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NIH Clinical Center
Adam Harrison
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Progressive and Multi-Path Holistically Nested Neural Networks for Pathological Lung Segmentation from CT Images
Pathological lung segmentation (PLS) is an important, yet challenging, medical imaging application. Pulmonary diseases are a major source of death and hospitalization worldwide, for which computed-tomography (CT) is the leading analysis modality. As such, there is great impetus to develop CT tools for automated disease detection and diagnosis. Yet, such tools, in turn, often rely on reliable PLS, i.e., an accurate delineation of pulmonary regions. As such, methodological simplicity and generality are key factors in a PLS tool's usability. Any tool must also handle the wide variability in appearance and shape of pathological lungs. State-of-the-art methods lack either simplicity or robustness. Along those lines, we present a bottom-up deep-learning based PLS method that is expressive enough to handle variations in appearance, while remaining unaffected by any variations in shape. We build off a deeply-supervised and fully-convolutional network (FCN) architecture called holistically nested networks (HNNs). To address the well-known coarsening problem of FCNs, we enhance the architecture by a simple, yet effective, progressive multi-path scheme, which more reliably merges outputs from different network stages and resolutions. Unlike other solutions to this problem, our multi-path scheme requires no extra parameters, and even has less parameters than standard HNNs, ensuring our method remains straightforward and usable. This results in a deep model able to produce finer detailed masks, which we call progressive holistically-nested networks (P-HNNs). Depending on the number of slices, our tool only takes 10-30s to process an entire CT volume. Using extensive cross-validation, our method is tested on multi-institutional datasets comprising 929 CT scans (848 publicly available) of pathological lungs, which include cases of interstitial lung diseases, chronic obstructive pulmonary disease, and various infections. This is the largest and most rigorous analysis to date of a PLS tool's performance. We report mean Dice scores of 0.985, significantly (p<0.001) outperforming standard HNNs and providing high-quality masks in instances where HNNs completely fail. As well, we also compare against a prior state-of-the-art, but non-deep-learning, PLS tool, demonstrating significant qualitative and quantitative (p<0.001) improvements. These results demonstrate the potential of our tool to contribute toward large-scale and high-throughput analysis of medical scans.

NIH Clinical Center
Andrew Mikhail
Research Fellow
Radiology/Imaging/PET and Neuroimaging

Drug dose mapping using imageable, drug-eluting embolic beads for transarterial chemoembolization in a preclinical rabbit tumor model

An estimated 40 000 new cases of primary liver cancer are expected in the United States in 2017. Regrettably, only 10-20% of patients with hepatocellular carcinoma (HCC), the most common form of primary liver cancer, are candidates for surgery. Transarterial chemoembolization (TACE) is a minimally-invasive treatment that consists of selective catheterization of tumor-feeding arteries under fluoroscopic image-guidance and delivery of embolic materials that cut off blood supply to the tumor. Drug-eluting embolic beads (DEBs) containing the chemotherapeutic agent doxorubicin (DOX) have been developed that serve both as an embolic agent and drug delivery vehicle for sustained tumor-localized delivery of chemotherapy. Recent development of drug-eluting radiopaque beads (DEROB) that are visible by fluoroscopy and CT may allow for optimization of therapy based on imaging feedback regarding DEROB distribution and density. Moreover, the image-ability of DEROB raises an intriguing potential for drug dosimetry, whereby relative levels of attenuation (image contrast) on CT may act as a surrogate for drug dose distribution during TACE facilitating greater customization of therapy.

The purpose of this study was to determine the correlation between DEROB x-ray attenuation on CT and DOX concentrations in the liver and to estimate drug dose following TACE in a preclinical tumor model. Rabbits bearing VX2 liver tumors underwent TACE with DEROB following which the livers were resected, frozen and imaged with a 16-slice multidetector CT (MDCT). For tissue sectioning, the frozen livers were inserted into liver-specific 3D printed molds containing cutting slots for precise radiologic-pathologic correlation of tissue sections and imaging. A linear correlation was found between DEROB attenuation in the liver determined by MDCT image segmentation, and the concentration of DOX measured by liquid chromatography (r2 = 0.971). Regression analysis of DOX predicted on CT vs. DOX measured in a subsequently treated liver demonstrated accurate and precise drug dose estimation (slope = 1.06, intercept = 2x10-4 mg DOX, R2 = 0.93). This relationship potentially estimates drug dose and drug distribution on post-embolization imaging, enabling identification of potentially under-dosed regions of tumor. The availability of such drug map estimates during TACE could inform treatment decisions, better define treatment endpoints and optimize therapy.

National Cancer Institute - Center for Cancer Research
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Abstract removed at request of author
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Biochemistry - Proteins
Hsp90 of E. coli modulates assembly of FtsZ, the tubulin homolog in E. coli

Heat shock protein 90 (Hsp90) is a highly conserved ATP dependent molecular chaperone involved in remodeling, activating and stabilizing numerous client proteins. Since many Hsp90 client proteins have been linked to cancer and other diseases, understanding the functions of Hsp90 is important. The Hsp90 homolog in E. coli, Hsp90Ec, has been shown to cause cell filamentation when overexpressed. By observing the cells under light microscopy, we observed that the filamentous cells had distinct nucleoids, indicating that Hsp90Ec overexpression does not affect chromosomal replication or segregation. To assess if overexpression of Hsp90Ec interferes with the cell division machinery, we tested if FtsZ, a tubulin homolog essential for cell division, assembled into ring-like structures at future sites of cell division as it does in cells not overexpressing Hsp90Ec. We observed by immunofluorescence of fixed cells that FtsZ rings were not detectable in Hsp90Ec overexpressing cells. We also found that FtsZ was present at normal levels in cells overexpressing Hsp90Ec. Together these results suggest high levels of Hsp90Ec affect FtsZ assembly. To test if the Hsp90Ec stabilized negative regulators of FtsZ, we singly deleted genes coding for negative regulators SulA, ClpX, MinC and SlmA but none of these mutants reversed the filamentous phenotype seen in Hsp90Ec overexpressing cells. We next tested the hypothesis that Hsp90Ec prevents FtsZ polymerization. Using purified proteins and fluorescence microcopy, we observed that fluorescently labeled FtsZ formed filaments and bundles in the absence of Hsp90Ec, but not in the presence. Additionally, we showed that light scattering by FtsZ polymers was inhibited when Hsp90Ec was added prior to polymerization. We further observed that an ATP hydrolysis defective Hsp90Ec mutant retained ability to inhibit FtsZ polymerization, consistent with the known ability of Hsp90 to interact with clients independent of ATP hydrolysis. Moreover, we observed that Hsp90Ec client-binding defective mutants exhibited reduced ability to prevent FtsZ polymerization in vitro. In summary, our data show that Hsp90Ec, when overexpressed, inhibits divisome assembly in vivo and prevents FtsZ polymerization in vitro. They suggest that Hsp90Ec may modulate of cell division by interacting and holding FtsZ, possibly slowing cell division during heat stress and other stresses.

National Cancer Institute - Center for Cancer Research
Defne Bayik
Visiting Fellow
Immunology - Autoimmune

Toll-like receptor 2/1 signaling induces human monocytes to differentiate into immunosuppressive macrophage: implications for the treatment of autoimmune and inflammatory diseases.

Autoimmune diseases and chronic inflammatory conditions are characterized by excess immune cell activation. Since inflammatory macrophage have been implicated in the development and progression of such diseases, generation of immunosuppressive macrophages capable of down-regulating pathologic immune responses could provide a therapeutic opportunity. Established methods of generating immunosuppressive macrophage involve stimulation of monocytes with cytokines, which are suboptimal for treatment of chronic conditions. Therefore, we sought alternative methods of immunosuppressive macrophage polarization. Our results demonstrated that Pam3CSK4 (PAM3), an agonist of the Toll-like receptor 2/1 heterodimer (TLR2/1), induces human monocytes to differentiate into immunosuppressive macrophage. The resulting macrophages were characterized by the expression of CD163, CD206, PD-L2, DC-SIGN and SR-A (established markers of suppressive macrophage) and the secretion of anti-inflammatory cytokines including IL-10 and IL-1Ra. Functionally, macrophages generated by treating

monocytes with PAM3 suppressed the proliferation of autologous CD4+ T cells and were highly endocytic. Microarray analysis coupled with inhibition studies demonstrated that PAM3-driven differentiation of monocytes involves the activation of NF-KB, Akt, Erk and p38 MAPK signaling pathways. Whereas inhibition of NF-KB and Akt prevented the general process of macrophage activation, blockade of Erk and p38 MAPK signaling specifically interfered with the differentiation of immunosuppressive rather than inflammatory macrophage. The therapeutic potential of PAM3 was evaluated on monocytes from patients with dermatomyositis. Unlike other tested TLR agonists, PAM3 did not induce production of pro-inflammatory cytokines IL-12 and TNFa, while it triggered differentiation of patient monocytes into immunosuppressive macrophages (with the same capacity as in healthy controls). Studies using Rhesus macaques verified that PAM3 has the same effects in vivo with no toxicity. PAM3 induced the local production the anti-inflammatory mediators (IL-10 and IDO) and a resulted in a systemic increase in immunosuppressive macrophage in the macaques. These findings describe a novel mechanism that promotes the polarization of primate monocytes into immunosuppressive macrophage via PAM3-induced activation of TLR2/1, p38 MAPK/Erk pathway, which could be of use in the treatment of inflammatory and autoimmune conditions.

National Cancer Institute - Center for Cancer Research
Cedric Belair
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Stem Cells - General

THE RNA EXOSOME REGULATES DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

The unique abilities of human embryonic stem cells (hESCs) to self-renew and to differentiate into all three germ layers (endoderm, mesoderm, ectoderm) are linked to their “open chromatin”, which allows high levels of transcription and contributes to pluripotency by keeping many tissue-specific genes in permissive transcriptional states. As a consequence, hESCs express many RNAs at higher levels than differentiated cells, including potentially harmful RNAs such as endogenous retrotransposons and mRNAs that encode proteins promoting differentiation. Although both the proteasome and RNA interference pathway contribute to controlling this pervasive transcription, the role of RNA surveillance pathways in degrading potentially deleterious RNAs is largely unknown.

Here we report that the RNA exosome, a major ribonuclease complex, restrains hESCs from differentiating into endoderm, mesoderm and ectoderm. We generated clonal hESC lines expressing doxycycline-inducible shRNAs directed against the core exosome subunit EXOSC3/hRRP40. Although EXOSC3 depletion does not affect the proliferation of hESCs or the expression of pluripotency markers, we uncovered a role for the exosome in preventing differentiation. In these experiments, we allowed hESCs to differentiate into embryoid bodies and assayed for the expression of markers corresponding to each of the three germ layers. Interestingly, embryoid bodies derived from EXOSC3-depleted hESCs exhibited up to 10-fold level increases in mRNAs encoding ectoderm, endoderm and mesoderm markers, compared to control cells. Consistent with the idea that the exosome is critical for preventing hESC differentiation, we found that downregulation of exosome subunits is an early event in the differentiation of wild-type hESCs. To determine the set of RNAs targeted by the exosome, we combined whole transcriptome analysis (RNA-Seq) with high throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP). We found that the exosome reduces the levels of specific miRNAs, long noncoding RNAs and mRNAs as well as potentially active retrotransposon transcripts.

Importantly, we showed that the exosome restrains differentiation in part by degrading pre-mRNA and mRNA encoding FOXH1, a transcription factor crucial for mesendoderm formation. Together, our data establish the RNA exosome as a new regulator of hESC differentiation and reveal the importance of RNA decay pathways in maintaining ESC pluripotency.

