56th ANNUAL MIDWEST STUDENT BIOMEDICAL RESEARCH FORUM

Saturday, March 8, 2025

ROOM 3042

1:30 p.m.	O-40	EXPLORING THE ROLE OF RABANKYRIN-5 IN PROMOTING HPV INFECTIONS Presenter: Madison Love
1:45 p.m.	O-27	UNRAVELING THE NOVEL ROLE OF BUBR1 IN REGULATING INTESTINAL EPITHELIAL HOMEOSTASIS AND MAINTAINING BARRIER INTEGRITY Presenter: Rodaina Hazem Monieb
2:00 p.m.	O-46	CD2AP AND CIN85 LOCALIZE TO TUBULAR RECYCLING ENDOSOMES AND REGULATE CLATHRIN-INDEPENDENT CARGO RECYCLING Presenter: Gunjan Misri
2:15 p.m.	O-50	MICAL2 REGULATES ENDOSOMAL FISSION Presenter: Ajay Murakonda
2:30 p.m.	O-58	EXTRACELLULAR VESICLES AND NANOPARTICLES RELEASED FROM ELECTRICALLY STIMULATED C2C12 MYOTUBES PROTECT H9c2 CARDIOMYOCYTES FROM OXIDATIVE STRESS-INDUCED DAMAGE Presenter: Sarah Pribil Pardun
2:45 p.m.	O-35	AXONAL TRANSPORT OF PRIONS Presenter: Sam Koshy
3:00 p.m.	O-23	PERFLUORODECANOIC ACID (PFDA) EXPOSURE IMPACTS CELL VIABILITY AND ACTIVATES AUTOPHAGY PATHWAYS IN A HUMAN GRANULOSA CELL LINE. Presenter: Rasha Ghorab
3:15 p.m.	O-21	INVESTIGATING THE ESTROGEN DEPENDENCE OF ESTROGEN RECEPTOR α AND Sle1b COOPERATION IN MODULATING BCR SIGNALING IN LUPUS Presenter: Misha Gansvind
3:30 p.m.	O-07	EHD1 AS A NOVEL REGULATOR OF MICROTUBULE DYNAMICS Presenter: Bazella Ashraf
3:45 p.m.	O-03	P-REX1 MODULATES POLARIZATION AND FUNCTIONS OF TUMOR-ASSOCIATED MACROPHAGES Presenter: Daniel Afolabi
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EXPLORING THE ROLE OF RABANKYRIN-5 IN PROMOTING HPV INFECTIONS

Madison Love, Pengwei Zhang (University of Nebraska Medical Center, Omaha, NE)

Background and Significance: Human papillomaviruses (HPV) are the causative agents of approximately 90% of cervical cancer and 5% of cancer worldwide, but many aspects of intracellular infection remain unknown. HPV must utilize endocytic mechanisms, multiple viral and cellular protein complexes, and be directed through retrograde transport for trafficking from the cell surface to the nucleus for viral replication. Previous findings have reported on the interaction of some of these protein complexes, but a complete picture remains undetermined. Mechanistically, it remains unclear how HPV-containing compartments associate with dynein, a microtubular motor protein, to successfully organize and initiate retrograde transport of the virus from the cell periphery to the nucleus for replication. Our recent study has explored a novel molecular basis for dynein recruitment to HPV-carrying early endosomal compartments. In this process, incoming HPV exploits the early endosomal small GTPase Rab5 and its effector Rabankyrin-5 to form a complex, establishing a connection between the HPV-carrying early endosome and dynein, thereby facilitating viral transport along microtubules. These observations suggest that HPV may repurpose Rabankyrin-5 as a potential dynein cargo adaptor during viral infection. Notably, known endosomal dynein adaptors do not participate in HPV entry. These findings imply a novel mechanism for HPV intracellular transport during the early stages of viral entry, involving the spatial and temporal coordination of endosomal coat complex formation and dynein recruitment and activation. With this knowledge, the long-term research objective would be to develop a novel targeted therapeutic using these findings for the treatment of clinical HPV infections, as no therapeutics currently exist for the treatment of HPV.

Hypothesis and Experimental Design: In this study, we hypothesized that Rabankryin-5 associates with dynein at the endosomal membrane to establish an essential connection during the initial stage of HPV infection. To test this hypothesis, we first tested Rabankyrin-5's requirement in HPV infection by using targeted siRNA in HeLa and HaCaT cells. This assay required an siRNA transfection targeting Rabankyrin-5 and a control, followed by flow cytometry analysis to determine relative infection levels. Following this, we utilized a series of immunofluorescence and viral tracking assays to determine the dynein recruitment to HPV-carrying vehicles and used live imaging to analyze the motion and displacement of the viral particles in the presence and absence of Rabankyrin-5. In these imaging assays, cells were transfected with siRNA targeting Rabankyrin-5 or not, then either mock-infected or infected with HPV16-FLAG PsVs, fixed, stained and imaged. We further tested the ability of Rabankyrin-5 to form a complex with the L2 minor capsid protein and dynein through a series of pulldown assays. These were conducted using bacterially expressed protein such as Rabankyrin-5 protein domains as well as respective controls incubated and immobilized on GSH resin along with a GFP-L2 fusion protein or GFP as a control. Following an incubation, the bound proteins were eluted from the resin and subject to immunoblotting for detection of protein-protein interactions.

