

## Ecto-5'-nucleotidase (CD73) mediates hepatocyte sensitivity to alcohol and alcoholic liver injury in mice

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Alcoholic liver disease (ALD) is a prevalent cause of chronic liver injury in the US, and current treatment relies solely on abstinence and supportive therapy. Ecto-5'-nucleotidase (CD73) is a promising novel molecular target for chronic liver injury. CD73 is a ubiquitous ecto-enzyme that generates adenosine from adenosine monophosphate. Previous studies on CD73<sup>-/-</sup> mice suggested that CD73 contributes to alcohol-induced steatosis, but the mechanisms remain unknown. Here, we generated the first conditional hepatocyte-specific CD73 knockout (CD73 cKO) mice, which are viable and fertile. We found that at 5-9 months of age, the CD73 cKO mice develop spontaneous liver injury marked by steatosis and elevated serum level of alanine aminotransferase (ALT). In female mice aged 5-9 months, the ALT values were 68±17 and 133±34 U/L in WT versus cKO mice, respectively (not statistically significant). Male WT and cKO mice had ALT levels of 67±20 and 195±53 U/L, respectively (p<0.05). Given these baseline phenotypes, we hypothesized that the male CD73 cKO mice will be predisposed to alcohol-induced liver injury. We used the NIAAA chronic/binge mouse model of ALD, which mimics acute-on-chronic-alcoholic liver injury in patients. The mice were fed a liquid ethanol-containing diet (5% v/v) for 10 days, given an ethanol gavage the following day (5g/kg), and euthanized 9h later. Control mice were fed an isocaloric liquid diet and gavaged with maltose dextrin. In parallel studies, primary mouse hepatocytes were isolated and treated with alcohol *ex vivo*. We found that total liver CD73 mRNA expression increased significantly in the livers from WT mice (p<0.001) but not CD73 cKO mice after ethanol treatment, suggesting that the increase in CD73 was specific to hepatocytes. This was confirmed *ex vivo*, as WT hepatocytes showed significant upregulation (p<0.005) of the CD73 transcript upon alcohol treatment. Although hepatic steatosis was comparable, the CD73 cKO mice showed more severe signs of hepatocyte injury and inflammation than WT mice after ethanol treatment, and had significantly elevated serum ALT levels (p<0.01). Blinded histological analysis of the livers revealed necrosis, vascular injury, and neutrophil infiltration in CD73 cKO, but not WT mice. These data demonstrate that hepatocytes upregulate CD73 in response to ethanol, and that this upregulation serves a protective function in male mice. Future studies will address the sex differences and mechanisms by which hepatocyte-specific CD73 exerts hepatoprotection.

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## **Diacylglycerol kinases eta and iota regulate lipid signaling and mediate responses to pruritogens**

Vicki Bartsch

Chronic pain and chronic itch severely impair daily function and quality of life. Current treatments lack sufficient efficacy and have serious side effects. Characterizing the mechanisms by which painful and pruritogenic (itch-causing) stimuli are processed may reveal candidate molecules to target with novel pharmacological treatments. Pain- and itch-sensing neurons (nociceptors) whose cell bodies lie in the dorsal root ganglia (DRG) detect noxious stimuli in peripheral tissues and transduce those signals to the central nervous system. Inflammatory mediators released after injury or in the context of skin disorders both activate and sensitize nociceptors, shifting patients toward a state of chronic sensitivity that can persist in the absence of a stimulus. These inflammatory mediators activate receptors on DRG neurons that can yield diacylglycerol (DAG) and monoacylglycerol (MAG) production. DAG and MAG are important signaling mediators in somatosensory neurons, as well as immune cells that respond to noxious stimuli. I have discovered that diacylglycerol kinase (DGK) can phosphorylate MAG in addition to phosphorylating DAG. Altering levels of DAG, MAG, or their metabolites has been shown to cause differences in pain signaling. Therefore, I am interested in determining if DGK's phosphorylation of DAG and MAG can lead to changes in somatosensation. I am investigating two DGK isozymes (eta,  $\eta$ , and iota,  $\iota$ ) that I found to be highly expressed in the pain- and itch-sensing DRG neurons of mice and humans. Using DGK $\eta$  and DGK $\iota$  knockout mice, I confirmed that kinase activity on DAG and MAG is reduced in DRG neurons. Behavior analyses demonstrate that these knockout mice have differential responses to pruritogens, while mechanical and thermal pain sensitivities are unaltered. *In vitro* signaling experiments coupled with *in vivo* behavioral assays reveal the role of DGK $\eta$  and DGK $\iota$  in somatosensation and their potential as targets for itch therapies.

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## **Unique phosphorylation sites on GFAP reveal Casein Kinase 2 as a novel target in Alexander Disease**

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Alexander Disease (AxD) is a rare and fatal leukodystrophy characterized by white matter abnormalities, developmental delays and seizures. AxD is caused by autosomal mutations in glial fibrillary acidic protein (GFAP), the major intermediate filament (IF) protein in astrocytes. The cellular hallmark of AxD astrocytes are Rosenthal Fibers (RFs), which are aggregates of GFAP, ubiquitin, and small heat shock proteins. Our major goal is to understand the triggers and consequences of RF formation. We hypothesized that AxD mutations promote aggregation by disrupting key post-translational modifications (PTMs) on GFAP. The goal of this study was to identify PTMs unique to mutant GFAP and to determine how these sites contribute to aggregate formation in the context of AxD. We