National Cancer Institute - Center for Cancer Research
Mike Beshiri
Postdoctoral Fellow
Clinical and Translational Research - Cancer
p38 MAPK is required for in vitro organoid culture of human prostate cancer
Metastatic castration-resistant prostate cancer (CRPC) is a genetically heterogeneous disease. Effective translational research demands a diverse and representative set of preclinical models. A major obstacle in the field is the limited capacity to culture prostate cancer-derived cells in vitro. Few cell lines exist. Those that are available do not well-represent the genetic diversity of the disease, nor do they accurately predict in vivo response. Recently, organoid culture techniques have increased our ability to grow metastatic tumor-derived prostate cancer cells in vitro. This represents a great step forward, but is restricted by limited availability of tissue and a success rate of less than 20% when establishing new lines. In the field of prostate cancer, patient-derived xenografts (PDXs) are the model that most broadly represents the clinical and genetic diversity of the disease. Like primary prostate tumors, PDXs have not been readily adaptable to in vitro culture, making them poorly suited for mechanistic studies or high-throughput drug screens. Therefore, there is great interest in adapting the prostate cancer PDX model to an in vitro culture system. The LuCaP series of PDXs is a well-characterized set of > 25 diverse CRPC specimens. Using LuCaP PDXs, we systematically tested 30 modifications to the organoid culture method. This includes the addition of factors to modify metabolism, growth and differentiation; removal of standard media components; and combinations thereof. We show p38 is required for growth and survival of almost all the LuCaP organoids. Removal of the p38 inhibitor component from standard prostate organoid media makes it possible to grow PDX-derived organoids. We successfully cultured 21 of 24 different LuCaPs attempted, for at least one generation, and 12 have been grown long-term. Additionally, our method improves the success rate for culturing samples directly from patient biopsies. We show that 2 of 3 biopsy-derived organoid lines that we have established, grow well in our conditions but not in standard published conditions. The third grows equally well in both. Further, our data suggest that p38 is a previously unappreciated target for CRPC treatment. Importantly, our method immediately and significantly increases the number of prostate cancer lines available to the community by making the LuCaP PDXs accessible to in vitro culture and by improving the success rate for establishing organoids directly from patient samples.

National Cancer Institute - Center for Cancer Research
Ritu Chaudhary
Visiting Fellow
Genetics
Genome-wide analysis of circular RNAs identifies circ-MDM2 as a novel regulator of p53-dependent cell cycle arrest after DNA damage

The tumor suppressor protein p53 is activated during cellular stress to induce cell cycle arrest, apoptosis and senescence. It is well-established that p53 mediates its effect by functioning as a master regulatory transcription factor that enhances the transcription of hundreds of mRNAs and several microRNAs. However, a role of circular RNAs (circRNAs) in the p53 pathway is not known. CircRNAs are a novel class of noncoding RNAs that, unlike mRNAs, form a covalently closed continuous loop, produced by a non-canonical form of alternative splicing called “backsplicing”. Of the handful of circRNAs studied so far, some function as microRNA (miRNA) sponges or bind to RNA-binding proteins to regulate their activity. Here, we identified novel p53-regulated circRNAs in response to DNA damage induced by Doxorubicin (DOXO) by performing RNA-seq from 3 pairs of p53+/+ and isogenic p53-/- cell lines (HCT116, RKO and SW48) from untreated and DOXO-treated conditions. In response to DNA damage, we found 811 circRNAs that were up-regulated (2- to 550-fold) in all 3 lines. Of the 811 circRNAs, only 26 were up-regulated in a p53-dependent manner, including circ-MDM2, an abundant circRNA expressed from the MDM2 locus. Given the central role of MDM2 in regulating p53 protein and p53 activity, we investigated the physiological function of circ-MDM2. Knocking down circ-MDM2 with 2 independent siRNAs followed by DNA damage identified a novel function for circ-MDM2 in regulating G1-arrest and cell proliferation in multiple cell lines. Because circRNAs can regulate gene expression, we identified the circ-MDM2-regulated transcriptome after knocking down circ-MDM2 followed by DOXO-treatment of HCT116 cells. We found a novel role of circ-MDM2 in regulating the expression of several cell cycle genes that control G1 arrest after DNA damage, including CDC25A, CCND1, CCNE2 and CCNA2. Our data indicates that circ-MDM2 plays a major role in regulating the expression of these genes in response to DNA damage. Collectively, our results uncover a previously unrecognized role of a circRNA during DNA damage and provide the first functional and mechanistic role of circ-MDM2 in the p53 network.

National Cancer Institute - Center for Cancer Research
Jennifer Cowan
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Myc activity in Thymic Epithelial Cells controls thymic size
Adaptive immunity declines with age. This age-associated decline results in increased susceptibility to infection, reduced vaccine response rates in the elderly, and lower success rates of bone marrow transplantation. A critical organ for adaptive immunity is the thymus, which is required for T cell production. Thymus size changes over time. The thymus rapidly grows until early adulthood, but thereafter declines in size, a process termed involution. This involution causes a decrease to T cell production, but it is yet to be demonstrated that restoring thymic size can improve immune function with age.

The decline in thymic size corresponds with a decrease in numbers of thymic epithelial cells (TEC). We aimed to identity genes that control thymic size, that hold potential for manipulation in reversing the involution process. We transcriptionally profiled TEC populations from mice at different ages, to identify transcriptional programs that change over time. We discovered that Myc-regulated genes decline with age in TEC. The Myc pathway controls organ size in many models, yet little is known about its role in TEC

biology. We hypothesized that Myc activity in TEC could contribute to the changes in thymic size with age.

To test this hypothesis, we generated a transgenic mouse model, driving continuous myc expression exclusively in TEC. The overexpression of myc in TEC resulted in a dramatic increase in thymus size. By 11 weeks of age the thymus is tenfold larger in myc transgenic mice, compared to wildtype controls. Moreover, continuous Myc activity in TEC resulted in increased numbers of T cells. We are currently generating a myc inducible mouse model, so we can evaluate the effect of inducing myc overexpression in an aged, involuted thymus. We hope to discover whether a reversal of thymic involution will restore adaptive immunity in aged mice.

National Cancer Institute - Center for Cancer Research
Lisheng Dai
Visiting Fellow
Molecular Biology - Eukaryotic

Genetic Dissection of Drosha Mediated microRNA Biogenesis Through Genome-scale CRISPR Screens

MicroRNAs (miRNAs) are a class of small non-coding RNAs, which collectively regulate more than half of protein encoding genes in mammals. RNase III enzyme Drosha together with its cofactor DGCR8 initiate miRNA biogenesis by cleaving primary miRNA transcripts (pri-miRNA) in the nucleus. Dysregulation of such process plays essential roles in cancer development. For example, BRCA1 and p53 regulate miRNA expression level via modifying Drosha activity. Despite its importance, little is known about how Drosha function is regulated in vivo.

Here, using CRISPR–Cas9 based genome-wide loss-of-function screen, we seek to identify additional cellular regulators of Drosha activity. We inserted pri-miRNA sequences into the 3'UTR of the mCherry reporter, rendering its mRNA as a target of Drosha cleavage. Stable cell lines were established in which such reporters were co-expressed with an eYFP control. After normalizing to eYFP, the signal of mCherry serves as a faithful indicator of Drosha activity in cells. As expected, reporters containing pri-miR-16 and pri-miR-155, but not the control lacking pri-miRNA sequences, exhibited robust de-repression of mCherry upon Drosha knockout. Next, we performed a genome-wide screen by randomly knocking out cellular genes via a treatment of CRISPR-Cas9 library. Cells in which Drosha activity was impaired were sorted out based on the ratio of mCherry to eYFP. sgRNA representation in the sorted and unsorted cells was enumerated by high-throughput sequencing. MAGeCk and HiTSelect were used to analyze combined results of 12 screens, generating high-confidence hits. The well-known components of pri-miRNA processing, Drosha and DGCR8, were top hits in both pri-miR-16 and pri-miR-155 reporter cells, establishing the sensitivity of this approach. Several novel factors, including SUGP2 (SURP and G-patch Domain Containing 2), IKZF1 (IKAROS Family Zinc Finger 1), and POLD4 (DNA Polymerase delta 4, Accessory Subunit), were identified in the screen. Loss-of-function of these genes de-repressed mCherry in both pri-miR-16 and pri-miR-155 reporter cell lines. Interestingly, some factors were uniquely identified in either pri-miR-16 or pri-miR-155 reporter cell lines, indicating miRNA-specific regulation.

Together, our results reveal a large number of previously unknown miRNA biogenesis regulators and provide novel targets for cancer therapeutics.

National Cancer Institute - Center for Cancer Research
Seham Ebrahim
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Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

A lattice-like septin cage is required for acto-myosin contractility on secretory granules during regulated exocytosis.

The constant remodeling of cellular membranes into various curved configurations is a requisite for myriad biological processes ranging from cytokinesis to intracellular trafficking. Membrane remodeling may be achieved by: 1) varying membrane lipid composition, 2) protein scaffolds that sense, induce or stabilize curved membranes, and/or 3) forces exerted by membrane-bending proteins, molecular motors and the cytoskeleton. However, the chronology and contribution of each of these processes is not well understood, particularly in vivo, and only a few key proteins have so far been identified. Considering the multitude of human pathologies associated with membrane remodeling defects, this leaves an essential gap unfilled.

We sought to elucidate the molecular machines and processes driving membrane remodeling under physiological conditions using the process of regulated exocytosis in specialized secretory cells of the murine salivary gland as a model system. We previously found in live animals that F-actin and non-muscle myosin (NMII) are recruited to the membrane of secretory granules undergoing exocytosis, and drive their integration into the plasma membrane to allow content release into the extracellular space.

We now show via immunofluorescence and superresolution microscopy that septins are a novel cytoskeletal component on the surface of fused secretory granules, where they form a striking cage-like lattice. Using transgenic mice expressing GFP-NMIIA, we discover that NMIIA colocalizes with the septin-lattice by forming connected triskelia of bipolar minifilaments, reminiscent of clathrin, that have not been previously observed. Pharmacological inhibition of septin 2 results in a significant decrease in the presence of activated NMII and of myosin light chain kinase (MLCK) on actin-coated fused granules. Conversely, disruption of F-actin assembly on the granule surface leads to an expansion in granule size without impairing the recruitment of NMII and septins.

We propose that the newly observed septin-lattice: 1) provides a molecular scaffold to recruit and curve actin filaments populating the surface of secretory granules, and 2) is needed for the activation of NMII, likely through MLCK-mediated phosphorylation. Finally, the pattern of organization of both septins and NMII on the granule surface provide new structure-function associated insights into the molecular mechanisms driving membrane remodeling during regulated exocytosis.

National Cancer Institute - Center for Cancer Research
Jessica Eisenstatt
Postdoctoral Fellow
Chromatin and Chromosomes
A Genome-Wide Screen Reveals a Replication-Independent Role of Dbf4-Dependent Kinase in Localization of the Centromeric Histone H3 Variant for Genome Stability
The evolutionarily conserved centromeric histone H3 variant (Cse4 in budding yeast, CENPA in humans) is essential for faithful chromosome segregation. CENPA is observed to be overexpressed and mislocalized in various cancers, which is associated with a poor prognosis. We therefore aimed to identify novel therapeutic targets for tumors overexpressing CENPA (CENPA-OE). Synthetic lethal (SL) interaction partners were identified in a genome-wide screen using budding yeast as a model system, an approach that has not yet been exploited. Strains with conditional mutant alleles of essential genes with overexpressed Cse4 (Cse4-OE) were assayed for colony growth size; smaller colonies indicated a SL interaction. Validation of the screen was provided by independent confirmation using growth assays for SL. Gene Ontology analysis of significant negative interactors identified categories related to ubiquitin-mediated proteolysis, chromosome segregation, chromatin binding, and DNA replication. Among the top fifteen hits of essential gene conditional mutant alleles with a negative interaction with Cse4-OE, we identified five alleles of the Cdc7-Dbf4 kinase complex, which is evolutionarily conserved and essential for DNA replication initiation. Biochemical analysis showed defects in degradation, ubiquitination, and localization of Cse4 in cdc7 mutants. Defects in Cse4 localization and chromosome segregation as well as SL in the presence of Cse4-OE were also observed in cdc7 mcm5 mutant strains which do not exhibit defects in DNA replication. These data indicate that Cdc7 regulates Cse4 localization independently of its role in the initiation of DNA replication. Furthermore, Cdc7 and Cse4 interacted in vivo, and in vitro kinase assays showed that the Cdc7-Dbf4 kinase complex phosphorylates Cse4. In summary, we have identified potential therapeutic targets for CENPA-OE tumors and provide insights into a novel mechanistic role of Cdc7 in regulating CENP-A/Cse4 levels for genome stability.

