and also reduced the effect of ADP and ristomycin. Arachidonic acid, in contrast to other inducers, is known to activate platelets directly penetrating into

Tuesday-492
the cell and reducing cyclic adenosine monophosphate concentration. The loss of this property in the presence of sulfoxide is explained by changes in permeability of platelet membranes associated with formation of additional intermolecular interactions between hydrophobic parts of the thieterpenoid molecules and the phospholipids of the membrane. Integration of the hydrophobic part of sulfoxide molecule into the cell membrane stabilized it and prevented using the molecules of phosphatidylcholine from the external layer of membrane as a formation source of lipid peroxidation products, which would lead to initiation of platelet aggregation mechanisms. Detailed NMR studies and molecular dynamics simulations using SDS membrane models indicated that the bicyclic fragment of sulfoxide was embedded in the SDS micelle whereas the -SO(CH2)2OH fragment remained on the surface of the micelle contacted with the solvent. It was demonstrated that the coagulation activity of sulfoxide was due to its ability to inhibit platelet activation and suppress the catalytic activity of phospholipid surface. The cell membrane was stabilized by van-der-Waals interactions of sulfoxide with phospholipids of the external layer which was involved in formation of coagulation clotting factor complexes. Considering the low toxicity, the ability to block induced and spontaneous aggregation, sulfur-containing terpene compounds are promising agents with possible use for platelet blood products stabilization, treatment and prevention of thrombophilia.

**Tissue Development and Morphogenesis 3**

**P3496**

**Board Number: B790**

**Src oxidation directs cell polarity to promote rapid embryonic wound healing.**

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Embryos exhibit a striking ability to rapidly repair wounds without inflammation or scarring. Embryonic wound healing is a conserved process, driven by redistribution of the cytoskeleton and cell-cell junctions around the wound. Immediately after wounding, both actin and the molecular motor myosin become polarized in the cells around the wound, accumulating at the wound margin to form a supracellular cable. The rapid contraction of this cable draws the surrounding cells together to close the wound. We recently showed that polarized endocytosis of the junctional protein E-cadherin from the wound margin is a prerequisite for actomyosin cable assembly and wound closure. However, the upstream signal that triggers redistribution of junctions and the cytoskeleton around wounds is unknown. Using quantitative time-lapse microscopy in *Drosophila* embryos, we measured a burst of reactive oxygen species (ROS) production immediately after wounding, exclusively in the cells that are eventually enclosed by the actomyosin cable. Blocking ROS production significantly inhibited wound healing, and severely impaired junctional trafficking and actomyosin accumulation around the wound. Together, our results suggest that ROS production drives wound healing by promoting E-cadherin trafficking and subsequent polarized actomyosin assembly. ROS can post-translationally modify proteins by oxidizing electron-rich cysteine residues. The *Drosophila* ortholog of Src kinase, Src42A, contains several putative redox-sensitive cysteines, and has been implicated in E-cadherin trafficking during morphogenesis. Src42A⁴⁷¹ is a conserved cysteine that is oxidized in zebrafish leukocytes upon wounding. We mutated Src42A⁴⁷¹ to generate an oxidation-resistant form of Src42A (Src42A⁴⁷¹). Src42A⁴⁷¹ exhibited reduced kinase activity in
vitro, and, importantly, embryos expressing Src42A	extsuperscript{OR} displayed impaired wound healing. We are currently investigating the phosphorylation targets of Src42A and how Src activation promotes wound healing. We propose that ROS act as a critical wound signal, orchestrating the polarity of junctions and the cytoskeleton through Src to drive rapid wound healing.

P3497

Board Number: B791

Actomyosin-based basal protrusions drive long range lateral inhibition via dynamic cell-cell contacts during epithelial tissue patterning.

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Here we investigate (1) the nature of cell-cell contacts mediated by basal cellular protrusions, and (2) whether the retraction of cellular protrusions and cortical tension generated by the actomyosin cytoskeleton contributes to the mechanical forces required for Notch signaling. Dynamic, actin-based cellular protrusions (e.g., cytonemes) mediate cell-cell signaling across several species and signaling paradigms, including Notch signaling, a conserved mechanism that drives cell fate determination. Activation of Notch receptor by Delta ligand requires a pulling force in ligand expressing cells. The pulling force required for Notch activation between neighboring cells is proposed to be generated through ligand endocytosis, but it is unclear how activation occurs via protrusions. (1) We use bristle patterning in the Drosophila notum as a model system for studying protrusion-mediated signaling. Notum epithelial cells radially extend long, actin-rich protrusions from their basal surface that contact cells > 10 microns away (i.e., distant neighbors). At the level of individual protrusions, we quantify cycles of extension/retraction in these dynamic, unbranched structures. Using GRASP and EM, we observe that contact between protrusions extends along their entire length, and that both Notch receptor and Delta ligand localize to areas of contact. We show that protrusions interact in dynamic ‘foci’, making complex contacts between several neighboring cells, which contribute to a gradient of cell response in the tissue. (2) Next we address the role of actomyosin contractility in Notch signaling. Using a genetic approach, we find that contractility is required in ligand and receptor expressing cells for bristle patterning. Expression of constructs aimed to decrease myosin activity lead to defects in bristle precursor cell spacing, indicative of defective protrusion-based Notch signaling. Expression of a transcriptional reporter of Notch signaling shows that loss of contractility in ligand expressing cells is sufficient to decrease Notch response in adjacent and distant cells compared to controls. Epistasis experiments in vivo and in cell culture suggest contractility drives Notch signaling in parallel with endocytosis. We find that moderate loss of myosin activity decreases tissue tension but does not affect protrusion morphology or ligand endocytosis, whereas strong loss of activity leads to irregularly shaped protrusions. Treatment of notum explants with Rho kinase inhibitor to decrease contractility similarly did not affect protrusion formation, but inhibited movement of contractile foci of interacting protrusions. Our results support a model in which actomyosin contractility in protrusions and at the cell cortex is required for Notch activation in vivo.
P3498
Board Number: B792
Erk-dependent control of epithelial morphogenesis.
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The role of Ras/Erk signaling in cancer, growth, and differentiation has long been appreciated. In contrast, the roles of Erk signaling in cell motility, collective cell migration, and tissue-level morphogenesis are complex and remain poorly defined. Here, we set out to define the Ras/Erk pathway’s role in orchestrating collective cell movement during gastrulation in the early Drosophila embryo. Using an optogenetic input to Ras, we found that Erk activity is sufficient to induce cells to adopt a contractile cell fate at nearly any illuminated location within the embryo. This tissue mimics gene expression and physical organization of the posterior midgut (PMG), a tissue normally patterned by the Torso receptor tyrosine kinase. We define the transcriptional network by which Erk programs PMG cell fate, leading to the accumulation of apical myosin and tissue contraction at gastrulation. By systematically varying the timing and duration of Erk activity, we define the spatiotemporal features of the Erk signal that is required to program these fates. We find that the early embryo responds to the cumulative load of Erk activity delivered over a two hour window in early embryogenesis, revealing a previously unknown long-term memory of signaling that spans multiple nuclear division cycles. Our work mechanistically defines an Erk-dependent cell fate choice and establishes a model system for interrogating how signaling pathway activity can program morphogenesis in vivo.

P3499
Board Number: B793
Unraveling multiciliated ependymal cell identity: Ubiquitin-proteasome and IKK2 control Foxj1 stability.
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Cellular differentiation and identity is often coupled to the presence of specific organelles and typically driven through genetic programming. For instance, proper multiciliated cell function requires a highly differentiated state that includes numerous motile cilia and polarized epithelial-like morphology. Multiciliated ependymal cells line brain ventricles, promote cerebrospinal fluid (CSF) flow, and form a barrier between CSF and the brain parenchyma. Foxj1 is an evolutionarily conserved transcription factor required for the development of multiciliated cells from Xenopus to mammals. Here we show that continued Foxj1 expression is required to maintain differentiation of mature multiciliated ependymal cells. Upon tissue-specific deletion of Foxj1 from mature animals, ependymal cells undergo multicilia loss, develop radial-glial-like basal projects that contact blood vessels, and re-enter the cell cycle. Environmental insult such as growth factors and viruses induce Foxj1 protein loss, dedifferentiation, and hydrocephalus. Live imaging combined with lineage tracing captures stages of ependymal transformation and cell division, while recovery experiments reveal a capacity for ependymal cell self-renewal. Surprisingly, we find that Foxj1 protein is inherently unstable and continually degraded by the ubiquitin-proteasome through activity of cullin-RING E3 ligases. Foxj1 stability is regulated by a highly conserved and protein stabilizing IKK2 phospho-site. Thus we uncover opposing IKK2 and cullin-RING
ligase pathways that regulate FoxJ1 and ependymal stability. These results have important implications for hydrocephalus research and post-translational control of cellular differentiation.

P3500
Board Number: B794

Nopo, the Drosophila ortholog of the microcephalic primordial dwarfism gene TRAIP, encodes a centrosomal E3 ubiquitin ligase specifically required for mushroom body development. R.S. O'Neill, B.J. Galletta, C.J. Fagerstrom, N.M. Rusan;

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Primary microcephaly and microcephalic primordial dwarfism (MPD) are a spectrum of genetic disorders characterized by reduced brain size and, in MPD, reduced body size. Most known primary microcephaly and MPD genes function at centrosomes or in DNA damage response (DDR). While some functional connections between centrosomes and DDR have been established, it is likely that a deeper and more direct link is at play. We believe that primary microcephaly and MPD genes are opportune candidates for uncovering functional connections between centrosomes and DDR pathways and, more generally, how these functions impact neurodevelopment. As part of a large screen for neurodevelopmental defects, we homed in on TRAIP, an MPD gene encoding an E3 ubiquitin ligase known to regulate DDR and apoptosis. Interestingly, the Drosophila melanogaster ortholog nopo (no poles) was named for its loss-of-function phenotype of acentrosomal spindles, suggesting that this DDR gene might function at centrosomes. We performed yeast two-hybrid analysis to reveal extensive interactions between Nopo and several core centrosome proteins, including Sas4, Ana2 and Plk4. Thus, we have established another potential link between a DDR gene and the centrosome. To explore the role of nopo in neurodevelopment, we analyzed both larval and adult brains from nopo mutant animals. We discovered that loss of nopo leads to defects in the mushroom body (MB), a brain region critical for memory formation. Nopo mutant α and β MB lobes are thin, fused, and are often missing. Axon guidance is also abnormal in nopo mutants as we find many misguided MB axons. Our studies of mutants for bendless, which encodes an E2 conjugating enzyme previously shown to interact with Nopo, reveal that 100% of MBs are fused, suggesting that Nopo functions with Bendless to ensure proper brain development and prevent MPD. We are currently focused on identifying the ubiquitination substrates of Nopo required for MB development; candidates include Nopo direct binding partners at the centrosome. Together, this work reveals an exciting new link between the DDR and the centrosome bridged by nopo. Furthermore, we have established D. melanogaster as a new model for understanding the role of TRAIP in neurodevelopment.

P3501
Board Number: B795

Negative feedback loops protect stem cell progenitors’ identity and architecture during embryonic skin development.

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Adult stem cells (SCs) are long-lived and can self-renew and differentiate to replenish the tissues where they reside. They exist in specialized niches, where they often remain quiescent until they are needed for homeostasis and wound repair. Their precise spatial organization and tight regulation of proliferation are essential to generate and maintain the complex architecture of adult tissues. The formation of SC niches and regulation of SC behavior by local signals are essential for the proper functioning of organs
and fundamental to the organism’s health. However, despite intensive investigation, it remains obscure how SC progenitors are embryonically specified in space and time, how they occupy their position, and how they dynamically interact with their neighbors while maintaining their own identity. We successfully developed innovative ex-utero live imaging, and together with immunofluorescence and in utero lentiviral transduction we showed that in developing hair buds, SCs are born from asymmetric divisions that differentially display WNT and SHH signaling. Displaced WNT low suprabasal daughters become SCs that respond to paracrine SHH and symmetrically expand. By contrast, basal daughters remain WNT high. Here we show that basal daughters use negative feedback loops to keep their number and identity. This is fundamental to distinguishing them from their immediate neighbors, preserving their identity, promoting hair follicle morphogenesis and surveilling epithelial architecture.

P3502
Board Number: B796
The interaction between calreticulin and Wnt/β-catenin signaling induces hair follicle neogenesis.
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Topical application of calreticulin (CRT), an endoplasmic reticulum stress response chaperone protein, to wounds in pig and diabetic mouse models profoundly enhances the rate and quality of cutaneous wound healing. Interestingly, wounds treated with CRT are repopulated with dermal appendages, including neogenic hair follicles; this has not been observed in adult mammals following full-thickness wound injury. In mice, we show evidence of hair follicle neogenesis in the neodermis as early as 3 days post-injury. Notably, hair follicle neogenesis has been shown to be dependent on the Wnt pathway. In addition, genetic deletion of CRT results in disruption of canonical Wnt signaling and thus, decreased downstream β-catenin transcriptional activity. Therefore, we hypothesized that CRT is critical for inducing stem cell differentiation for hair follicle neogenesis. However, a direct and/or indirect relationship between Wnt/β-catenin and CRT signaling has not been investigated. Our studies show that CRT treatment increases nuclear localization of β-catenin, and induces Wnt/β-catenin signaling in Human Foreskin Fibroblasts (HFFs) transfected with a vector containing β-catenin/Tcf/Lef binding sites linked to a Luciferase reporter gene. Moreover, inhibition of glycogen synthase kinase 3 beta (GSK3β), which targets β-catenin for proteasomal degradation, with lithium chloride enhances CRT-mediated increase in nuclear β-catenin by 3-fold. Furthermore, CRT dose-dependently induces Wnt5a, 10a, 10b, and FGF9 mRNA expression, as well as Wnt3a protein expression, all of which have been shown to be involved in hair follicle neogenesis. Accordingly, in CRT-treated mouse wounds, Wnt5a, β-catenin, and LRP-1 (CRT receptor) were expressed by day 7 post-wounding. Interestingly, treatment of CRT-null mouse embryo fibroblasts (MEFs) with exogenous CRT failed to upregulate mouse Wnt ligand (WLS/GRP177) mRNA whereas wild-type MEFs were responsive, suggesting that intracellular CRT is required for Wnt induction by exogenous CRT. Additionally, the treatment of HFFs with a Wnt agonist dose-dependently increased both CRT mRNA and protein levels indicating a reciprocal feedback effect between CRT and Wnt signaling. Whereas we show that CRT treatment induces hair follicle neogenesis via the Wnt pathway, transgenic mice expressing constitutively active β-catenin in the dermis have less hair follicles, increased dermal thickening and fibrosis post-wounding. Taken together, these findings suggest a complex mechanism by which CRT interacts with the Wnt/β-catenin pathway to modulate wound regeneration and hair follicle neogenesis. Revealing this mechanistic interaction may lead to the development of potential therapies for wounds, degenerative skin conditions, and hair loss.
P3503
Board Number: B797
Zebrafish models of RASopathies: the impact of PTPN11 mutations on early embryogenesis.
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Mutations that activate the RAS/ERK signaling pathway contribute to human disease; de novo somatic mutations in pathway components cause cancer, while mutations transmitted through the germline cause a spectrum of developmental disorders, collectively termed RASopathies. PTPN11 missense mutations cause either Noonan Syndrome (NS) or Noonan Syndrome with Multiple Lentigines (NSML). NS patients exhibit congenital heart defects, craniofacial malformation, neurocognitive delay and an increased predisposition to cancer. NSML patients display the same symptoms, with the addition of lentigines – freckle-like lesions – and sensorineural deafness. While NS mutations increase the phosphatase activity of PTPN11 by destabilizing the inactive conformation, NSML mutations decrease phosphatase activity but cause sustained pathway activation. Modeling the comparative impact of these mutations could be informative for predicting symptom risk in patients. Using transient overexpression of variant PTPN11, we have compared the strength of patient mutations as measured by the effect upon early embryogenesis in zebrafish. The aspect ratio of the 11 hpf-old embryo provides a convenient readout of pathway activity, with stronger mutations presenting an increasingly oval morphology due to defects in convergence and extension. Injected embryos are also shorter at 3 dpf, display craniofacial malformations at 5 dpf and exhibit heart defects, reduced heart rate and cardiac edema, phenocopying many of the characteristic symptoms of NS and NSML. We have performed quantitative analysis and generated a rank of mutation impact for each phenotype. Intriguingly, preliminary data suggest that the rank of mutation strength generated at 11 hpf is not predictive for phenotype severity at later stages, indicating that mutations may differentially impact the development of distinct structures.

P3504
Board Number: B798
Interplay of ubiquitination and palmitoylation in trafficking of Fat-Hippo signaling pathway components.
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Proper coordination of growth and morphogenesis during development is critical to formation of appropriately proportioned organs. The evolutionarily conserved protocadherins, Dachsous (Ds) and Fat constitute a signaling pathway that coordinates growth and morphogenesis by regulating the Hippo pathway and planar cell polarity (PCP) respectively. The atypical myosin Dachs is a key downstream effector of Fat signaling that mediates both of these effects and apical localization is critical for Dachs function. Fat regulates growth and PCP by modulating the levels and polarity of Dachs at the apical membrane. However, how Dachs localization is regulated is not well understood. A palmitoyl transferase, Approximated (App) is required for proper Dachs localization. But the exact mechanism by which it regulates Dachs is unknown. Recently, I have identified a novel gene, Vamana (Vam), which encodes an SH3 domain containing adapter protein that plays a critical role in apical membrane localization of Dachs. Further, Vam functions as an adapter by physically connecting Dachs to Ds and Fat. In order to identify additional regulators of this signaling pathway, I conducted a genetic screen using
RNAi targeted against the ubiquitin ligases encoded by the Drosophila genome and isolated a novel RING domain E3 ligase, Elgi, which when depleted results in tissue overgrowth and PCP defects, phenotypes reminiscent of mutations in Fat signaling. At the cellular level, depletion of Elgi results in significant increase in Dachs and Vam levels. Interestingly expression of a dominant negative Elgi results in failure of Dachs and Vam to localize to the apical cortex and their accumulation in the cytoplasm in punctate structures. Similar accumulations are observed when Elgi and App are depleted simultaneously. These accumulations are reversible in nature and do not arise from concentration dependent phase separation of misfolded proteins. Further localization analysis revealed that they arise from trafficking defect in Dachs, where it is observed to traffic in random directions instead of trafficking apically. Taken together these results indicate that Elgi and app coordinately regulate the trafficking of Dachs and Vam to the apical membrane. Our current efforts are aimed at elucidating the mechanism by which loss of Elgi and App disrupt the vectorial trafficking of these key effectors of Fat-Hippo signaling.

P3505  
**Board Number: B799**  
**Fish scales dictate the pattern of adult skin innervation.**  
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The skin is profusely innervated by axon endings of touch-sensing neurons. As animals mature from embryonic to adult stages, the skin grows and adds epithelial strata, specialized cells, and dermal appendages, like hairs, feathers and scales. How cutaneous sensory axons adapt to these dramatic changes is poorly understood. By characterizing remodeling of skin innervation in zebrafish, we discovered that sensory axons are delivered to the adult epidermis in strikingly organized nerves patterned by features in bony scales. These sensory axons associate with Schwann cells, blood vessels and osteoblasts. Osteoblasts create paths that independently guide nerves and blood vessels, during both development and regeneration. By preventing scale regeneration and examining mutants lacking scales, we found that scales dictate the pattern of axon distribution in the epidermis, suggesting a new mechanism for achieving comprehensive innervation of the adult skin. Thus, scales coordinate a metamorphosis-like transformation of the skin with sensory axon remodeling. We speculate that regularly spaced dermal appendages in other animal classes may also pattern skin innervation.