Data and Results: Our results indicate that Rabankyrin-5 is a requirement during early HPV infection, specifically, without Rabankyrin-5, cells produced a relatively 65% less productive HPV infection than controls. The immunofluorescence assay results indicated significant colocalization between the HPV16 PsVs and Rabankyrin-5 in HPV infected cells, but not in the mock infected cell lines, indicating a complex formation between Rabankyrin-5 and L2. Live image acquisition of cells during viral infection demonstrated viral particle movement exhibiting halted or slowed movement along microtubules in the absence of Rabankyrin-5, with an average displacement of 0.7 ± 0.5 μm compared to control cells at 1.3 ± 0.7 μm. Similarly, in Ranbankyrin-5 depleted cells the velocity of viral particles was found to be 0.12 ± 0.08 μm/s and 0.26 ± 0.09 μm/s for virus particles in control cells, both indicating slower and less movement of HPV particles in the absence of Rabankyrin-5. Lastly, pulldown assays revealed both Rabankyrin-5 and dynein directly bind to the L2 minor capsid protein of HPV.

Conclusion: We can conclude that Rabankyrin-5 is required for early HPV infection, it is in complex with the HPV L2 protein, as well as recruits and affects the actions of cytoplasmic dynein. These results confirm the hypothesis that Rabankyrin-5 is specifically required for the recruitment and interaction of the L2 minor capsid protein and dynein during early HPV infection. This novel role for Rabankyrin-5 provides a more complete understanding of the mechanism of action for HPV infections and lays the groundwork for the first targeted therapeutic development for HPV infections. Further study will aim to illuminate the mechanism behind these findings and elucidate additional required binding factors.

UNRAVELING THE NOVEL ROLE OF BUBR1 IN REGULATING INTESTINAL EPITHELIAL HOMEOSTASIS AND MAINTAINING BARRIER INTEGRITY

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Background and Significance: The intestinal epithelium is the largest surface barrier protecting our body from the harsh external environment by performing critical functions in nutrient absorption and immune defense. Every 3-5 days, it self-renews in an intricate process of balanced proliferation and differentiation, termed intestinal homeostasis. Intestinal epithelial cells comprising the villi, are derived from a heterogenous population of multipotent stem cells at the crypt base called Intestinal Stem Cells (ISC). ISCs self-renew and fuel epithelial cell turnover, contributing to the overall barrier integrity and absorptive functions of the intestinal epithelium. Upon intestinal tissue injury, regenerative cycling of ISCs rapidly repairs the epithelial barrier; however, injury resulting in ISC loss impairs their function in repairing epithelial damage. Aging significantly perturbs intestinal homeostasis as ISCs are lost with age, and promotes a pro-inflammatory environment fueled by the disruption of important immune defense mechanisms. Inflammatory Bowel Diseases patients such as Ulcerative Colitis and Crohn's Disease, have an altered ISC niche, suggesting that ISC dysregulation may contribute to the chronic nature of these diseases. *BubR1* is a known regulator of organismal aging. One of the defining features of *BubR1* is its natural decline with age which triggers cellular senescence in tissues, highlighting its important role in maintaining cellular and tissue homeostasis. However, its role in rapidly renewing tissues, such as the intestinal epithelium, and regulation of tissue-resident stem cells, remains poorly understood. Understanding the role of *BubR1* in regulating epithelial homeostasis and ISC function will broaden our knowledge of how aging impacts epithelial homeostasis, barrier integrity and elucidate mechanisms underlying IBD pathophysiology.

Hypothesis: We hypothesize that loss of *BubR1* in the intestinal epithelium leads to reduced survival of mice and dysregulation of epithelial homeostasis due to loss of barrier integrity and impaired regenerative function of ISCs.

Experimental Design: To test the impact of *BubR1* on organismal survival, we utilized the Cre-loxP system for tissue-specific knockout of *BubR1* using *cre* driven by the *Villin* promotor. We monitored these mice for overall health, survival, and measured their weights weekly to obtain a growth trajectory. To determine the role of BubR1 in maintaining intestinal epithelial homeostasis, we utilized immunohistochemistry/immunofluorescence techniques along with bulk-RNA sequencing and bioinformatic analysis. We assessed the expression of multiple markers for epithelial cells including ISCs, to determine the role of *BubR1* in regulating epithelial homeostasis.

Results: We found that intestinal-specific *BubR1* knockout (ISBK) mice suffer from significant postnatal growth delay, and have a median survival of 25 days. We also observed that *BubR1* loss results in a reduction of $Olfm4^{+}$ intestinal stem cells despite an upregulation of crypt proliferation which is coupled with increased apoptotic cell death. In addition, ISBK mice have significant morphological changes observed along their crypt-villus axis, noted by epithelial hypertrophy, villous atrophy and disorganized stratification. Moreover, we observed a striking downregulation in defensin genes, coupled with upregulation of pro-inflammatory pathways and upregulation of the Wnt/β-catenin pathway, which is necessary for maintaining crypt proliferation and ISC homeostasis.

Conclusion: Our results support the hypothesis that intestinal epithelial loss of *BubR1* reduces survival of mice and dysregulates the intestinal epithelial barrier integrity. Our mouse model provides a novel platform to dissect the molecular underpinnings of epithelial barrier dysfunction, a core driver of IBD pathogenesis, and ISC regulation under chronic inflammation. Further research will direct us to implicate *BubR1* as a potential therapeutic target for treating aging-induced barrier dysfunction and provide better treatment options for IBD.