National Cancer Institute - Center for Cancer Research
Marina Feric
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Biophysics
Mitochondrial dysfunction in the premature aging disease Hutchinson-Gilford Progeria Syndrome
Aging is attributed to the accumulation of cellular damage. Altered biochemical pathways can accelerate the normal aging process and have the potential to cause premature aging diseases. For the case of the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS), a single point mutation in the lamin A/C gene leads to the aberrant splicing of its pre-mRNA and the production of the disease-causing protein progerin. Similar to the wild-type lamin A, progerin remains localized to the nuclear envelope, but appears to have numerous global downstream effects on nuclear-structure and function. In particular, one organelle that has long been implicated in aging is the mitochondrion, due to its generation of reactive oxygen species (ROS). Whether mitochondria play a role in HGPS is unclear. We

use confocal microscopy and high-throughput microscopy with quantitative image analysis, as well as next generation sequencing (NGS), to determine how mitochondria biophysically contribute to the premature aging phenotype in HGPS. We find that progerin causes mitochondria to become swollen and fragmented, leading to altered spatial distribution of mitochondrial DNA (mtDNA) nucleoids. Live cell imaging shows differences in dynamics, and mitochondria from HGPS become more unstable due to increased depolarization events. These increased flickering events can release the mitochondrial contents, including ROS, and can cause damage to the cytoplasm. Using techniques from multiple particle tracking, the mtDNA nucleoids are observed to have decreased mobility in HGPS cells. Furthermore, the swollen, isolated mitochondria are seen via FRAP to undergo less fusion events with the surrounding mitochondrial network, potentially limiting exchange of material and proper maintenance. Moreover, while progerin is known to increase oxidative stress, further oxidative challenges via either light-induced ROS production or addition of hydrogen peroxide cause HGPS cells to appear less resilient than control cells with the nucleoids coalescing more rapidly and the mitochondria failing to maintain their membrane potential. Next generation sequencing determines the amount of damage (mutations and deletions) to mtDNA, which can further exacerbate the premature aging phenotype, and correlates the damage to the time course of progerin accumulation. These findings implicate mitochondria in HGPS pathogenesis and have applications to normal aging.

National Cancer Institute - Center for Cancer Research
Elizabeth Finn
Postdoctoral Fellow
Chromatin and Chromosomes

High-Throughput Imaging to Study Patterns of Variability in Genome Organization

The genome is spatially organized on many levels in vivo. Chromosomes are distinct nuclear territories, which are made up of co-regulated chromatin domains, which in turn are formed by loops and fibers of coiled and clumped nucleosomes. The spatial organization of the genome is disrupted in both aging and cancer, suggesting a crucial role in genome function. The study of genome organization has a long history of studies using low-throughput imaging techniques such as DNA fluorescence in situ hybridization (FISH), which have recently been complemented by high-throughput sequencing approaches based on chemical crosslinking such as 3C and Hi-C. While these biochemical methods provide largely unbiased and high-resolution maps of interactions, the relation the resulting data to physical distance is unclear, and these methods cannot address cellular variability since they are based on population averages. To bridge this gap, we systematically compared biochemical interaction data to spatial distance measurements in individual cells by high-throughput imaging. We identified a set of more than 200 interaction pairs on several chromosomes separated by 0.25 to 250 Mbp. We used high-throughput FISH combined with an automated image analysis pipeline to determine spot localization in at least 1,000 cells for each interaction pair. We observe overall excellent correlation between Hi-C capture frequency, genomic distance, and FISH association frequency at all length scales and detected interactions in 0.25 to 60% of spots, highlighting the variability present at every interaction. We examined cell-specific and allele-specific variation at these loci and observed that in most cells, pairs behaved independently of each other, suggesting that variability in the population is due to intrinsic variation in the folding of the chromatin fiber, rather than variation on the cell- or allele- level. Finally, we compared regions in different chromatin contexts and observed that interaction partners in gene

rich chromatin form longer-range contacts and have a greater overall range between ‘interactors’ and ‘non-interactors’. Our results indicate that the overall folding landscape of a chromatin fiber is characterized by variation, some of it due to genomic distance, some of it due to sequence and chromatin context, some of it due to cell-to-cell and allele-to-allele variation, and some of it simply due to stochasticity.

National Cancer Institute - Center for Cancer Research
Ying Fu
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Hematology/Oncology, Tumor Immunology, and Therapy
Glypican-3 Specific Antibody Drug Conjugate for Hepatocellular Carcinoma
Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death. Therapeutic outcomes in HCC remain unsatisfactory, novel treatments are urgently needed. Glypican-3 (GPC3) is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein consisting of a core protein and two heparin sulfate (HS) chains. GPC3 is a potential target for HCC given the facts that: 1) GPC3 has been reported to be highly expressed in more than 70% of HCC, but not in normal adult tissues; 2) relationship between elevated GPC3 expression level and poor HCC prognosis has been established; 3) GPC3 can mediate internalization after antibody binding; 4) Codrituzumab, a humanized monoclonal antibody (mAb) against GPC3 has shown a good safety profile in phase I study, but failed to show efficacy in phase II. The hypothesis of using anti-GPC3 antibody drug conjugate to treat HCC patients has not been tested in clinical trials. Here we report the development of hYP7-DC, a humanized anti-GPC3 antibody conjugated to a highly potent duocarmycin DNA damaging agent through cysteine via protease-cleavable linker. In the preclinical test, hYP7-DC showed potency at picomolar range against a panel of GPC3-positive liver cancer cell lines and was more than 100-fold selective against GPC3-negative cell lines. Mechanistic studies indicate the cytotoxic effect involves GPC3 induced ADC internalization and apoptosis. hYP7-DC showed antitumor effect in vivo in two mouse models. To improve the ADC efficacy, we screened two libraries of drugs (NCATS Pharmaceutical Collection and Mechanism Interrogation PlatE) in clinical trials or approved by FDA against Hep3B liver cancer cell line which overexpresses GPC3. Pyrrolobenzodiazepine dimer (PBD) is identified as the most potent small molecule, which was used to construct a new anti-GPC3 ADC, hYP7-PC. hYP7-PC showed approx. 10 times more potency and selectivity than hYP7-DC against a panel of liver cancer cell lines in vitro. Moreover, hYP7-PC (single dose, 5mg/kg) caused tumor remission in Hep3B HCC xenograft model. Together, these data suggest that hYP7-PC has GPC3-directed antitumor activity and support clinical testing of this novel therapeutic in patients with GPC3-posivitve liver cancer.

National Cancer Institute - Center for Cancer Research
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Tumor Biology and Metastasis
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Abstract removed at request of author

National Cancer Institute - Center for Cancer Research
Berkley Gryder
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Epigenetics
Miswiring of Enhancer Logic Circuitry Causes Childhood Sarcoma
Super enhancers (SEs) are regulatory regions with unusually large deposits of active histone marks, chromatin regulators and transcriptional coactivators. Chromosomal rearrangements allowing SEs to drive oncogene expression is an emerging mechanism in tumor biology. An aggressive myoblastic cancer of childhood, alveolar (fusion-positive) rhabdomyosarcoma (FP-RMS), universally possesses a chromosomal translocation, involving PAX3 and FOXO1. PAX3 initiates specification of the muscle lineage, but is shut off during myogenic differentiation, which is in turn dominated by master regulators MYOD and finally MYOG. FP-RMS has the master regulators needed to trigger muscle differentiation, but are halted in an early myoblastic state. We hypothesized that the translocations miswire regulation of the fusion oncoprotein in FP-RMS by hijacking SEs and creating new topologically associated domains (TADs) which allow for continued expression of PAX fusions, thus circumventing normal myogenic enhancer logic.

We recently completed the first epigenetic landscape of FP-RMS and uncovered a strong dependence on SEs for tumor survival, with PAX3-FOXO1 being a chief determinant of SE formation. Importantly, we discovered a key SE 300 kb distal of FOXO1 which was occupied by all four of these master regulators.

Further, we found that PAX3-FOXO1 is driven by this novel translocated SE forming a key TAD structure which was necessary to directly influence PAX3 upon translocation, with CTCF analysis in FP-RMS cells confirming the predicted boundaries. We demonstrate these elements to physically interact only in the presence of the translocation by chromatin conformation followed by sequencing (4C-seq). Exon-level expression via RNAseq in primary tumors revealed that the final exon of PAX3, not involved in the translocation, was unexpressed, indicating that only the allele influenced by the FOXO1 SE is activated in patients. Finally, CRISPR/Cas9 technologies were employed to functionally interrogate the relative contributions of the enhancer elements and CTCF looping sites at TAD boundaries. Together these data suggest that these newly juxtaposed enhancer elements initiate and continually drive PAX3-FOXO1 expression, implying that enhancer miswiring is at the heart of the oncogenic process in FP-RMS. Thus, late myogenic factors (MYOG/MYOD) are contributing to drive an early factor (PAX3), changing a “progressive” enhancer logic into an “infinite loop” enhancer logic.

National Cancer Institute - Center for Cancer Research
Emine Guven-Maiorov
Postdoctoral Fellow
Informatics/Computational Biology
Prediction of novel host-pathogen interactions for Helicobacter pylori through interface mimicry and their implications to gastric cancer
About 20% of the cancer incidences worldwide have been estimated to be associated with infections. There is a strong correlation of some pathogens with various cancer types, such as Helicobacter pylori with gastric cancer. However, the molecular mechanisms how they trigger cancer in the host is generally

unknown/incomplete. Pathogens interact with the host mainly through proteins. To subvert host defense, they mimic host proteins at different levels: sequence, structure, motif and interface -binding surface-. Interface mimicry seems to be the most common type. This similarity in interfaces permits pathogenic protein to compete with host proteins to bind to a target protein, alter physiological signaling and cause persistent infections, as well as cancer. Detection of host-pathogen interactions (HPIs) and mapping the re-wired HPI network – along with its structural details – is critical for in-depth understanding of the underlying pathogenesis mechanisms of infections, pathogen-triggered cancers, and developing efficient therapeutics. Host-pathogen interaction (HPI) data including structural details is far from complete and experimental characterization of the large-scale inter-species interactions is challenging. Thus, computational tools are becoming increasingly important in enriching the HPI data, uncovering their complex (bound) structures, and complementing the experiments. Here, we developed the first computational approach to identify novel HPIs that utilizes solely interface mimicry. Employing interface mimicry is promising to identify more HPIs than utilizing sequence or complete structural similarity since interface mimicry is more common. We applied our interface-based approach to H. pylori, dominant species in gastric microbiome that greatly increases gastric cancer risk in order to understand how they modulate host immunity and lead to tumorigenesis. We found that its proteins interfere with the functioning of host apoptosis pathway, cytokine and chemokine pathways, and also cell-cell adhesions. Our results shed light on the molecular mechanisms of resistance to apoptosis, immune evasion and loss of cell junctions that are seen in H. pylori-infected host cells. In conclusion, HPIs can help us unravel which human pathways are targeted by pathogenic proteins and how they contribute to pathogenesis of infections and pathogen-triggered cancer. With a better grasp on virulence strategies, we can develop better therapies.