P3506  
**Board Number: B800**  
**Building a barrier: survival of the fittest in the developing skin.**  
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A key feature of animal embryogenesis is the creation of a permeability barrier that protects internal organs and tissues from physical damage, dehydration, pathogen invasion, and other harmful environmental stresses. In mammals, this function is fulfilled by the epidermis, a tissue comprised of stratified layers of specialized epithelial cells. During embryonic development, the epidermis expands dramatically to accommodate the growth and increase in surface area of the organism. Concomitantly, the skin undergoes a differentiation program from a monolayer of progenitors to become a functionally specialized, multi-layered permeability barrier at birth. This rapid process of patterning and growth is vulnerable to deleterious genetic errors and epigenetic and/or post-translational misregulation that could be propagated throughout the tissue and thus compromise both barrier function and the fitness
of the organism. Therefore, we sought to understand the quality control mechanisms that operate during the complex process of skin development. Cell competition is a phenomenon by which less fit “loser” cells are eliminated by more fit “winner” cells. Could such a mechanism exist in embryonic skin to ensure that only the fittest progenitor cells persist to establish and maintain a functional epidermal barrier? Using a combination of functional genetics and time-lapse imaging, we establish a model for cell competition in the mouse embryonic epidermis. Early in epidermal development, loser cells are eliminated from the growing epithelium via apoptosis and engulfment by surrounding winner cells. Strikingly, we observe a developmental switch in loser cell elimination mechanisms; upon the emergence of stratified tissue layers, loser cells are eliminated via hastened differentiation rather than apoptosis. Abrogating the function of factors required for loser cell clearance leads to compromised barrier integrity and differentiation defects. Altogether, our studies raise the intriguing possibility that cell competition acts as a selective force during epidermal development to maximize tissue fitness. Moreover, our data illuminates how the strategy a tissue uses to preserve fitness can evolve as architectural complexity increases during morphogenesis.

P3507
Board Number: B801
Examining Carmil3’s role in Kupffer's vesicle morphogenesis and function.
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CARMIL family proteins have traditionally been thought of as regulators of actin capping protein; recent work has also begun to examine their role as scaffolds within signaling networks. Vertebrates express three CARMIL isoforms, and CARMIL3 is the least well studied. Zebrafish have three genes and express the three isoforms throughout development. Fish homozygous for loss-of-function alleles of Carmil3, generated via genome editing or ENU mutagenesis, exhibit a delay in development and an increase in dextrocardia. The nodal-related gene southpaw, required for left-right patterning, was no longer strictly expressed in the left lateral plate mesoderm; instead, it was frequently expressed in the right lateral plate mesoderm or bilaterally. Preliminary data suggests that this phenotype is a result of a decrease in Kupffer’s vesicle formation and lumen inflation; and a loss of primary cilia in the Kupffer’s. Overexpression of Carmil3, induced by microinjection of RNA, leads to different defects in embryo patterning, and these gain-of-function phenotypes require Carmil3’s ability to bind capping protein. These results demonstrate a critical role for CARMIL3 in regulation of the Nodal signaling pathway establishing left-right asymmetry in the developing animal.

P3508
Board Number: B802
Distinct Signaling Roles for Type I Receptors Bmpr1 and Acvr1l, and the Type II Receptors Bmpr2 and Acvr2 within the BMP Receptor Complex.
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The Bone Morphogenetic Protein (BMP) pathway patterns dorsal-ventral (DV) axial tissues during gastrulation. The zebrafish embryo is an excellent system to investigate the mechanism of BMP signal transduction during DV patterning, as many BMP pathway mutants are available and, unlike in
mammals, these mutants survive to show DV patterning defects. When signaling, a dimeric BMP ligand assembles a receptor complex composed of two type I and two type II receptors. Type II receptors phosphorylate and activate type I receptors, which in turn phosphorylate Smad proteins. Phosphorylated Smad then regulates gene expression. This model, however, is overly simplistic as there are two conserved classes of type I receptor, Bmpr1 and Acvr1l, and two conserved classes of type II receptor Bmpr2 and Acvr2, all of which are necessary for vertebrate development. Our previous findings demonstrate that BMP2/7 heterodimers are the only ligands that signal in DV patterning. This sufficiency arises from the heterodimer’s unique ability to integrate both type one receptors into the BMP receptor complex, as Bmpr1 preferentially binds the BMP2 ligand, and Acvr1l exclusively binds BMP7. We have also found that kinase dead Acvr1l cannot rescue acvr1l knockdown, while, surprisingly, kinase dead Bmpr1 can rescue bmp1 knockdown. Additionally, Bmpr1’s intracellular domain (ICD) cannot function in the place of Acvr1l’s. These results suggest Acvr1l and Bmpr1’s ICDs play separate functional roles in BMP signaling. I aim to determine the location and nature of the motifs within Bmpr1 and Acvr1l that impart these specific functions through a series of domain swap experiments. We do not currently know the contribution of the two BMP type II receptor classes, Bmpr2 and Acvr2, to the signaling complex acting in DV patterning. While experiments in mice suggest that both classes are necessary for early embryonic development, these embryos die before DV patterning begins. I aim to create zebrafish mutants null for each entire type II receptor class, allowing us to determine whether both classes have independent, necessary signaling functions in DV patterning.

P3510
Board Number: B804
Caenorhabditis elegans BMP Signaling Determines Body Size Via Transcriptional Regulation of Collagen Genes.
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Body size is a tightly regulated phenotype in metazoans that depends on both intrinsic and extrinsic factors. While signaling pathways are known to control organ and body size, the downstream effectors that mediate their effects remain poorly understood. In the nematode C. elegans, a Bone Morphogenetic Protein (BMP)-related signaling pathway is the major regulator of growth and body size. We investigated the transcriptional network through which the BMP pathway regulates body size and identified cuticle collagen genes as major effectors of growth control. We demonstrate that cuticle collagens can act as positive regulators (col-41), negative regulators (col-141, col-142), or dose-sensitive regulators (rol-6) of body size. Moreover, we find a requirement of BMP signaling for stage-specific expression of cuticle collagen genes. We show that the Smad signal transducers directly bind conserved Smad binding elements in regulatory regions of col 141 and col-142, but not of col-41. Hence, cuticle collagen genes are directly and indirectly regulated via the BMP pathway. Our work thus connects a conserved signaling pathway with its critical downstream effectors, advancing insight into how body size is specified. Since collagen mutations and misregulation are implicated in numerous human genetic disorders and injury sequelae, understanding how collagen gene expression is regulated has broad implications.
P3511
Board Number: B805
Determination of Novel BMP-Smad1/5 Signaling Interactions in Fibrodysplasia Ossificans Progressiva.
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Fibrodysplasia ossificans progressiva (FOP) is a rare and debilitating human genetic disorder that perturbs skeletal development and induces heterotopic ossification. Classical FOP is caused by a single nucleotide substitution in the BMP/TGFβ cell surface receptor, ACVR1 (617G>A, R206H). This mutation results in over-activation of receptor signaling through the Phospho-Smad1/5 (pSmad1/5) pathway. However, the mechanism through which the mutant receptor confers enhanced signaling activity remains uncertain. To assay for mutant ACVR1 activity, we used zebrafish embryonic dorsoventral (DV) patterning, which is established by a gradient of BMP signaling activity that specifies ventrolateral cell fates. We confirmed that ACVR1-R206H misexpression causes over-activation of pSmad1/5 activity and ventralization of zebrafish embryos. We tested several rare ACVR1 mutations of FOP for signaling activity in the zebrafish embryo assay and found similar results. Recent studies suggest that ACVR1-R206H and other FOP variant mutant receptors may have altered ligand affinity compared to WT ACVR1. We confirmed that BMP ligand enhances pSmad1/5 signaling through ACVR1-R206H. Surprisingly, Activin A, a ligand that normally binds ActR1 and signals through pSmad2/3, also enhances pSmad1/5 signaling by ACVR1-R206H. In addition to testing ligand enhancement, we examined if other receptors are required with ACVR1 to induce signaling. We found that BMPR1, a receptor normally required for pSmad1/5 signaling and DV patterning in the zebrafish, is not required for pSmad1/5 over-activation by ACVR1-R206H or the variant mutant ACVR1-G328R. These data suggest that BMPR1 is not required for the pathogenesis of FOP. These and further studies of the signaling interactions of ACVR1-R206H will allow for identification of novel therapeutic targets to treat FOP and give us unique insight into how this fundamental cell signaling pathway functions in development.

P3512
Board Number: B806
Expression pattern and potential role of placental Psg genes in sex-specific immune response during pregnancy.
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Placenta is essential organ for sustaining survival and growth of fetus during gestation. It mediates maternal-fetal exchange and produces hormones that alter maternal physiology and protect the fetus against the maternal immune system during pregnancy. These placental roles are necessary for normal pregnancy outcomes, and defects in its functions lead to adverse pregnancy outcomes including intrauterine growth restriction (IUGR) and pre-eclampsia (PE), spontaneous preterm delivery, and abortion as well as long-term effects on health and behavior of adult offspring in sex-specific manners. The pregnancy-specific glycoproteins (PSGs), members of the immunoglobulin (Ig) superfamily, are the most abundant fetal proteins produced by placenta during pregnancy and play pivotal roles in anti-platelet, pro-angiogenesis and anti-inflammation. There is accumulating evidence that prenatal maternal stress or infection show differential effects on immune activation and inflammation depending on fetal sex. Especially, our previous work demonstrated that administration of synthetic glucocorticoid, dexamethasone, on pregnant mice induces sex-specific activation of immune-related genes including...
many Psg genes. Therefore, we hypothesized that the placental Psg genes are regulated differently in a sex-dependent manner, contributing to immune response during pregnancy. Here we first examined differential Psg gene expression patterns at different gestational days by RT-PCR and further determined the possible roles of Psg genes in sex-specific immunoregulatory responses during pregnancy.

**P3513**

**Board Number: B807**

*Role of HAND1 in collagen expression and post-translational modifications in the long bone.*

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Temporal and/or spatial modifications in gene regulation result in a multitude of long bone defects. The long bone develops through endochondral ossification from the ossification center of the diaphysis. The basic helix-loop-helix transcription factor HAND1, which is expressed in the developing long bone, is involved in morphogenesis and mineralization of the bone collar. To understand the underlying mechanism of HAND1 in bone development, we characterized and examined the expression of fibril-forming collagens from *Hand1*-overexpressing mice. Overexpression of *Hand1* in osteochondral progenitors resulted in hypoplastic ossification in the diaphyses of long bones. In *Hand1*-overexpressing mice, expression of *Runx2*, a master molecule for osteoblast differentiation, was significantly decreased. Mass spectrometric analysis revealed that the expression of types V and XI collagens was down-regulated in the diaphyses of *Hand1* mutants. In addition, *Hand1* overexpression led to an increase in collagen-specific post-translational modifications for type I collagen. Our results demonstrate that HAND1 plays a role in the temporal expression and post-translational modifications of collagens involved in endochondral ossification, which may result in the development of long bone defects.

**P3514**

**Board Number: B808**

*Tbx1* knockout mice exhibit dysregulated expression of genes associated with cleft palate in humans.

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*TBX1*, the T-box transcription factor gene, is considered the causative gene for 22q11.2 deletion syndrome, which manifests as velocardiofacial syndrome, DiGeorge syndrome, or conotruncal anomaly face. 22q11.2 deletion syndrome is characterized by craniofacial anomalies including facial muscle hypotonia, velopharyngeal insufficiency, and various subtypes of cleft palate, namely, complete cleft palate, incomplete cleft palate, soft palate cleft, and submucosal cleft palate. Ablation of *Tbx1* in mice results in different subtypes of cleft palate similar to those observed in humans. To examine the function of TBX1 in palatal development, we performed gene profiling of developing palatal shelves from wild type and *Tbx1* knockout embryos. Gene ontology analysis indicated that genes associated with...
development of muscle and the nervous system were dysregulated in Tbx1 knockout palatal shelves. Furthermore, in Tbx1 knockout palatal shelves, genes associated with human cleft palate, specifically, gamma-aminobutyric acid (GABA) A receptor subunit beta 3 (Gabra3), collagen type IX alpha 2 (Col9a2), myosin heavy chain 3 (Myh3), andnebulin (Neb) were downregulated. These results demonstrate that TBX1 maintains normal palatogenesis, mediated at least partly through the regulation of genes involved in the GABAergic system, development of muscle and the nervous system, and collagen synthesis. We conclude that multiple factors in Tbx1 knockout mice lead to different subtypes of cleft palate.

P3515
Board Number: B809
Delineation of neuro-ontogenic mechanisms of schizophrenia using induced Pluripotent Stem Cells (iPSCs).
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Schizophrenia is a neurodevelopmental disorder featuring complex aberrations in the structure, wiring, and chemistry of multiple neuronal systems. The abnormal developmental trajectory of the brain appears to be established during gestation, long before clinical symptoms of the disease appear in early adult life. In order to understand the effect of schizophrenia on neuronal differentiation, we utilized induced Pluripotent stem cells (iPSCs) derived from both schizophrenia disease (SCZD) patients (n=3) and normal patients (n=4). iPSCs were differentiated into neuronal progenitor cells. In order to elucidate the effects of schizophrenia on NPCs we preformed global RNA-sequencing to quantify differences in RNA expression. RNA-seq analysis shows 1375 dis-regulated genes in all SCZD iPSCs compared to control. Many of these genes are involved in important developmental pathways such as WNT/B-Catherin signaling, or in neuronal pathways, such as axonal guidance and glutamate receptor signaling. The majority of the deregulated genes are targeted by nuclear form of FGFR1, supporting the role of the Integrative Nuclear FGFR1 Signaling in schizophrenia. Experiments utilizing iPSC-derived cerebral organoids support an early (preneuronal) developmental-genomic etiology of schizophrenia. Supported by NYSTEM (C026415, C026714), National Science Foundation (CBET-1555720), Clinical and Translational Science Institute NIH (2T35AI089693-06), and Patrick P. Lee Foundation.

P3516
Board Number: B810
The Formin, DIAPH1, Contributes to Epidermal Structure and Differentiation By Enabling Basal Keratinocyte Crowding.
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To maintain a stratified, epidermal barrier, basal layer keratinocytes proliferate, stratify and differentiate to replace cells shed from the skin surface. Basal cell morphology impacts the production of differentiated, suprabasal layers, with prior cell crowding and micropattern studies suggesting that minimization of cell-matrix contact enables differentiation. While these studies implicated Rho-dependent, cortical actin accumulation upstream of serum-response factor (SRF), it remains unclear what actin nucleators partake. Identifying specific nucleators which participate in differentiation may aid in resolving the conundrum that arises in determining whether, in a physiological setting, the production of cortical actin represents a response to confinement/crowding or enables it by driving the columnar
shape associated with basal keratinocytes in vivo. Towards addressing these issues, we investigated the canonical formin, DIAPH1, a Rho-activated actin nucleator/elongator, known to support SRF signaling, but little studied with regard to epidermal architecture. Our results suggest that DIAPH1-deficient 2D and 3D cultures stratify but fail to differentiate. Depletion of DIAPH1 disrupted basal layer morphology, yielding large, heterogeneously shaped cells defective with respect to accumulation of E-cadherin along the apical surface. In 2D, knockdown cells also failed to induce differentiation markers and SRF targets in response to increasing cell density. We hypothesize that DIAPH1 supports epidermal differentiation by driving basal cells into a columnar morphology, thereby, increasing basal cell density, minimizing matrix contact and enabling differentiation. Potential effects on cell-matrix adhesion, cell-cell adhesion and cortical mechanics are being evaluated as possible contributors to the DIAPH1-phenotype.

P3517
Board Number: B811
Role of ROOT UVB SENSITIVE 1 in Vitamin B6 Homeostasis Modulation.
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Vitamin B6 (vitB6) is essential for all life. In plants, vitB6 can be either synthesized via a highly conserved biosynthesis pathway or regenerated by salvage pathways. VitB6 is consumed in a number of physical and physiological processes. VitB6 in its bioactive form, pyridoxal 5'-phosphate (PLP), can be photolytically destroyed by ultraviolet-B (UVB) light. As a potent antioxidant, PLP is utilized to quench out reactive oxygen species. A good portion of cellular PLP is expected to be conjugated at the active site serving as a cofactor for more than 140 enzymes. How cellular vitB6 homeostasis is maintained to regulate development is not understood. An Arabidopsis protein called ROOT UVB SENSITIVE 1 (RUS1) was shown to play a key role in modulating vitB6 homeostasis. When mutated, rus1 displays a vitB6 deficient phenotype that can be partially rescued by either supplying vitB6 or by specific mutations in Aspartate Aminotransferase 2 (ASP2), a vitB6 using enzyme. The endogenous vitB6 level is comparable with that in the WT but any physiological conditions that perturb vitB6 homeostasis greatly enhance rus1 phenotype. Here we report mutations in genes that are required in vitB6 salvage pathways result in severe developmental arrest in rus1. Loss-of-function of the pyridoxal kinase gene called SOS4 (SALT OVERLY SENSITIVE 4) gives minor phenotype as the de novo vitB6 biosynthesis pathway is expected to supply vitB6 and maintain vitB6 homeostasis. Surprisingly, our genetic analyses show that the rus1 sos4 double mutant is embryonic lethal, suggesting that RUS1 is required to maintain vitB6 homeostasis that is directly linked vitB6 biosynthesis pathway. Our study strongly suggest that RUS plays a critical role in regulating development via modulating vitB6 homeostasis in Arabidopsis.

P3518
Board Number: B812
On the male disadvantage: generation and characterization of a male specific mouse model of bronchopulmonary dysplasia.
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Tuesday-505
France

The proper development of both human and murine lungs relies on a precise temporal-spatial expression of growth factors including WNT, FGF, and TGF-beta family of ligands. Among these, Tgf-beta family is well known for its functions in relation to cell proliferation, differentiation, apoptosis, and epithelial-mesenchymal transition, which play key roles not only during development but also during tumor growth. Sex is a risk factor in several human lung diseases. Male infants have higher risk of developing bronchopulmonary dysplasia (BPD) as compared to females and idiopathic pulmonary fibrosis has higher incidence and worse outcome in male patients than in female patients. Sex hormones have been shown to modulated the severity of lung disease in animal models. For instance, the male hormone androgen exacerbates the bleomycin-induced lung fibrosis in female mice. Herein, we have induced the activation of the TGF-beta signaling pathway specifically in embryonic lung epithelium by crossing Ca-Tgf-beta-r1-Stopfl/fl (Constitutively active Tgf-beta-receptor-1) female mice with Ttf1-Cre male mice which produced a complex and lethal phenotype only in male fetuses. The phenotype displayed by the mutant male resembles the bronchopulmonary dysplasia disease displayed by human preterm infants and was not compatible with life. In contrast, mutant females were viable and showed either a weak phenotype or no phenotype at all. The lung epithelium of the mutant males showed increased number of progenitor Sox9 positive cells and genetic deletion of one allele of the Sox9 gene significantly reduced the severity of the phenotype in mutant male mice. Transcriptome profiling delivered important insights on the mechanism responsible for the lung phenotype in mutant male animals. Among the genes deregulated in the mutant male lung we recognized genes already known to be critical for the lung physiopathology such as Spock2 and Amantsl2 which are implicated to the pathogenesis of BPD in human and bronchial dysplasia in mice, respectively, Adams18 which is a critical regulator of murine lung development and Adam1s2 which is over-expressed in patients with sarcoidosis. Finally, we showed that injection of estrogen in pregnant female mice was able to reverse the phenotype in the male lungs which suggests a hormonal dependency of the phenotype in the lung of the transgenic male mice. In conclusion, we have established a novel male sex-specific model of the TGF-beta signaling pathway that will be useful in studying how male sex can adversely affects lung development as well as neonatal pulmonary diseases in humans and to identify the sex-specific signaling pathways that give to male patients bearing lung diseases a prognostic disadvantage compared to female patients.

P3519

Board Number: B813

Sox2 inhibits lef1 expression in the trailing zone to facilitate FGF-dependent neuromast formation.