performed proteomic phospho-mapping on GFAP extracted from human control (non-diseased) and AxD brain tissue. In parallel studies, wild type (WT) or AxD associated GFAP mutants (R79H, R79C, R88C, R239H, R239C, E373K, R416W) were expressed SW13 vimentin-negative cells, which lack all endogenous cytoplasmic IFs and GFAP was analyzed by confocal imaging, and biochemically by high salt extraction followed by isoelectric focusing (IEF) and tandem mass spectrometry (MS/MS). GFAP phosphorylation was modulated by site-directed mutagenesis and pharmacologic kinase inhibition. We found that ectopic expression of GFAP mutants led to prominent intracellular aggregates and promoted cell death. IEF analysis revealed charged GFAP species unique to the AxD mutants, but absent from the WT protein, while MS/MS analysis identified Serine 16 (S16), located in the N-terminal “head” domain of GFAP, as a common modified residue among multiple AxD GFAP mutants in cells and in patient samples. Mutagenesis of S16, which is a predicted casein kinase 2 (CK2) target, blocked AxD mutant GFAP aggregation when rendered non-phosphorylatable (S16A) and promoted aggregation when it was substituted for phospho-mimetic residue (S16D). Furthermore, the CK2-selective inhibitor tetrabromocinnamic acid (TBCA) reduced total GFAP expression and blocked mutant GFAP aggregation in an over-expression system. In AxD patient brains, but not normal controls, we found significant ( $p < 0.01$ ) dimerization of the regulatory subunit of CK2 (CK2 $\beta$ ), which is known to be a key factor in sustaining CK2 activity. In conclusion, our study identified a novel CK2 phosphorylation site on GFAP that promotes GFAP aggregation and implicates CK2 as a novel target for AxD.

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### **Centrosome Number Affects Endothelial Cell Junctions and Polarity**

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Although the role of centrosomes in spindle orientation during mitosis has been well established, their importance in interphase cells remains less clear. Also called the microtubule organizing center (MTOC), the centrosome consists of two centrioles surrounded by a pericentriolar material from which microtubules are nucleated. Centrosome homeostasis in endothelial cells (EC) is disrupted when centrosomes are lost or when there are too many centrosomes (>2) present within a cell. Excess centrosomes have been seen in tumor microvasculature, which is often leaky and has improperly formed lumens. Our previous work revealed that excess numbers of centrosomes in EC leads to increased microtubule catastrophes and repolarization

defects in sprouting angiogenesis. To better understand the importance of centrosome homeostasis in non-dividing cells, we sought to further characterize the effects of excess centrosomes and centrosome loss on human umbilical vein endothelial cells (HUVEC). We found that when cultured on micropatterns, EC with >2 centrosomes displayed repolarization defects and had increased VE-cadherin area, suggesting more activated adherens junctions. Furthermore, we found that centrosome overduplication in a zebrafish model resulted in a lack of lumen formation. In addition, we found that pharmacological centrosome depletion via centrinone, a Plk4 inhibitor, led to defects in a sprouting angiogenesis assay. Understanding how the centrosome regulates various EC functions will provide insights on the importance of this structure both pathologically and during normal vascular development.

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### **Studying the functions of alternative splicing regulation of *Clathrin Heavy Chain (Cltc)* and *Clathrin Light Chain A (Clta)* in muscle development using CRISPR mice**

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Alternative splicing is a posttranscriptional mechanism that produces multiple protein isoforms from a single gene. Trafficking genes, such as, *Clathrin Heavy Chain (Cltc)* and *Clathrin Light Chain A (Clta)* employ this mechanism to regulate splice variants specifically in striated muscle<sup>1</sup>. *Cltc* and *Clta* work in conjunction in the cell to mediate endosomal sorting and endocytosis<sup>2</sup>. The *Cltc* gene has a splice variant that includes a 21-nucleotides (nt) exon near the C-terminus of the protein. Likewise, the *Clta* gene contains two alternatively spliced exons (36 nt and 54 nt) located between the *Cltc* binding region and the calmodulin-binding domain<sup>2</sup>. Our aim is to identify the physiological roles of the regulation of these exons by alternative splicing in a developmental-stage and tissue-specific manner. Using CRISPR/Cas9 technologies, we can address this question by blocking fetal-to-adult splicing transitions in a mouse model. We generated mice where the alternative spliced exons are deleted at the genomic level. Thus, homozygous mice can only express the fetal isoforms, while their wild type littermates gradually include the alternatively spliced exons (Giudice, Koushiuk, Blue, Cooper, in prep). We first characterized the splicing patterns of these Clathrin specific exons during development in different tissues including heart, various skeletal muscles, liver, brain, pancreas, intestine, spleen, lung, and kidney (unpublished data). These experiments revealed that *Cltc* (exon 21nt) was spliced out during neonatal stages and included in adulthood specifically in heart, skeletal muscles, and brain. Additionally, our data showed that inclusion of *Clta* (exon 54nt) is brain specific, whereas *Clta* (exon 36nt) is gradually included during development of the skeletal muscles, heart, and brain. At the physiological level our first

observations indicate that *Cltc* CRISPR homozygous mice exhibit protection to cardiac hypertrophy induced by transverse aortic constriction, while also showing a slight increase in muscle weights in comparison with their wild type littermates. This difference in muscle weight may be associated with the increase in the myofiber area isolated from the *flexor digitorum brevis* (FDB) muscles of homozygous vs wild type littermates. We intend to fully characterize these animals in terms of their physiology. We also aim to explore the cell and molecular biology angle of this project because it will allow us to learn new aspects of *Cltc* and *Clta* functions (and the different splice variants) in muscle and neuronal contexts.