National Cancer Institute - Center for Cancer Research
Zhen Han
Postdoctoral Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
Development of a curative TheraVac immunotherapy for large mouse hepatocellular carcinoma
HCC is one of the most common cancers with no curative therapy available except surgical resection followed by liver transplantation. The recently FDA-approved checkpoint inhibitors or RTKIs are only effective for some patients with limited duration, necessitating the development of improved therapies for HCC. We have developed an immunotherapeutic vaccination (TheraVac) regimen to treat large established HCC in mice. TheraVac consists of two arms: one aimed at activating tumor-infiltrating dendritic cells (DCs) and the immune response via a combination of high-mobility group nucleosome binding protein 1 (HGMN1) - a TLR4 agonist, and R848 - a TLR7/8 ligand since they synergistically promoted DC production of IL-12; the other aimed to curtail tumor-associated immunosuppression by using one of the checkpoint inhibitors (e.g. anti-CTLA4, anti-PD-L1, or low dose Cytoxan). Treatment of mice harboring large (1 cm in diameter) Hepa1-6 hepatomas with intratumoral (i.t.) injection of HMGN1 and R848 and systemic administration of one of the mentioned checkpoint inhibitors twice every week for 2~3 weeks eradicated the tumors. TheraVac-treated Hepa1-6-bearing mice elevated CXCL9 and CXCL10 expression, CD4+ and CD8+ T cell infiltration, and IFNg production in the tumors, and increased Hepa1-6-specific CD8+ CTLs in the draining lymph nodes, all pointing to successful induction of antitumor immune responses. Strikingly, the resultant tumor-free mice were resistant to challenge with

Hepa1-6, but not to B16 melanoma, demonstrating the acquisition of hepatoma-specific immunity. Since i.t. route of administration is difficult to use on HCC patients, we further established a gold (Au) nanoparticle (NP) platform to deliver HMGN1 and R848 systemically. We succeeded to attach HMGN1 and R848 to PEGylated AuNP to form PEG-Au-HMGN1-R848 complexes, which, not only preserved their DC-activating capacities, but also remained stable for up to 3 weeks. The majority of intravenously (i.v.) injected PEG-Au-HMGN1-R848 went to Hepa1-6 tumors rather than liver or spleen, demonstrating the preferential tumor-targeting feature of the resultant AuNP. Importantly, i.v. injection of PEG-Au-HMGN1-R848 and a systemic administration of Cytoxan also cured C57BL/6 mice bearing large Hepa1-6 hepatomas. Overall, we have developed an unprecedented TheraVac treatment of HCC, which can potentially be translated into the clinic due to its high effectiveness, few side effects, and no antigenicity.

National Cancer Institute - Center for Cancer Research
Carrie House
Postdoctoral Fellow
Tumor Biology and Metastasis

Divergent NF-kB signaling supports distinct phenotypes necessary for ovarian cancer tumorigenesis via classical regulation of cells with high proliferative activity and alternative regulation of ALDH+ cells with low proliferative activity

Chemotherapy resistance and cancer relapse provide strong evidence for tumor-initiating cells (TICs) in epithelial carcinomas. Understanding the mechanisms supporting this subpopulation is vital for combating advanced stage ovarian cancer, the deadliest gynecological cancer. We previously showed that specific components of classical NF-kB signaling are required for aggressive tumor behavior in ovarian cancer. NF-kB signaling is a complex network that follows both classical and alternative cascades, defined by activation of either RelA (classical) or RelB (alternative) transcription factors. Elevated classical NF-kB signaling has been observed in TICs of some solid tumors, however the role of alternative NF-kB signaling is not established. My preliminary data show differential activation in TICs versus non-TICs. I investigated the hypothesis that classical and alternative NF-kB support distinct phenotypes of ovarian TICs. To investigate this pathway, I designed a novel method to enrich TICs in cell lines and patient samples by culturing non-adherent, floating cells in TIC-enriching conditions. These free-floating spheroids mimic malignant cells found in patient ascites. I found that these cells have higher TIC markers, resist carboplatin, and are more tumorigenic in nude mice compared to their adherent counterparts. Using specific inducible shRNA targeting RelA or RelB I measured changes in spheroid formation, proliferation, drug resistance, TIC marker expression, and tumorigenesis. I show here that NF-kB signaling through RelB supports TICs by directly regulating aldehyde dehydrogenase (ALDH), an enzyme with high activity in TICs. Spheroid formation, ALDH expression and activity, chemoresistance, and tumorigenesis in both subcutaneous and intrabursal xenograft models significantly diminished with loss of RelB. Using ChIP-qPCR I further show that RelB directly regulates the expression of ALDH1A2. Interestingly, classical NF-kB signaling through the RelA transcription factor was equally important for tumorigenesis but had no effect on ALDH. Classical signaling was essential for proliferating cells, whereas alternative signaling was not. I conclude that NF-kB sustains diverse cellular phenotypes through differential classical and alternative signaling pathways to promote tumorigenesis.

Given the broad role of NF-kB in cancer biology these findings could notably impact our understanding of the molecular basis for disease recurrence and therapeutic response.

National Cancer Institute - Center for Cancer Research
Hiroki Ishii
Visiting Fellow
Tumor Biology and Metastasis
miR130a and miR145 reprogram immature myeloid cells and inhibit tumor metastasis via improvement of anti-tumor immunity.
Immature myeloid cells, identified as Gr1+CD11b+ cells or myeloid derived suppressor cells (MDSCs), are expanded in tumor bearing host, and promote distant organ metastasis. Previous studies showed that transforming growth factor beta (TGFß) type 2 receptor (T?RII) is overexpressed in these myeloid cells under tumor conditions compared to those from normal condition. Depletion of myeloid specific T?RII in several mouse models inhibits tumor metastasis. However, it is not clear how T?RII is regulated. We found that miR130a and miR145 directly target myeloid TßRII and are down-regulated under tumor conditions. Ectopic expression of miR130a and miR145 in bone marrow stem cells driven by CD11b decreased lung metastasis of 4T1 mammary tumors. Additionally, miR130a transgenic mice also decreased metastasis of Lewis lung carcinoma. Functionally, miR130a and miR145 reduced Th2 cytokine production by myeloid cells and increased IFN?-producing CD8 cytotoxic lymphocytes. Therapeutically, miR130a and miR145 mimics skewed a pro-tumor microenvironment (Th2) to an anti-tumor microenvironment (Th1) through reprogrammed Gr-1+CD11b+ myeloid cells, and enhanced the anti-tumor effect of Paclitaxel. Mechanistically, miR130a and miR145 targeted multiple molecular networks including TGFß and insulin like growth factor 1 receptor (IGF1R) pathways that correlated with higher tumor stage in cancer patients. Lastly, miR130a and miR145 mimics, as well as IGF1R inhibitor NT157 improved anti-tumor immunity and enhanced efficacy of paclitaxel treatment in preclinical mouse models. These results demonstrated that miR130a and miR145 can reprogram tumor-associated myeloid cells by altering the cytokine milieu and metastatic microenvironment, thus enhancing host antitumor immunity.

National Cancer Institute - Center for Cancer Research
Sameer Issaq
Postdoctoral Fellow
Clinical and Translational Research - Cancer
Characterization of pediatric sarcoma metabolic dependencies identifies a glutamine synthetase-dependent mechanism of adaptation to glutamine deprivation
Sarcomas represent a diverse group of malignancies with unique molecular and pathological characteristics. In order to improve sarcoma treatment, a better understanding of the alterations associated with specific sarcoma subtypes is critically important. Renewed interest in the altered metabolic properties of cancer cells has led to an exploration of targeting metabolic dependencies as a novel therapeutic strategy. In this study, we have characterized the dependency of human pediatric sarcoma cells on key metabolic substrates and identified a mechanism of adaptation to metabolic stress

by examining cell proliferation and bioenergetic properties under varying concentrations of glucose and glutamine. We utilized 3 human rhabdomyosarcoma cell lines and 5 human Ewing sarcoma cell lines. While all cell lines were completely growth-inhibited by lack of glucose, the majority of cell lines tested were able to adapt to glutamine deprivation and restore proliferation following an initial period of reduced growth. We show that the expression of glutamine synthetase (GS), the enzyme responsible for de novo glutamine synthesis, increased in all cell lines that adapted to glutamine deprivation, and that pharmacological or shRNA-mediated inhibition of GS abolished the ability of glutamine-deprived cells to proliferate, while having no effect on cells grown under normal culture conditions. Furthermore, glutamine deprivation significantly reduced mitochondrial bioenergetic function. The effects of glutamine deprivation on proliferation and bioenergetics were rescued by the re-introduction of glutamine into the culture media. Moreover, the GS substrates and glutamine precursors glutamate and ammonia were able to restore proliferation of glutamine-deprived cells in a GS-dependent manner, further emphasizing the necessity of GS for adaptation to glutamine stress. Our findings suggest that GS mediates proliferation of glutamine-deprived pediatric sarcoma cells, and that GS inhibition and metabolic dependencies should be further investigated in order to identify vulnerabilities that could be targeted for potential therapeutic benefit.

National Cancer Institute - Center for Cancer Research
WEI LI
Postdoctoral Fellow
Immunology - Infectious Disease
One-domain CD4 Fused to Human Anti-CD16 Antibody Domain Mediates Effective Killing of HIV-1-Infected Cells
Bispecific killer cells engagers (BiKEs) which can bind to natural killer (NK) cells through the activating receptor CD16A and guide them to cells expressing the HIV-1 envelope glycoprotein (Env) are a promising new weapon for elimination of infected cells and eradication of the virus. Here we report the design, generation and characterization of BiKEs which consist of CD16A binding human antibody domains fused through a flexible linker to an engineered one-domain soluble human CD4. In presence of cells expressing HIV-1 envelope glycoproteins (Envs), these BiKEs activated specifically CD16A-expressing Jurkat T cells, degranulated NK cells, induced cytokine production and killed the Env-expressing cells. They also effectively mediated killing of chronically and acutely HIV-1 infected T cells by human peripheral blood mononuclear cells. Assessment of the in vivo HIV-1 eradication capacities for these BiKEs are undergoing on mouse and monkey models. The presumed ability of these CD4-based BiKEs to bind all HIV-1 isolates, their small size and fully human origin, combined with high efficacy suggest their potential for HIV-1 eradication.

National Cancer Institute - Center for Cancer Research
Yang Liu
Visiting Fellow
Cell Biology - General
Microenvironmental mitochondria transfer support chemo-resistance in cancer cells
Mitochondria is the powerhouse in eukaryotic cells that plays vital roles in cellular metabolism.

Emerging evidence indicates that in addition to inherited from parent cell, mitochondria could be horizontally transferred among different cell lineages, which not only supports metabolism but also relieve metabolic stress in recipient cells. Cancer cells exploit mitochondrial function by diverting ATP generation to synthetic pathways, as such to meet the substantial demands for fast cellular proliferation and redox balance. However, it is still obscure on whether microenvironment-derived mitochondria affect cancer biology and disease outcome. In this study, we established in vitro and in vivo model to explore the mitochondria transfer in tumor microenvironment. By co-culture and conditional media system, we demonstrated that mitochondria are released from normal human astrocyte (NHA) via extracellular vesicles (EVs). The release of mitochondria is governed by cADPR/CD38 pathway and concomitant intracellular calcium signaling. Microenvironment-derived mitochondria could be internalized by glioma cells that shared the same microenvironment. Mitochondria transfer support metabolism in recipient glioma cells, characterized by significantly increased oxidative metabolism and glycolysis. Moreover, mitochondria transfer supports the synthesis of macromolecules with biologic importance, such as ATP and NADH/NAD+, and therefore potentiates the cellular resistance against chemotherapies by prompting PARP-associated base excision DNA repair (BER) pathway. Our findings suggest that EVs-derived mitochondria component in microenvironment benefit cancer cells with both metabolic capability and chemo-resistance. Targeting microenvironment-derived mitochondria transfer may become a novel avenue in cancer therapies which not only diminish crucial metabolic pathways in cancer cells, but also augment the therapeutic effect with combination with other anti-cancer agents.