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The posterior Lateral Line (pLL) primordium, a group of approximately 100 cell, migrates under the skin, periodically depositing sensory organs called neuromasts, to pioneer formation of the zebrafish posterior Lateral Line system. During its migration, primordium cells sequentially reorganize themselves to form nascent neuromasts. Previous studies have shown that establishment of polarized signaling systems, Wnt in a leading zone and FGF in the trailing zone, coordinate systematic changes in cell organization and migratory potential within the migrating primordium. Wnt signaling inhibits FGF signaling in cells of the leading zone preventing them from reorganizing to form epithelial rosettes. On
the other hand, FGF signaling promotes morphogenesis of epithelial rosettes to form nascent neuromasts in the trailing zone. Sox2 is expressed in maturing protoneuromasts in the trailing zone. We now show that Sox2 whose expression is inhibited by Lef1-mediated Wnt signaling in the leading zone, contributes to the stability of maturing neuromasts in the trailing zone by inhibiting expression of the Wnt effector, Lef1. Knock-down of Sox2 with morpholinos results in a consistent loss or delay in the formation of the first deposited neuromast. However, the loss of the first neuromast is much more variable in sox2 loss-of-function mutants. We show that this weaker phenotype, is likely to be related to compensation by sox1a, another sox homologue, whose expression is normally restricted to the leading zone of the primordium. In sox2 mutants, sox1a expression expands from the leading zone into the trailing zone, where sox2 is normally expressed. When sox1a is knocked down in a sox2 mutant background, there is consistent loss of the first neuromast, as observed in sox2 morphants. This observation supports a compensatory role for sox1a in a sox2 mutant background. However, it remains unclear why sox1a does not adequately compensate for loss of sox2 function in a sox2 morphant, where, surprisingly, its expression also expands into the trailing zone.

P3520
Board Number: B814
Low-level copper exposure causes neurodevelopmental and cardiac defects in the embryonic zebrafish.
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Neuronal and cardiac birth defects are correlated with pesticide application and water quality. Human exposure to these pollutants may occur through drinking water, or during contact with surface water, but the molecular pathways that cause these defects are not well defined. GPCR signaling pathways constitute one primary mechanism for cells to respond to their environment. Using the zebrafish retinotectal projection as a model system, we previously showed that GPCR signaling is required for axon guidance during normal development. Retinal ganglion cell (RGC) axons that express a dominant negative G-protein subunit Ga\alphaS fail to cross the midline and misproject. Our aim was to use this system to dissect cell signaling events that translate exposure to environmental pollutants into neuronal developmental defects. We measured elevated copper levels at sites along Newtown Creek, one of the most polluted waterways in New York City and designated Superfund Site validating our findings with EPA data. We report generalized developmental as well as cardiac and behavioral deficits in zebrafish embryos transiently exposed to low levels of copper ions during development. To discover the molecular and cellular basis of copper toxicity we identified potential gene targets of copper by analyzing GEO2R datasets, and refined our candidate list to targets expressed in the right time and place to direct growing retinal axons. Using Isl2b transgenic embryos, as well as lipophilic dye tracings of retinal axons, we will assess the affect of copper on axonal trajectory correlating these developmental changes with alterations in expression of canonical GPCR signaling components.
P3521

Board Number: B815

Endoderm Nitric Oxide Focally Elevated “Hotspots” at the Heart Forming Regions (HFRs) Signals in Early Cardiogenesis in Chicken Embryos.
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The chicken embryo expresses three nitric oxide synthase (NOS) isoforms: eNOS, nNOS, and iNOS, each binding L-arginine and converting it to the nitric oxide (NO) messenger gas molecule that signals through a NO canonical pathway in cardiogenesis. However, at the earliest stages of embryo cardiogenesis, NO signaling has not been well-studied for its role in signaling heart development. To investigate, gastrula staged chick embryos (HH3 – H7) were placed on agarose plates, labeled by 5 μM DAF-2DA fluorescent NO indicator, and embedded in agarose for imaging by confocal microscopy (n=23). In HH3 embryos, NO was elevated in the endoderm and early cardiogenic mesoderm (n=4) in the bilateral heart fields which are anterolateral to the cranial-most primitive streak. In HH4 and HH4+ embryos, endoderm NO signaling to subjacent cardiac splanchnic mesoderm was shown to be focal in cranial left and right bilateral Heart Forming Regions (HFRs) over an elliptical area with a diameter of 200 ± 20 μm (n=6). By HH5, endoderm and cardiac mesoderm NO signaling was observed in maturing HFRs where cardiac progenitors differentiate and cardiomyocytes migrate cranial-ward and fuse at the embryo midline to form the cardiac crescent. (n=3) Moreover, the NO signal was elevated along the cardiac crescent just prior to final heart tube formation, as well as in the HFRs in the posteriorly adjacent splanchnic mesoderm of the developing myocardium of HH6 and HH7 embryos (n=3). Focal elevated patterns of NO signal are found to be confined to anterior regions of the embryo during heart tube formation. Furthermore, HH 4-5 embryos were microinjected with NOS inhibitor, L-NAME (1 or 10 mM), into the subterminal cavity in ovo and developed to heart tube formation (HH 8 -10) stages. L-NAME embryos were observed to develop 20 ± 5% smaller heart tubes (n=4) than comparable heart tubes from control embryos. L-NAME embryos also showed noticeably reduced myosin heavy chain expressed in cardiomyocytes compared to controls (n=4). We conclude that NO is produced as dynamic focal “hotspots” in endoderm that correlate with HFRs and early cardiogenic mesoderm differentiating to cardiomyocytes, suggesting its role in early embryo heart development. These are the first studies to show in the whole chicken embryo NO signaling correlation with early heart formation and that NO is an added biomolecular regulator functioning in early heart development. NSF STC CCC: 1548297

Cell Fate Determination

P3522

Board Number: B816

Mechanical cues control alveolar epithelial cell differentiation.
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The mammalian pulmonary alveolar epithelium is essential for the gas exchange between blood and the outside environment. The pulmonary alveolar epithelium is composed of two types of epithelial cells: alveolar type I (AT1) cells and type II (AT2) cells, which both differentiate from bipotent alveolar progenitor cells. AT2 cells are cuboidal in shape, and are alveolar stem cells in adult lungs. AT1 cells, in contrast, are thin and flat in shape, and function mainly in gas exchange via their close contact with vascular endothelial cells. Despite the vital importance of alveolar epithelial cell differentiation for
essential lung function, we know very little about the cellular and molecular mechanisms that drive developmental process. Here, using mouse genetics, in vivo and ex vivo live imaging, and quantitative cell biology, we investigated the genetic programs and cellular mechanisms that control alveolar epithelial cell differentiation. We discovered that some bipotent progenitor cells are able to protrude from the basal side of the airway epithelium. The cell protrusion process is accompanied by apical constriction, which leads to myosin enrichment at the apical side of protruded progenitor cells. During fetal breathing movements, the mechanical forces in alveolar sacs are increased by inhaled amniotic fluid. All non-protruded bipotent progenitor cells were flattened by increased mechanical forces and then became AT1 cells, whereas all protruded bipotent progenitor cells were able to maintain their cuboidal cell shape and became alveolar stem cells. We show that the enriched apical myosin function to protect protruded cells from mechanical stretching. The cell protrusion process ensures the alveolar stem cell fate specification by protecting alveolar stem cells to structurally withstand the mechanical forces. Our study revealed an intricate developmental process in which cell fate choices are controlled by the levels of mechanical forces.

P3523

Board Number: B817

Metalloprotease activity shapes BMP signaling output in the developing zebrafish embryo.

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Bone Morphogenetic Proteins (BMPs) act in an extracellular signaling gradient to specify dorsoventral (DV) axial tissues in invertebrates and vertebrates. Though the regulation of the BMP gradient across space and time is fundamental to proper tissue patterning, the molecular mechanisms that underlie this process in vivo remain unknown. The shape of the BMP gradient across the DV axis specifies distinct cell fates at precise BMP signaling levels. This critical BMP distribution is shaped by secreted modulators such as Chordin, an essential BMP antagonist. Chordin activity is extracellularly regulated by the related astacin metalloproteases Tolloid (Tld) and Bmp1a, which cleave and inactivate Chordin. Tld and Bmp1a are further regulated by Sizzled (Szl), their competitive inhibitor that prevents Chd cleavage and thus protects Chd function. We found that zebrafish embryos deficient for Tld and Bmp1a are severely defective in DV axial tissues. This indicates that Tld and Bmp1a function redundantly and reveals that astacin metalloproteases play an essential role in establishing the BMP gradient. We developed a quantitative immunofluorescence assay of nuclear phosphorylated Smad1/5 (a direct intracellular readout of BMP signaling) and used it to show that the BMP gradient in these embryos is abolished. This indicates that Chd, when unrestricted by metalloproteases, inhibits BMP embryo-wide and suggests that Tld/Bmp1a are required to spatially limit Chd activity. We also discovered, surprisingly, that the BMP signaling gradient in szl mutants is significantly altered at late blastula stages although patterning is unaffected. This underscores the importance of properly regulated metalloprotease activity in establishing the BMP gradient and suggests that Szl may play a previously unrecognized role in this process. Additionally, we discovered that the BMP gradient dramatically and rapidly steepens between mid and late gastrulation. Furthermore, tld and szl mutants display distinct alterations in BMP gradient shape at the end of gastrulation, suggesting region-specific roles for Tld and Szl. Our data indicate that Tld maintains the steepness of the late BMP gradient, while Szl restricts the highest levels of BMP signaling in lateral regions. Together, these data support fundamental, stage-specific roles for Tld/Bmp1a to differentially shape the BMP gradient, positioning astacin metalloprotease regulation as a central mechanism for modulating BMP signaling in space and time.

Tuesday-509
P3524

Board Number: B818

Loss of function of Seven-In-Absentia (SINA) E3 ligase impedes proper RAS signaling and alters peripheral nervous system (PNS) development in Drosophila.

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The RAS signal transduction cascade is a pivotal signaling pathway that controls numerous fundamental cellular processes, such as cell proliferation, differentiation, motility, and apoptosis. Seven-IN-Absentia (SINA) is an evolutionarily conserved E3 ubiquitin ligase that is the most downstream signaling module identified in the RAS signaling pathway. Under scoring the importance of SINA is its high evolutionary conservation with over 83% amino acid identity shared between Drosophila SINA and its human SINA homologs (SIAHs). As a major signaling hub and the downstream signaling “gatekeeper” in the RAS pathway, SIAHs are uniquely positioned to inhibit “undruggable” oncogenic K-RAS activation that is prevalent in high-grade metastatic cancer. Thus, it is important to delineate the enzymatic activity, molecular regulation, and substrate targeting mechanism(s) of this highly conserved family of SINA/SIAH E3 ligases. By deploying the elegant and powerful Drosophila photoreceptor cell development system, we conducted a genetic modifier screen and identified 28 new sina mutant alleles that exhibit a range of mutant phenotypes. For example, sina complete loss of function (null alleles) is cell lethal, suggesting that SINA is an essential gene in development. All other sina mutant alleles exhibit stronger phenotypes than the previously published sina2 and sina3 alleles, providing us with an opportunity to study sina loss-of-function mutant phenotypes. Sequencing analysis of these newly identified mutant alleles reveals the critical roles of several immutable amino acid residues essential for SINA function. To demonstrate the functional conservation of SINA/SIAH proteins, we have generated a collection of transgenic fly lines that carry either wild-type (WT) or dominant negative (DN) DmSINA and human SIAH. The altered DmSINA/hSIAH expression under the control of sev-, GMR-, and dpp-GAL4 drivers revealed the biological consequences of gain of function and/or loss of function of DmSINA/hSIAH1/2 in transmitting RAS signaling in PNS development. Immunofluorescent staining of developing imaginal discs and electron micrographs of adult tissues show that ectopic expression of SINAWT/DN/SIAHWT/DN in neurons resulted in dramatic changes in neuronal cell fate in the developing eye and notum. The results from these transgenic animals suggested that Drosophila SINA and human SIAH1/SIAH2 are evolutionarily conserved and functionally interchangeable. Through this developmental neurobiology-based study, we aim to dissect the molecular action of SINA/SIAH1/2 in RAS signaling. Furthermore, we hope to translate these findings to demonstrate the anticancer efficacy of the next-generation anti-SIAH-based anti-K-RAS strategy against high-grade metastatic human cancer in the future.

P3525

Board Number: B819

Different role of YAP1 during early and late stages of mesenchymal cells differentiation.

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Yes-associated protein 1 (YAP1), a transcriptional co-activator, regulates cell proliferation and differentiation. In this study, we sought to investigate the role of YAP1 in the differentiation of limb bud mesenchymal cells in micromass culture. As chondrogenesis proceeds, expression of YAP1 decreased while phosphorylation of YAP1 increased. YAP1 localized both in the cytosol and nucleus on 1 day of...
culture and became excluded from the nucleus on day 3 of culture. Inhibition of YAP1 with verteporfin resulted in the suppression of chondrogenesis and cell aggregation. In addition, verteporfin also inhibited the expression of N-cadherin and fibronectin. Incubating the constitutively active YAP1-overexpressing cells for 3 days had no effect on the cell aggregation and differentiation. However, Alcian blue staining decreased in 7-day cultured YAP1 overexpressing cells. When cells were cultured for 3, 6, or 8 days in normal media and treated with verteporfin for 1 or 2 more days, there was no change in Alcian blue staining. Interestingly, when cells were cultured for 8 days in normal media and incubated for 2 more days in the presence of verteporfin, Alizarin red S staining was increased. Taken together, these results suggest that YAP1 activity is essential for cell aggregation by the expression of N-cadherin and fibronectin at the early stage of chondrogenesis and that downregulation of YAP1 activity at late stage is not required for chondrogenesis but for bone formation.

P3526
Board Number: B820
A new transgenic Xenopus reporter line reveals dynamic expression of snail2 during cranial neural crest development.
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During vertebrate embryogenesis, the cranial neural crest (CNC) is induced at the neural plate border (NPB) and subsequently migrate and differentiate into various cells and tissues. In Xenopus, the transcription factor Snail2 is induced at the NPB by canonical Wnt signaling and is required for CNC induction and migration, but the roles of Snail2 at later stages remain unclear. To monitor CNC development in live embryos, we generated a transgenic X. tropicalis line expressing Green Fluorescent protein (GFP) driven by the snail2 promoter. Using whole genome sequencing, we mapped the transgene insertion to a noncoding region. Live imaging of the transgenic embryos reveals that snail2 is expressed in pre-migratory and migrating CNC but is lost in CNC cells that have arrived at their destinations. However, snail2 is re-expressed during CNC differentiation in all CNC derivatives that were examined. Defects in CNC differentiation, in addition to induction and migration, can be readily detected using this transgenic snail2 reporter line. Comparison with a transgenic Wnt reporter line shows that Wnt signaling is consistently activated prior to snail2 expression, suggesting that the Wnt-Snail2 axis may play important roles in multiple stages of CNC development. We are also developing a new transgenic line with a photoconvertible protein instead of GFP that can be used to for lineage tracing of CNC derivatives.

P3527
Board Number: B821
Enhanced Wnt signaling in Mesenchymal stem cells can induce cell differentiation but reduce cell viability.
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Mesenchymal stem cells (MSCs) are multipotent adult stem cells, which are potentially applicable for cell therapy to treat various diseases, such as osteoarthritis (OA). For application, further understanding
the mechanism of MSC differentiation is necessary. MSC osteogenic differentiation and adipogenic differentiation process are controlled by many signaling pathways, and one of these is Wnt signaling pathway. We previous studies have shown that a Wnt downstream effector, Fhl1 can promote osteogenic differentiation of a pre-osteoblastic cell, MC3T3-E1. Here we present our studies in investigating the role of Wnt and Fhl1 in differentiation of the earlier cell lineage, Mesenchymal stem cells. Firstly, we mimic the Wnt signaling by LiCl treatment, and indicating that the expression of Fhl1 and the osteogenic markers, Runx2, OCN, and OPN were increased accompanying with the osteogenic induction; conversely, the adipogenic markers, PPARγ2 and FABP4 but not Fhl1 were down-regulated on the second day, of MSC adipogenesis. On the other hand, MSC cell viability was also increased in the low dose of LiCl, but was decreased in the high dose of LiCl treatment. We then questioned whether high dose but not low dose of LiCl induces Fhl1 expression, leading to the induction of differentiation and inhibition of cell growth. Examining by qRT-PCR indicated that Fhl1 expression could be induced by 25 mM LiCl but not 5 mM LiCl treatment to promote osteogenesis and to inhibit adipogenesis of MSC. The influence of Wnt signaling induced by the high dose of LiCl can be abolished by treatment of the Wnt inhibitor, Dkk. Overexpression of Wnt3a and Fhl1 gene could directly induce osteogenesis and inhibit adipogenesis. In addition to the MTT assay, cell viability was also tested by PCNA and p21 expression level. Interestingly, although each of over-gene expression could reduce cell viability, overexpressed Wnt3a increased PCNA and p21 expression, yet Fhl1 decreased PCNA gene expression. In conclusion, High Wnt signaling induces osteogenesis and to repress adipogenesis of MSC. Simultaneously, inhibition of cell viability of MSC may be because of the elevation of p21 and the down-regulation of PCNA gene expression.

P3528
Board Number: B822
Six3 suppression of R-spondin 2-Wnt signaling is required during mammalian neuroretina development.

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The mammalian neuroretina is a sensory organ that consists of multi-layered neuronal cell types. It has an important role during visual recognition where light is converted into neural signals in the visual cortex. Retinal abnormalities and retinal degenerative diseases cause severe vision loss in millions of people worldwide. Recent advances in 3-dimensional (3D) tissue cultures of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) provided an additional in vitro model that recapitulates many aspects of the in vivo developmental steps. In this study we took advantage of this 3D eye organoid culture system as a faster and reliable tool to identify and characterize some of the molecular events controlling early steps of mammalian eye development. We validated these results in vivo in mouse embryos. From genome-wide microarray data, we first identified R-spondin 2 (Rspo2) as a novel candidate gene in neuroretina differentiation in vitro. Next, we determined that Six3 binds to Rspo2 regulatory elements using ChiP analysis. Using newly generated Six3−/− iPSCs and conditional Six3
knockout ESCs, we found that Six3 repression of Rspo2 is a novel required step during optic vesicle morphogenesis and neuroretina differentiation in vitro. Finally, we validated these results in vivo by showing that transient ectopic expression of Rspo2 in the anterior neural plate of transgenic mouse embryos was sufficient to arrest neuroretina differentiation. Additionally, using a chimeric eye organoid assay we determined that Six3-null cells exert a non-cell autonomous repressive effect during neuroretina differentiation. Our results further validate the organoid culture system as a reliable and fast alternative to identify and evaluate genes involved in eye morphogenesis and neuroretina differentiation in vivo.

**P3529**
**Board Number: B823**
Epithelial-mesenchymal micro-niches integrate signaling effectors to drive dynamic chromatin remodeling during stem cell lineage choices.
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Tissue regeneration relies on resident stem cells, whose activity and lineage choices are influenced by microenvironment. Once activated, stem cells typically give rise to short-lived progenitors, which then progress to differentiate into their lineages. Exploiting the synchronized, cyclical bouts of tissue regeneration in hair follicles, we investigated when and how microenvironment dynamics shape the emergence of stem cell lineages. Using temporal single-cell RNA-seq, we unearthed unexpected heterogeneity among stem cells and their progeny and found that the ability of stem cells to make lineage choices (plasticity) becomes restricted in a sequentially and spatially choreographed program. We traced the roots of lineage restriction to micro-niches located along epithelial-mesenchymal borders, each of which receive slightly different signaling inputs. Whether these signals are WNTs, BMPs or NOTCH, each has its own effector that alters the activity of a downstream transcription factor. Employing epigenetic and ChIP-seq profiling, we uncover how signal-dependent transcription factors couple spatio-temporal cues to chromatin dynamics, thereby choreographing stem cell lineages. Using enhancer-driven reporters, mutagenesis and genetics, we define the signaling inputs involved and show how stem cells establish chromatin platforms permissive for diversification, and how new signaling inputs progressively compartmentalize lineage options in vivo by modifying chromatin dynamics.