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### Determining the Role of Fragile X-Related Protein 1 (FXR1) on Muscle Cell Differentiation and How it is Regulated by Alternative Splicing

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**Background** Alternative splicing is a posttranscriptional process that allows a single gene to produce multiple proteins. Fragile X syndrome, which affects 1 in 5000 people, is the most common cause of inherited mental retardation in the world. It is caused by a mutation in the gene encoding for the RNA-binding protein Fragile X Mental Retardation 1 (FMR1). The Fragile X-Related Protein 1 (FXR1) is the autosomal homolog of FMR1 and the member of the family most highly expressed in muscles. FXR1 is required for postnatal survival and during the early stages of mouse embryogenesis is highly expressed but the functional significance of the protein is still unclear. Four regions of the FXR1 gene can be alternatively spliced: an 87 nt insert within exon 12, the first 78 nt of exon 13 (alternative 3' splice site), and the cassette exons 15 (81 nt) & 16 (92 nt). Abundance of FXR1 isoforms vary across cell and tissue types with some of the highest expression being in skeletal muscle and heart. Misregulation of FXR1 splicing is present in patients with muscular dystrophy and diabetes. **Hypothesis** We hypothesize that FXR1 regulates striated muscle development through translational control of specialized cytoskeletal structures and that alternative splicing is one of those mechanisms. **Results** To test this hypothesis, we performed reverse transcription PCRs and found that FXR1 is regulated by alternative splicing during postnatal development and that the balance

between the splice isoforms vary from tissue to tissue. Notably, exons 15 and 16 are progressively included in different skeletal muscle types following the transition from neonatal to adult stages. We confirmed the presence of different protein isoforms through Western blots. Depletion of FXR1 prevents differentiation of C2C12 myoblasts into multinucleated myotubes confirming the essential role of FXR1 in muscle biology. Currently we are characterizing the phenotypes that arise from the various splice variants through knocking down FXR1 in C2C12 cells and also modulating splicing decisions using antisense oligonucleotides. We are performing gene expression analysis to explore potential targets of the different FXR1 splice variants. **Conclusion** Elucidating the role of FXR1 on muscle cell differentiation and how splicing affects that role will be a step towards improving diagnosis for muscle related diseases.

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### **Calcitonin receptor-like receptor regulates intestinal lymphatic lipid homeostasis**

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Dietary fat constitutes a vital macronutrient and its absorption requires a regulated concert between the intestinal lymphatic vessels, epithelium, enteroendocrine cells, and the enteric nervous system. Most studies explore these systems in isolation, therefore the exact nature of their interaction to promote satiety, fat absorption or dysregulation of the process in metabolic syndrome remains unclear. Calcitonin receptor-like receptor (gene: *Calcrl*) is a GPCR that can bind either adrenomedullin (with coreceptor Ramp2) to regulate lymphangiogenesis or the neuropeptide CGRP (with coreceptor Ramp1) to alter satiety, intestinal blood flow and smooth muscle contractility. We found that deletion of this critical receptor from the lymphatic and enteroendocrine cells using the inducible *Prox1-CreERT2* system in adult mice leads to inefficient absorption of dietary fat. While high fat diet consumption remained the same, MRI scans of *Calcrl<sup>fl/fl</sup>/Prox1-CreERT2* animals showed significantly reduced fat mass but no change in lean mass compared to *Calcrl<sup>fl/fl</sup>* controls. *Calcrl<sup>fl/fl</sup>/Prox1-CreERT2* mice also had significantly reduced gonadal fat, subcutaneous fat, and white adipose cell size. Oil red O staining showed fewer lipids entering the intestinal lacteals in *Calcrl<sup>fl/fl</sup>/Prox1-CreERT2* mice compared to controls. BODIPY FL C16 (long-chain fatty acid analogue) gavage in these animals shows the accumulation of fatty acid binding proteins towards the intestinal lumen side of enterocytes in *Calcrl<sup>fl/fl</sup>/Prox1-CreERT2* animals compared to being dispersed throughout in controls. Furthermore, the enteric nerves that innervate the submucosal collecting lymphatic vessels demonstrate patterning defects in the *Calcrl<sup>fl/fl</sup>/Prox1-CreERT2* animals compared to controls. Collectively, our data demonstrates that *Calcrl* critically balances fat processing and absorption through its role in both the intestinal lymphatic system and nervous system.

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## Studying the functions of SNAP23 protein and its regulation by alternative splicing in muscle cell differentiation

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Alternative splicing impacts over 90% of human genes, which allows for a breadth of proteins produced from a finite number of genes<sup>1,2</sup>. Within tissues, developmental splicing networks are specific for certain cell-types and/or regions of the tissue<sup>3</sup>. It was previously demonstrated that in heart development, genes involved in vesicular trafficking and membrane remodeling are the most significantly regulated by alternative splicing, primarily in cardiomyocytes<sup>4</sup>.

Within this trafficking network, splicing transitions have been identified in the *Snap23* gene<sup>4</sup>. SNAP23 is the synaptosomal associated protein-23, a member of the SNARE complex. SNAP23 is involved in exocytosis, particularly in the fusion of transport vesicles with the plasma membrane before cargo release<sup>5</sup>. SNAP23 controls glucose metabolism through the regulation of the glucose transporter GLUT4 dynamics in adipocytes<sup>6</sup>. Furthermore, SNAP23 is involved in regulation of insulin sensitivity through the fatty-acid-mediated pathway<sup>7</sup>.

Here, we aimed to characterize SNAP23 functions in muscle cell differentiation and the role of each splice isoform in a striated muscle context. We demonstrated that *Snap23* alternative exon containing 33 nucleotides is gradually included throughout development in heart and different types of skeletal muscles. This exon is not included in other tissues that we tested, such as brain, pancreas, intestine, liver, and spleen. Furthermore, we showed that alternative splicing of *Snap23* gene is conserved in human heart and skeletal muscle postnatal development. This data suggests that alternative splicing of *Snap23* might have striated muscle specific functions that are evolutionarily conserved.

Knockdown experiments in C2C12 muscle cells revealed that SNAP23 is essential for cell viability and differentiation of myoblasts into myotubes. We are seeking to determine the molecular mechanisms linking SNAP23 with the observed cellular phenotypes. We hypothesize that *Snap23* is involved in the secretion of insulin-like growth factors (IGFs), which are required for C2C12 cell viability and differentiation. We plan to treat SNAP23 depleted cells with exogenous IGFs to determine whether we can rescue the observed defects in cell viability and differentiation.