National Cancer Institute - Center for Cancer Research
Haibin Liu
Postdoctoral Fellow
Virology - DNA
Regulation of long noncoding RNA lnc-FANCI-2 in cell proliferation by HPV oncoprotein E7 and host transcription factor YY1
Long noncoding RNAs (lncRNA) play diverse roles in biological processes. To explore whether lncRNAs are involved in the development of cervical cancer induced by human papillomaviruses (HPV), we identified by RNA-seq and verified by qRT-PCR significant increase of a long noncoding RNA, lnc-FANCI-2, in cervical cancer over that in the normal cervix or cervical pre-cancer lesions. We characterized that lnc-FANCI-2 encoded from the chromosome 15 is transcribed from two alternative promoters, contains six or seven exons and uses two alternative polyadenylation sites for its RNA polyadenylation. By alternative RNA splicing, the lnc-FANCI-2 gene produces up to 35 isoforms of the polyadenylated RNAs. Host transcription factor Yin-Yang 1 (YY1) overexpressed in cervical cancer tissues has been found being a transcriptional activator for the expression of lnc-FANCI-2 through two mapped YY1 binding sites in the lnc-FANCI-2 promoter. High-risk, but not low-risk, HPV infections increase lnc-FANCI-2 expression and viral E7 is responsible for the upregulation. Knockdown of YY1 expression prevents lnc-FANCI upregulation by HPV infection and reduces cell growth. To further investigate the roles of lnc-FANC-2 in cervical cancer carcinogenesis, we knocked down the expression of lnc-FANC-2 or HPV16 E7 in HPV16-postive cervical cancer cell line CaSki cells and compared the differential expression of host genes from CaSki cells to the cells knocked down of lnc-FANCI-2 or viral E7 expression by RNA-seq analysis. Subsequently, we discovered that the expression of a substantial amount of coding and noncoding genes were affected by the knockdown of either lnc-FANC-2 or E7 expression, indicating the important

roles of lnc-FANCI-2 in E7-dependent tumorigenesis. Several dozens of these genes related to lnc-FANCI-2 or HPV16 E7 expression were verified by NanoString technology and most of them are associated with cell division and cell cycle controls. In conclusion, we have identified lnc-FANCI-2 expression being regulated by high-risk HPV infection and host YY1 and elucidated its function in cell proliferation, shedding important light on understanding how high-risk HPV infection leads to development of cervical cancer.

National Cancer Institute - Center for Cancer Research
Poulami Majumder
Visiting Fellow
Chemistry
Mighty Misty miRNA Modality: Peptide Hydrogels as Sprayable miRNA Depot for Mesothelioma
Malignant Pleural Mesothelioma (MPM) is a rare but highly aggressive asbestos-related tumor that develops within the chest cavity. Due to long period of latency, insensitivity towards radiotherapy and extremely challenging surgical procedures, finding treatments for MPM has been challenging. Surgical resection of MPM leaves a large surface area of tissue that can possibly be directly treated with therapy to prevent recurrence. However, this necessitates the use of a drug delivery vehicle that can cover large areas of tissue having complex surface topology. We are developing a biodegradable hydrogel that can encapsulate therapeutics and be delivered as a misty spray directly to the pleural cavity to allow uniform coverage of the tissue surface with drug. The gel is prepared from self-assembling peptides that allow the direct encapsulation of nanoparticles containing miR-215, a miRNA shown to induce a p53 positive feedback loop causing apoptosis in MPM cells. Fabrication of the delivery platform begins by first condensing miR-215 with the positively charged amphiphilic peptide MAX1 affording nanoparticles capable of trafficking the RNA into cells after being delivered by the spray gel to the tissue. We show that these nanoparticles ferry miR-215 into human MPM cells via endocytosis and endosomal escape, resulting in coordinated downregulation of multiple cell cycle transcripts. We next show that RNA-nanoparticles can be stably encapsulated into sprayable gels to achieve sustained miRNA release for about a month. Appropriate control of release rate is possible by varying the net charge of the sprayable peptide matrix. Based on these results, the efficacy of the sprayable miRNA-215 depot will now be evaluated under pre-clinical settings. Together, our work describes a simple and effective delivery platform that could be extended for a diverse array of locoregional therapies.

National Cancer Institute - Center for Cancer Research
Kerrie Marie
Visiting Fellow
Tumor Biology and Metastasis
Melanoma cells co-opt hardwired embryonic pathways to facilitate metastasis
Re-expression of embryonic pathways occurs in tumors, however the significance in cancer progression is unknown. Metastatic melanoma (cancer of melanocytes) is responsible for most skin cancer deaths, therefore understanding the pathways that facilitate melanoma metastasis is critical to devise new therapeutic targets. Cell characteristics that mediate metastatic competence are analogous to those required during embryonic melanocyte (melanoblast) migration and colonization. We hypothesized that

melanoma cells co-opt innate melanoblast pathways to facilitate metastasis, and reasoned these pathways would be upregulated in melanoblasts and metastatic melanoma, but downregulated in mature melanocytes. We used an iDct-GFP mouse model with melanocyte-specific GFP to isolate melanoblasts and melanocytes during development (E15.5, E17.5, P1, P7) and performed RNAseq. This yielded a novel 149-gene melanoblast transcriptome, which was filtered for upregulation in melanoma to yield 16 putative metastasis genes. 8/16 genes were upregulated in human metastatic melanoma in 3 patient databases, and we identified a novel melanoblast-derived biomarker that predicts metastatic melanoma patient outcome. To investigate how re-expression of embryonic genes might promote metastasis, we characterized our top-ranked candidate, KDELR3, an Endoplasmic Reticulum (ER) retention receptor of the ER-stress response. shRNA/siRNA KDELR3 knockdown (KD) impaired lung colonization in tail vein metastasis assays and anchorage-independent growth in soft agar, which was rescued by expression of mutated KDELR3 that cannot be targeted by shRNA. Moreover, KDELR3 KD did not affect cell cycle or subcutaneous tumor growth, confirming its metastasis-specific function. Acute tunicamycin-induced ER-stress in KDELR3 KD metastatic melanoma cells resulted in 14-fold more cell death than controls, suggesting KDELR3 protects against ER stress-mediated cell death. RNAseq analysis of stable KDELR3 KD vs. control cells showed upregulation of UGT1A genes that link protein folding and ER-Associated Degradation (ERAD). ERAD was impaired in KD cells, and, immunoprecipitation and confocal imaging identified a novel interaction between KDELR3 and the ERAD protein gp78. Our data indicates KDELR3 is a novel regulator of ER stress and ERAD pathways in metastatic melanoma, demonstrating the importance of exploring new, now-uncovered melanoblast pathways as targets for therapeutic intervention in metastatic melanoma.

National Cancer Institute - Center for Cancer Research
Stephen Miller
Postdoctoral Fellow
Chemistry

Tunable Protein Release from a Peptide Hydrogel

Protein therapeutics have become a significant source of new drugs in recent years due to high affinity and selectivity towards specific biological targets. However, proteins can't be delivered orally and the need for constant IV injections can reduce patient compliance with treatment regimens. Hydrogels, polymer networks that contain large amounts of water, are one type of material that researchers have been attempting to use for improved protein delivery. Over the last 15 years, our lab has been a pioneer in the development of peptide-based hydrogels guided by protein design principles. One notable advantage, compared to many other types of hydrogels or delivery platforms, is that our gels composed of self-assembling beta-hairpin peptides have shear-thin recovery properties that makes them capable of syringe delivery directly to a site of interest. A single, local injection of therapeutic agents encapsulated in gel can have the same efficacy as daily, systemic injections by IV. Here we report an extension of this work to precisely control the rate of protein release from one of our peptide gels. Release can be modulated by pairing a protein and a gel that have either the same or opposite net charge to achieve rapid or very slow release, respectively. Though these extremes have found uses in some applications, others require sustained release over the course of a few weeks and such a mechanism has been difficult to design and accomplish in the field of hydrogel-mediated protein delivery. We have been able to alter the release timelines using a protein and peptide of the same net

charge, with a genetically incorporated N-terminal fusion domain on the protein that contains a complementary charge to the gel. The fusion tail electrostatically binds to the gel, while the protein itself is repelled, and the rate of release can be controlled by adjusting the number of charges in the fusion domain. Using the model protein EGFP, we have demonstrated a diverse set of release profiles from a single peptide gel ranging from very fast, to intermediate and very slow. To date, this is the first hydrogel delivery strategy to display such control of protein release and allows us to tailor our design for specific clinical applications for optimal therapeutic delivery timelines. Ongoing work is being devoted to achieving sustained release for in vitro and in vivo cancer models.

National Cancer Institute - Center for Cancer Research
Kyster Nanan
Visiting Fellow
Epigenetics

Enhanced in vivo binding of CTCF to 5-carboxylcytosine occurs consequent to thymine-DNA glycosylase knockout and results in modest changes in alternative splicing

Methylation of the 5th carbon of cytosine produces 5-methylcytosine (5mC), the most common form of DNA methylation observed in eukaryotic genomes. This epigenetic modification has many nuclear roles, including regulation of gene expression, pre-mRNA splicing, and maintenance of genomic stability. In addition to 5mC, the epigenetic landscape is further altered by TET enzymes to produce the oxidized derivatives 5-hydroxy-methylcytosine, 5-formylcytosine, and 5-carboxylcytosine (5caC). Predictably, changes to the “epigenome” have pleiotropic effects, some of which promote diseases like cancer.

Previous reports have linked alternative mRNA splicing to 5mC via the transcription factor CTCF. Specifically, CTCF binding promoted RNA pol II pausing and spliceosome assembly at weak exons, favoring their inclusion in processed transcripts. Overlapping 5mC precluded CTCF binding, pol II pausing, and spliceosome assembly, resulting in exon exclusion. This sensitivity of CTCF to 5mC prompted us to ask whether this property was shared with other cytosine derivatives. Gel-shift assays showed that, in stark contrast to 5mC, CTCF interacted more strongly with DNA probes containing 5caC vs. cytosine. Intrigued by this novel finding, we next asked whether this observation was recapitulated in vivo. To this end, we used mouse embryonic stem cells (mESCs) whose genomes contained increased 5caC due to ablation of the enzyme thymine-DNA glycosylase (Tdg KO). CTCF chromatin immunoprecipitation-sequencing performed in Tdg KO mESC identified 53,018 CTCF binding sites, a 26% increase in the number of sites observed in control mESC. RNA-seq analysis showed that Tdg KO resulted in up- and downregulation of 4,167 and 4,238 genes, respectively. It is important to note, however, that the observed gene-expression changes are likely due to altered promoter methylation found in Tdg KO mESC, rather than CTCF-specific effects. Next, splicing analysis revealed a modest increase in the number of “cassette” exons whose inclusion was favored in Tdg KO mESC relative to control cells. Finally, CTCF binding was observed within 1 kb of 45 exon-inclusion events in Tdg KO mESC. Ongoing work in our laboratory is aimed at characterizing the biophysical basis for the interaction between CTCF and 5caC. Our investigations will help to identify the mechanism by which the enhanced interaction between CTCF and 5caC is achieved and will illuminate the biological relevance of this interaction.