CD2AP AND CIN85 LOCALIZE TO TUBULAR RECYCLING ENDOSOMES AND REGULATE CLATHRIN-INDEPENDENT CARGO RECYCLING

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Background: There is enormous diversity in the repertoire of cell surface receptors essential for signaling and cellular communication. Once internalized from the plasma membrane via clathrin-dependent or clathrin-independent endocytosis, receptors on early/sorting endosomes are generally sorted to the lysosome via late endosomes for degradation or recycled back to the plasma membrane through recycling endosomes. Tubular Recycling Endosomes (TREs) are a specialized set of endosomes primarily involved in the transport and recycling of cargo internalized via clathrin-independent endocytosis such as Major Histocompatibility Complex Class I, or large neutral amino acid transporter CD98 receptors. However, the mechanisms and molecular players that regulate cargo transport from these compartments are not completely understood. A key scaffold protein known as Molecule Interacting with CasL-Like 1 (MICAL-L1) is essential for orchestrating receptor recycling via interaction with various proteins and lipids, including phosphatidic acid, the BAR domain-containing protein Syndapin2, the microtubule-associated protein CRMP2, and the endosome fission catalyst and ATPase, EHD1. To identify additional MICAL-L1 interaction partners potentially involved in clathrin-independent receptor recycling we undertook an unbiased yeast two-hybrid screen with MICAL-L1 as bait. We identified two homologous actin regulatory proteins, CD2-associated protein (CD2AP) and the SH3 domain-containing kinase-binding protein 1 (known as SH3KBP1 or CIN85), as novel MICAL-L1 interaction partners. In this study, we characterize the interaction of CD2AP and CIN85 with MICAL-L1 and investigate the roles of these proteins at TREs in facilitating clathrin-independent receptor recycling.

Significance: Efficient receptor recycling is essential for fundamental cellular processes such as cell polarity, cell migration and cell division. Consequently, dysregulation of proteins involved in the overall process of receptor transport/trafficking is associated with various diseases such as neurodegenerative disorders, and different types of cancers including breast, non-small cell lung, and prostate cancers. Therefore, it is crucial to elucidate the molecular players and mechanisms involved in receptor recycling.

Hypothesis: We hypothesize that CD2AP and CIN85 are recruited to TREs by MICAL-L1 to facilitate recycling of clathrin-independent cargo. MICAL-L1 contains fourteen proline-rich sequences, whereas CD2AP and CIN85 each have three Src Homology 3 (SH3) domains. We hypothesize that one or more SH3 domain of each protein is required for the interaction.

Experimental Design: We first asked whether MICAL-L1 interacts with CD2AP and CIN85 in mammalian cells, and to test this we carried out co-immunoprecipitations using HeLa cell lysates. Since CD2AP and CIN85 each have three SH3 domains, we asked whether these domains are required for their interaction with MICAL-L1, and if so, which SH3 domains are involved in the binding. To map the binding of the SH3 domains, we used: a) selective yeast two-hybrid assays, or b) GST-pulldown assays. We then asked whether CD2AP and CIN85 localize to TREs and if so, does MICAL-L1 facilitate the recruitment of these proteins. To this aim, we knocked-down MICAL-L1 using siRNA and quantified TRE-associated CD2AP and CIN85 using immunofluorescence. We next addressed whether CD2AP and CIN85 are required for the recycling of clathrin-independent CD98 receptors. In CD2AP- and CIN85-depleted cells, anti-CD98 antibody was used to stimulate receptor internalization, followed by incubation at 37°C to allow receptor recycling. The percentage of intracellular CD98 remaining was quantified by immunofluorescence as an inverse measure of receptor recycling.

Results/Data: MICAL-L1 interacts with both CD2AP and CIN85 in HeLa cells. All three SH3 domains of CD2AP and the second and third SH3 domains of CIN85 facilitate binding to MICAL-L1. Both CD2AP and CIN85 localize to TREs in a MICAL-L1-dependent manner. We also demonstrated that depletion of CD2AP and CIN85 led to impaired recycling of clathrin-independent CD98 receptors.

Conclusion: Our studies suggest that the interaction of CD2AP and CIN85 with MICAL-L1 is required for their recruitment to TREs, and we speculate that the actin regulatory activities of these proteins may control key endosome functions such as cargo sorting and fission, thus facilitating clathrin-independent receptor recycling. Ongoing studies are aimed at addressing the molecular roles of CD2AP and CIN85 at tubular endosomes, further advancing our understanding of the basic mechanisms regulating endocytic sorting and receptor recycling, critical for various cellular and physiological processes.