**P3530**
**Board Number: B824**
The Tumor Suppressor p53 Mediated Apoptotic Pathway Plays An Essential Role in HSF4 Regulation of Lens Fiber Cell Differentiation.
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The Tumor suppressor p53 is a multi-functional regulator of cell proliferation, differentiation, apoptosis and transformation. Our previous studies have revealed that PP-1 directly modulates p53 functions by dephosphorylating S15 and S37 in both cell differentiation and apoptosis (Li et al., Oncogene, 2006). More recently, we have demonstrated that p53 can regulates 3 types of gene to control lens differentiation: lens major transcription factors such as c-Maf and Prox-1, lens differentiation related
genes, members of alpha, beta and gamma-crystallin genes, and apoptotic genes, Bak and Bax (Mao et al. 2001. JBC; Li et al. 1998. Eur J. Biochem., Deng et al., 2010. CMM., Liu et al. 2012; CMM., Ji et al., 2013, Hu et al., 2014. CMM. Huang et al., 2015. BBA.). The heat shock transcription factor 4 (HSF4) is a major regulator for normal lens development. Mutations in HSF4 have been associated with cataract, but the mechanisms regarding how mutations in HSF4 cause cataract are still obscure. In a recent study, we found that HSF4 can stabilize p53 to regulate cell cycle progression (Huang et al., 2015. BBA.). Here, an hsf4 knockout zebrafish line was generated. The mutant zebrafish developed an early onset cataract with multiple developmental defects in lens. Mechanistically, it was found that the lens epithelial cells were over-proliferated at the germinal zone, resulting in the overabundance of lens fiber cells in hsf4null zebrafish lens. As a result, the arrangement of the lens fiber cells became more disordered and irregular with age. More importantly, the terminal differentiation of the lens fiber cell was interrupted since the organelles including nucleus, mitochondridial, lysosome and endosome cannot be cleaved in due time. In exploring the molecular mechanisms, the cultured human lens epithelial cells were used to test HSF 4 functions. It was revealed that HSF4 could stabilize and retain p53 in the nucleus to activate its target genes Fas and Bax, members of the extrinsic and intrinsic pathways. In the hsf4null zebrafish, both p53 and activated-caspase3 were significantly decreased. Combined with the finding that the denucleation defect could be partially rescued through microinjection of p53, fas and bax mRNA into the mutant embryos, these results demonstrated that HSF4 promotes lens fiber cell differentiation by activating p53 and its downstream regulators. HSF4 functions in the upstream to activate these genes highlighted the new regulatory modes of HSF4 action in the terminal differentiation of lens fiber cell.

P3531

Board Number: B825

The Impact of Flame Retardant Exposures on Human Pregnancy.
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Classes of flame retardants (FRs) such as polybrominated diphenyl ethers (PBDEs) and organophosphorus FRs (OPFRs) are used in consumer products and subsequently released into the environment. While in humans, the health consequences associated with FR exposures remain undefined, in animal models, specific FR exposures may be developmentally toxic, e.g., endocrine disruption, alter nervous system development. Recently, FRs have been identified in human placental tissues. Due to the importance of placental development during pregnancy, environmental chemicals may adversely impact prenatal development. Specialized placental cells known as cytrophoblasts (CTBs) differentiate and are responsible for uterine invasion and maternal vascular remodeling, two very important components of placentaion. In culture, primary human villous CTBs display characteristics of CTB differentiation representative of their inherent functional behaviors in vivo. Preliminary data from our laboratory suggests that PBDEs may disrupt CTB functions that are integral to normal differentiation. Here, we will test the hypothesis that FRs significantly impair CTB function, resulting in compromised placental development. In Aim 1, using cultured CTBs as a model of placentaion, we will evaluate the ability of FRs to alter CTB functions that are a proxy for their differentiation, e.g., migration and invasion. In Aim 2, we will analyze the effects of FRs on the expression of molecules that regulate these functions. In Aim 3, we will select a subset of these molecules and use an immunolocalization approach to profile their expression in placental biopsies in relationship to pre-identified FR placental levels. My project will result in the novel characterization of the effects of FRs on CTB differentiation and the molecules that regulate these processes. Proteins identified to be altered by FRs may be used as biomarkers of FR
exposure in future environmental health studies. In summary, this work will help us understand how environmentally-induced toxicity may contribute to placental pathologies during human pregnancy.

P3532
Board Number: B826
Cell fate under N-acyl dopamines control: to die or to differentiate?
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Bioactive lipids N-acyl dopamines (NADA) are conjugates of dopamine with fatty acids, which usually are unsaturated, and belong to the endocannabinoid/endovanilloid family. They are synthesized in mammals, as well as in some more primitive organisms. The known molecular targets of NADA are the GPCRs CB1, CB2 and several non-CB1/CB2 receptors, ion channel TRPV1 as well as some other proteins (dopamine receptors, potassium and calcium channels). The objective of the study was to evaluate the long-term effects of NADA on cell proliferation and death and to elucidate the underlying mechanisms. As far as most of the known NADA effects occur within nervous system, we used PC12 pheochromocytoma, HT22 neuroblastoma and C6 glioma cells as a model. For each cell line, the LC50 of a set of NADA was determined, and after that the cultures were incubated with various concentrations of NADA for two weeks. Cell death was assessed using MTT, LDH tests, caspase activity and annexin staining. The differentiation degree was determined by the count and length of the processes and by the mRNA expression of several stem, neural progenitor, astrocyte and “neuron markers. The molecular target of NADA and signal transduction pathways were determined using inhibitor screening, siRNA knockdown or using reporter systems. The LC50 values for NADA with arachidonic, oleic and docosahexaenoic fatty acid residues, as well as for arachidonic acid amides of tyramine and norepinephrine were in range 2-30 μM. The prolongation of cell culture treatment with NADA did not increase cell death. Instead, the concentrations below 1 μM stimulated cell proliferation, and those in range 1 μM–LC50 induced neurite outgrowth in PC12 and C6 cells with a shift towards neuronal and astrocytic marker expression, accordingly. In HT22 cells, no response other than cell death was observed for intermediate and high NADA concentrations. The molecular target of the NADA action in the PC12 cells was found to be the non-CB1/CB2 receptor GPR55, which transduced signal through PLC, IP3R and calcium to the CaMKIV, and then to the CREB transcription factor with NO synthase expression induction and a supposed IL-6 secretion; the latter is expected to induce differentiation via the JAK-STAT signaling pathway. Thus, on the model of the rat C6 glioma and the PC12 pheochromocytoma cells, we demonstrated for the first time that the low concentrations of N-acyl dopamines (below 1 μM) stimulate cell proliferation, intermediate ones (1-20 μM) induce differentiation, and higher ones lead to apoptosis after a specific receptor activation. The work is partially supported by the RFBR grant 16-04-00729a.
P3533
Board Number: B827
The tumor suppressor Lkb1 controls cell fate through pyruvate-alanine transamination.
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The tumor suppressor LKB1 (also named STK11) codes for a serine/threonine kinase. Germline mutations of LKB1 are responsible for the Peutz-Jeghers syndrome, a dominantly inherited cancer disorder. Somatic mutations of this gene have been associated with various cancers including lung and cervical tumors. Lkb1 acts as a key regulator of energy metabolism through the activation of the AMP-activated protein kinase (AMPK), a sensor that adapts energy supply to the nutrient demands of cells facing situations of metabolic stress. To achieve metabolic adaptations, AMPK phosphorylates numerous substrates which inhibit anabolic processes while activating catabolic reactions. In particular, AMPK inhibits the nutrient-sensor kinase mTOR. In addition to AMPK, Lkb1 also phosphorylates 12 AMPK-related kinases that regulate cell polarization, axon branching of cortical neurons and hepatic neoglucogenesis.

To determine how Lkb1 coordinately regulates cell polarity and energy metabolism during cell fate decision, we spatio-temporally deleted the Lkb1 gene in embryonic multipotent neural crest cells (NCC). These cells originate from the neural tube and give rise to various tissues including the peripheral nerves and the enteric nervous system (ENS). We showed that Lkb1 governs several aspects of cephalic NCC development that are crucial during vertebrate head formation (Creuset et al., 2016). We also reported that mutant mice exhibited hypopigmentation, hindlimb paralysis and intestinal pseudo-obstruction. We described that Lkb1 is crucial for the differentiation and maintenance of two NCC-derivatives, Schwann cells and the ENS. Using a model of neural crest stem cell line, we demonstrated that Lkb1 is key for glial differentiation. Mechanistically, Lkb1 loss led to increased alanine levels as also observed in vivo. Interestingly, inhibition of pyruvate-alanine transamination rescued glial differentiation of Lkb1-null NCC, in a dependent manner of mTOR. Furthermore, AICAR rescued glial differentiation of Lkb1-deficient NCC and treatment of Lkb1-deficient mice with AICAR corrected the Schwann cells and ENS phenotypes (Radu et al., submitted).

Altogether, these findings highlighted the novel and crucial role of Lkb1 during neural crest cell fate and uncovered a link between Lkb1-mediated pyruvate-alanine cycling and glial differentiation. These results provide new insights to understand the metabolic regulations exerted by Lkb1 during development and tumorigenesis.
Cell fate determination is critical for cell survival and development. Importantly, cell fates are shaped by the integration of multiple signals such as external cues, intracellular nutrient storage, and the overall state of the cell. Our research is using budding yeast to study a model differentiation system: entry to meiosis/sporulation. When diploid yeast is exposed to medium without nitrogen and fermentable carbon, it can activate a specific transcriptional program that allows yeast to irreversibly commit to meiosis and sporulate. To be able to commit to meiosis, yeast has to accumulate nutrients, especially storage carbohydrates (Kane & Roth 1974). However, how upstream nutrition level regulates cell commitment remains poorly understood. Here, we investigate how storage carbohydrates affect sporulation using combinations of single cell microscopy and biochemical methods. Using fluorescently tagged enzymes that regulate the storage carbohydrates trehalose and glycogen, we can predict whether individual cells will sporulate with high (~90%) accuracy. Ongoing work aims to link such single cell measurements to the actual levels of the storage carbohydrates to verify our working hypothesis that entry to meiosis is determined by some fixed threshold of storage carbohydrates.


Background: Pulmonary fibrosis is a debilitating disease that is poorly understood. Carbon nanotubes (CNTs) have been shown to induce pulmonary fibrosis in animals, which is of concern given their widespread use in consumer products and other applications. While animal models of CNT exposure are well characterized, a robust in vitro model of particle induced fibrosis would enable testing of the fibrotic potential of these particles more quickly and efficiently. Due to the important role the extracellular matrix plays during fibrosis, an in vitro model based in a three-dimensional extracellular matrix such as collagen is necessary to best recapitulate the disease state in the body. The current study uses a 3D assay of cell contractile ability, the gel contraction assay, in conjunction with investigation of biomarkers of fibrosis to test the fibrotic potential of several CNTs of different physical dimensions.

Methods: WI38-VA13 human pulmonary fibroblasts were treated with various CNTs of differing physical properties. Exposed cells were examined for markers of myofibroblast differentiation, an important step of the fibrotic process, including α-Smooth Muscle Actin (α-SMA) expression and cellular contractile ability in a 3D in vitro model.

Results and Conclusion: Fibroblasts treated with CNTs displayed increased contractile ability compared with untreated controls. Additionally, CNT treated fibroblast expressed markers of α-SMA, a marker of myofibroblast differentiation. The fibrogenic potential of CNTs tested varied, with CNTs of more extreme physical dimensions, the largest and smallest aspect ratios in the group of CNTs tested, having a greater
effect on fibroblasts in the gel contraction assay. These results indicate that the physical dimensions of CNTs may affect their fibrogenic potential. The collagen gel contraction assay used allows for efficient measurement of the contractile ability of fibroblasts, an important component of fibrosis. Additionally, cells can be directly examined for markers of myofibroblast differentiation such as α-SMA. Finally, while not attempted in this study, the gel contraction model can be expanded to include other cell types, allowing for a more robust study of the cell interactions at play during induction and progression of fibrosis.

P3536
Board Number: B830
Understanding programming logic of motor neurons from differentiated and undifferentiated cells.

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The safety concerns for transplantation of tissues derived from iPSCs/ESCs based differentiation protocols propose in vitro or in vivo transdifferentiation of somatic cells as a substitute for clinical applications. Although programming from somatic cells has the potential to be instrumental for cell replacement therapies, transdifferentiation is inefficient. The goal of this study is to understand how transcription factors (TFs) program rapidly-dividing pluripotent stem cells into post-mitotic motor neurons and then to apply this knowledge to efficiently program motor neurons from differentiated cell types.

Viral expression of 7 TFs (Ascl1, Brn2, Myt1l, Ngn2, Isl1, Lhx3 and Hb9) is able to transdifferentiate primary mouse embryonic fibroblasts (pMEFs) to spinal motor neurons (sMNs) with low efficiency (4%). Comparison of this approach to an efficient (>90%) ESC based sMN programming protocol that expresses three transcription factors (Ngn2-Isl1-Lhx3, the NIL factors) lead to an increase of efficiency (20%) in pMEF based sMN programming with maintaining 1:1 stoichiometry between Isl1 and Lhx3. NIL factors induce secondary TFs, Ebf and Onecut families during programming in ESC and colocalize with these secondary TFs at sMN specific regulatory regions. Preliminary results demonstrate that NIL factors fail to induce Ebf and Onecut TFs in pMEFs, and thus, propose a potential mechanism causing inefficient sMN programming in pMEFs. To further improve the efficiency of transdifferentiation, I will investigate if forced expression of other TFs required for sMN differentiation improves the sMN programming efficiency from pMEFs. Ration ally improving an inefficient transdifferentiation protocol by comparing to an efficient differentiation approach from ESC can lead to TF combinations that will be applicable to transdifferentiate other cell types. In the future, novel programming methods can be used to generate homogenous neuronal fates for in vitro disease modeling, drug studies and in vivo cell replacement therapies.
Host-Pathogen/Host-Commensal Interactions 2

P3537
Board Number: B832
Molecular mechanisms of sterol transfer in intracellular coral-algal symbiosis.
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Many animals establish symbioses with microorganisms to gain an ecological advantage. A remarkable example is the endosymbiosis between corals and dinoflagellates (genus Symbiodinium), which provide photosynthetically fixed nutrients to enable coral survival in nutrient-poor tropical oceans. Corals are unable to endogenously synthesize cholesterol, an essential membrane component and regulator of developmental and signaling pathways, whereas coral symbionts (like their fellow dinoflagellates) synthesize cholesterol and many related sterols, but the basis and function for this breadth remains unknown. Here we use Aiptasia, a marine sea anemone, as an emerging laboratory model for coral endosymbiosis to dissect the role of the evolutionary highly-conserved cholesterol-binding proteins Niemann-Pick Type C (NPC2) in sterol transfer from the symbiont to the host. We find that the NPC2 gene family is expanded from one to six or more genes in corals and Aiptasia when compared to humans. Some NPC2 gene sequences fall outside the group of canonical NPC2s, leading to the hypothesis that these ‘non-canonical’ NPC2s are an adaptation to exploit the variety of symbiont-produced sterols for cellular use. Accordingly, gene expression of various NPC2 genes is upregulated in response to symbiosis in Aiptasia and corals and semi-quantitative GC-MS analysis reveals that indeed symbionts transfer cholesterol as well as many other sterol derivatives to their hosts. Interestingly, sterol composition in the hosts varies with different symbiont types housed. We generated polyclonal antibodies to those non-canonical NPC2 proteins and using western blotting could confirm the presence of NPC2 proteins in symbiotic animals but not in non-symbiotic ones. Currently, we are exploiting our antibodies to analyze protein localization and are establishing lipidomics techniques to directly test the binding patterns between the non-canonical NPC2 proteins and distinct sterols. Taken together, our data suggest that functional adaptation of existing molecular machineries may have contributed to forming a functional holobiont and thus the successful radiation of reef-building corals into nutrient-poor habitats.

P3538
Board Number: B833
Type III interferon signaling affects Cryptosporidium parvum infection of human intestinal epithelial cells.
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Interferons are a family of cytokines that were initially discovered and named for their ability to interfere with viral replication. Type III interferons, interferon lambda subtypes 1-4, are members of the most recently discovered class. IFN lambda shares all upstream components of signaling with type I interferons, interferon alpha and beta, a well characterized relative. In contrast to IFN-a/b controlling viral replication systemically, IFN lambda specifically prevents viral replication within cells of epithelial origin. The specificity of the type III interferon response is based on the restricted expression of the IFNLR1 subunit of the receptor which is almost exclusively expressed on epithelial cells.
Cryptosporidium is an obligate intracellular protozoan parasite, and the second leading cause of severe diarrhea and diarrheal-related death in children worldwide. There are currently no vaccines and the only drug available has low efficacy in immunocompromised individuals who need it most. A lack of tools to study Cryptosporidium including continuous culture, molecular genetics, and immune competent animal models have impeded progress towards development of novel therapeutics. We conducted a genome-wide CRISPR/Cas9 knockout screen to discover host genes necessary for Cryptosporidium parvum infection. Type III interferon signaling was the most highly enriched pathway in our screen. Human intestinal epithelial cells, HCT-8s, stably expressing Cas9 were transfected with a lentiviral sgRNA library targeting every gene in the human genome. Selection for resistance to cell death was accomplished by three consecutive 72-hour C. parvum infections. After each challenge, cells were removed for gDNA extraction and sequenced to determine the abundance of each sgRNA. The top 25 enriched genes after three replicates indicate that type III interferon signaling is important for C. parvum infection. Investigation into the role of type III interferon signaling in C. parvum infection will reveal how a viral defense pathway seems to promote parasite infection.

P3539
Board Number: B834
A possible role for unconventional splicing enzymes in nonreplicative recombination of poliovirus.
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RNA recombination is one of the major driving forces in RNA virus evolution. Two mechanisms of this process are known to date. A recombinant RNA molecule may be formed due to the replicative template switching. Recently, there are increasing data about another, nonreplicative mechanism of viral RNA recombination. However, roles of host factors in this process remain poorly understood. We investigated the role of unconventional splicing enzymes in the process of viral RNA recombination, particularly IRE1 endonuclease. IRE1 was shown to be involved in Xbp1 mRNA intron excision in cytoplasm during UPR (unfolded protein response). A chimeric virus with a structure mimicking Xbp1 intron was inserted in a 3D-polymerase coding sequence. These mutations lead to failed viral reproduction due to the open reading frame shift. But reactivation of IRE1 endonuclease activity by chemical inducers of UPR lead to intron excision and restoring viral reproduction. A similar sequence with point mutations disrupting the IRE1 excision site was inserted into the 2A-protease coding sequence of poliovirus. Constructed virus also showed increased viability upon IRE1 activation by chemical inducers of UPR. Taken together our data shows colocalization of poliovirus RNA with IRE1 and a possible role for unconventional splicing enzymes in viral RNA recombination. We also discuss the role of cellular tRNA ligase HSPC117 in the process of non replicative poliovirus recombination. To study fundamental aspects of RNA recombination by the use of poliovirus derived RNAs, a cell culture based RNA recombination system has been established. This system allowed the generation of infectious viral RNA genomes by nonreplicative RNA recombination between two synthetic, replication incompetent transcripts. Knockdown of genes, which are essential for viral recombination will lead to failed recombinants production. Since we have in vivo system for nonreplicative recombination of poliovirus, we can observe the effect of gene knockdown on the production of recombinants. Future studies will elucidate the role of nonreplicative RNA recombination and bring new insights into the molecular mechanism of this process.
P3540
Board Number: B835
Non-silencing miRNAs promotes autophagic viral degradation by inhibiting retroviral Gag assembly.
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MiRNAs are a class of short non-coding RNAs that are recognized for their functions in silencing gene expression through base-pairing with target mRNAs. In this study, we have found that miRNAs can generally inhibit the assembly and budding of retroviruses MLV and HIV-1 through a mechanism not involving gene silencing. We showed that this blockade occurs by miRNA binding to retroviral Gag protein, the major structural protein of all retroviruses. This interaction disrupts Gag assembly at the plasma membrane, causing endocytosis of the misassembled viral complexes and ultimately their degradation in lysosomes through an intermediate pathway involving LC3, p62, ULK1 and ATG5, which are essential components of autophagy. Overall, these results support the idea that miRNAs can exert functions besides gene silencing, and that, in the context of retroviral biology, miRNAs and autophagy can work synergistically to block retroviral production. Additionally, these findings also raise the possibility of developing a universal strategy to combat retrovirus infection based on synthetic oligonucleotides. Finally, the observed pathway leading to the degradation of misassembled viral complexes could manifest in other cellular and pathological contexts where regulation of misassembled RNA-protein complexes are critical.