National Cancer Institute - Center for Cancer Research
Ville Paakinaho
Visiting Fellow
Biophysics
Simultaneous Tracking of Multiple Transcription Factors in Living Cells by Single-Molecule Imaging
Population-based assays such as ChIP-seq, DNase-seq and FAIRE are widely used to investigate the interactions of transcription factors (TFs) with chromatin and are often interpreted in terms of sequential and static binding. However, fluorescent microscopy techniques have revealed a more dynamic binding behavior of these proteins in living cells. One such protein, glucocorticoid receptor (GR), is a hormone-regulated TF that plays a central role in metabolic regulation and anti-inflammatory activities. Recently, a new and critical fluorescent microscopy technique, single-molecule tracking (SMT) has emerged. This technique enables the real-time tracking of individual TF molecules, making it possible to measure the distribution of TF binding times. I have intensively investigated the application of this approach to characterization of the binding behavior of TFs. I and my colleagues have shown, using Halo or Snap tags labeled with bright and stable fluorophore JF549, that TF residence time at specific response elements ranges between 6 and 14 sec. This supports transient rather than stable TF chromatin interactions. In addition to short residence time at chromatin, our data also show that only a small proportion of GR and its cofactors are functionally bound at any given time. Interestingly, similar behavior can be seen with classical pioneer factor FoxA1, indicating that its action is more dynamic that has been led to believe by literature. These single-molecule observations affirm the general model that many TFs are highly dynamic in their chromatin binding activity. These studies were performed using a single-color HILO microscopy. To take the next step in understanding real-time dynamics of TF action, we have developed 3-color HILO microscopy system that allows simultaneous tracking of multiple TF and cofactors in living cells. To understand the real-time behavior of GR action, simultaneously tracking of GR+GR dimer as well as GR+cofactor pairs will be performed. Studying the simultaneous action of these pairs at colocalized binding sites, enables us to decipher the sequential arrival/departure sequence for a series of factors. Preliminary tracking of Halo-GR+Snap-GR indicated that at colocalized sites GR on average arrives and departs at the same time. Thus, our approach offers the potential to observe for the first time the real-time action TFs at enhancers and promoters.

National Cancer Institute - Center for Cancer Research
Manoj Rajaure
Visiting Fellow
Microbiology and Antimicrobials
Tweaking the T7 Tail Fibers to Target Pathogenic Bacteria
The extensive use of antibiotics can cause the dysbiosis of normal gut microbiota affecting the human health as well as selecting for the drug-resistance bacterial pathogens during treatment. These effects have emphasized the need for new and innovative antimicrobial strategy. The use of bacteriophages to address these problems is viewed as an alternative strategy. However, there are number of challenges using phages for clinical application, such as the potential of phage to transmit toxins or resistance genes and the ability of bacteria to develop resistance by altering their receptors. In order to address these concerns we proposed to develop an engineered multivalent phage using a well-characterized E.

coli phage T7 as a single platform to target various bacterial pathogens. T7, a member of Podoviridae family of the tailed phages, strictly depends on E. coli for its growth. Host specificity of T7 is governed by the six copies of a tail fiber, each homo-trimers of gene product 17, that are attached to the phage tail complex. These tail fibers play an essential role to initiate infection by attaching the virus, albeit reversible, to the surface exposed lipopolysaccharide (LPS) of E. coli. We envisioned that by incorporating additional tail fibers of different phage into the T7 genome, a multivalent T7 that can target multiple host could be developed. In order to test this idea, we first assembled a chimeric tail fiber gene encoding a product comprised of tail-binding N-terminal domain of T7 fiber and receptor binding C-terminal domain of Pharr, a T7-like phage that is specific for a pathogenic strain of Klebsiella pneumonia. This chimeric gene was then introduced into the T7 genome by means of homologous recombination resulting into a T7 phage product assembled with two different types of tail fibers. Upon testing on both host E. coli and K. pneumonia, the purified hybrid phage, which we named kT7, was able to effectively kill Klebsiella as well as E.coli, suggesting the targeting of multiple bacteria by incorporating biologically active additional tail fibers is feasible. This synthetic approach of phage engineering will leverage us in targeting multiple pathogenic bacterial strains using a well characterized single phage platform, such as T7. We anticipate that this technique can be expanded to target other know human pathogens as well as to target multiple surface receptors of a single pathogen, making bacterial escape very unlikely.

National Cancer Institute - Center for Cancer Research
Revathy Ramachandran
Postdoctoral Fellow
Molecular Biology - Prokaryotic
Coordination of DNA replication in bacteria with multiple chromosomes: the Vibrio cholerae example
Introduction: In all organisms, chromosomes replicate once per cell cycle to avoid aneuploidy and genome instability, the cause of many diseases including cancer. Although bacteria mostly have one chromosome, about ten percent have multiple chromosomes whose replication must be coordinated to ensure complete genome duplication before cell division. Most well-studied among such bacteria is the human pathogen Vibrio cholerae. It has two chromosomes, Chr1 and Chr2. Unlike eukaryotes where a common set of proteins replicate all chromosomes, Chr1 and Chr2 possess their own initiators of replication. Chr1 is three times larger than Chr2, yet the two terminate replication at the same time. This suggests the presence of a mechanism to delay replication of the smaller Chr2 until the right time.

Motivation: The first clue that Chr1 and Chr2 might communicate was the identification of a 150-bp site on Chr1 that controlled Chr2 replication. The timing of replication of the Chr1 site correlated with the initiation of replication of Chr2. The site was named crtS, for Chr2 replication triggering site but the triggering mechanism remains unknown. Here we address how the replication of a site on one chromosome licenses replication of another chromosome.

Results: We devised a conditional system to selectively block replication of Chr1 and found that blocking Chr1 replication blocked Chr2 replication. The dependence on Chr1 replication is relieved by providing

the crtS site in a plasmid, indicating that the site alone is the relevant player from Chr1. Strikingly, two copies of unreplicated crtS could also trigger Chr2 replication, suggesting that the unreplicated site has significant basal activity and a role of replication is to increase the site dosage. How the passage of the replication fork across crtS activates its function remains unclear. We find that crtS remodels the Chr2 initiator RctB to activate its initiator function. The crtS site thus appears to be an allosteric modulator of RctB, a function that is best served upon replication of the site.

Significance: The activation of RctB by crtS, itself activated by passage of the replication fork, provides a novel example of replication-triggered activation of a protein function. The licensing of Chr2 replication by crtS is also the first example of communication between chromosomes for replication.

National Cancer Institute - Center for Cancer Research
Christopher rice
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses

Tumor-elicited neutrophils generate immune-inhibitory reactive oxygen species through utilization of mitochondrial oxidative metabolism

Neutrophils are a vital component of the immune system with multiple roles in both physiology and disease. In cancer however, neutrophils can play major roles in tumor progression and establishment of the metastatic niche. Here they suppress anti-tumor immunity by generating reactive oxygen species (ROS) which disrupts lymphocyte function. Recently, there has been a resurgence of interest in metabolism and how this controls leukocyte function. Neutrophils are generally considered to be a homogenous population dependent on glycolysis. However, we show that during hematopoiesis, immature neutrophil subsets possess a greater capacity for mitochondrial oxidative metabolism, which is maintained by the receptor tyrosine kinase c-Kit. In healthy mice, circulating c-Kit+ neutrophils are a marginal population, however in mammary tumor (4T1) bearing mice, this population is greatly expanded in the periphery. This elevated c-Kit expression corresponds with increased mitochondrial fitness in peripheral neutrophil populations, and was reversed after in vivo blockade of c-Kit. Furthermore, we demonstrate that neutrophils that possess a greater mitochondrial capacity are able to support ROS production independently of glucose utilization, via fatty acid-dependent mitochondrial activity. This is achieved via a mitochondrial source of NADPH which is required to fuel NADPH-oxidase (NOX) activity and subsequent ROS generation. Consistent with these findings, tumor elicited oxidative neutrophils were able to maintain ROS production and suppression of T-cells following the inhibition of glucose utilization. Our data suggests that 4T1 tumors use c-Kit signaling to maintain an oxidative neutrophil population which is able to utilize a greater variety of fuels to maintain immune-suppressive activity in the glucose-depleted tumor microenvironment. Therefore, modulation of c-Kit signaling may pose an attractive approach for therapeutic intervention, where significant neutrophil populations are often a poor prognostic marker.

National Cancer Institute - Center for Cancer Research
Christina Ross
Postdoctoral Fellow
Tumor Biology and Metastasis

Metastatic breast cancer cells hijack mRNA turnover to regulate levels of metastasis-suppressor factors

Breast cancer is a leading cause of cancer-related deaths in women in the US, and most deaths are due to metastatic disease. Despite this little is understood regarding the underlying mechanisms of metastatic breast cancer, making it difficult to identify and treat at-risk patients. To address this, transcriptional networks associated with inherited predisposition to metastasis were investigated. Integration of gene expression analysis and susceptibility genetics studies identified a gene network associated with metastasis that is centered on the Cnot2 gene, a member of the CCR4-NOT deadenylase complex which removes mRNA poly-A tails to initiate RNA degradation. Subsequent analysis validated the role of the CCR4-NOT deadenylation activity in mammary tumor metastasis. However, considering the essential yet seemingly non-specific function of this complex, it is unclear how the CCR4-NOT complex specifically promotes metastatic disease. To examine the role of CCR4-NOT in metastasis, upregulated transcripts following shRNA knockdown (KD) of the CCR4-NOT catalytic subunit were analyzed. Interestingly, we found significant enrichment of transcripts bound by RNA binding proteins (RBPs) Pum2, Nanos1, and Cpsf4. Furthermore, individual KD of these RBPs reduced in vivo metastasis of mouse mammary cancer cells. This suggests that the RBPs may downregulate specific metastasis-suppressor factors by targeting their mRNAs for CCR4-Not guided degradation. To test this, RNAseq data from the RBP KD cells was filtered for commonly upregulated mRNAs containing RBP recognition sequences. mRNA stability assays were then performed to identify probable direct targets of the RBPs. The mRNA for Smarcd1, a SWI/SNF protein that regulates chromatin structure and pluripotency, contains all three RBP binding sites and was significantly stabilized upon RBP KD suggesting a possible role in metastatic progression. Current efforts focus on measuring the effect of Smarcd1 protein levels on metastasis in vivo and characterizing how Smarcd1 mRNA is regulated in metastatic cells. This study highlights the very complex role of mRNA decay machinery in modulating discreet pools of mRNAs that encode modulators of metastasis. Understanding these transcriptomic alterations will be significant for the development of metastasis-targeted therapies to combat this devastating disease.

National Cancer Institute - Center for Cancer Research
Chringma Sherpa
Postdoctoral Fellow
Virology - RNA and Retroviruses

A single nucleotide change in a functionally undefined region of the RRE impairs HIV replication through an RRE conformational switch.

The HIV-1 Rev Response Element (RRE) is a cis-acting RNA element with multiple stem-loops. Binding and subsequent mutlimerization of the HIV-1 Rev protein on the RRE are essential steps in HIV replication. Most of our understanding of the Rev-RRE regulatory axis comes from studies on a few lab-adapted HIV clones. However, from a therapeutic standpoint, in a rapidly evolving virus like HIV,

mechanistic studies of naturally occurring Rev and RRE sequences are very critical. A recent study on Rev-RRE variation in serum samples from HIV infected individuals reported that, in one patient, the predominant RRE sequence at around seroconversion (early SC3) and after AIDS onset (late SC3) differed only by 4 isolated nucleotide changes in functionally undefined regions of the RRE. The viral construct with late SC3 was found more active than that containing the early SC3 by a Gag-Pol reporter assay and viral growth assays. Interestingly, the Rev sequence remained unchanged between the two time-points, suggesting RRE variation as the driver of this change in activity. To understand the mechanism behind this functional difference between the two RREs, these RRE RNAs were in-vitro transcribed and their secondary structure determined using CE-SHAPE (capillary electrophoresis based selective 2' -hydroxyl acylation analyzed by primer extension) and SHAPE-MaP (high throughput sequencing based SHAPE-mutational profiling) technologies. The late RRE assumed a canonical 5 stem-loop structure whereas the early RRE formed a structure with differential folding in stem-loops I, IV, and V. To further investigate the contribution of each nucleotide change in RRE folding, early SC3 RRE mutants, carrying different combination of the four signature late RRE nucleotides sequence, were created and their RNA structure determined by SHAPE-MaP. We found that only one nucleotide change (G to C) in the functionally undefined central loop region of the RRE was responsible for the structural switch between the early and late RRE. By Gag-Pol reporter assay, we found that the same mutation alone restored the RRE activity of the early RRE to the late RRE level. Thus, using naturally occurring RRE variants, we show for the first time, that a single nucleotide change in the functionally undefined central loop region of the RRE can affect global RRE structure and function. Studies investigating the effect of these RRE mutations on Rev binding and multimerization are underway.