MICAL2 REGULATES ENDOSOMAL FISSION

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Background: Endocytosis is a conserved process across species that involves the internalization of cell surface receptors, nutrients, and lipids from the plasma membrane. Following endocytosis, the internalized vesicles derived from the plasma membrane fuse with a membrane-bound organelle called the early endosome. Depending on the receptor type, the receptor cargos are typically directed to lysosomes for degradation via late endosomes or recycled back to the plasma membrane through recycling endosomes. A crucial endosomal event required for receptor recycling and degradation is endosome fission, which generates cargo-laden transport vesicles. Key components of the fission machinery, including the endocytic membrane scaffold MICAL-L1, the ATPase EHD1, and the actin cytoskeleton, have been implicated in endosomal fission. However, a complete list of the players involved is lacking and how their functions are coordinated remains incompletely understood. For over a decade, the actin cytoskeleton has been hypothesized to play a major role in regulating endosomal fission events. Studies have shown that actin branching at endosomes is necessary to constrict the membrane to form buds, while subsequent actin debranching is required to recruit EHD1 to the endosomal neck, facilitating endosomal fission. Nonetheless, actin regulation is complex and the key proteins that regulate actin at endosomes remain to be fully elucidated. Actin assembly and disassembly involve a plethora of proteins, including the MICAL (Molecule Interacting with CasL) family (MICAL1–3). MICALs are actin-regulatory oxidation-reduction enzymes capable of directly binding to and disassembling filamentous actin. While MICAL1 and MICAL3 remain inactive due to their auto-inhibitory C-terminal coiled-coil domains, MICAL2 is constitutively active, in part due to the absence of its C-terminal coiled-coil domain. This study elucidates the novel role of MICAL2 in endosomal fission and receptor recycling.

Significance: The trafficking of internalized receptors, such as EGFR and transferrin, to the plasma membrane is a tightly regulated process involving coordinated events like endosomal sorting, budding, and fission. Defective receptor trafficking is associated with various neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. Similarly, aberrant expression of MICAL2 has been implicated in several cancers, such as pancreatic and head and neck cancers. While MICAL2 is known to promote actin disassembly, its molecular mechanism in receptor recycling remains unexplored. Based on our findings, we propose a novel role for MICAL2 in orchestrating endosomal fission for receptor recycling. This insight not only advances our understanding of endosomal fission but also highlights MICAL2 as a potential therapeutic target for cancer treatment.

Hypothesis: We hypothesize that MICAL2 plays a crucial role in regulating actin depolymerization and/or debranching at endosomes, potentially facilitating endosomal fission.

Experimental design: To elucidate the functional role of MICAL2, we depleted MICAL2 using siRNA in HeLa cells and evaluated its impact on endosomal size and actin polymerization. Endosome size typically exhibits a negative correlation with fission, but it is also influenced by fusion. To assess the impact of MICAL2-depletion on endosome fission, we performed a novel endosome fission assay developed in our lab. Briefly, mock and MICAL2-depleted cells were incubated with a phosphoinositide 3-kinase (PI3K) inhibitor to induce enlarged endosomes and synchronize fission events upon inhibitor washout. This assay allows us to assess the degree of fission by measuring the decrease in endosome size over time. Furthermore, to establish the role of MICAL2 in receptor recycling, we performed pulse-chase experiments using fluorescently labeled transferrin and analyzed transferrin retention in cells upon MICAL2-depletion as a readout for recycling.

Results: MICAL2-depletion:1) led to the formation of enlarged endosomes. 2) caused an accumulation of affiliated branched actin at endosomes. 3) impaired endosomal fission and delayed transferrin recycling.

Conclusion: Endosome fission is a crucial process for recycling receptors and lipids back to the plasma membrane, playing a vital role in maintaining cellular homeostasis. However, the key players and mechanisms underlying this process remain poorly understood. Our results show that MICAL2 knock-down leads to endosome enlargement accompanied by accumulation of affiliated branched actin, suggesting both aberrant actin regulation and impaired endosome fission. Moreover, depletion of MICAL2 delays the recycling of internalized transferrin receptor, consistent with impaired endosome fission. Our current studies aim to rescue the defects caused by MICAL2-depletion (by employing an siRNA-resistant version of MICAL2) and ultimately analyze how an enzymatically inactive mutant of MICAL2 affects actin filamentation at endosomes and endosomal function. Overall, our data support a novel role for MICAL2 in endosomal fission and receptor recycling, likely through its regulation of endosomal actin.

EXTRACELLULAR VESICLES AND NANOPARTICLES RELEASED FROM ELECTRICALLY STIMULATED C2C12 MYOTUBES PROTECT H9c2 CARDIOMYOCYTES FROM OXIDATIVE STRESS-INDUCED DAMAGE