C2C12 cell differentiation robustly reproduced the gradual inclusion of exon of 33 nucleotides that we observed in heart and skeletal muscle development. Therefore, we plan to modulate *Snap23* endogenous splicing by using morpholino antisense oligonucleotides. Morpholinos bind to either 5' or 3' alternative splice sites, blocking their

recognition. In consequence, the alternative exon will always be skipped. Using this strategy, we will determine the functions of *Snap23* splicing cell viability and myoblast differentiation into myotubes. In conclusion, this combined data may elucidate the molecular functions of each SNAP23 splice isoform in myogenesis.

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## Implementation of patient-derived cell culture models for use in a multiple-assay platform to advance precision medicine strategies in cystic fibrosis

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Cystic Fibrosis (CF) is one of the most widespread life-shortening genetic diseases with autosomal recessive inheritance. CF leads to lung and digestive system damage. The CFTR gene, which is mutated in CF patients, codes for a chloride channel that is important for proper hydration of epithelial surfaces. Advancements of CF research, therapeutic discovery, and clinical treatments have provided a proof of principle that research-based understanding can guide strategies that predict pharmacological interventions that successfully target CFTR chloride channel function in CF patients. There are more than 2,000 CFTR variants and recent insights into mechanisms that govern CFTR function and structure have supported development of effective CFTR therapeutics, e.g., corrector and potentiator compounds. Correctors partially repair defective protein processing and increase trafficking of mutant CFTR to the cell surface, while potentiators increase the activity of defective CFTR at the cell surface. The FDA approval of such CFTR therapeutics, which are suitable for many patients, is a breakthrough towards the ultimate goal to provide a cure for all CF individuals. Because so many different CFTR mutations can cause disease, and even patients with the same mutation can respond differently to the same treatment, reliable screening tools capable of predicting drug efficacy to support individualized treatment plans in the clinic are essential. We have developed a sophisticated assay platform that utilizes bronchial, nasal, and rectal tissues from individual CF patients for drug testing in *in vitro* assays, including evaluation of ion and fluid movement in 3D spheroid/organoid cultures,

biochemical evaluation of CFTR folding, and electrophysiological measurements of CFTR activity, all of which advance the prediction of patient-specific responses. In addition, incorporation of novel assays that monitor other aspects of successful CFTR rescue such as restoration of mucus characteristics, which is important for predicting mucociliary clearance, allow for better prognosis of success of therapies *in vivo*. Furthermore, for precise prediction of drug effects, advanced key *in vitro* models of CFTR rescue have been implemented that 1) incorporate the inflamed CF airway environment and 2) mimic the complex tissue architecture of airways, both of which affect responses to CFTR drugs.

In conclusion, we have developed a powerful patient cell-derived multiple-assay platform to achieve efficient usage of donor specimens by incorporating numerous physiological readouts for precision medicine analyses of drug efficacy (Cholon and Gentsch 2017 *Journal of Cystic Fibrosis*, <https://doi.org/10.1016/j.jcf.2017.09.005>, *Epub ahead of print*). This multiple-assay platform is an important translational approach as it effectively bridges laboratory research with clinical outcomes, promoting substantial progress towards improved quality of life for all CF patients.

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Reem Hakeem  
PI: James E. Bear

Directional cell motility is indispensable for embryonic development, immune response, wound repair, and cancer metastasis. Cells encounter a variety of cues that can serve as navigational guides, directing the cell where to migrate. We have previously harnessed micro-fabrication techniques to elucidate how a cell responds to soluble cues (chemotaxis) and substrate-bound cues (haptotaxis). We determined that both mechanisms of directional migration require distinct molecular ‘first responders,’ though different cues can employ alternative signaling pathways. For example, Arp2/3, a key regulator of actin dynamics, is critical for haptotaxis but not for chemotaxis<sup>1</sup>. While progress has been made understanding the molecular underpinnings of chemo- and haptotaxis, how a cell can sense and respond to changes in the mechanical properties of its microenvironment (durotaxis) remains elusive. Durotaxis is founded on the observation that cells seem to move by ‘feel’ from soft to stiff matrix<sup>2</sup>. The lack of an efficient platform that allows us to fully investigate cytoskeletal dynamicity as cells undertake a range of physical cues has hindered the field’s understanding of durotactic behavior. We address this issue by engineering photopolymerizable hydrogels that possess tunable gradients of stiffness. We hypothesize that hydrogel stiffness gradients present a more robust model for investigating durotactic migration than current ‘gradient-free’ model systems, as it allows us to track the transformation of molecular machinery with spatial precision. Using our UV-based system, we have obtained stiffness gradients that range from 1 to 70 kPa. Contrary to recent findings that argue for collective durotactic migration<sup>3</sup>, our preliminary data demonstrates that individual cells can durotax across a rigidity gradient, showing highest persistence between 8-25 kPa. Our aim is to

dissect the key molecular players of durotaxis with the intent of furthering the field's understanding on how cells navigate their environment. In addition, we hope that providing mechanistic insight on durotaxis will help bridge the role of physical cues in the development of metastasis and fibrotic diseases, to eventually pave the way for novel targeted therapeutics.