National Cancer Institute - Center for Cancer Research
Kenta Shinoda
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Identification and potential role of atypical rearrangements at the mouse immunoglobulin kappa locus
The defining event of B lymphopoiesis is immunoglobulin gene recombination. V(D)J recombination is initiated by lymphocyte-specific recombination activation gene (RAG) proteins that generates DNA double strands breaks (DSBs) at recombination signal sequence (RSS) associated with rearrangeable gene segments. RSS consisting of conserved heptamer and nonamer sequences separated by either a 12 or a 23 base pair spacer. Recombination is only efficient when one 12RSS and one 23RSS are engaged by RAG, a restriction known as the 12/23 rule. Human and mouse immunoglobulin kappa light-chain locus consist of Vk and Jk gene segments. Vk segments are flanked with 12RSSs and the Jk segments with 23RSSs. RAG-mediated recombination beyond the traditional boundaries of V(D)J recombination is inherently dangerous. Here we described atypical rearrangements that apparently violate the 12/23 rule in VJ recombination at the mouse Igk locus. We deleted the Jk gene region including all Jk gene segments with 23RSSs by CRISPR/Cas9 mediated gene editing in v-abl-transformed pre-B cells. By using a novel approach termed END-seq which enable genome-wide mapping of broken DNA ends generated by DSBs, we found that RAG dependent DSBs were still observed in 12RSS sites of Vk gene segments in

Jk-deleted clones. Surprisingly, the level and distribution of Vk breaks were similar in wild-type and Jk-deleted clones. As we could not find enrichment of DSBs out of Igk locus to explain the DSBs seen in Jk-deleted clones, we next analyzed rearrangement in Jk-deleted clones. We applied high-throughput genome-wide translocation sequencing (HTGTS) to generate genomic maps of Igk rearrangements in Jk-deleted clones. By this approach, we sequenced the rearrangements observed from Vk 1-117, which is one of highly used Vk gene in the initial Igk repertoire. Interestingly, we detected rearrangements to other Vk gene segments from Vk 1-117 in an RAG-dependent manner. These Vk-Vk rearrangements were detected not only in Jk-deleted clones, but also in wild-type cells. Moreover, the Vk-Vk rearrangement occurred more frequently than Vk-Jk rearrangement from Vk 1-117, suggesting that Vk-Vk rearrangement is not a rare event as compared to traditional VJ recombination. These results suggest that Vk-Vk rearrangement might have a role in shaping the repertoire of VJ recombination in Igk locus. Our novel approach to recovering atypical Igk rearrangement provides insights into the stringency of V(D)J recombination.

National Cancer Institute - Center for Cancer Research
Shweta Singh
Postdoctoral Fellow
Hematology/Oncology, Tumor Immunology, and Therapy

Loss of Id1 protects hematopoietic stem cells from stress-induced exhaustion and aging

Hematopoietic stem cells continuously replenish the blood and immune system throughout the life of an individual. Inhibitor of DNA binding (Id) proteins are important transcriptional regulators of cell growth and differentiation during this process. They are required for normal muscle, nerve and hematopoietic cell development in the adult, and their deregulation has been linked to the initiation and progression of multiple cancer types. We found that Id1 is induced in hematopoietic progenitors by growth factors, such as IL-3, which suggest that Id genes may regulate the response of normal hematopoietic stem cells (HSCs) and progenitor cells to stress. We examined the requirement for Id1 in HSCs during bone marrow transplantation (BMT), genotoxic and inflammatory stress, and aging assays. Using serial bone marrow transplantation assays, we determined that HSCs which lack Id1 have enhanced self-renewal and long term engraftment capacity, suggesting that these HSCs were uniquely protected from stress-induced exhaustion. We then accessed the level of Id1 in various stem and progenitor populations and found that Id1 is expressed at low levels in the HSCs during steady-state hematopoiesis, but is induced by conditions mimicking hematopoietic stress. To determine if the loss of Id1 affected the self-renewal or maintenance of HSC, or was associated with increased HSC quiescence, we performed cell cycle analysis and assessed indicators of biological stress. We found that not only do the Id1-/- HSCs cycle less, they also have reduced DNA damage, mitochondrial biogenesis/stress indicators and lower ROS levels. Single cell division assays revealed that the HSCs that lack Id1 have reduced proliferation and show increased quiescence. Furthermore, we found that small molecule inhibition of cytokine signaling in vivo prevents Id1 induction in HSCs, suggesting a potential mechanism to reduce Id1 levels in HSCs during the proliferative stress of BMT or other states of hematopoietic stress. Altogether, our data suggest that HSCs lacking Id1 are protected from exhaustion by physiological stress including chronic genotoxic and inflammatory stress, and aging. Thus, targeting Id1 to inhibit its activity may be therapeutically useful to improve HSC survival and function during bone marrow transplantation, chronic stress and aging.

National Cancer Institute - Center for Cancer Research

NAYOUNG SONG

Visiting Fellow

Carcinogenesis

Determining the signaling pathway of epithelial-IKKa-deletion-mediated symbiotic bacterial and fungal infection in carcinogenesis

Bacteria and fungi, two major components of the microbiota, generally share niches and develop both antagonistic and symbiotic relationships, regulating the pathological impacts on the host. The epithelium is where the bacterial-fungal interaction occurs most abundantly, but the relationship between the epithelium, bacteria, and fungi on the pathogenesis, particularly tumorigenesis, is poorly understood. IKKa is one of the crucial factors regulating the homeostasis of squamous epithelial tissues. Recently, our lab has established a mouse model that develops esophageal squamous cell carcinomas associated with IKKa reduction, inflammation and chronic fungal infection. Cladosporium cladosporioides was a major type of fungi identified in this mouse model. Because IKKa deletion in the keratinocytes causes impaired skin barrier, we hypothesized that loss of epithelial IKKa may control fungal colonization through regulating the barrier integrity and inflammation. We generated IKKaf/f mice with inducible K15.Cre (IKKaf/f/K15.Cre) specifically expressed in keratinocytes in hair follicles which is considered as skin stem cells. After deleting IKKa in K15 cells in oral mucosa and skin, IKKaf/f/K15.Cre mice were orally inoculated with Cladosporium cladosporioides. We found that epithelial IKKa deletion increased bacterial colonization in oral mucosa and skin. Moreover, Cladosporium infection further promoted bacterial and fungal colonization in oral cavity and development of skin tumors, particularly sebaceous gland carcinomas. Interestingly, we found that impaired epithelial IKKa led to reduction of sebaceous lipid on the top of the epidermis which can be produced by sebaceous gland and protect skin against bacterial infection. Taken together, our data suggest that loss of epithelial IKKa induces the bacterial-fungal symbiosis in oral mucosa, promoting skin tumors, possibly due to reduced sebaceous lipid production. This study will shed light on the importance of the epithelial-bacterial-fungal interaction in the pathogenesis, proposing epithelial IKKa as a novel regulator of the bacterial-fungal interaction.

National Cancer Institute - Center for Cancer Research

Shogo Takahashi

Visiting Fellow

Pharmacology and Toxicology/Environmental Health

Farnesoid X receptor protects against chemically-induced liver injury through the taurocholate-JNK pathway

Hepatotoxicity is of major concern for human exposure to industrial chemicals and drugs, and clarifying the molecular pathogenesis of chemically-induced liver damage may lead to novel therapeutic interventions. Farnesoid X receptor (FXR), a ligand-activated transcription factor and a member of the nuclear receptor superfamily, is expressed in intestine and liver where it is activated by bile acid metabolites and controls bile acid homeostasis. Disruption of FXR was reported to enhance the sensitivity to acute liver injury in mice after toxicant exposure, but the precise mechanism remains unclear. A single low-dose intraperitoneal injection of carbon tetrachloride (CCl4), an inducer of acute

hepatitis in mice, resulted in more severe hepatocyte damage and higher induction of pro-inflammatory mediators, such as chemokine (C-C motif) ligand 2 (Ccl2), in Fxr-null mice compared with wild-type mice. Serum metabolomics analysis revealed marked increases in circulating taurocholic acid (TCA) and tauro-beta-muricholic acid (T-b-MCA) in these mice, and forced expression of bile salt export protein by adenovirus in Fxr-null mice ameliorated CCl4-induced liver damage and increases in these serum bile acids. Treatment of Fxr-null hepatocytes with TCA, but not T-b-MCA, significantly increased c-Jun-N-terminal kinase (JNK) activation and Ccl2 mRNA, and up-regulation of Ccl2 mRNA was attenuated by co-treatment with the JNK inhibitor SP600125, indicating that TCA directly amplifies hepatocyte inflammatory signaling mainly mediated by JNK in the absence of FXR signaling. Additionally, pre-treatment with SP600125 or restoration of hepatic FXR, by forced expression with recombinant adenovirus, attenuated CCl4-induced liver injury in Fxr-null mice. Collectively, these results suggest that the TCA-JNK axis is likely associated with increased susceptibility to CCl4-induced acute liver injury, and provide clues to the mechanism by which FXR and its downstream targets such as bile salt export protein, protects against chemically-induced hepatotoxicity.

National Cancer Institute - Center for Cancer Research
Yihan Wan
Postdoctoral Fellow
Gene Expression
Capturing transcription and splicing dynamics across the human genome with single-molecule sensitivity in living cells
Transcription and splicing are dynamic processes which reflect the synergy between chromosome structure changes, epigenetic modifications and stochastic molecular activities. These dynamic processes of RNA synthesis and processing contribute to the gene expression heterogeneity in a cell population. Importantly, heterogeneity is a dynamic phenomenon: the complement of RNA expressed in each cell depends on when the cell is observed. Although a continuous time-lapse quantification across human genome in single cells would be required to detect heterogeneous and stochastic behavior, the current measurements of transcription and splicing dynamics are mostly restricted to individual reporter genes. To obtain a broader view for understanding dynamic gene regulation, we present a platform for genome-scale measurement of transcription and splicing kinetics. By labeling endogenous genes with MS2 and/or PP7 stem loops in the introns, we recorded the nascent RNAs production process for dozens of endogenous loci. This approach relies on several methodological advances. First, we developed an approach for labeling genes at their endogenous loci with random insertion, followed by mapping of the labeling sites in a high-throughput manner. Second, we developed robust high-throughput single-molecule imaging and analysis. Finally, we implemented stochastic time-series analysis on a massive scale. The view that emerges from these studies is that transcription is episodic and often very infrequent. This combined approach can in principle be scaled to thousands of genes. We hope to establish a reference model and general framework for studying the property of RNA synthesis with single-molecule resolution.

National Cancer Institute - Center for Cancer Research
Limin Wang

Postdoctoral Fellow
Carcinogenesis
Nitric Oxide (NO•) Enhances IDO/Kynurenine Pathway in Pancreatic Cancer Progression
Pancreatic cancer is one of the most lethal malignancies and is the fourth leading cause of death due to cancer in the United States. The dismal prognosis in pancreatic cancer is due to the lack of early detection markers and its resistance to available treatments. Therefore, development of novel therapeutic target and treatment strategies are of utmost importance. We have recently shown that inducible nitric oxide synthase (NOS2)/NO• signaling enhances tumor progression and disease aggressiveness in pancreatic ductal adenocarcinoma (PDAC), which is the most common form of pancreatic cancer. Metabolic reprogramming is one of the hallmarks of cancer, and several metabolic adaptations are discovered in PDAC, which help pancreatic tumor cells to thrive under low nutrient and hypoxic condition. Therefore, metabolic vulnerabilities may be exploited to develop novel therapeutic strategies. Furthermore, inflammatory signaling pathways are implicated in regulating cellular metabolism. We hypothesized that NOS2/NO• signaling regulates metabolic reprogramming in PDAC. To test this hypothesis, we compared metabolic profile of tumors with NOS2-high and NOS2-low expression from resected human PDAC patients, and found that a tryptophan metabolite, Kynurenine, is associated with high NOS2/NO• expression, and a higher level of Kynurenine is associated with poor survival in PDAC patients (N=69, p<0.01). Gene expression profiling in PDAC tumors (N=78) showed positive correlation between the expression of NOS2 and the Tryptophan/Kynurenine pathway genes, including aryl hydrocarbon receptor (AHR), indoleamine-2,3-deoxygenase 1 (IDO1), tryptophan-2,3-deoxygenase (TDO). Treatment of pancreatic cancer cell lines with the NO• donor drug, not only induced the expression of AHR, IDO1, TDO and ARNT, but also induced many aryl hydrocarbon receptor (AHR) target genes including IL1b, IL6, IL8, CYP1A1, CYP1B1, TIPARP, ALDH1A3, SERPINB2. Furthermore, kynurenine treatment enhanced invasion/migration of pancreatic cancer cell lines in vitro. These findings identified a novel NOS2/NO•-induced signaling pathway which regulates the IDO/TDO/kynurenine metabolic axis to promote pancreatic cancer progression, which can be targeted for potential therapeutic significance.