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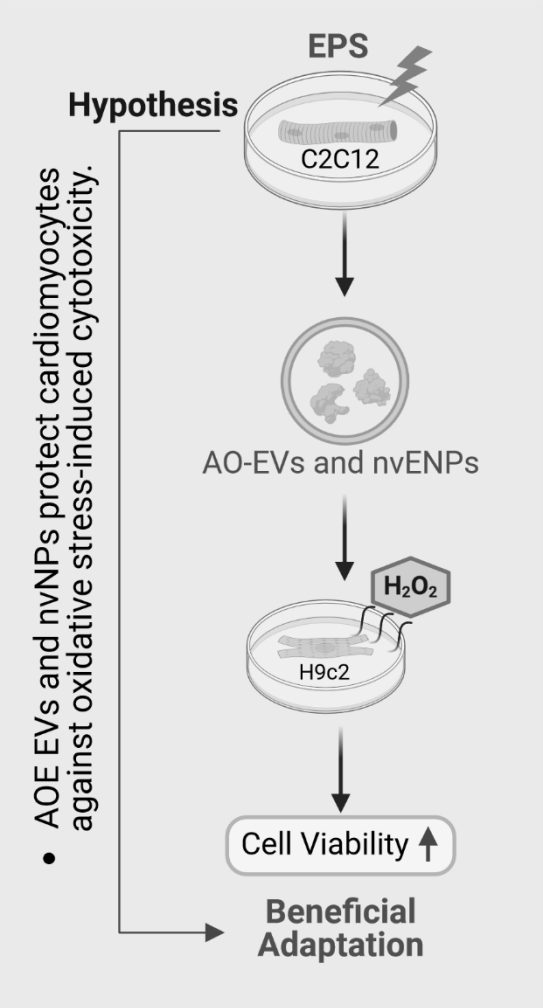
Background, Significance, Hypothesis: While the benefits of exercise are well-ackowledged, the underlying molecular mechanisms remain to be fully understood. Previous research in our laboratory demonstrated that electrical pulse simulation (EPS) of cultured C2C12 myotubes activates the Nrf2 pathway. This activation evokes an upregulation of key antioxidant enzymes that protect the myotubes from oxidative stress-induced cytotoxicity. In this current study, we hypothesized that these upregulated antioxidant enzymes could be secreted via extracellular vesicles (EVs) and non-vesicular extracellular nanoparticles (nv-ENPs). We also hypothesized that these particles could protect H9c2, a rat cardiac cell line, from hydrogen peroxide (H_2O_2)-induced damage.

Experimental Design: EVs/nvENPs were isolated from the conditioned medium of EPS-treated C2C12 cells (one hr/day for six consecutive days) and compared to those from non-stimulated cells using differential ultracentrifugation (dUN). These EVs/nvENPs and their effects on H9c2 cell viability were analyzed via transmission electron microscopy (TEM), western blot analysis, and CCK-8 cell viability assay.

Data and Results: Transmission electron microscopy (TEM) revealed that the isolated particles displayed a typical morphology, with EVs showing a cup-shaped structure and lipid bilayer membrane, and nvENPs exhibiting a pill-like and amembranous structure with high electron density. Western blot analysis demonstrated that EVs/nvENPs from EPS-C2C12 myotubes contained higher levels of antioxidant proteins downstream of the Nrf2 pathway (NQO1 and GSTA2) than those isolated from non-stimulated myotubes. Using a CCK-8 cell viability assay, we found that H9c2 cells pretreated with EPS-C2C12 derived EVs/nvENPs showed significantly higher survival rates following H_2O_2 challenge compared to cells treated with particles from non-stimulated C2C12 cells ($p < 0.05$, n = 3/group).

Conclusion: Our findings suggest that electrically stimulated C2C12 myotubes release antioxidant-enriched EVs/nvENPs that protect cardiomyocytes from oxidative stress. This reveals a potential novel mechanism by which exercise benefits cardiovascular health, wherein contracting skeletal muscles release EVs/nvENPs enriched with antoxidants into the bloodstream, which are then taken up by the heart to enhance its antioxidant defenses and mitigating oxidative stress-related damage, such as that seen in ischemia/reperfusion injury.



EPS: electrical pulse stimulation
AOE: antioxidant enriched
EV: extracellular vesicle
nvENP: nonvesicular extracellular nanoparticle

AXONAL TRANSPORT OF PRIONS

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Background, Significance, Hypotheses: Disease associated prions (PrP^{Sc}) spread via defined neuroanatomical pathways through the peripheral and central nervous systems, but the mechanisms of transport are still unknown. Current studies indicate PrP^{Sc} slow axonal transport, but the methodology utilized to investigate this has low sensitivity and includes the confound of newly replicated PrP^{Sc}. Also, the role of the endogenous cellular prion protein (PrP^{C}) in PrP^{Sc} transport is debated, despite being necessary for PrP^{Sc} replication and disease propagation. Deciphering the mechanisms of prion transport can pave the way for new therapeutic targets to limit the spread of disease. We hypothesize that PrP^{Sc} utilizes fast axonal transport and undergoes PrP^{C} independent axonal transport.

Experimental Design: Our goal was to directly visualize and measure PrP^{Sc} transport in the presence or absence of endogenous PrP^{C}. PrP^{Sc} strains were purified from terminally infected brains and conjugated to Alexa Fluor 647 (AF^{647}). $PrP^{+/+}$ or $PrP^{0/0}$ mice were anesthetized, and the right sciatic nerve (ScN) was surgically exposed. Using a 30-gauge Hamilton syringe, Dextran-AF^{647} or PrP^{Sc}-AF^{647} was inoculated into the ScN by reciprocating the needle along the individual nerve fibers under the epineurium. The nerve was then immobilized and imaged using two photon microscopy. Spectral scans were acquired in multiple ScN axons confirm the presence of AF conjugated material and timeseries were acquired to track particle motion. Using the Fiji/ImageJ plugin, Trackmate, individual particles were localized and tracked to acquire transport velocities.

Data and Results: PrP^{Sc}-AF^{647} strains were inoculated into mouse ScN, and fluorescent particles were successfully imaged in multiple nerve segments and individual axons. Particles were observed to be in close association with ScN axons. Particles were also found to be motile and velocity measurements were acquired. PrP^{Sc}-AF^{647} particle velocities were observed to be in the fast axonal transport range (2-5 μm/sec) in both $PrP^{+/+}$ and $PrP^{0/0}$ ScN in live mouse ScN imaging.