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CBP Research Day

#### **Abstract**

Effective cell migration, which plays an essential role in many physiological events, including tissue morphogenesis, wound healing and inflammatory responses, is dependent on dynamic remodeling of the actin-cytoskeleton. Type I coronins, a conserved class of WD40-repeat proteins, have been shown to facilitate this process by modulating the turnover of branch-actin networks at the leading edge. Recent work suggests that the Type I coronins, Coro1B and Coro1C, mediate the formation of membrane protrusions during cell migration and the stabilization of branched-actin networks within these protrusions. Despite these novel findings, however, our understanding of the function of Coro1B and Coro1C in directed migration remains incomplete. We therefore *hypothesize* that Coro1B and Coro1C regulate branched-actin dynamics during chemotaxis and haptotaxis. Using mouse tail fibroblasts that conditionally expressed Coro1B and Coro1C, we established matched-pair cell lines with and without coronins. To assess the effect of Coro1B and Coro1C on the locomotory behavior of cells during chemotaxis and haptotaxis, we utilized microfluidic chambers that generate chemically diffused and substrate-bound gradients, respectively. In addition, we used live-cell imaging and kymography to examine lamellipodia morphodynamics in the matched-pairs. In the absence of Coro1B and Coro1C, cells lack

directionality and migrate more slowly than their WT counterparts. Coro1B and Coro1C null cells also have lower protrusion durations and increased protrusion rate as compared to WTs. We conclude that Coro1B and Coro1C are highly involved in regulating actin dynamics at the leading edge which is required for effective lamellipodial formation and directed migration.

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## **The role of endothelial MERTK in leukocyte transendothelial migration and the inflammatory response**

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### **Abstract:**

Leukocyte transendothelial migration (TEM) is an important process during inflammation and immune response. During leukocyte transmigration, endothelial cells (ECs) form apical membrane protrusions called transmigratory cups surrounding the adherent leukocyte to facilitate TEM. The morphological similarity between the transmigratory cup and phagocytic cup led us to MERTK, a receptor tyrosine kinase known to regulate actin reorganization in macrophages during phagocytosis. Despite extensive documentation of MERTK's involvement in immune response and tumor progression, few studies have illuminated its function in the vasculature system. My study evaluates the role of **endothelial** MERTK in leukocyte TEM and the inflammatory response both *in vitro* and *in vivo*. My preliminary analysis with *in vitro* TEM demonstrated that more transmigration events were observed when MERTK is removed from ECs. Further examination revealed that ECs devoid of MERTK failed to restore tight junctions after leukocyte TEM. Consistent with these *in vitro* findings, *in vivo* experiments assessing the lung endothelium permeability in healthy mice demonstrated that vasculature in *Mertk*<sup>-/-</sup> mice were more leaky than that of wild type mice. Based on these data, I hypothesized that endothelial MERTK affects leukocyte TEM by regulating junction integrity and actin cytoskeleton. To further test my hypothesis, I will evaluate EC permeability changes during TEM *in vitro* using siRNA knockdown or MERTK inhibitors. I will also analyze lung endothelium permeability and leukocyte transmigration during pneumonia in EC-specific *Mertk*<sup>-/-</sup> mice. Results from my study will characterize novel functions of MERTK in ECs and identify potential pharmaceutical targets for inflammatory diseases.

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## Role of B Cell Receptor signaling in Pancreatic Ductal Adenocarcinoma

Dan Michaud

### Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) features a prominent pro-tumorigenic microenvironment mediated by intercellular signaling between cancer cells and the stroma. A prominent result of intercellular signaling in PDAC tumors is the suppression anti-tumor immune responses to allow for rapid tumor growth. Current immunotherapeutic approaches have been unsuccessful in improving PDAC patient outcomes, leaving a need for better understanding of immunomodulatory signaling mechanisms in PDAC tumors. We have demonstrated that a subset of the immune infiltrate consists of regulatory (CD5<sup>+</sup>CD1d<sup>Hi</sup>) B cells, characterized by their expression of the immunosuppressive cytokine IL35, suppress anti-tumor immune responses within the microenvironment. Genetic deletion of IL35 from B cells lead to significantly decreased pancreatic tumor growth, therefore demonstrating that IL35 B cells are highly important in PDAC. However, the exact mechanisms how tumor cells cause regulatory B cells to secrete IL35 in the tumor microenvironment is not understood. My preliminary data suggests that immunosuppressive cytokine release is exacerbated by engagement of the B cell receptor (BCR) on regulatory B cells *in vitro*. Furthermore, using a mouse model harboring a BCR incapable of sensing mouse antigen, I observed both significantly smaller pancreatic tumors and decreased IL35<sup>+</sup> intratumoral B cells than wild-type controls underlying the importance in BCR signaling in PDAC tumor growth. Here we show preliminary data and outline a plan for establishing a signaling axis between PDAC cells and regulatory B cells resulting in immunosuppression of the tumor microenvironment via IL35. We hypothesize that the production of tumor-derived factors capable of engaging BCR's on regulatory B cells leads to induction of cytokine expression. Our experiments will determine the molecular circuitry that underlies induction of IL35 expression in regulatory B cells.

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## The role of CD73 in Non-Alcoholic Fatty Liver Disease

Marquet Minor, Monea Richardson, Karel Alcedo and Natasha Snider

Non-alcoholic fatty liver disease (NAFLD) is a spectrum disorder with the most severe form being non-alcoholic steatohepatitis (NASH), which is characterized by fat accumulation in the liver (steatosis), chronic inflammation, and fibrosis. NAFLD is highly prevalent, but there are no effective treatments. Ecto-5'-nucleotidase (CD73) represents a novel molecular target for liver diseases of multiple etiologies. CD73 is a ubiquitously expressed glycosylphosphatidylinositol (GPI)-anchored enzyme that generates