P3541
Board Number: B836
Fulicin-like immunoreactivity in Biomphalaria glabrata, an intermediate host for schistosomiasis.
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About 10% of the world’s population live at risk of contracting the parasitic disease schistosomiasis. Schistosoma mansoni, the digenetic trematode that causes the most widespread form of intestinal schistosomiasis, employs the freshwater snail Biomphalaria glabrata as its primary intermediate host. It has been shown that infection of B. glabrata by S. mansoni causes profound behavioral changes in the snail host, including parasitic castration, a reduction in reproductive behaviors. In this study, a neural transcriptomics approach was undertaken to identify precursor prohormones that could encode neuropeptides involved in Biomphalaria reproductive behaviors. A transcript (1616 nucleotides) was found to encode a putative precursor polypeptide (316 amino acids) that could give rise to the neuropeptide fulicin (Phe–D–Asn–Glu–Phe–Val–NH₂) and five additional related peptides. For this investigation, affinity purified polyclonal antibodies were generated against the predicted fulicin neuropeptide. Antibody specificity was confirmed with dot blots and preabsorption control experiments. Fulicin–like immunoreactivity was observed throughout the central nervous system (CNS) and in specific peripheral tissues. Double-labeling experiments (biotin backfill x fulicin immunohistochemistry) identified three groups of fulicin-like immunoreactive neurons that project to penile nerve. Immunoreactivity was present in the ovisperm duct, sperm duct and prostate gland, tissues associated with the male reproductive system. These results indicate that fulicin and other
peptides derived from the fulcin precursor could regulate male reproductive behaviors that are altered during the course of infection in this host-parasite system.

P3542
Board Number: B837
Identification of inflammatory biomarkers in dengue disease severity in eastern India.
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Background: Dengue is the most common arboviral infection with an estimate of 390 million infection per year and about 3.6 billion people at risk. Notably, 34% of the total global infections of dengue reported is from India alone. The detail mechanisms of dengue pathogenesis are not established yet. Host immune response could contribute to disease severity. There are four serotypes of dengue virus (dengue-1, -2, -3 and -4). There are limited data on the circulating strains in this region. Information on the circulation strains and identification of inflammatory biomarkers will help in devising effective interventionalal strategies. Objective: To determine the dengue virus circulating strains, immune biomarkers involved in disease severity in eastern India Methodology: Eight nine subjects of suspected dengue subjects (WHO criteria) presented to the SCB Medical College & Hospital, Cuttack, India, during the 2016 outbreak, were enrolled in the study. Dengue virus detected by serology and RT-PCR. Dengue serotype discrimination and was made by types-specific PCR and Sequencing. Multiplex cytokine bead array for 41 cytokine panel was used to measure the expression of the inflammatory biomolecules.
Results: RT-PCR for dengue detected in 49% samples. Dengue serotype-2 was predominant (58%) followed by serotype-1 (24 %), serotype-3 (8 %) and serotype- 4 (in 10 %) subjects. Mixed infections were seen in 39% subjects. Mixed infections were seen in 39% subjects. The levels of IFN-γ, GM-CSF, IL-10, IL-1Rα, IL-6 TNF-α, MIP-1β and MCP-1 were significantly elevated in severe dengue. Conclusions: Dengue virus serotype-2 was predominant type in this outbreak. This study demonstrated the association of serotype specific immunity in determinants of severe dengue. The involvement of the host inflammatory biomarkers, could serve as potential predictor for therapeutic interventions. Acknowledgement: This study was funded by the Science Engineering Research Board, Department of Science and Technology, Government of India.

P3543
Board Number: B838
The pathogenic yeast, Cryptococcus neoformans, alters the transcriptome of host macrophages.
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Cryptococcus neoformans (Cn) is a ubiquitous fungal pathogen that is a leading cause of mortality among the immunocompromised with almost 625,000 deaths per year worldwide. While Cn infections usually begin as acute pulmonary infections that can progress to fungal meningitis, Cn can also establish long-lasting chronic infections by residence inside host macrophages. However, the mechanisms Cn utilizes to circumvent the host immune response are only partially understood. Some intracellular pathogens, including Cn, are able to attenuate the innate immune response through inhibition of host cell protein synthesis, altering host cellular functions, or by compromising macrophage polarization. Classically-polarized (M1) macrophages exhibit enhanced microbicidal activity and are associated with a more
favorable clinical outcome in cryptococcal infections, while alternatively-activated (M2) macrophages are less able to destroy ingested pathogens. However, the mechanisms intracellular Cn uses to disrupt host macrophage function to survive are poorly understood. Although altered host macrophage function by intracellular Cn could be explained as a byproduct of cellular stress, we hypothesized that there would be Cn-induced changes in the gene expression of host macrophages that lead to the attenuated immune response. Using RNA sequencing to assess the transcriptome profile of M1-polarized Cn- and non-infected RAW 266.7 murine macrophages, we first analyzed the data using R-based tools to generate hierarchical clusters and multidimensional scale plots to visualize similarities of overall gene expression. The data were then analyzed for differential gene expression between Cn-infected and non-infected macrophages. Gene ontology enrichment analysis was then used to determine over-represented functional pathways and processes. Our data suggest that there are unique gene expression profiles, and that intracellular Cn causes altered expression of ribosomal and mitochondrial oxidative phosphorylation genes. These differences corroborate previously reported inhibition of host cell translation and mitochondrial dysfunction upon Cn-infection. Furthermore, our data show that even in an M1-polarized environment, there is an up-regulation of M2-specific markers, including M2-associated Arg1 and M2-exclusive Myc, after 24 hours of infection. Additionally, using western blotting, our data show an up-regulation of c-Myc after 24 hours of infection. Therefore, our data suggest that intracellular Cn alters macrophage function through changes at the transcriptional level to attenuate fungicidal activity and survive long-term within host cells.

P3544
Board Number: B839
Role of Viperin Interaction with Human Cytomegalovirus Tegument Protein pp28 in Virion Assembly.
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Viperin is a multifunctional interferon (IFN)-inducible protein that is directly induced in cells by human cytomegalovirus (HCMV) infection. Viperin normally localizes to the endoplasmic reticulum (ER) and has antiviral activity. However, this protein re-localizes from ER to mitochondria by interacting with a HCMV protein vMIA at early stage of infection and modulates cellular metabolism to enhance viral infectivity. Viperin finally localizes to the virus assembly compartment (AC) at late stage of infection, although its function remains unexplored. Viperin interaction with HCMV viral proteins is the key factor to determine viral replication status. Nevertheless, HCMV viral proteins which interact with viperin have not yet been identified except for vMIA. Here, we screened interacting partners for viperin using yeast two hybrid assay. We selected a HCMV tegument protein pp28 that is accumulated at the AC at late stage of infection and essential for viral replication. The pp28 contains a stretch of acidic amino acids (aa 44-59) that is required for its trafficking to the AC and the virus assembly. Recently, we have shown that this acidic cluster of pp28 has distinct functional domains. The first half (aa 44-50) of the acidic cluster is sufficient for pp28 trafficking and the native acidic cluster containing the second half (aa 51-59) is required for the virus assembly. We confirmed that viperin interacts with pp28 in transient transfection and viral infection and co-localizes with pp28 to the AC at late stage of infection. We generated a panel of pp28 mutants and identified a binding domain (aa 51-61, the second half of acidic cluster of pp28) of pp28 interacting with viperin. Recombinant viruses expressing this pp28 deletion mutant (delta aa 51-61) but not pp28 acidic cluster deletion mutant (delta aa 44-59) produced infectious virions. We also observed that the pp28 deletion mutant (delta aa 51-61) localizes to the AC at late stage of infection.
However, the recombinant viruses exhibited delayed viral growth kinetics and decreased viral yield. The results suggested that viperin interaction with pp28 plays a critical role in the virus assembly and the production of infectious viruses, and the second half of acidic cluster of pp28 is required for their interaction.

P3545

Board Number: B840

Computational analyses of the ExoS protein, a component of Sinorhizobium meliloti ExoR-ExoS/ChvI pathway.

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The Sinorhizobium meliloti periplasmic ExoR protein and the ExoS/ChvI two-component system form a regulatory mechanism, the RSI Invasion Switch, which directly controls the transformation of free-living to host-invading cells. In the absence of crystal structures, understanding the molecular mechanism of interaction between ExoR and the ExoS sensor, which is thought to drive the key regulatory step in the invasion process, remains a major challenge. Here we present for the first time a three-dimensional representation of S. meliloti Rm1021 ExoS periplasmic sensing domain generated through computational methods. Our model suggests the Per-ARNT-Sim (PAS)-like fold for ExoSp. Even though ExoSp shares a similar overall structure as other PAS domains, it does not bind small ligands and therefore probably uses a different mechanism to recognize and bind the periplasmic binding protein, ExoR. We suggest that elongated structural elements that are unique to ExoSp might provide flexibility to this fold to accommodate binding of ExoR, an elongated multi-repeat protein. This study lays the foundation for future work including experimental analysis that target key proposed functional sites of ExoSp and the interface of the ExoSp-ExoR complexes so as to validate the ExoSp model and uncover the essential residues employed in ExoSp-ExoR interactions.

P3546

Board Number: B841

SERINC5 Inhibits HIV-1 Fusion Pore Formation by Promoting Functional Inactivation of Env Glycoproteins.

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SERINC5 (SER5) is a newly discovered multi-transmembrane domain protein that inhibits HIV-1 infectivity upon incorporation into budding virions in the absence of HIV/SIV Nef expression. The cellular functions of SER5 and the mechanism by which this protein restricts HIV-1 infection are poorly understood. The SER5-sensitivity maps to the HIV-1 envelope (Env) glycoprotein, with different isolates exhibiting a broad range of sensitivities to this host factor. Based upon the earlier observation that SER5 more potently blocks viral infection than fusion, it has been proposed that SER5 impairs enlargement of the fusion pore and thereby prevents the release of viral nucleocapsid. Here, by using live imaging assays, we show that incorporation of SER5 into virions in the absence of Nef inhibits the formation of small fusion pores between viruses and cells. We also demonstrate that SER5 restricts both virus-mediated fusion between adjacent cells (“fusion-from-without”) and cell-cell fusion between Env-expressing and CD4/coreceptor-expressing target cells. Importantly, we found that SER5 promotes
spontaneous functional inactivation of sensitive, but not resistant Env glycoproteins, an effect that phenocopies the SER5 resistance phenotype. We found that the accelerated loss of Env function upon SER5 incorporation occurs through exposure of the conserved domains of the transmembrane gp41 subunit of Env, as evidenced by the enhanced virus sensitivity to neutralizing antibodies against cryptic gp41 epitopes and to gp41-derived inhibitory peptides. Together, our results imply that SER5 blocks HIV-1 infection at a step upstream of small fusion pore formation by selectively inactivating Env and delaying conformational transitions of the HIV-1 gp41 en route to fusion. Efforts are on the way to detect SER5-Env interactions within virions and to assess the possibility that this restriction factor interferes with the formation of functional Env clusters on the viral envelope. This work was partially supported by the NIH R01 grant GM054787 to G.B.M. The first two authors contributed equally to this work.

P3547
Board Number: B842
Behavioral Response of Caenorhabditis elegans with Pseudomonas aeruginosa Infection Controlled by Iron Variables.
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Caenorhabditis elegans is a model organism that has a life span of 2 to 3 weeks; it’s widely studied within laboratories because of its host pathogen interaction. This organism expresses unique characteristics such as defense mechanism against pathogenic virulence. A known resistant pathogen is Pseudomonas aeruginosa, a common bacteria found in various amounts of environments such as plants and animals. P. aeruginosa thrives and colonizes to form clusters of biofilm that affects patients with a disease known as cystic fibrosis. Cystic fibrosis is a disease where mucus builds up in the lungs in which affects the respiratory system. Previous studies have shown that three mechanisms are known to killing C. elegans; NG medium, increase the iron concentration of high-osmolality, and brain- heart infusion medium. Studies have shown that the NG medium has a “slow killing” of this organism and high osmolality has a “fast killing.” In this research, we are studying the environmental behavior that interacts between the biofilm and C. elegans when being exposed to various levels of the medium. Current studies suggest that increasing the osmolality of iron would extend the slow killing process of C. elegans.

P3548
Board Number: B843
Multiple Epstein-Barr viral microRNAs downregulate human NF-kB pathway transcripts.
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The Epstein-Barr virus (EBV) is a gamma-herpesvirus infecting nearly 95% of humans worldwide. EBV infection is the primary cause of infectious mononucleosis and has been linked to specific malignancies including Burkitt’s lymphoma and nasopharyngeal carcinoma. Through divergent expression of 49 mature microRNAs (miRNAs), EBV disrupts cellular mechanics to enable pathogenesis. The targets of the EBV miRNAs are not completely understood. High-throughput sequencing and crosslinking immunoprecipitation (HITS-CLIP) and bioinformatic predictions identified targets of EBV and human miRNAs in Burkitt’s lymphoma cells and demonstrated a significant enrichment of at least seven NF-kB pathway transcripts. To confirm the extent of downregulation and specificity of miRNA base pairing to
these transcripts, luciferase reporter assays and endogenous protein western blots were conducted for each potential miRNA-mRNA pair. Many, but not all predicted, EBV BART miRNAs significantly downregulate NF-kB pathway proteins, most likely to fine tune expression of the pathway.

P3549
Board Number: B844
Adhesion of Providencia stuartii onto Cultured Bladder Epithelial Cells.
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Providencia stuartii are opportunistic bacteria related to the pathogens of nosocomial urinary tract infections. However, so far little is known about the mechanisms of pathogenesis used by these bacteria. The initial step of the infectious process is the ability of microorganisms to adsorb or to adhere onto sensitive cells and such interaction involves the specialized structures on their surfaces. According to modern concepts, adhesins are surface proteins or glycoproteins of the bacterial cell. Data on the adhesive potential of P. stuartii are not widespread in the literature and some basic questions remain unanswered. The purpose of this work is to study the adhesiveness of opportunistic bacteria P. stuartii using bladder epithelial cell line. The clinical isolate of P. stuartii NK has been used in the work. First of all, by using quantitative adhesion assay it has been demonstrated that the bacteria are able to adhere onto T24 eukaryotic cell line. To determine the nature of the bacterial components responsible for binding to eukaryotic cells, the microbial culture was treated with trypsin. It has been established that a preliminary 10-minute enzymatic treatment of P. stuartii NK at 37°C ensured the reduction of adhesion index by 94% compared to the control. The adhesion of P. stuartii NK onto eukaryotic cell line has been visualized by scanning electron microscopy (Carl Zeiss, Germany). The cell culture was grown on coverslips until a subconfluent monolayer was reached and infected with the daily culture of P. stuartii NK. Samples were taken after 30, 60, 90 minutes of incubation at 37°C and fixed in 1% glutaraldehyde solution. Microphotographs have showed the presence of bacterial cells on the eukaryotic surface and the formation of contacts between them. Thus, it has been shown that bacteria P. stuartii NK are able to adhere onto the bladder epithelial cells. The adhesion index of P. stuartii NK has been significantly reduced after the pretreatment of bacteria with trypsin, which indicates the participation of protein substances in the attachment process onto eukaryotic cells. The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

P3550
Board Number: B845
Viperin exerts antiviral function against Junín mammarenavirus at different subcellular localizations.
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Junín arenavirus infections are associated with high levels of interferons in both severe and fatal cases. Upon Junín virus (JUNV) infection a cell signaling cascade initiates, that ultimately attempts to limit viral

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replication and prevent infection progression through the expression of host antiviral proteins. The interferon stimulated gene (ISG) viperin has drawn our attention as it has been highlighted as an important antiviral protein against several viral infections. The studies of the mechanistic actions of viperin have described important functional domains relating its antiviral and immune-modulating actions through cellular lipid structures. In line with this, through silencing and overexpression approaches, we have identified viperin as an antiviral ISG against JUNV. In addition, we found that lipid droplet structures are modulated during JUNV infection, suggesting its relevance for proper virus multiplication. Furthermore, our confocal microscopy images, bioinformatics and functional results also revealed viperin-JUNV protein interactions that might be participating in this antiviral pathway at lipid droplet level. Altogether, these results will help to better understand the factors mediating innate immunity in arenavirus infection and may lead to the development of pharmacological agents that can boost their effectiveness thereby leading to new treatments for this viral disease.

Organ/Disease Biology and Therapeutic Targets 2

P3551
Board Number: B847
Synaptotodin-2, a novel promyogenic marker required for the development of sarcomeric Z-disk in zebrafish.
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Myogenesis is a differentiation-dependent cell-cell fusion process that involves migration and fusion of uninucleated myoblasts to form multinucleated myotubes that develop further into striated contractile myofibers. This multi-step process also takes place during muscle regeneration and muscle injury, and several proteins have been identified to play a role in these processes. Deficiency or mutation in muscle-related proteins leads to different kinds of muscular dystrophies leading to weakened and non-functional muscle. Individual muscle cells have to migrate to the site of regeneration and fuse with existing muscle fibers to repair them. Both the migration and fusion process requires extensive remodeling of the actin cytoskeleton. We have discovered a new pro-myogenic actin regulatory protein, Synaptotodin-2 (SYNPO2), that differentially affects myoblast migration and myotube formation. Three isoforms of SYNPO2 was isolated from the mouse myoblast cell line, C2C12, and its functional role in the different stages of myogenesis was studied. Both ectopic expression and knockdown did not affect the expression of myogenic-specific markers, suggesting that the SYNPO2 isoforms function downstream of these proteins, and does not alter the differentiation pathway. Ectopic expression of SYNPO2As enhanced myotube formation, whereas SYNPO2A and SYNPO2B inhibited myotube formation. Knockdown of SYNPO2As, but not SYNPO2A or B, reduced myotube formation by 50% when compared to mock transduced cells. SYNPO2A and SYNPO2B significantly increased directional migration and SYNPO2As decreased directional migration pre-differentiation, and SYNPO2As, significantly decreased migration post-differentiation when compared to mock transduced cells. Therefore, SYNPO2As acts as a negative regulator of myoblast migration increasing the chances of cell-cell contact, thereby acting as a positive regulator of myoblast fusion. To gain further insights into the in vivo function of SYNPO2As, we identified the zebrafish homolog, synpo2b. CRISPR knockout of synpo2b in zebrafish resulted in developmental defects such as tail-coiling and bending of the body axis. When examined by fluorescent actin staining, knockout embryos showed disorganized actin fibers and reduced myotube formation.
Ultrastructural analysis of the knockout tail muscles by electron microscopy showed shortened myofibres, disorganized Z-disk filaments, accumulation of mitochondria and ribosomes between muscle fibers, and aggregation of autophagic vacuoles, the pathologies seen in a muscular dystrophy model. These results suggest that synpo2b is an essential promyogenic protein required for the normal development and maintenance of muscle integrity and function.

P3552
Board Number: B848
Role of metavinculin in actin reorganization and force transmission.
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Vinculin (Vcn) is an essential cytoskeletal protein that acts as a scaffold to link transmembrane receptors to actin filaments, thereby playing a crucial role in cell adhesion, motility, and force transmission between cells. While Vcn is ubiquitously expressed, metavinculin (MVcn), a larger isoform of Vcn, is selectively expressed in smooth and cardiac muscle cells. Similar to Vcn, MVcn can directly associate with actin and remodel the actin cytoskeleton. However, distinct from Vcn, MVcn contains an additional exon which encodes a 68-residue insert. Point mutations in the 68-residue insert have been associated with altered actin organization and heart disease, notably dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). Both DCM and HCM are diseases of myocardium, or the heart muscle, that prevent the heart from pumping blood normally because of disruption in force transmission. MVcn expression is higher in muscle cells that require greater force transmission. Given these observations, we postulate that MVcn plays an important role in force generation and transmission through its interaction with the actin cytoskeleton. We have now verified that the wild-type (WT) MVcn tail domain (MVt) does not bundle actin filaments and that the cardiomyopathy-associated MVt mutant ΔL954 shows slightly enhanced actin bundling in vitro through actin cosedimentation assay and negative-stain electron microscopy (EM). We are expanding these studies to investigate how MVcn regulates actin organization in the presence of Vcn. We have observed that WT MVt inhibits Vt-mediated actin bundling via negative-stain EM and actin cosedimentation assay. We have additionally succeeded in expressing MVcn in Vcn null mouse embryonic fibroblasts (MEF) and observed that MVcn localizes to focal adhesions, similar to Vcn. In Vcn null MEFs that transiently express MVcn, focal adhesion size was slightly larger and focal adhesion number per cell slightly fewer compared to cells transiently expressing Vcn. Cell area, on the other hand, did not seem to differ significantly between the cells transiently expressing each isoform. We have more recently generated stable Vcn null MEFs that express either MVcn or Vcn, and are currently conducting similar studies to determine differences between the two isoforms. We also plan to make MVcn disease-associated mutant expressing stable cells to determine differences in adhesion, motility, and force transmission compared to WT MVcn expressing cells. Results from these studies will provide the groundwork for how Vcn and MVcn differ and how MVcn disease mutants contribute to associated cardiomyopathies.
P3553
Board Number: B849
Altered cardiac expression of genes involved in iron metabolism is driven by an iron-independent process in heart failure.
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Chronic heart failure (HF) is a multi-system disease characterized by primary cardiac dysfunction associated with neuro-humoral and systemic inflammatory responses to diminished blood flow that affect other organ systems. Systemic iron deficiency was observed in 50 percent of HF patients and myocardial iron levels have been shown to be decreased in HF patients. This suggests that relative deficiency of iron might play an important role in the pathophysiology of HF. To understand the connection between cardiac iron metabolism and heart failure we studied expression of genes involved in iron metabolism in a rat model of HF. In this model, HF is induced by volume overload due to surgically created aorto-caval fistula (ACF). We manipulated iron status of ACF and control (sham-operated) animals using defined diets with different iron content - LOW iron diet (5 ppm Fe), NORMAL iron diet (50ppm Fe) and HIGH iron diet (500 ppm Fe).