Conclusion: We were able to directly image fluorescently tagged PrP^{Sc} *in vivo* in live mouse ScN. All rodent-adapted prion strains tested associated in proximity to axons in $PrP^{+/+}$ and $PrP^{0/0}$ ScN and appear to undergo axonal transport. This indicates PrP^{C} independent axonal transport of PrP^{Sc}. The recorded velocities were consistent with the known rates of fast axonal transport. Moreover, all prion strains had similar axonal transport velocities, indicating that PrP^{Sc} transport is independent of the biochemical or biological phenotypes of prion strains. Future work will probe the mechanistic aspects of this process including PrP^{Sc} associated transport vesicles, specific molecular motors, and involvement of other cofactors.

PERFLUORODECANOIC ACID (PFDA) EXPOSURE IMPACTS CELL VIABILITY AND ACTIVATES AUTOPHAGY PATHWAYS IN A HUMAN GRANULOSA CELL LINE.

Rasha Ghorab, Hannah Wood, Kendra L. Clark (UNMC Omaha, NE)

Background, Significance, Hypothesis: Perfluorodecanoic acid (PFDA) is a member of the per- and polyfluoroalkyl substances (PFAS) family, chemicals that are widely used in industrial applications such as water- and stain-repellent coatings, fire-fighting foams, and other chemical manufacturing processes. While the use of other PFAS have decreased, PFDA concentrations are increasing in human serum, raising concerns due to its persistence in the environment and potential for bioaccumulation. This study investigates the effects of PFDA exposure on cell viability and autophagy-related markers in a non-cancerous human granulosa cell line (HGrC1). We hypothesized that exposure to PFDA exposure compromises cell viability and alters granulosa cell function in HGrC1 cells.

Experimental Design: Cells were exposed to (0-.01-.1-1-10uM) concentrations of PFDA for 24, 48, 72, and 96 hours, followed by assessments of cell viability by MTT. Dose-dependent changes in autophagy and DNA damage were assessed at 48 hours via immunoblotting by examining the levels of key markers such as NRF2, p62, KEAP1, HO-1, LC3A/B, and pH2AX. RT-qPCR was completed to evaluate expression of genes related to oxidative stress such as *SOD1*, *GSR*, *GPX1*, and *CAT*.

Data and Results: The results revealed dose-dependent changes in cell viability beginning at 24 hours. Additionally, cells treated with 10uM PFDA display altered morphology, characterized by a spindle-shaped appearance and reduced cell density. 10uM PFDA exposure led to a notable accumulation of P62 ($p<0.001$), HO-1 ($p<0.05$), and LC3A/B ($p<0.01$). Concurrently, a significant decrease in KEAP1 ($p<0.05$) protein levels was observed. Interestingly, the increase in autophagy did not result in changes in pH2AX ($p>0.05$) protein abundance, a marker for DNA double strand breaks. Further, no significant changes were observed in mRNA transcripts for *SOD1* and *GSR* levels across treatment groups. However, *GPX1* ($p>0.1$) and *CAT* ($p>0.1$) transcript levels exhibited a slight decrease at 10uM.

Conclusion: The results support the hypothesis that exposure to 10uM PFDA for 48 hours significantly reduced cell viability, alters cell morphology, and initiated autophagy in HGrC1 cells. The altered expression of autophagic markers and antioxidant enzymes suggests PFDA may disrupt autophagic flux and cellular oxidative stress responses. These findings underscore the potential reproductive health risks of PFDA exposure due to its adverse effects on cell viability, morphology, oxidative stress, and autophagic processes. Further studies should be done on the long-term impact of PFDA exposure on reproductive health and granulosa cell function.

INVESTIGATING THE ESTROGEN DEPENDENCE OF ESTROGEN RECEPTOR α AND *Sle1b* COOPERATION IN MODULATING BCR SIGNALING IN LUPUS

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Background: Lupus is an autoimmune disease in which there is a loss of immune tolerance due to the presence of autoreactive B cells, leading to the production of anti-nuclear antibodies that cause chronic tissue inflammation and organ damage. Ninety percent of lupus patients are women. Previous research has demonstrated that estrogens contribute to the female sex bias of this disease. We have shown that the effects of estrogen on lupus are mediated via estrogen receptor alpha (ERα) acting in a B cell intrinsic manner. Genetic factors also contribute to the development of lupus. *Sle1b* is a lupus susceptibility allele that controls the initial loss of tolerance event in lupus, loss of tolerance to chromatin. B6.*Sle1b* mice carry the *Sle1b* allele on an otherwise non-autoimmune background display loss of tolerance, immune cell hyperactivation, and spontaneous germinal center formation with a strong female sex-bias. *Sle1b* results in expression of autoimmune isoform of Ly108, a protein that modulates B cell receptor (BCR) signaling. Our lab demonstrated that ERα deficiency abrogates B cell hyperactivation and loss of tolerance, decreases of spontaneous germinal center formation, and an eliminates the female sex-bias of these phenotype in B6.S*le1b* mice. Furthermore, we found that ERα deficient B cells from B6.*Sle1b* female mice display attenuated BCR activation-induced phosphorylation of intracellular proteins involved in the BCR signaling cascade, such as p38 MAPK and SHP2 and decreased proliferation. These conclusions are based on experiments in which B cells were cultured in media containing phenol red (an estrogen) and supplemented with fetal bovine serum (which contains estrogens). Thus, we could not determine if simultaneous activation of estrogen-dependent ERα signaling was required for these effects on BCR signaling.