extracellular adenosine through the metabolism of adenosine monophosphate. The goal of the present study was to evaluate the role of CD73 in NAFLD models. In WT C57BL/6J mice fed a high fat diet (HFD), we found that total CD73 protein expression increased by 2-fold in response to HFD ( $p < 0.05$ ). In contrast, CD73 ecto-AMPase activity significantly decreased, as determined by a qualitative enzyme histochemistry assay. To examine the cell-specific roles of CD73 in the liver, we generated hepatocyte-specific CD73 knockout mice, which develop age-dependent spontaneous liver injury marked by steatosis. Given the baseline phenotype of the mice, we hypothesized that hepatocyte-specific loss of CD73 will augment HFD-induced liver injury. In female mice 16-week HFD feeding increased body weight, but decreases the liver/body weight ratio ( $p < 0.0001$ ) in both the WT and CD73KO mice that were fed the HFD compared to mice that were fed a control diet. Serum analysis revealed that cholesterol levels increased in both WT and CD73KO mice ( $p < 0.0001$ ), while serum albumin levels increased only in CD73KO mice given the HFD ( $p < 0.001$ ). Liver enzymes, such as alanine aminotransferase (ALT) and alkaline phosphatase (ALP), displayed differential effects, with ALT ( $p < 0.001$  in WT,  $p < 0.01$  in CD73KO) levels increasing and ALP ( $p < 0.0001$ ) levels decreasing in both WT and CD73KO mice fed the HFD. In conclusion, total CD73 expression and enzyme activity exhibit opposite regulation in the setting of hepatic steatosis, suggesting potential non-enzymatic functions. Ongoing histological and biochemical evaluation of the hepatocyte-specific CD73 KO mice will reveal additional mechanisms by which CD73 may regulate hepatic steatosis. Ultimately, further insight into the molecular mechanism of CD73 will lead to foundational knowledge that can be applied to chronic liver diseases including NAFLD.

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## Modeling Myocardial Edema in Mice

Natalie Nielsen

The heart is covered with a vast network of lymphatic vessels that help drain interstitial fluid and regulate myocardial fluid balance. In many disease states or surgical interventions, such as pulmonary hypertension, myocardial infarction, coronary bypass surgery, and cardioplegic arrest, the forces that regulate fluid homeostasis in the heart are disrupted. This causes accumulation of fluid within the interstitium and causes swelling, or edema, in the heart. Surgically induced myocardial edema in large animal models, through ligation of the coronary sinus, has been shown to increase cardiac fibrosis and decrease total cardiac output. Here we show that we can model myocardial edema in mice through coronary sinus cauterization (CSC), both through wet:dry ratios and histologically. In echocardiograms, CSC does not appear to have any impact on cardiac function, but does seem to increase the number of visible lymphatic vessels in the heart. This study establishes a novel method to study myocardial edema in mice without massive cell death, as is seen with traditional myocardial infarction models. Once

established, this injury model in combination with genetically modified mice will offer new insights into genes regulating cardiac lymphatics, injury repair, and heart function.

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### **Dissecting the function of classical cadherins in stratified epithelial morphogenesis.**

Carlos Patino Descovich

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 (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.

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## LYMPHATIC MIMICRY PROMOTES PLACENTAL SPIRAL ARTERY REMODELING

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Spiral arteries (SA) of the placenta tightly regulate blood flow to meet fetal nutritional and oxygenation needs. As pregnancy progresses, SAs remodel into low resistant, high flow vessels to support increased fetal demands. Poor SA remodeling contributes to a highly prevalent and potentially fatal hypertensive disease called pre-eclampsia that is associated with fetal growth restriction and long-term health complications for mom and baby. Using a combination of *in vitro* and *in vivo* techniques, we demonstrate that SAs express lymphatic markers, including VEGFR3, during SA remodeling and that this process is dependent on VEGFR3 signaling. We also find that essential promoters of SA remodeling – uterine natural killer cells, which secrete the VEGFR3 ligand VEGFC – stimulate endothelial VEGFR3. Consistently, mice lacking uterine natural killer cells demonstrate blunted ERK1/2 signaling, which occurs downstream of VEGFR3 activation. We then turned to adrenomedullin, a peptide that is essential for placental development and SA remodeling, as a potential factor that may interact with VEGFR3. By proximity ligation assay, we find that adrenomedullin signaling is capable of transactivating VEGFR3 via c-Src-mediated phosphorylation in cultured endothelial cells. Having identified a novel mechanism by which the placenta promotes SA remodeling, this work reveals new targets that may lead to new treatments against the pathological origins of pre-eclampsia.

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

Melissa Plooster, Guendalina Rossi, Martilias Farrell, Patrick Sullivan, Stephanie Gupton and Patrick Brennwald

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.

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## **A novel function of the inflammasome in axon pruning**

**Selena Romero**

During nervous system development, neurons undergo constant remodeling, both creating and destroying axons and axon branches. Axons/axon branches are degraded through a process called axon pruning, which results in the degeneration of specific

axons/axon branches while the neuronal cell bodies remain intact and healthy. Axon pruning is not only important during nervous system development, but is also associated with many neurodevelopmental diseases such as Parkinson's and Alzheimer's disease.

While axon pruning has received considerable attention in recent years, the exact mechanisms that control axon pruning remain unknown. Specifically, the mechanisms by which caspases are activated during axon pruning remain a mystery. During apoptosis, where the entire neuron degenerates, caspases are activated by a protein complex called the apoptosome. However, we have previously shown that the apoptosome is not required during axon pruning. Thus, the mechanisms of caspase activation during axon pruning require further study.

While investigating this question, I discovered a novel function of Caspase-1 (an inflammatory caspase), and the inflammasome pathway, in axon pruning. Using an *in vitro* microfluidic model of axon pruning I have shown that inhibition or deletion of Caspase-1 prevents axon pruning. Caspase-1 is normally activated by a group of protein complexes known as inflammasomes, thus initiating an inflammatory response to pathogen exposure. While Caspase-1 and inflammasomes have been studied in the context of innate immunity, the Caspase-1 and inflammasome pathways have not been investigated in the context of axon pruning. Interestingly, I have found that deletion of NLRP1, an inflammasome protein, similarly inhibits axon pruning *in vitro*. These exciting findings may provide a mechanism by which caspases are activated during axon pruning.