Heart failure developed in all ACF cohorts regardless of diet, cardiac function and morphology were not significantly affected by iron content. Animals fed LOW iron diet (both control and ACF) were anemic and had markedly reduced liver iron levels compared to animals fed NORMAL iron diet. Animals fed HIGH iron diet had markedly increased liver iron compared to rats fed NORMAL diet. We observed only very mild increase (cca 10-20 percent) in myocardial iron content in response to increased iron content in diet. Cardiac iron content tended to be marginally lower in ACF animals compared to control sham-operated rats fed the same diet. Despite comparable iron content, mRNA expression of most genes critical for iron metabolism was significantly increased in ACF rats compared to control sham-operated animals. Most of the iron-related genes were upregulated in ACF animals (hepcidin, ferrooxidases ceruloplasmin and hepehaestin, iron exporter ferroportin, iron reductase Dcytb, Tfr2, mitoferrin1 and Voltage-dependent L-type calcium channel subunit alpha-1D) regardless of nutritional iron status.

Upregulation of reductase Steap 3, Hfe, and mitoferrin 1 was significant in ACF animals in NORMAL and HIGH iron cohorts. Expression of H-ferritin and neogenin mRNA was not affected by presence of heart failure neither by iron status. Expression of Tfr1 mRNA positively responded to low iron in diet. The observed marked dysregulation of most genes involved in iron metabolism in failing myocardium suggests presence of an iron-independent process driving the expression. Hypoxia, stress response or other processes and pathways can be hypothesized. Increased expression of Bmp6 mRNA (the key regulator of hepcidin expression) in ACF suggests involvement of TGF-beta/Smad signaling.

P3554
Board Number: B850
Analysis of fusion-related genes in ethanol-treated C2C12 muscle cells.
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A significant event in the formation of skeletal muscle is the fusion of myoblasts to form multinucleated muscle fibers. Fusion of myoblasts with existing muscle fibers continues to be essential throughout life for successful growth and repair of damaged skeletal muscle. Long-term alcohol use has been
associated with the development of skeletal myopathy, which is characterized by progressive muscle weakening accompanied by atrophy of skeletal muscle tissue. In the first part of this study, we assessed the effect of ethanol on the differentiation of C2C12 skeletal muscle cells in culture, utilizing the fusion index calculation as a measurement of differentiation. Our results demonstrate a significant reduction in the fusion of myoblasts to form multinucleated myotubes when treated with a physiologically relevant concentration of ethanol at the start of differentiation. This is consistent with additional published evidence demonstrating that ethanol reduces the differentiation capability of skeletal muscle cells, in part by preventing fusion of myoblasts to form mature muscle fibers. In the second part of this study, we are investigating the expression of fusion-related genes to determine if there are specific factors that are impacted by ethanol treatment. We performed a literature search to identify potential gene candidates involved in the fusion process and are examining their expression in ethanol-treated C2C12 cultures. For these experiments, RNA was extracted from cultures that were either untreated or treated with 100 mM ethanol over five days of differentiation. RNA was converted to cDNA and PCR was performed using gene-specific primers. For the gene candidates analyzed so far, preliminary results do not indicate consistent expression changes in ethanol-treated cultures as compared to untreated cultures. In continuing work, we are refining this analysis to contribute to a more comprehensive understanding of the effect of ethanol treatment on fusion-related gene expression in C2C12 muscle cell culture. Results from these studies may provide further insight into the mechanism by which ethanol exposure leads to the development of skeletal myopathy.

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**Board Number: B851**

**SNP analysis of a mixed population reveals associations with osteoarthritis.**

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Of the arthritic disorders, osteoarthritis is the most common. In the United States, an estimated 30 million adults have osteoarthritis of varying degrees. The etiologic factors that determine susceptibility to osteoarthritis and the subsequent course of the disease are quite varied. Generally, genetic, acquired, and environmental factors together determine susceptibility to the disease. Single nucleotide polymorphisms (SNPs) constitute one type of genetic variations that have been explored as players in osteoarthritis and many other diseases. For the current report, we analyzed 32 SNPs located in 19 genes, and putatively linked with arthritic disorders. Using the TaqMan genotyping assay (Life Technologies), we conducted this analysis on a gender and race mixed population of 1000 individuals, 500 osteoarthritis patients and 500 healthy (control) individuals. Multiple regression logistic models controlled for gender, age, and race were employed to reveal any SNP associations with osteoarthritis. For the overall population, three SNPs showed statistically significant association with osteoarthritis (p < 0.05): rs2294984 (COL9A3, G>A, missense, G17E), rs1800629 (TNFA, G>A, promoter), and rs1800796 (IL6, G>C, promoter). In comparison to its homozygous G/G genotype, the G/A of rs2294984 showed significant association with osteoarthritis (OR = 1.36; p = 0.049). Likewise, in comparison to the G/G genotype of rs1800629, the G/A had significant association (OR = 1.39; p = 0.03). The homozygous genotype A/A for both SNPs had p-values greater than 0.05, suggesting lack of association, a result that is likely due to the small sample size in this genotype category. For rs1800796, the G/C and C/C genotypes in comparison to G/G had odds ratios of 0.67 and 0.34, respectively, suggesting negative association with osteoarthritis, although only the G/C genotype had statistical significance (p = 0.03).
In the age group 40-59 years (n = 536), rs2227306 (IL8) homozygous T/T associated with osteoarthritis, with odds ratio of 2.59 in comparison to C/C (p = 0.008). Among the Hispanic population (n = 118), rs143383 (GDF5) homozygous C/C associated with the disease (OR = 4.93; p = 0.005), but not among the White subjects (n = 561). Overall, 17 SNPs showed association with osteoarthritis in the total population or various subgroupings (gender, race and age).

P3556
Board Number: B852
Cell Size and Nuclear Scaling Relationships in Multinucleated Muscle Fibers.
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Different cell types can be identified by their overall size and the relative size, i.e. scaling, of their organelles. In particular, nuclear size and position are characteristic features and reflect cell state and activity. Both, the intrinsic regulation of cell size and nuclear scaling, and the contribution of physical dimensions to cell function are poorly understood – specially in skeletal muscle fibers, which are among the largest cell types and can contain hundreds of nuclei. We have developed a *Drosophila* system to analyze cell size and nuclear scaling relationships in fully differentiated multinucleated muscle fibers *in vivo*. With this system, we are further able to genetically manipulate individual cellular components, and to quantify the impact of these manipulations on muscle size and function. We find that, while nuclear number and DNA content increase with muscle cell size, the cumulative size of all nuclei in the cell is the most precise global nuclear scaling parameter. Combining our *in vivo* data with mathematical modeling further suggests that size scaling relationships are established locally by coordinating the size and the position of individual nuclei within a cell. However, differences in nuclear size scaling and transcriptional/translational activity in distinct sub-cellular domains indicate that cell size regulation might differ dependent on nuclear position along the length of each muscle fiber. We confirm these observations using genetic manipulations to alter nuclear size and DNA content. Moreover, our data show that changes in nuclear size scaling affect locomotion and thus correlate with reduced muscle function. Our study provides the first comprehensive approach to unraveling the intrinsic regulation of size and scaling in multinucleated muscle fibers. Ultimately, identifying the underlying molecular mechanisms will provide insights into how disruption of sub-cellular organization results in muscle disease.

P3557
Board Number: B853
Genes Differentially Expressed During Reversion of Androgen-Dependent Skeletal Muscle Atrophy.
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Muscle wasting or atrophy is a condition associated with major human systemic diseases including, diabetes, cancer, and kidney failure, among others. There is accumulating evidence from comparison of transcriptional profiles that a common set of genes, termed atrogene, are modulated in atrophying muscles. However, the transcriptional changes that trigger reversion or attenuation of muscle atrophy
have not been characterized at the molecular level. To identify key factors involved in the recovery of skeletal muscle mass, we have used cDNA microarrays to investigate genes differentially expressed during the atrophy reversion of the androgen-sensitive levator ani muscle (LA), in the well-established model of castration and testosterone replacement. As expected, most of the differential expressed genes behave as atrogenes and responded to castration-induced atrophy. Strikingly, 7 genes did not respond to castration but exclusively to the testosterone replacement. Considering that almost all proteins encoded by these genes are associated to reversion of atrophy and may function as regulators of cell proliferation/growth, our results open new perspectives in signaling pathways on atrophy-related syndromes field.

P3558
Board Number: B854
High throughput screening to identify mechanisms regulating endothelial fatty acid uptake.
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Type II diabetes is marked by hyperglycemia brought on by severe insulin resistance, a condition caused in part by intramuscular lipid accumulation. We have recently uncovered that the muscle-borne paracrine metabolite 3-hydroxyisobutyrate (3-HIB) plays a novel role in transporting serum fatty acids across the endothelium both in vitro and in vivo. However, the molecular mechanisms behind both 3-HIB-mediated and basal endothelial fatty acid uptake remain unclear. To begin understanding how this regulation works, I conducted a high-throughput screen in which a large chemical compound library was administered, along with 3-HIB and luciferin-tagged fatty acids, to luciferase-expressing endothelial cells. Through the inhibition of various intracellular pathways, some of these drugs inhibited or enhanced fatty acid uptake, as measured by luminescence. I then carried out secondary screening on the top 1% hits, and further orthogonal assays for validation. These ongoing studies will help us uncover the pathways modulating endothelial fatty acid uptake and transport, and holds promise for developing novel therapeutics against insulin resistance.

P3559
Board Number: B855
Determining the role of endothelial Notch signaling in vascular regeneration in the mouse hindlimb ischemia model.
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Ischemic disease such as peripheral arterial disease predicts to be a major cause of morbidity in the future; with few late stage treatment options. Emerging therapeutic approaches involve the transplantation of endothelial progenitors or localized release of pro-angiogenic factors to enhance neovessel formation and to re-vascularize ischemic tissue. Our previous work revealed the significance of the Hif1a-Etv2-Notch signaling axis in differentiation of functional arterial endothelial cell from embryonic stem cells. Hypoxic differentiation of embryonic stem cells led to increased differentiation of arterial endothelial cells and improved outcomes when transplanted in both myocardial infarction and hindlimb ischemia models in mice. As Notch signaling is situated downstream, compared to Hif and Ets signaling pathway, during vascular development, and is a gatekeeper in maintaining endothelial identity, i.e. tip vs stalk cell, we looked to investigate if modulating Notch signaling in existing vasculature could enhance regeneration by preferentially specifying the generation of arterial endothelial cells following
acute hindlimb ischemia. Notch signaling was conditionally inhibited in 8-12week old male, DNMAML:Cdh5/CReRT, mice prior to inducing ischemia. We observed decreased functional recovery, as measured by Laser Doppler Imaging, in hindlimbs of DNMAML:Cdh5/CReRT mice compared to DNMAML:Cdh5/CReRT negative littermates. This lead us to hypothesize that Notch signaling is required to adaptively generate new or remodel pre-existing vessels to perfuse ischemic tissue. Ongoing studies characterizing the temporal regulation of Notch signaling will help delineate the role of Notch signaling in therapeutic arteriogenesis and angiogenesis.

P3560
Board Number: B856

Optogenetic stimulation of pericytes lacking alpha smooth muscle actin produces a decrease in capillary blood flow in the living mouse brain.
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Vascular mural cells surrounding the cerebrovasculature are responsible for modulating blood supply in accordance with the metabolic demands of the brain. Although it is accepted that vascular smooth muscle cells on arterioles can regulate blood flow, the capacity for pericytes on capillaries to regulate blood flow is debated. Contributing to this debate is the finding that only some pericytes express alpha smooth muscle actin, a protein believed to be required for the ability to change blood vessel diameter (Grant, et al. JCBFM 2017). This has led to a burning question in the field: Does alpha smooth muscle actin expression in pericytes dictate their ability to modulate blood flow? To answer this question, we stimulated channelrhodopsin (ChR2) in individual pericytes using two photon illumination, as done previously (Hill, et al. Neuron 2015). Although we used offspring of PDGFRBeta-Cre and Ai32 mice, which possess ChR2 in both smooth muscle cells and pericytes, the spatial specificity of two photon excitation allowed selective stimulation of individual pericytes, thus avoiding incidental activation of nearby vascular smooth muscle cells that also express ChR2. Simultaneously, with the same two photon laser used for excitation of ChR2 in pericytes, we measured capillary diameter and red blood cell velocity using intravenous dextrans. We found that pericyte excitation for 60 seconds produced, on average, a ~20% reduction in diameter and velocity throughout the cortical microvasculature. Surprisingly, the magnitude and kinetics of this decrease in blood flow did not differ between pericytes with and without alpha smooth muscle actin expression. Importantly, identical stimulation parameters did not produce hemodynamic changes in control mice with cytosolic YFP or membrane-bound GFP in vascular mural cells, indicating that our observed reductions in blood flow in ChR2 mice were not an artifactual result of animal movement or tissue damage. Further, the observed decrease in velocity and diameter in response to pericyte ChR2 stimulation was inhibited when we applied fasudil, a Rho kinase inhibitor and vasodilator, to an intact dura (dose of 10 mM). Our results to date suggest that pericytes, even those without alpha smooth muscle actin, have the capacity to modulate blood flow. These findings will advance our understanding of mural cell biology, and could be relevant for disease processes known to involve deficits in capillary blood flow, such as stroke and dementia.
P3561
Board Number: B857
The necroptosis machinery in normal gut development, and in the pathogenesis of neonatal necrotizing enterocolitis (NEC).
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Necrotizing enterocolitis (NEC) is a devastating disease that causes the breakdown of the gastrointestinal barrier of infants, leading to sepsis and death in up to 50\% of cases. NEC is characterized by extensive cell death and inflammation in the intestine, which the Hackam Lab has shown is mediated in part by toll-like receptor 4 (TLR-4)-dependent apoptosis. However, since apoptosis is generally a non-inflammatory form of cell death, we seek to investigate alternative, more inflammatory forms of programmed cell death that may contribute to NEC and may be targets for prevention and treatment of NEC. Necroptosis, a receptor-interacting protein kinase 1 and 3 (RIPK) and mixed lineage kinase domain-like protein (MLKL)-dependent pathway is a key mediator of cell death which is also activated downstream of TLR4, and is a strong candidate for involvement in the pathogenesis of NEC. Here, using a combination of pharmacologic and genetic inhibition of the necroptosis signaling pathway both in vivo in mice and in vitro in enteroid culture, we show that necroptosis gene expression is dynamically regulated in the gut during the transition from late prenatal to early postnatal development, and that inhibiting RIPK1 function with Nec-1s or by using an mlkl-deficient mouse in our NEC model both result in decreased inflammation as measured by inflammatory cytokine production, leading to partial attenuation of disease.

P3562
Board Number: B858
Levels of CSF amyloid-\textit{β} 40 and 42 are decreased in amyloid PET (-) normal pressure hydrocephalus patients.
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Coexistence of Alzheimer Disease (AD) in Normal Pressure Hydrocephalus (NPH) is known to be associated with shunt unresponsiveness and usually suspected by a decrease in the CSF Aβ42 level. In the recent studies, it has been reported that the well-established CSF AD biomarkers could be misled in NPH. In this study, we investigated levels of CSF AD biomarkers in amyloid PET (+) AD, amyloid PET (-) NPH, and cognitively normal (CN) subjects. Ten patients diagnosed with probable AD, 10 probable NPH, and 8 CN subjects were included in the experimental groups. 18F-florbetaben PET was performed and CSF was collected and analyzed for Aβ40, Aβ42, total tau and phosphorylated tau (p-tau) by ELISA method. All AD subjects were amyloid (+) and all NPH subjects were amyloid (-). Levels of CSF Aβ40 and Aβ42 were significantly decreased in NPH subjects compared to CN subjects. The level of Aβ42 was significantly decreased in the AD group compared to NPH and CN groups while there was no significant difference in the level of Aβ40 between AD and NPH groups. Total tau and p-tau levels were significantly increased in the patients with AD compared to NPH and CN. There was no statistical difference in the levels of total tau and p-tau between NPH and CN groups. This study demonstrates for the first time that CSF Aβ40 and Aβ42 are decreased in amyloid PET (-) NPH.
P3563
Board Number: B859
Control of high-fat-diet induced obesity by the mouse mSeg1 salivary protein.
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Abstracts: The increasing incidence of obesity is a growing global public health concern, potentially controlled by therapeutic reductions in fatty acid biosynthesis. Here we report that the mouse Seg1 (mSeg1) salivary protein controls obesity. We show that mSeg1 null mice (mseg1Δ/Δ) do not exhibit the increases in fatty acid biosynthesis or obesity characteristically induced by high-fat diets. We also show that mSeg1 activates the Srebp pathway to regulate liver lipogenesis genes. Whilst the native mSeg1 is a glycosylated protein, a non-glycosylated recombinant E. coli-expressed mSeg1 antagonizes the function of native mSeg1. Most importantly, feeding wild-type mice with recombinant mSeg1 protects them from high-fat-diet induced obesity. In conclusion, mSeg1 is a novel salivary liver lipogenesis regulatory factor and a potential therapeutic target for the prevention of obesity.