Significance of Problem: Lupus impacts over five million people worldwide, with an estimated 16,000 new cases annually in the United States alone. Treatment includes corticosteroids and immunosuppressive drugs. Two FDA approved biological drugs are also available. However, lupus therapies have many side effects due to their nonspecific nature, are often ineffective, and can be extremely costly. Developing a deeper understanding of the mechanisms underlying lupus pathogenesis will lead to the development of novel treatments with improved efficacy, specificity, and outcomes.

Hypothesis: We hypothesize that in females, ERα acts in an estrogen-dependent manner to cooperate with *Sle1b* to promote BCR-induced activation of p38 MAPK and SHP2, thereby promoting B cell proliferation.

Experimental Design: Naïve B cells isolated from ERα wildtype and ERα deficient B6.*Sle1b* mice (males and females) were placed in phenol-red free media supplemented with charcoal stripped FBS. Cultures received vehicle, 10 nM 17β-estradiol (the most abundant endogenous estrogen), 10 μM fulvestrant (an ERα antagonist), or 10 nM 17β- estradiol plus 10 μM fulvestrant. The BCR cascade was activated by the addition of an anti-IgM antibody F(ab')2 fragment. Phosphorylation of p38 MAPK and SHP2 were analyzed by flow cytometry and western blotting. Cell proliferation is being analyzed by CFSE and flow cytometry.

Results: In the cultures of naïve B cells from B6.Sle1b females expressing ERα, BCR activation-induced phosphorylation of p38 MAPK and SHP2 was enhanced in cultures treated with 17β-estradiol compared to those treated with 17β-estradiol and fulvestrant. Thes data suggest that both estrogen and ERα are required for enhanced BCR activation-induced p38 MAPK and SHPS2 phosphorylation. In fulvestrant treated cultures, BCR activation-induced p38 MAPK and SHP2 phosphorylation is diminished compared to that in the vehicle treated cultures. This later comparison is meaningful because it demonstrates that in vehicle treated B cells, residual intracellular estrogens, which persist in cells through the culture period, act through ERα to enhance BCR activation-induced p38 MAPK and SHPS2 phosphorylation, an effect disrupted by fulvestrant. Neither 17β-estradiol nor fulvestrant impacted BCR activation-induced phosphorylation of p38 MAPK or SHP2 in B cells from ERα deficient B6.Sle1b females or in B6.Sle1b males.

Conclusions: Our results show that contemporaneous estrogen-dependent ERα signaling is required for ERα to cooperate with *Sle1b* to enhance BCR activation-induced p38 MAPK, and SHP2 phosphorylation. Studies to determine if estrogen-dependent ERα signaling is needed for ERα to cooperate with *Sle1b* to enhance BCR activation-induced proliferation are ongoing. ERα is not a kinase, so our future directions seek to understand the molecular mechanisms through which ERα interacts with proteins in the BCR signaling cascade and promotes p38 MAPK, and SHP2 phosphorylation.

EHD1 AS A NOVEL REGULATOR OF MICROTUBULE DYNAMICS

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Background: Microtubules are dynamic, polar, polymeric filaments assembled from tubulins that are indispensable for intra-cellular trafficking, cell division, motility and sensory functions. Microtubules also comprise the primary cilium, a solitary, immotile structure that serves as a sensory organelle in almost every cell type. A series of microtubule associated proteins (MAPs) including kinesins, dyneins, End Binding Proteins and others regulate microtubule stability, assembly and disassembly, cargo transport, and cell division. Impairment of microtubules or MAPs has a significant impact on cell function, highlighting the need to identify additional microtubule regulators. New evidence supports the role of Eps15 Homology domain containing protein 1 (EHD1), a key endocytic regulatory protein, as a potential microtubule regulatory protein.

Significance of the problem: EHD1's overarching effects on microtubules can be gleaned from effects on both ciliogenesis and endocytic trafficking. Defective primary ciliogenesis is implicated in a variety of diseases (ciliopathies) including cancer. Interestingly, a mutation in EHD1 (R398W) also leads to a form of ciliopathy. Thus, understanding how EHD1 precisely regulates microtubules will provide mechanistic insights into both ciliogenesis and trafficking. This will also help inform our understanding of various ciliopathies and unveil therapeutic targets.

Hypothesis: While EHD1 functions as an ATPase in the fission of endosomes and promotes receptor recycling, we have observed that its depletion also results in aberrant microtubule-based transport, including coalescence of endosomes in the peri-nuclear region of the cell and the dispersal of centriolar satellites, which are proteinaceous aggregates that are crucial for primary ciliogenesis. We therefore hypothesize that EHD1 is a novel microtubule regulatory protein that interacts with microtubules and regulates their dynamics to control endocytic transport and centriolar satellite movement.