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### **Banana Pudding Flavored E-liquid, Ethyl Vanillin, Vanillin, and Ethyl Maltol Alter Store-Operated Ca<sup>2+</sup> Entry in Airway Epithelia**

*Temperance R. Rowell\**, Bryan T. Zorn, James Keating, Gary L. Glish, Stephen B. Shears,  
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CBP Research Day 2018

E-cigarettes are available in over 7,000 flavors, yet there is still much to understand regarding their cellular effects. We purchased e-liquids from commercial vendors and characterized their biological effects on primary human bronchial epithelial cultures, CALU3 airway epithelia, and HEK293T cells. Intracellular Ca<sup>2+</sup> signaling is tightly regulated and dysregulation is associated with autoimmune disease and cancer. Specifically, store-operated Ca<sup>2+</sup> entry (SOCE) functions downstream of cell surface receptors [i.e., G protein-coupled receptors (GPCR), receptor tyrosine kinases (RTK)], allowing extracellular Ca<sup>2+</sup> entry through STIM1/Orai1 protein aggregation and activation of downstream kinases (e.g., PKC $\alpha$ ). We therefore measured changes in Ca<sup>2+</sup> signaling for new biomarkers of e-liquid exposure and their individual flavoring chemicals.

Cells were loaded with cytosolic and mitochondrial  $\text{Ca}^{2+}$  dyes and changes were recorded after exposure to e-liquids diluted in media, aerosol generated from heated e-liquids, or flavoring chemicals. Total inositol phosphate (InsP) accumulation was determined by measuring [ $^3\text{H}$ ]InsP species in cells. STIM1 and Orai1 puncta were visualized in transfected HEK293T cells. Kinase phosphorylation was detected by Western blot. Chemical constituents were identified by gas chromatography-mass spectrometry. 100 e-liquids were screened for cytosolic  $\text{Ca}^{2+}$  responses and combined with chemical constituent data in a random forest classification model to predict chemicals that may cause the cytosolic  $\text{Ca}^{2+}$  responses.

Banana Pudding (BP) flavored e-liquid acutely elicited increases in InsP production that triggered downstream release of ER  $\text{Ca}^{2+}$  and subsequent SOCE. These changes caused aggregation of STIM1/Orai1 and altered phosphorylation of PKC $\alpha$  acutely, but decreased SOCE after 3h. InsP production was independent of RTK activation, likely meaning that BP e-liquid either activated an upstream GPCR or phospholipase C directly. Additionally, we found that 42 of 100 e-liquids screened elicited a cytosolic  $\text{Ca}^{2+}$  signal. Ethyl vanillin, vanillin, and ethyl maltol (caramel) flavoring chemicals were the top constituents identified from our model and were able to dose-dependently elicit cytosolic  $\text{Ca}^{2+}$  signal when tested individually. These chemicals were also detected in the BP e-liquid.

We demonstrated that the BP e-liquid elicited significant changes in  $\text{Ca}^{2+}$  signaling, likely downstream of a GPCR. We also showed that SOCE responses were blunted with longer BP exposures, and that cytosolic  $\text{Ca}^{2+}$  responses were elicited by many e-liquids in our screen. Moreover, chronic changes in  $\text{Ca}^{2+}$  homeostasis are predicted to have dramatic effects on the lung including chronic inflammation (e.g., increased cytokine/mucin secretion) and altered cell growth/proliferation. Taken together, these data show e-liquids and their constituents can alter important airway cell signaling pathways. These data could be used to inform regulations regarding e-cigarette flavorings and toxicity.

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## Characterizing the Intratumoral Heterogeneity in Mucoepidermoid Carcinoma

Kshitij Sharma

Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland cancer (affecting 1 in 100,000 >50yrs) and is characterized by significant *intra*-tumoral cellular heterogeneity. This heterogeneity is marked by the presence of at least 6 distinct cell types (as identified by H&E staining), with mucus, squamous and intermediate cells reflecting the predominant populations. The former two are thought to be terminally differentiated whereas the intermediate cells are considered to be mitotic and the source of these differentiated cell types. Thus, the relative proportions of intermediate cells

correlates with disease severity however the molecular basis underlying the regulation of this population remains elusive. Notably, >55% of MEC's are attributed to a chimeric CRTC1/MAML2 (C1/M2) oncoprotein created by a recurrent t(11;19) chromosomal translocation. C1/M2 is comprised of the CREB binding domain of the CREB transcriptional co-activator CRTC1 fused to the TAD of the NOTCH transcriptional co-activator MAML2. While the role of NOTCH signal dysregulation in MEC currently remains unclear, we and others have established that C1/M2 functions as a constitutively active CREB co-activator and also identified an unexpected and novel *de novo* gain of function interaction between C1/M2 and MYC oncoprotein. Furthermore, the lack of additional genomic alterations or somatic mutations in C1/M2 positive MEC, highlights the importance of transcriptional rewiring driven by C1/M2 as a key player in this cancer. Since C1/M2 can directly bind and upregulate both CREB (classically associated with differentiation) and MYC (classically associated with proliferation) regulated genes, it is important to dissect the functional contributions of these two pathways in fueling MEC heterogeneity and growth. Given the canonical roles of CREB and MYC signaling in maintaining a differentiated versus stem like state respectively, *we hypothesize that C1/M2 mediates distinct transcriptional profiles in the intermediate (MYC driven) versus the mucus/squamous (CREB driven) cell types.* Understanding the difference in the transcriptional profiles of these distinct cell types and the key pathways that maintain the differentiated/stem like state will enable us to improve MEC therapy by specifically targeting the intermediate cells and driving them towards a terminally differentiated state. To elucidate these cell type-specific transcriptional profiles, we performed unbiased single cell RNA sequencing (DDseq) on subcutaneously derived xenografts established from human MEC cell lines. PCA and tSNE was performed to separate out distinct cell types based on their transcriptional states. As a complementary approach, we are also optimizing protocols to isolate the mucus, squamous and intermediate cell types based on their cellular phenotypes (presence of mucus granules, large cytoplasm, and small nuclear:cytoplasm ratios respective).