P3564
Board Number: B860
Intermittent hypoxia induces inflammation in 3T3-L1 adipocytes by stabilization of hypoxia-inducible factor 1α (HIF-1α).
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Obstructive sleep apnea (OSA), characterized by cyclic intermittent hypoxia (IH) during sleep, is associated with systemic inflammation. Adipose tissue is now recognized as an endocrine organ that secretes a wide variety of bioactive peptides. We previously showed that IH suppressed the secretion of adiponectin, an anti-inflammatory adipokine and that hypoxia-inducible factor 1α (HIF-1α) increased interleukin-6 (IL-6) adipose secretion. Here, we hypothesized that IH will induce inflammation in adipocytes via stabilization of HIF-1α. 3T3-L1 adipocytes cultured in 100 mm dishes were exposed to IH at 12 cycles/h for 8 h/d to simulate the hypoxic stress similar to that encountered in OSA. Control adipocytes were exposed to 21% O2 under identical conditions. After 48 h, 3T3-L1 cells exposed to IH have increased HIF1α mRNA expression, which was associated with an increase in mRNA expression and protein secretion of the inflammatory cytokine IL-6 (p < 0.05). There were no significant changes in tumor necrosis alpha (TNF-α) expression or protein secretion. Pre-treatment of adipocytes with PX-478, a known specific inhibitor of HIF-1α, reversed the inflammatory phenotype of IH-exposed adipocytes, with a decrease in IL-6 expression and protein secretion to control levels. These findings suggest a mechanism of how IH directly leads to adipose tissue inflammation through HIF-1α stabilization and subsequent IL-6-upregulation. The HIF1α pathway appears to be a promising target to reduce adipose tissue inflammation in OSA patients.
Kidney disease is the ninth leading cause of death in the U.S., with an estimated 26 million adults afflicted with chronic kidney disease. Chronic kidney disease is a significant public health problem due to the high incidence rate of causal comorbidities, the scarcity of kidneys for transplants, and the growing needs for dialysis. Unfortunately, neither the tools for early detection of renal decline nor clinical interventions to preserve kidney function exist, due to a limited repertoire of molecular markers of the mammalian kidney. Using an unbiased screening approach, we combined peptide phage display technology and high-resolution imaging techniques to identify molecular signatures and spatial localization patterns of ligand:receptor interactions in normal kidney and decellularized renal scaffolds. Three iterative selection rounds were performed to enrich for peptide ligands that bound components within the renal extracellular matrix (ECM). A separate protocol was performed in vivo to compare enriched peptide phage populations between whole kidney and decellularized kidney matrices. The ECM- and in vivo-enriched libraries demonstrated robust binding throughout the renal matrices and whole kidneys, respectively. Importantly, phage were internalized by renal tubules under in vivo conditions, suggesting that phage can traverse the glomerular filtration barrier and/or migrate through ECM to target epithelial components. We next evaluated individual clone enrichment and chose three unique peptide phage candidates from the ECM library for in vivo delivery. Clones and control, fd-tet insertless phage were individually injected via tail vein, and kidneys and control organs were harvested to compare ex vivo and in vivo binding patterns. In decellularized scaffolds, Ligand 1 revealed intense binding to glomerular vascular poles, Ligand 2 bound robustly throughout glomerular matrices, and Ligand 3 decorated the vascular network. These data demonstrate the utility of the approach for identifying distinctive molecular regions within the renal ECM. In vivo, Ligands 1, 2, and 3 displayed binding within the renal interstitium, with Ligand 2 also demonstrating internalization by tubular epithelia. We predict that glomerular targeting may require injury-induced exposure of the ECM; these studies are ongoing. In sum, our strategy has potential to lead to applications such as the basic evaluation of renal cell structure-function relationships, identification of diagnostic biomarkers, and ligand-directed targeting of small molecules to specific sub-cellular regions of the kidney for diagnostic and treatment purposes.
P3566
Board Number: B862

GENERATION OF AN EGFR ISOFORM D SPECIFIC KNOCKOUT MODEL.
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The Epidermal Growth Factor Receptor (EGFR) is a receptor tyrosine kinase that is a clinically validated target in cancer and arthritis patients. In addition to the full-length receptor, the human EGFR gene encodes three shorter variants known as isoforms B, C, and D, that are comprised of the EGFR extracellular domain along with short regions of unique aa sequence not found in EGFR. EGFR Isoform-D is produced from a 3.0 kb EGFR transcript that arises from an alternate splice event. The alternatively spliced Isoform D transcript encodes a unique 78 aa carboxyl-tail and 3' UTR sequences. The Isoform-D transcript is expressed in normal adult tissues where its expression is tightly regulated to maintain a constant high ratio of full-length to Isoform-D. In cancer cells, Isoform-D expression is regulated by the Inc-AS-1-RNA, and is linked to ‘super responsiveness’ to EGFR-targeted inhibitors (i.e., gefitinib). Both the human and murine homologs of EGFR Isoform-D are intrinsic membrane proteins expressed on the cell surface. This Isoform-D membrane protein, p110 sEGFR, is proteolytically released from the cell surface into the bloodstream, where it is the major circulating EGFR isoform. Quantitative assays suggest that changes in blood concentration of EGFR Isoform D have diagnostic potential in certain cancer and rheumatoid arthritis patients. The extraordinary level of regulation imposed on EGFR Isoform D synthesis, cell-surface expression, and proteolytic release into the circulation suggest this protein has unsuspected functions in systemic maintenance of health and homeostasis in the body. We have shown that an antibody directed against the unique 78 aa domain of human Isoform D promotes cell:cell and cell:matrix interactions, in vitro. However, the endogenous functional roles of this novel EGFR isoform in normal and diseased tissues remain unknown because no animal model has been available to study its function. This is because generation of isoform-specific mouse knock out models has not been feasible using standard homologous recombination approaches.
Here, we used CRISPR gene editing to selectively delete the 2.8 kb transcript that encodes the murine homolog of EGFR Isoform D. This new animal model offers opportunities to test the functional roles of this highly-regulated normal serum protein (i.e., a matriline) and putative serum biomarker. It also provides an in vivo system for exploring the mechanism of action of certain EGFR-directed therapeutics, including the ability of some patients to exhibit exceptional responsiveness to EGFR-targeted drugs.

Therapies: Design and Mechanisms for Normal and Diseased Organs 2

P3567
Board Number: B863

Effects of rho-associated protein kinase inhibitor Y-27632 on scarring formation after glaucoma filtration surgery.
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Glaucoma filtration surgery usually fails because of post surgical scarring, a process in which fibroblasts play a prominent role. To elucidate the effects of rho-associated protein kinase (ROCK) inhibitor Y-27632...
in post surgical scarring (fibrosis), we have now investigated the molecular mechanisms with human tenon fibroblasts. So, human tenon fibroblasts were cultured with Y-27632 or various antiglaucoma drugs for indicated periods. After cultivation, we have prepared total RNA and protein samples from tenon fibroblasts. Using Multi RT-PCR array, we examined the factors respond to Y-27632. Also, we have examined the three-dimensional collagen gels cultivation for gel contraction by various antiglaucoma drugs. Collagen gel contraction by tenon fibroblasts was blocked in the presence of Y-27632. In Multi RT-PCR array using fibrosis-related genes, the expression of MMP-3 was down-regulated in tenon fibroblasts by additional Y-27632. Furthermore, Immunoblot and immunofluorescence analysis revealed that the expression of fibrosis markers was down-regulated in the presence of Y27632. These results suggest that the rho-associated protein kinase inhibitor Y27632 may block scarring formation with interaction MMP-3 after glaucoma surgery. And, it will be possible that ROCK inhibitors and MMP-3 may have potential to be developed for treatment of glaucoma and other ocular diseases.

P3568
Board Number: B864
Metformin ameliorates progressive nephritis in a mouse model of Alport syndrome.
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Alport syndrome (AS) is a hereditary kidney disease caused by a dysregulation of type IV collagen, a crucial component of the glomerular basement membrane (GBM). Abnormal GBM structure due to a mutation in one of the type IV collagen (COL4A3, COL4A4, and COL4A5) genes leads to glomerular aberration, which eventually results in chronic progressive nephritis and end-stage renal disease (ESRD). Importantly, early inhibition of Renin-Angiotensin-Aldosterone-System (RAAS) in AS delays renal failure and improves life expectancy; however, most AS patients finally develop ESRD and have higher risk of hyperkalemia, a life-threatening side effect of RAAS inhibitors. Therefore, identification of novel therapeutic targets and agents that have different mechanisms from RAAS inhibitors is crucial to establish therapeutic approaches for progressive nephritis such as AS. Here, we isolated glomerulus from AS model mice (Col4A5-G5X) and analyzed global protein expression by LC-MS/MS. Proteomics analysis showed a dysregulation of metabolic pathways in AS glomerulus. Notably, treatment of AS model mice with metformin (6-11 wk; 5 mg/mL, 12-20 wk; 2.5 mg/mL, free drinking water), a metabolic stressor and popular anti-diabetic drug, significantly suppressed proteinuria and glomerular injury as well as renal fibrosis to the same extent as a RAAS inhibitor losartan (6-11 wk; 250 μg/mL, 12-20 wk; 125 μg/mL, free drinking water). Consistently, metformin also reduced the expression of kidney injury markers (Lysosome, Kim1), pro-inflammatory cytokines (Il-6, Il-1β, KC) and pro-fibrotic genes (Tgfβ, Mmp9/12), and the effects was better than losartan, possibly due to alteration of a variety of metabolic signals by metformin but not losartan. Finally, metformin also suppressed proteinuria and ameliorated kidney injury in a mouse model of acute progressive nephritis induced by adriamycin treatment (10 mg/kg, i.v.), implying that metformin directly targets glomerulus to protect against progressive nephritis. Together, the study provides a novel concept that dysregulation in metabolic pathways may play important role in the pathogenesis of progressive nephritis of AS, and suggest that metformin treatment is a novel therapeutic option for AS.
P3569
Board Number: B865
Beneficial effect of midazolam against vascular endothelial growth factor-induced vascular leakage in the retinas of diabetic mice.
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Anesthetics, including midazolam, are widely used for induction and maintenance of anesthesia, anxiolysis, and sedation; however, their preventive effects against diabetic complications, including diabetic retinopathy, are unknown. Here, we investigated the beneficial effects of midazolam against vascular endothelial growth factor (VEGF)-induced vascular leakage and its mechanism of action in human retinal endothelial cells (HRECs) and the retinas of diabetic mice. Midazolam inhibited VEGF-induced elevation of intracellular Ca2+, generation of reactive oxygen species (ROS), and transglutaminase activation in HRECs; these effects were reversed by the GABAA receptor antagonist flumazenil, but not by the translocator protein antagonist PK11195. Midazolam also prevented VEGF-induced disassembly of adherens junctions and in vitro permeability. Intravitreal injection of midazolam prevented hyperglycemia-induced ROS generation, transglutaminase activation, and subsequent vascular leakage in the retinas of diabetic mice, and these effects were reversed by flumazenil. The roles of flumazenil were further supported by identifying GABAA receptors in mouse retinas. Thus, midazolam prevents hyperglycemia-induced vascular leakage by inhibiting VEGF-induced intracellular events in the retinas of diabetic mice, suggesting that midazolam has a therapeutic potential for treating diabetic retinopathy.

P3570
Board Number: B866
Protective effects of chrysin on visual cycle impairment in diabetic retina.
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Diabetes-associated visual cycle impairment has been implicated in diabetic retinopathy and chronic hyperglycemia causes detrimental effects on visual function. Chrysin, a naturally occurring flavonoid found in various herbs, carrot and propolis, has anti-inflammatory, antioxidant and anti-cancer properties. The current study investigated retinoprotective effects of chrysin on visual cycle-related proteins and photoreceptors in 33 mM glucose-exposed human retinal pigment epithelial cells (HRPEC) and db/db mouse retinae. HRPECs treated with glucose temporally reduced retinal pigment epithelium-specific 65 protein (RPE65), a critical enzyme in the visual cycle located in retinal pigment epithelium. When 1-20 μM chrysin was treated to HRPEC, the RPE65 level was near-completely restored. In addition, oral administration of 10 mg/kg chrysin enhanced tissue levels of RPE65, cellular retinol-binding proteins, interstitial retinol-binding protein, and cellular retinaldehyde-binding protein diminished in db/db mouse eyes. Furthermore, chrysin enhanced the level of rhodopsin present in outer segments of visual cells and involved in visual signal transduction. These results demonstrated that chrysin restored the levels of visual cycle-related proteins in glucose-exposed HRPEC and diabetic mouse eyes. Therefore, chrysin may be a potent agent combating diabetes-associated retinal visual cycle impairment.
P3571

Board Number: B867

Protective effect of cysteamine against vascular leakage by inhibiting VEGF-induced transglutaminase activation in diabetic mice.

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Cysteamine (an aminothiol), which is derived from coenzyme A degradation and metabolized into taurine, has beneficial effects against cystinosis and neurodegenerative diseases; however, its role in diabetic complications is unknown. Thus, we sought to determine the preventive effect of cysteamine against hyperglycemia-induced vascular leakage in the retinas of diabetic mice. Cysteamine and ethanolamine, the sulfhydryl group-free cysteamine analogue, inhibited vascular endothelial growth factor (VEGF)-induced stress fiber formation and vascular endothelial (VE)-cadherin disruption in endothelial cells which play a critical role in modulating endothelial permeability. Intravitreal injection of the amine compounds prevented hyperglycemia-induced vascular leakage in the retinas of streptozotocin-induced diabetic mice. We then investigated the potential roles of reactive oxygen species (ROS) and transglutaminase (TGase) in the cysteamine prevention of VEGF-induced vascular leakage. Our findings suggest that cysteamine protects against vascular leakage through inhibiting VEGF-induced TGase activation rather than ROS generation in diabetic retinas.

P3572

Board Number: B868

Chrysin inhibits advanced glycation end products-associated glomerulosclerosis in glucose-exposed mesangial cells and db/db mice.

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Diabetic nephropathy (DN) is one of the major diabetic complications and the leading cause of end-stage renal diseases, which is characterized by glomerular hypertension and basement membrane thickening, and mesangial expansion due to matrix protein deposition in kidneys. Advanced glycation end products (AGEs) play a causative role in the development of DN via induction of extracellular matrix accumulation. The present study investigated the effects of chrysin (5, 7-dihydroxyflavone) present in bee propolis and herbs, on glomerulosclerosis in glucose- or AGE-exposed renal mesangial cells and db/db mice. The in vitro study employed human mesangial cells exposed to 33 mM glucose for 72 h in the absence and presence of chrysin nontoxic at 1-20 μM. Chrysin suppressed the induction of collagens, α-smooth muscle actin (α-SMA) and fibroblast-specific protein-1 (FSP-1) enhanced by glucose. In addition, chrysin inhibited the induction of AGEs and receptor for AGEs by glucose that led to increased production of mesangial collagens and matrix metalloproteinases. The in vivo study explored the demoting effects of 10 mg/kg chrysin on glomerular fibrosis in a type 2 diabetic model. Oral supplementation of chrysin inhibited the collagen fiber accumulation in damaged renal tissues of db/db mice, evidenced by PAS staining and masson trichrome staining. The induction of α-SMAand FSP-1 was blunted in chrysin-treated diabetic glomeruli. Moreover, treating db/db mice with chrysin diminished the level of AGEs increased in diabetic glomeruli. These results demonstrate that chrysin attenuated the acquisition of myofibroblast phenotype of glucose- or AGE-exposed mesangial cells and the glomerular accumulation of extracellular matrix proteins in diabetic kidneys. Therefore, chrysin may be a potential renoprotective agent targeting glucose-mediated AGEs-associated glomerulosclerosis and fibrosis.

Tuesday-540
P3573
Board Number: B869
Upregulation of glucose uptake by (+) lariciresinol, a lignan isolated from Rubia philippinensis, through the activation of AKT and AMPK/GLUT4 pathway in C2C12 cells.
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Diabetes is one of the most prevalent diseases worldwide, and plays the pivotal role in developing various life threatening diseases such as stroke, hyperlipidemia, and cardiovascular disease. In the present study, we investigated that the upregulation of glucose uptake by (+)lariciresinol (LRS), a lignan isolated from Rubia philippinensis, along with the molecular mechanisms. The α-glucosidase inhibitory activity was found to be 80% at 50 μM dose by functioning as noncompetitive inhibitor, and improved the glucose uptake in a dose-dependent fashion which is confirmed by 2NBDG assay in C2C12 cells. RT-PCR analysis revealed that, LRS treatment was augmented the mRNA expression of Irs-1, Glut4, and Ppar, whereas mitigated the mRNA expression of As160. Moreover, the protein level of GLUT4 was increased by LRS treatment, which was confirmed by western blot analysis. Furthermore, LRS treatment also activated the phosphorylation of both AKT and AMPK. Taken together, the present data show that LRS stimulate AKT and AMPK phosphorylation, which led to upregulate the GLUT4 expression, and increase the glucose uptake in C2C12 cells.

P3574
Board Number: B870
Upregulation of the GLUT4 expression by Spatholobus suberectus extracts via the activation of AMPK and AKT signaling and mitigates the type II diabetic symptoms.
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Nowadays, the rapid increases in the incidence of diabetes have severely threatened in human health. In the present study, we investigated the antidiabetic effect of Spatholobus suberectus (Ss) Dunn belongs to Leguminosae family which is used as a Chinese traditional herb medicine. Foremost, the aqueous (AeSs) and ethanolic (EeSs) extract showed strong antioxidant activities in various in vitro methods in a concentration-dependent manner. Moreover, both extracts had a potent α-glucosidase inhibitory activity with an IC50 value of 6.42 ± 1.45 and 2.81 ± 0.48 g/ml for AeSs and EeSs, respectively. Treatment of EeSs significantly enhanced the glucose uptake, mediated through the upregulation of GLUT4 via the stimulation of AKT and AMPK pathways in C2C12 cells. Moreover, EeSs significantly lowered the postprandial blood glucose levels in STZ-induced diabetic mice, associated with increased the expression of GLUT4 and the activation of AKT and/or AMPK-mediated signaling cascade in skeletal muscle. Furthermore, RT-PCR analysis revealed that administration of EeSs significantly boosted up the expression of various antioxidant enzymes such as Sod-1, Gpx-1, Nox-1 and Ho-1, and also mitigated the gluconeogenesis enzyme such as Pepck and G-6-Pase enzyme expression in liver tissue of a STZ-induced diabetic mice model. Taken together, all these data indicated that Ss extract has potential application in preventing and/or treating type 2 diabetes.
P3576
Board Number: B872
Increased C-reactive protein (CRP) by placenta-derived mesenchymal stem cells is involved in angiogenesis and Wnt signaling pathway in hepatic failure model.
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Hepatic failure is a severe and irreversible disease although the liver has a certain regenerative capacity. This disease is associated with dysfunction of the liver vessels. Placenta-derived mesenchymal stem cells (PD-MSCs), which originate from the fetus, have their distinctive features such as recycling of the placenta removed as waste after birth, ease of accessibility, abundant cell numbers and strong immunosuppressive properties. Recently, we demonstrated that PD-MSCs can regenerate the liver in hepatic failure through anti-fibrotic and autophagic mechanism. Furthermore, the β-catenin is a key molecule for homeostasis in a number of tissues, regulating the Wnt signaling pathway through transcriptional activity. C-reactive protein (CRP) is produced in the hepatocytes and secreted via vessel for metabolism. Therefore, the objectives of this study were to compare the expression of CRP, vascular and Wnt signaling-related factors in hepatic failure model (bile duct ligation and CCl4-injured rat model) by PD-MSCs transplantation and evaluate their correlation between CRP, Wnt signaling and angiogenesis. The expression of CRP in transplanted PD-MSCs (TTX) group was increased compared with control (p<0.05) and non-transplanted (NTX) group in protein level of rat hepatic failure model. Moreover, Wnt signaling and vascular-related factors significantly increased in TTX group than control and NTX group (p<0.05). Likewise that of in vivo model, CRP, vascular-related and Wnt signaling factors were significantly increased in co-cultured PD-MSCs rat hepatocytes (WB-F344) treated CCl4 or LCA. Furthermore, the proliferation of WB-F344 was higher in co-cultivated PD-MSCs group than non-co-cultured PD-MSCs group through BrdU staining. The findings suggest that increased CRP by PD-MSCs promotes angiogenesis in hepatic failure through Wnt signaling and involves in proliferation of the hepatocytes. Therefore, CRP enhanced by PD-MSCs has therapeutic potential for regeneration in degenerative disease. This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI17C1050020017).

P3577
Board Number: B873
Hypoxic preconditioned human bone marrow–derived mesenchymal stromal cell therapy in a rat model of renal ischemia–reperfusion injury.
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INTRODUCTION AND OBJECTIVES: Preclinical studies suggested that administered cultured stem cells protected kidney function against renal IRI. The purpose of this study was to determine the therapeutic effects of hypoxic preconditioned hBMSC and the optimal route for cell delivery in a rat model of renal IRI. METHODS: Sixty male Sprague-Dawley rats were randomly divided into 6 groups (10 animals per group): sham, nephrectomy control, IRI control, renal arterial injection, renal parenchymal injection and
tail venous injection. To induce renal IRI, the left renal artery was clamped with a vascular clamp for 40 minutes, and the right kidney was removed. Serum creatinine, BUN and glomerular filtration rate were evaluated 1 day prior to IRI, and 1, 2, 3, 4, 7 and 14 days after IRI. For histological studies, the kidney was removed 14 days after IRI. RESULTS: Two and one of mortality cases were observed in the renal parenchymal and tail venous injection groups, respectively. All hBMSC injections significantly reduced the extent of elevation in serum creatinine compared with the IRI control group 1, 2, 7 days after IRI. Renal arterial injection significantly reduced the extent of elevation in serum BUN compared with the IRI control group 1, 14 days after IRI. Renal arterial injection significantly reduced the extent of decrease in glomerular filtration rate compared with the IRI control group 2, 4, 7 days after IRI. Sirius red stain for the degree of fibrosis showed that kidney of renal arterial injection group was significantly less fibrotic than that observed in the IRI control group. The number of TUNEL positive cells increased after IRI, suggesting apoptosis or necrosis had occurred. TUNEL assay showed significantly decreased apoptosis in renal arterial, renal parenchymal and tail venous injection groups compared to the IRI control group. A greater increase in glutathione reductase and glutathione peroxidase was observed in renal arterial, renal parenchymal and tail venous injection groups than in the IRI control group. These findings further suggest that anti-oxidative responses were elicited by IRI and hBMSC treatment contributed to further anti-inflammatory and anti-oxidative effects after IRI in this study. CONCLUSIONS: Our study showed that renal function is most effectively rescued from renal IRI through renal arterial injection of hypoxic preconditioned hBMSC. Source of Funding: This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C0925).