Experimental Design: To understand how EHD1 affects microtubule dynamics, microtubule regrowth was measured in a nocodazole washout assay upon EHD1 depletion. Herein, cells were treated with nocodazole to disrupt microtubules. Microtubule regrowth was then initiated by drug washout and the cells were fixed at increasing time points to measure the dynamics of microtubule regrowth. Regrowth was quantified by assessing the mean area of radial microtubule extension in both mock and EHD1 siRNA-treated conditions. Next, an interaction between EHD1 and microtubules was demonstrated by coimmunoprecipitating EHD1 and tubulins (α, β1, β3, and γ) in HeLa and retinal pigmented epithelial cells. We then assessed various EHD1 interaction partners, including MICAL-L1, Rab8, Rab10 and Cep215 as potential connectors for EHD1-tubulin interactions, using coimmunoprecipitation upon siRNA-mediated depletion of the aforementioned connectors. To evaluate the possibility of direct interactions and identify the potential domains involved, GST-EHD1 fusion proteins (including GST attached to the EHD1 EH domain) will be used for *in vitro* binding assays with αβ tubulin heterodimers.

Results: Our results show that the knockdown of EHD1 in HeLa cells impedes microtubule regrowth after nocodazole-induced depolymerization. Additionally, we identified novel interactions between EHD1 and the predominant tubulins: α, β1, β3, and γ. These interactions remain preserved even upon microtubule depolymerization. In addition, all of the candidate connectors, including MICAL-L1, Rab8, Rab10 and Cep215 are dispensable for EHD1-tubulin interactions, suggesting a likely direct interaction.

Conclusions: Our observations demonstrating EHD1-tubulin interactions and the effects of EHD1 on microtubule dynamics will help elucidate the role of EHD1 in regulating microtubule growth leading to centriolar satellite movement in ciliogenesis, as well as endosomal trafficking. The identification of EHD1 as a novel MAP may also have clinical implications, providing mechanistic insights into ciliopathies, including a missense mutation (R398W) in EHD1 that causes ciliopathic effects in patients. Addressing whether this mutant binds tubulin and fosters microtubule regrowth may be key to uncovering its pathogenic properties.

P-REX1 MODULATES POLARIZATION AND FUNCTIONS OF TUMOR-ASSOCIATED MACROPHAGES

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Background, Significance and Hypotheses: Tumor-associated macrophages (TAM) are a key subset of macrophages that infiltrate the tumor microenvironment, often adopting an M2-like phenotype. TAMs contribute to tumor progression by promoting angiogenesis and immune suppression, including inhibiting cytotoxic CD8+ T-cell activity and inducing T-cell exhaustion. Aberrant expression of P-Rex1 (Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchanger 1) in cancer cells has been implicated in tumor progression by promoting growth and metastasis. Interestingly, while P-Rex1 is highly expressed in macrophages, its role in the polarization and function of TAM remains unclear. From preliminary data, we have found that, TAM related markers were reduced in our WT mice model compared to KO mice model. Based on this, **our central hypothesis** states that P-Rex1 deletion promotes the polarization and propagation of Tumor-associated macrophages and has an immunosuppressive role in the tumor microenvironment of lung cancer.

Experimental Design: Primary bone marrow-derived macrophages (BMDMs) from wild-type (WT) and P-Rex1 knockout (KO) mice, as well as RAW 264.7 macrophage-like cells, were used. CD8+ T cells were isolated from mouse spleens. Tumor-conditioned medium (TCM) from Lewis lung carcinoma (LLC) cells was used to simulate the tumor microenvironment. Quantitative RT-PCR (qRT-PCR) assessed the mRNA expression of M1 (iNOS) and M2 (Arg1) macrophage markers, as well as T-cell exhaustion markers (PD-1, CTLA-4, and TIM-3). Transwell migration assays were conducted to measure macrophage migration toward tumor cells and macrophage-mediated suppression of CD8+ T-cell migration. Phagocytic activity was evaluated using a red zymosan phagocytosis assay. Genetic manipulations, including siRNA knockdown and P-Rex1 overexpression in RAW cells, were performed using electroporation. Western blotting was used to assess P-Rex1 expression, and RNA-seq to evaluate the impact of P-Rex1 deletion on macrophage gene expression and signaling.

Data and Results: TCM from LLC cells induced TAM-like differentiation in both BMDM and RAW cells, characterized by a marked upregulation of Arg1 and vascular endothelial growth factor, a key factor in angiogenesis and tumor progression, with minimal changes in iNOS expression. TCM treatment also downregulated P-Rex1 expression in BMDMs. Notably, P-Rex1 deletion in BMDMs and P-Rex1 silencing in RAW cells enhanced TCM-induced TAM-like differentiation. In contrast, overexpression of P-Rex1 attenuated this differentiation. Although P-Rex1 deletion did not affect the phagocytic ability of naïve macrophages, it significantly reduced the phagocytic capacity of TAM-like macrophages. P-Rex1 deletion also promoted macrophage migration toward tumor cells, enhanced TAM suppression of CD8+ T-cell migration and contributed to T-cell exhaustion. RNA-seq combined with gene set enrichment and pathway analysis revealed that P-Rex1 deletion not only augments macrophage polarization but also activates several pathways associated with tumor progression, including angiogenesis, immune suppression**,** and inflammation.

Conclusion: Our findings suggest that P-Rex1 plays a pivotal role in regulating the polarization and functional plasticity of TAM within the tumor microenvironment. Loss of P-Rex1 promotes TAM differentiation, impairs their phagocytic capacity, and exacerbates immune evasion by enhancing TAM-mediated suppression of CD8+ T-cell migration and promoting T-cell exhaustion. Taken together, these results highlight the potential of P-Rex1 as a therapeutic target for regulating TAM polarization and enhancing anti-tumor immunity. *(Supported by Nebraska State LB595 Research Program)*