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Michael Sidorov

Angelman syndrome (AS), a neurodevelopmental disorder associated with intellectual disability, is caused by loss of maternal allele expression of *UBE3A* in neurons. Mouse models of AS faithfully recapitulate disease phenotypes across multiple domains, including behavior. Yet in AS, there has been only limited study of behaviors encoded by the prefrontal cortex, a region broadly involved in executive function and cognition. Because cognitive impairment is a core feature of AS, it is critical to develop behavioral readouts of prefrontal circuit function in AS mouse models. One such readout is behavioral extinction, which has been well-described mechanistically and relies upon

prefrontal circuits in rodents. Here we report exaggerated operant extinction in male AS model mice, concomitant with enhanced excitability in medial prefrontal neurons from male and female AS model mice. Abnormal behavior was specific to operant extinction, as two other prefrontally-dependent tasks (cued fear extinction and visuospatial discrimination) were largely normal in AS model mice. Inducible deletion of *Ube3a* during adulthood was not sufficient to drive abnormal extinction, supporting the hypothesis that there is an early critical period for development of cognitive phenotypes in AS. This work represents the first formal experimental analysis of prefrontal circuit function in AS, and identifies operant extinction as a useful experimental paradigm for modeling cognitive aspects of AS in mice.

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### **Determining the Role of SPAG1 in the Cytoplasmic Assembly of Axonemal Dynein Arms**

Amanda J. Smith, Ximena Bustamante-Marin, Weining Yin, and Lawrence E. Ostrowski

#### **Abstract:**

Functional motile cilia are essential for mucociliary clearance in the respiratory tract, fertilization, and left-right patterning of the body. Dysfunctional cilia are the cause of a rare genetic disorder known as primary ciliary dyskinesia (PCD), which is characterized by neonatal respiratory distress, chronic sino-pulmonary disease, infertility, and laterality defects in about 50% of affected individuals. Over 30 genes associated with ciliary structure, function, and biogenesis have been shown to cause PCD when mutated, but the majority of these proteins' functions are unknown. One gene associated with PCD that has not been extensively studied in airway epithelium is *sperm-associated antigen 1 (SPAG1)*. Mutations in *SPAG1* are known to lead to defective or absent inner and outer dynein arms and a "classic" PCD phenotype [Knowles *et al.* (2013) *Am. J. Hum. Genet.* 93, 711-720]. To help further elucidate the function of SPAG1 and its role in ciliogenesis, we examined SPAG1's expression and localization in primary human airway epithelial cells (hAEC) using droplet digital PCR (ddPCR), immunoblots, immunofluorescence, and structured illumination microscopy. In differentiating hAEC, expression of the full-length isoform of SPAG1 correlates with ciliogenesis, but SPAG1 is not detected in isolated ciliary axonemes. A novel isoform of SPAG1 that may be transcribed from an alternative start site is expressed in both undifferentiated and differentiated hAECs, and therefore, could have a function that is distinct from ciliogenesis. In differentiated multiciliated airway cells, SPAG1 localizes throughout the cytoplasm and specifically near the basal bodies. Thus, due to the dynein arm defects caused by *SPAG1* mutations in PCD-affected individuals, SPAG1's expression pattern,

and SPAG1's localization, we have hypothesized that SPAG1 plays a role in the cytoplasmic assembly of axonemal dynein arms before they are transported into ciliary axonemes. In order to further examine SPAG1's role in this complex process, co-immunoprecipitation studies for SPAG1 in hAECs followed by proteomic analysis were performed. Our data suggests that SPAG1 interacts with other known dynein axonemal assembly factors, including DNAAF1/LRRC50 and DNAAF2/KTU, and a potentially novel dynein axonemal assembly factor, PIH1D2. In examining the expression of these factors using droplet digital PCR (ddPCR) in differentiating hAEC, these proteins, including PIH1D2, follow an expression pattern similar to SPAG1. Further studies examining the co-expression and co-localization of these proteins with SPAG1, along with co-immunoprecipitation studies of exogenous wild-type and truncated SPAG1 with these interactors, will be performed to confirm direct interactions with SPAG1 and to determine SPAG1's interaction domains. Other future directions include creating a SPAG1 knockout cell culture model to use in studies to determine which isoforms of dynein arms require SPAG1 to be assembled and which steps in the dynein arm assembly process require SPAG1.

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## **Investigation of Coronin 1C expression levels on melanoma growth and metastasis**

**Alicia Tagliatela – Bear lab**

### **Abstract**

Melanoma is the deadliest form of skin cancer, claiming an estimated 10,000 lives in the United States alone this year. Although an increased rate of metastasis is the likely explanation for the heightened mortality rate in this disease, we lack a molecular understanding of this process, hindering our ability to effectively prevent metastasis from occurring. Coronin 1C is upregulated in a variety of metastatically aggressive cancers, including melanoma, and is associated with poor prognosis. Coronin 1C is the least-well studied of the Type 1 Coronins, a family of proteins that impact lamellipodia formation and cell migration through regulation of the actin cytoskeleton. Our lab has developed two genetically engineered mouse allowing for tight temporal and spatial control of genetic recombination events leading to melanoma progression with or without Coronin 1C expression in order to identify changes in growth and distant metastases. Preliminary results suggest that the loss of Coronin 1C expression in developing melanoma results in increased occurrence of distant metastases, although expansion of our current *in vitro* work utilizing cell lines derived from these GEMs is necessary to understand the mechanisms driving the phenotypes observed.

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**Automated detection, classification, and verification of distinct modes of exocytosis**

Fabio Urbina and Stephanie L. Gupton

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 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.