P3578
Board Number: B874
Efficacy analysis of functional enhancement human mesenchymal stem cells depends on gene delivery systems.
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Mesenchymal stem cells (MSCs) have significant potential for regenerative medicine because of self-renewal, differentiative and immunomodulatory effect. Nevertheless, powerful MSCs generation via gene manipulation by viral or non-viral gene delivery systems should be enhanced the therapeutic effect in degenerative medicine. Recently, we reported that increased phosphatase of regenerating liver-1 (PRL-1) promotes migration of MSCs into injured target tissues as well as liver regeneration in hepatic failure models. Therefore, the major objectives are to generate stable placenta derived MSCs (PD-MSCs) and bone marrow derived MSCs (BM-MSCs) overexpressed with PRL-1 (PRL-1+) using lentiviral and AMAXA system, which is non-viral gene delivery system. Their characterizations by comparing between naïve and PRL-1+ were analyzed. The expression of stemness markers, differentiation potential and teratoma formation were analyzed by qRT-PCR, IF and chemical staining. Cells surface markers for CD34, 13, 90, 105, HLA-family in naïves and PRL-1+ cells were analyzed by FACS. Moreover, migration ability through Rho family by qRT-PCR and mitochondrial function by Western blotting, ATP assay, XF mito stress assay were evaluated in both PRL-1+ cells. The expression of stemness markers was observed in PRL-1+ cells compared to naïves in gene delivery systems (p<0.05). Differentiation abilities into multilineages and surface markers in the PRL-1+ cells are similar to those of naïves regardless of transfection system. There is no teratoma formation in NOD/SCID mice until 14 weeks after PRL-1+ cells

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transplantation. Moreover, migration ability of PRL-1+ cells was improved through Rho family. Interestingly, MSCs with PRL-1+ using viral and non-viral systems have a significant increase in mitochondrial DNA (mtDNA) copy number and ATP production in cell lysate (p<0.05). In addition, the protein expression of mitochondrial respiration-related genes (e.g. PD, SHDA, VDAC etc.) is higher than naïves. Furthermore, although BM-MSCs were relatively influenced metabolic state than PD-MSCs and PRL-1+ using lentiviral system, PRL-1+ in PD-MSCs using AMAXA system have more energetic mitochondrial function by upregulating ATP production and spare capacity for cellular respiration in live conditions than PRL-1+ in BM-MSCs. Taken together, functional enhancement MSCs with PRL-1+ were successfully not only generate using lentiviral and AMAXA systems but up-regulation of PRL-1 can improve self-renewal ability and significantly increased mitochondrial function compared to naïves. Furthermore, these data suggest that gene delivery system using non-viral AMAXA system provides a useful and safe strategy on MSCs manipulation based on gene reinforcement for degenerative medicine.

P3579
Board Number: B875
Attenuation of UVB-induced skin senescence by Nypa fruticans bud extract: involvement of the suppression of MMP activity through downregulation of NF B & AP1 signaling via the activation of MAP kinase.
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Ultraviolet (UV) radiation is one of the major causative factors to develop extrinsic skin aging and to change the various pathological condition of skin such as thickening, wrinkling, erythema, and dryness. The mechanisms underlying skin photo-aging are related to the degradation of collagen via activation of matrix metalloproteinase (MMP) activity, which is induced by reactive oxygen species (ROS) production. Nypa fruticans belongs to Arecaceae family and traditionally, used to treat asthma, tuberculosis, sore throat, and liver disease. The present study was undertaken to investigate the protective effect of Nypa fruticans bud (NFB) extract on skin aging induced by UVB irradiation in vitro and in vivo along with the mechanistic study. The results revealed that NFB dose-dependently inhibited the elastase activity. Pretreatment of NFB has increased the cell viability compared to non UVB-irradiated group along with dose dependent suppression of UVB-induced cellular ROS generation without showing any cellular toxicity in HaCaT cells. In addition, topical application of NFB was attenuated lesions of UVB-induced photoaging such as skin erythema and skin thickening in photo-aged BALB/C mice dorsal skin. Treatment of NFB also restored the UVB-induced decrease of collagen contents in dermis along with the augmentation of SIRT-1 expression as well as mitigation of the MMP-1 and IL-1 expression. Furthermore, RT-PCR analysis revealed that NFB treatment was markedly downregulated the mRNA expression of MMP-1, 8 and 13, whereas Col1a1 and Sirt-1 were upregulated. Moreover, western blot analysis revealed that treatment of NFB drastically suppressed the protein expression of MMP-1, NF- B and p-c-Jun compared to UVB-induced control group. In addition, NFB treatment has also regulated the activation of MAP kinase protein such as p-38, JNK, and ERK1/2. Together, we demonstrated that NFB had potential in attenuating UVB-induced skin senescence through the suppression of MMP-1 activity along with the down-regulation of NF- B and AP-1 signaling via the activation of MAP kinase proteins, suggesting that NFB is a promising natural agent to protect against skin photo-aging.
**P3580**

**Board Number: B876**

Mushroom anti-tyrosinase activities of Nymphaea nouchali flower extract attenuates melanogenesis in vitro and in vivo: involvement of cAMP/p-CREB, MAPKinase and proteasomal degradation machinery.

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Medicinal plants are employed in the treatment of human ailments from time immemorial. Several studies validated the use of medicinal plant products in hyperpigmentation disorder. The aim of the present study was to investigate the anti-melanogenic effects of Nymphaea nouchali flower extracts, the mechanisms responsible for its inhibition of melanogenesis in in vitro and in vivo. Ethyl acetate fraction of Nymphaea nouchali flower (NNFE) significantly inhibited mushroom tyrosinase activity in both mono- and di-phenolase activities, and markedly inhibited melanin production and intracellular tyrosinase activity in in vitro and in vivo. In addition, NNFE abolished the expressions of tyrosinase, TYRP-1, TYRP-2, and MITF, thereby blocking melanin production and interfering with cAMP/p-CREB as well as the phosphorylation of ERK1/2, JNK, and p38. Furthermore, specific inhibitors of ERK1/2, JNK and p38 prevented melanogenesis inhibition by NNFE, and the proteasome inhibitor (MG-132) prevented NNFE-induced reductions in cellular tyrosinase levels. Taken together, NNFE was found to suppress intracellular CAMP production with subsequent down-regulation of phosphorylation of CREB as well as stimulate MAP-kinase (ERK1/2, JNK and p38) phosphorylation and the proteolytic degradation pathway, which led to the degradations of MITF and tyrosinase, and to suppress the productions of melanin in both in vitro and in vivo demanding further attention.

**P3581**

**Board Number: B877**

Attenuation of melanogenesis by jineol through the activation of MAP-Kinase mediated suppression of MITF and its downregulating proteins and the proteasomal degradation of tyrosinase.

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The aim of the present study was to investigate the anti-melanogenic effects of 3,8-dihydroxyquinoline (jineol) isolated from Scolopendra subspinipes Mutilans in melan-a cells along with its molecular mechanism of action. Jineol exhibited significant, dose-dependent antioxidant effects in various in vitro methods. Jineol significantly inhibited mushroom tyrosinase activity by functioning as an uncompetitive inhibitor with an inhibition constant of 103 M⁻¹. Treatment of jineol markedly abolished melanin production and intracellular tyrosinase activity in melan-a cells. In addition, jineol lessened the expressions of tyrosinase, TYRP-1, TYRP-2, and MITF, thereby hindering melanin production and interfering with the phosphorylation of ERK1/2 and p38. Furthermore, specific inhibitors of ERK1/2 and p38 prevented melanogenesis inhibition by jineol, and the proteasome inhibitor (MG-132) prevented jineol-induced decreases in cellular tyrosinase levels. Taken together, jineol was found to stimulate MAP-kinase (ERK1/2 and p38) phosphorylation and the proteolytic degradation pathway, which led to the degradations of MITF and tyrosinase resulting in to quench melanin production.
Bioactive oligopeptides are a promising class of therapeutic molecules, which possess diverse activities, high specificity and low toxicity. The use of oligopeptides as potentially selective and safe antioxidants is of particular interest in treatment and prevention of inflammatory, cancer, and degenerative diseases. Conjugation of bioactive oligopeptides with carrier molecules is an effective approach to improving their pharmacokinetic properties at tissue and cellular levels and enhancing a specific action. We studied the effect of triphenylphosphonium (TPP) moiety as an established intracellular and mitochondria-directed carrier on antioxidant and cytoprotective properties of a model tetrapeptide composed of repetitive aromatic and basic amino acids, e.g. Tyr-Arg-Phe-Lys. The tetrapeptide and its N-derivatives with different carboxyalkyl TPP compounds were obtained by a solid phase peptide synthesis from Fmoc-protected amino acids. Antioxidant activity of the starting oligopeptide and the modified ones was assessed in the Fenton-type reaction of cobalt chloride (CoCl$_2$) and hydrogen peroxide (H$_2$O$_2$). It was found that the modified oligopeptides at a concentration of c.a. 1 mM completely inhibited generation of oxygen radicals in the reaction similarly to the tripeptide glutathione, whereas the unmodified tetrapeptide was inactive at a concentration as high as 5 mM. Introduction of the TPP moiety into the tetrapeptide increased its antioxidant effect towards PC-12 rat pheochromocytoma cells subjected to H$_2$O$_2$-induced oxidative stress upon short-term exposition. During culturing of PC-12 cells in the presence of CoCl$_2$ as a hypoxia-mimicking agent, the modified oligopeptides effectively prevented cobalt-induced cytotoxicity to a higher extent compared with the initial tetrapeptide or glutathione at the same concentration. Our results suggest that modification with TPP moiety can enhance antioxidant and cytoprotective activity of oligopeptides for mammalian cells, presumably, due to promoting their anti-radical capacity and cellular availability.


This work was co-funded by the Russian Foundation for Basic Research (project No. 16-33-60146) and performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

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**P3583**

**Board Number: B879**

**Jabuticaba peel extract favors the recovery of fertility and antioxidant activity in the testicle of high-fat-fed aging mice.**

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Senility and obesity are often associated with dysfunction of various biological systems, including the male reproductive apparatus. Jabuticaba, a Brazilian fruit rich in phenolic compounds with antioxidant, anti-inflammatory and anti-carcinogenic activities, could potentially counteract disorders related to
aging and weight gain. In the present study, we evaluated the testicular function, using stereological, spermatological and physiological methods, in senile mice after high-fat diet intake and investigated the possible beneficial effect of the treatment with jabuticaba peel extract (JPE). For that, 140 FVB mice were distributed in 7 groups: YG (Young), SE (Senile), SHD (Senile+Hyperlipid diet), SJI (Senile+Jabuticaba peel extract [JPE], [0.003mL/gJPE]), SJI (senile+0.006mL/gJPE), SHJI (Senile+Hyperlipid diet+JPE [0.003mL/gJPE]), and SHJII (senile+hyperlipid diet+0.006mL/gJPE). After 60 days, the animals were euthanized and their testes were collected for several analyses. Histological sections, stained with HE, were photographed with an Olympus BX41 microscope coupled to the Olympus Q Color 3 digital camera to be used for stereological and spermatologic analysis. Analysis of oxidative stress was performed using commercial kits read on the SynergyHT, Bioktek (Winooski, USA). The data were analyzed with Gen5™2.0 data analysis software. Other analyses applied the IMAGE PRO PLUS 6.0 software, while the statistical analysis was done with the PRISM 5 STATISTIC software. Considering oxidative stress evaluation, the treatment with JPE significantly increased the activity of GSH, GPx, GR and SOD in SJI and SJII groups in comparison with SE levels. In relation to SHJI and SHJII, we observed an increase in the activity of GR, SOD and Catalase, but lower GSH activity, when compared to SHD group. In conclusion, our data corroborate the anti-inflammatory activity of phenolic compounds attributed to the JPE and suggest that this JPE can prevent the deleterious effects of senility and obesity on male reproductive function.

P3584
Board Number: B880
Zebrafish modeling defines complex innate immune mechanisms in sepsis and repetitive intestinal injury.

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Inflammatory bowel disease (IBD) is a complex chronic intestinal human disease composed of genetic, environmental and host immune factors. Establishing a robust and predictive in vivo animal model to study cellular function during acute and chronic inflammation has been challenging. Our previous genome-wide association study (GWAS) showed strong association of increased IBD risk with SNPs in innate immunity and autophagy. Live in vivo imaging of intestinal innate immunity can be uniquely performed on zebrafish because of their transparency and the presence of solely innate immunity over its initial weeks of life. For these reasons, we examined the effects of intestinal injury on time-course and cell-type dependent responses, with a particular focus on prostaglandin E2 (PGE2). Dextran sodium sulfate (DSS) induces chemical intestinal inflammation and has been extensively utilized to study intestinal innate immunity. We compared the effects of single and repeated DSS treatment in zebrafish.
and reported the mortality, lysosomal function and mucin production. Using previously reported doses, we observed dose-dependent mortality with repeated injury (Oehlerls et. al., 2012, 2013). Since neutral red accumulation indicates normally functioning acidic lysosomes, we quantified neutral red intensity in the mid gut with single and repeated DSS treatment for one and two days post DSS. Our results showed that the initial damage to lysosomal function fully recovered in two days after single injury but remains impaired after repeated injury. Mucin production was quantified from alcian blue stained fixed zebrafish. Alcian blue intensity increased in single injury but decreased from 60 to 40% after repeated injury. We observed high mortality with impaired recovery of lysosomal function and mucin production after repeated injury. We then applied PGE2 to the single injury model. By adding 1μM PGE2 for 1, 3, and 6 hours after DSS removal, PGE2 promoted recovery of neutral red intensity, positively correlated to earlier PGE2 treatment. PGE2 treatment dose-dependently increased alcian blue intensity. We further applied fluorescent killed E. coli to the single injury model and detected a high mortality rate similar to repeated injury. 90 minutes after E. coli treatment, fluorescence signals were detected in the dorsal aorta and the posterior cardinal vein of DSS-treated zebrafish, suggesting that high mortality may be due to sepsis. The bacteria-induced mortality was significantly decreased with PGE2 treatment during recovery or co-treatment with DSS. Phagocytosed E. coli co-localized with autophagosome in mFAP4+ intestinal macrophages. Together, we showed that PGE2 prevents sepsis by increasing mucin production and recovering lysosomal function in intestinal macrophages.

**P3585**

**Board Number: B881**

**Hepatocyte exosomes are anti-fibrotic in the liver.**

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Exosomes are nanovesicles that arise by inward budding of multivesicular bodies and which are released extracellularly when multivesicular bodies fuse internally with the plasma membrane. Exosomes contain a complex mixture of miRs, mRNAs and proteins that reflect the transcriptional and/or translational activity of the donor cell. Following their release, exosomes traverse the intercellular space and may be taken up by neighboring cells into which the components of their molecular payload are delivered, resulting in epigenetic re-programing and phenotypic alterations. In this study, we have investigated how experimental liver fibrosis is regulated by exosomes from normal hepatocytes. Exosomes were purified by ultracentrifugation from the conditioned medium of mouse AML12 hepatocytes cultured under serum-free conditions for 48 hrs and identified by nanoparticle tracking analysis and Western blot for CD81. In a short-term in vivo model of liver fibrogenesis in which BAC reporter transgenic connective tissue growth factor-enhanced green fluorescent protein (CCN2-EGFP) Swiss Webster mice expressing EGFP under the control of the CCN2 promoter received 30μl CCl4/25g i.p. every other day for 10 days, expression of EGFP, CCN2, or αSMA in the liver was dose-dependently blocked following administration of purified exosomes over the last 6 days (20-80 μg exosomal protein i.p., q.o.d). In a long-term in vivo model of liver fibrosis in which wild-type Swiss Webster mice received 30 μl CCl4 /25g, i.p. 3 times/wk for 5 wks, collagen deposition and expression of CCN2, αSMA, or collagen α1 were blocked following administration of purified exosomes over the last 10 days (80 μg exosomal protein i.p., q.o.d). Target cells in mouse livers were identified as hepatocytes and hepatic stellate cells (HSC) based on the in vivo localization of iv-administered PKH26- or PKH67-labelled exosomes. When tested in vitro, hepatocyte exosomes diminished expression of fibrosis-associated molecules (αSMA, collagen α1(I)), CCN2) in HSC and reversed ethanol-mediated cytotoxicity in hepatocytes. Thus hepatocyte exosomes carry a payload that is instrinsically anti-fibrotic and reverses HSC fibrogenesis or toxin-mediated hepatocyte damage.
P3586
Board Number: B882
Gomisin N inhibits an early stage of adipogenesis in 3T3-L1 preadipocytes.
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Gomisin N (GN) is a physiological lignan derived from Schisandra chinensis. In the present study, we investigated the inhibitory effects of GN on differentiation of 3T3-L1 preadipocytes. Incubation with GN significantly inhibited the differentiation of 3T3-L1 preadipocytes in a dose-dependent manner. This inhibitory effect primarily occurred at an early adipogenic stage through impairment of mitotic clonal expansion (MCE) caused by cell cycle arrest at the G1/S phase transition. GN inhibited the extracellular signal-regulated kinase and phosphoinositide 3-kinase/protein kinase B signaling in the MCE process and activated AMP-activated protein kinase. Furthermore, GN downregulated CCAT/enhancer-binding protein β (C/EBPβ) and histone H3K9 demethylase JMJD2B during early stages of adipogenesis, and therefore repressed the expression of C/EBPβ-targeted cell cycle genes. Collectively, these findings suggest that GN has potential as a novel agent for the prevention and treatment of obesity.

P3587
Board Number: B883
Shockwaves decrease PPARgamma and suppress adipocyte differentiation.
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ObjectiveWhite adipose tissue is a fat storage organ for keeping energy and an endocrine organ secreting adipokines such as leptin, adiponectin, and resistin. Adipogenesis which generates mature adipocyte is important in obesity. Shockwaves are mechanical pressure disturbances which transmit signals for biological responses. The goal of this study is to investigate the effect of shockwaves to adipocyte differentiation.

MethodsMouse fibroblast cell line, 3T3L-1 was (American Type Culture Collection (ATCC, CL-173)) cultured following the manufacturer’s instruction. 3T3L-1 cells at confluency were treated with insulin (1ug/mL), dexamethasone (DEXA, 1uM) and 3-isobutyl-1-methylxanthiane (IBMX, 0.5 mM). After 48 hours, the media was replaced with DMEM containing insulin (1ug/mL). Then, the medium was replaced with adipocyte maintenance medium (DMEM supplemented with penicillin and streptomycin) every 2-3 days until terminal differentiation into adipocytes Shockwaves (0.04mJ/mm2, 5Hz, 1000 impulses) were applied to the cells at the time when we changed the medium.

ResultsWe revealed that intracellular lipid accumulation was 20 % less in shockwave-treated 3T3L-1 than untreated control cells after adipocyte differentiation. Key adipogenic transcriptional factors, PPARgamma and C/EBPalpha were 40 % less in shockwave-treated cells compared to the untreated 3T3L-1 after 14 days of differentiation process. Shockwave treatment decreased other adipogenesis markers such as Perilipin, FAS, ACC in 3T3L-1 derived adipocytes. We found that shockwave-treatment activated Wnt signaling pathway and induced stabilization of beta-catenin and decreased PPARgamma which finally suppressed preadipocyte differentiation into adipocytes.

ConclusionsShockwaves suppressed 3T3L-1-derived preadipocyte differentiation into adipocytes by decreasing PPARgamma. These findings may provide a new therapeutic strategy for the treatment of obesity.