National Cancer Institute - Center for Cancer Research
Cen Xie
Visiting Fellow
Biochemistry - General and Lipids
Activation of intestinal hypoxia-inducible factor-2alpha during obesity mediates non-alcoholic fatty liver disease
Non-alcoholic fatty liver disease (NAFLD) is becoming the most common chronic liver disease in industrialized countries. Persistent NAFLD triggers an increased risk of non-alcoholic steatohepatitis and end stage liver diseases such as cirrhosis and hepatocellular carcinoma. Pharmacologic therapy that targets NAFLD remains extremely limited. Accumulating reports indicate that liver hypoxia-inducible factors (HIF-1alpha and HIF-2alpha), members of the basic helix-hoop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor family, have a role in modulating the pathogenesis of NAFLD. However, the role of intestine HIF-alpha on NAFLD is poorly understood. Human intestine biopsies from patients with or without obesity revealed a relationship between activated HIF-2alpha but not HIF-1alpha, and increased body mass index and hepatic toxicity. Mice with an intestine-specific knockout (Hif2a-dIE) were examined to clarify the role of intestine HIF-alpha in obesity and NAFLD development. Wild-type mice

fed a 60% high-fat diet (HFD) had marked obesity and hepatic steatosis, while Hif2a-dIE mice on a HFD had diminished obesity and hepatic steatosis. PT2385, a HIF2alpha-specific inhibitor in clinical trials for kidney cancer, had preventive and therapeutic effects on metabolic disorders dependent on intestine HIF-2alpha. Decreased steatosis in Hif2a-dIE mice was further found to be independent of adiposity. Amelioration of these HFD-induced adverse metabolic phenotypes was correlated with reduced intestine and serum ceramide levels. Mechanistically, intestine HIF-2alpha regulates ceramide metabolism as revealed by identification of a novel HIF-2alpha target gene, neuraminidase 3 (Neu3), encoding a key enzyme in the ceramide salvage pathway. Direct inhibition of intestinal NEU3 mimicked the effects of HIF-2alpha inhibition on weight loss and NAFLD. Further, ceramide administration to Hif2a-dIE mice reversed the improvement in hepatic steatosis and obesity. This study uncovered an essential role for intestine HIF-2alpha in regulating obesity-related NAFLD, and provided a viable new target for NAFLD therapy.

National Cancer Institute - Center for Cancer Research
Hongping Zheng
Postdoctoral Fellow
Carcinogenesis
Single cell analysis reveals cancer stem cell heterogeneities in hepatocellular carcinoma
Tumor heterogeneity represents a major obstacle to effective cancer treatment and personalized medicine. Hepatocellular carcinoma (HCC) is clinically and biologically heterogeneous, which is partly attributed to the presence of hepatic cancer stem cells (CSCs). CSC surface makers can have overlapping expression or exclusive expression of different subpopulations of hepatic CSCs, and these cells may contain different oncogenic changes. The existence of various CSC surface-marked subpopulations might pose a problem for CSC targeted therapeutics. Little is known about whether there are shared or distinct pathways in different subpopulations. Moreover, it remains unknown whether heterogeneity exists within certain subpopulations. Thus, we propose to profile the global transcriptome of surface-marked CSCs (EpCAM+, CD133+ and CD24+) at the single-cell level using index flow cytometric sorting and single-cell RNA sequencing technology. We statistically compared mRNA transcriptomes of individual single cells of triple positive (EpCAM+/ CD133+ /CD24+) CSCs and triple negative (EpCAM-/ CD133-/CD24-) cells. Further, we compared the triple positive and the triple negative cells at the single cell level in terms of their self-renew capability in both normoxic and hypoxic conditions. Our results show that at the single cell level, the transcriptomic profiles show a high-degree of heterogeneity in HCC cell lines, meanwhile, there is a dramatic difference between triple positive and triple negative cells in their transcriptomic profiles. There is a high-degree of intra-tumor and inter-tumor heterogeneity observed in single cell transcriptomes of HCC patients' samples. Triple positive CSC single cells show continuous rather than discrete stemness-related gene expression patterns. In parallel, the self-renewal capability of triple positive cells also exhibits heterogeneity, while triple negative cells show little self-renewal capability. Thus, our single cell analysis study reveals molecular and biological heterogeneity of cancer stem cells in HCC, which may provide insight into the underlying mechanisms of how CSCs contribute to tumor heterogeneity and underscore key pathways and novel targets for hepatic CSC therapy.

National Cancer Institute - Center for Cancer Research

Dali Zong

Postdoctoral Fellow

DNA-binding Proteins/Receptors and DNA Repair

RNF168 guards the genome against BRCA1 mutation-induced instability

BRCA1 coordinates DNA double strand break repair by homologous recombination (HR). Such genome maintenance functions are thought to be critical for embryonic development and tumor suppression. Complete loss of BRCA1 results in embryonic lethality in the mouse, while human patients that inherit a single mutated allele of BRCA1 are markedly predisposed to developing breast and ovarian cancer over their lifetime. Despite intensive research efforts, why heterozygous BRCA1 germline mutations predispose to cancer remains unclear, as they produce no apparent phenotype in cultured cells. Here, using mouse genetics, we identify the E3 ubiquitin ligase RNF168 as a critical factor that maintains genome stability in mice that harbor heterozygous BRCA1 mutations similar to those found in human patients. Strikingly, deletion of RNF168 in BRCA1 heterozygous mice and cells results in embryonic lethality and hypersensitivity to DNA damaging agents, respectively. The finding that one functional copy of BRCA1 gene becomes inadequate in the absence of RNF168, a condition known as haploinsufficiency, is particularly unexpected, since earlier studies have shown that deletion of 53BP1, which requires RNF168 for its function, produces the opposite phenotype and rescues embryonic lethality in BRCA1-deficient mice. Further dissection of this 53BP1-independent function of RNF168 revealed that RNF168 is essential for the stable recruitment of the recombination mediator PALB2, which loads RAD51 at sites of DNA damage to initiate HR. Interestingly, the contribution of RNF168-mediated PALB2 recruitment is normally masked in BRCA1-proficient cells, because BRCA1 itself can bind PALB2. However, when BRCA1 expression and/or function is compromised by mutation, cells become reliant on RNF168 for PALB2 recruitment. Consistent with this, forced tethering of PALB2 to sites of DNA damage bypasses the requirement for RNF168 and stabilizes the genome of RNF168-/-BRCA1+/- cells. Intriguingly, RNA-seq analysis revealed that RNF168 expression is significantly lower in normal human mammary epithelial cells compared to several other cell types. These data provide a plausible explanation to the curious tissue specificity of tumors that arise as a result of germline BRCA1 mutations.

National Cancer Institute - Cancer Prevention Fellowship Program

Megan Clarke

Cancer Prevention Fellow

Clinical and Translational Research - Cancer

HPV DNA methylation as a biomarker for improving risk stratification and clinical management of HPV-positive women

Background:Human papillomavirus (HPV) DNA testing is being widely evaluated for cervical cancer screening. While HPV DNA testing has greater sensitivity compared to cytology, specificity is lacking, and triage tests are required to distinguish benign HPV infections from precancers. A promising option is HPV DNA methylation (DNAm). Increased HPV DNAm has been associated with precancer in four major carcinogenic types (HPV16, 18, 31, 45). We hypothesize that DNAm is an important step in carcinogenesis common to all HPV types. To test this hypothesis, we conducted a nested case-control study evaluating the association of HPV DNAm with cervical precancer for 11 carcinogenic HPV types.

Methods:For each HPV type (16, 18, 31, 33, 35, 39, 45, 51, 52, 58, and 59), we selected 30 cases with cervical precancer and 30 controls without abnormalities. HPV DNAm in viral L1 and L2 genes (about 9 CpG sites per type) was measured using next-generation bisulfite sequencing. We calculated odds ratios (OR) using logistic regression for the association of DNAm with precancer and assessed the possible discrimination of DNAm between infection and precancer using areas under the curve (AUC). For each HPV type, we compared the sensitivity (Se) and specificity (Sp) of DNAm to that of cytology, a test currently used for triage of HPV-positive women.

Results:We observed significant associations of higher DNAm with precancer in all but 3 sites (OR range 4-28.0). For each HPV type, the highest AUCs were 0.91 (HPV59), 0.86 (HPV18), 0.85 (HPV39), 0.84 (HPV16), 0.82 (HPV45), 0.81 (HPV35), 0.77 (HPV52), 0.74 (HPV58), 0.75 (HPV31), 0.73 (HPV33) and 0.71 (HPV51). At fixed Sp based on cytology (Sp range 33-60%), Se was equal or higher for DNAm compared to cytology for all types except HPV51, and 58 (Se range 88-100%).

Conclusions:We observed a strong association of increased HPV DNAm with precancers across 11 HPV types, suggesting that DNAm is a general phenomenon in the transition from infection to precancer. For most types, clinical performance of DNAm was comparable to or exceeded that of cytology. Next, we plan to analyze a combined panel of DNAm sites from each HPV type in a large screening population. We will develop an assay that provides risk stratifying information based on HPV genotyping and DNAm for the clinical management of HPV-positive women, which can be measured in a variety of specimen types, including self-collected samples, which are not amenable for cytology.

National Cancer Institute - Cancer Prevention Fellowship Program
Subhadip Kundu
Postdoctoral Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
Thymic precursor cells generate acute myeloid leukemia in NP23-NHD13 double transgenic mice
Abstract: Oncogenic fusion genes have been identified in a large number of hematologic malignancies. We previously generated mice that expressed either a NUP98–PHF23 (NP23) or NUP98-HOXD13 (NHD13) fusion in the hematopoietic compartment. Both NP23 and NHD13 mice develop a wide variety of leukemia at 9-14 months of age. Surprisingly, 100% of the NP23-NHD13 double transgenic mice developed acute myeloid leukemia (AML) within 3 months. The leukemias were characterized by extraordinarily high WBC and replacement of the thymus with Mac1+/Gr1+ myeloid cells; the percent of malignant myeloid cells in the thymus was often higher than the bone marrow (BM). These findings led to the intriguing hypothesis that the AML in NP23-NHD13 mice arose in the thymus, as opposed to the BM. To investigate this possibility, we transplanted unfractionated cells or residual CD4-/CD8- double negative (DN) thymocytes from the thymus of a NP23-NHD13 mouse invaded by AML cells irradiated recipients. All mice developed AML within 26 days, indicating that the AML was aggressive and transplantable, and could be transmitted by DN thymocytes. To rule out the possibility that the leukemia was transmitted by rare, contaminant AML cells, we repeated the experiment, twice, using DN thymocytes from 4-5 wk old mice with no signs of leukemia. DN thymocytes again transmitted AML.

Fractionating DN thymocytes into DN1-DN4 sub-populations revealed that AML initiating cells were found in the DN1 and DN2 compartments. DN thymocytes from non-leukemic NP23-NHD13 mice were cultured on an OP9 stromal layer, which has been shown to support myeloid differentiation in vitro. These studies revealed that DN thymocytes from non-leukemic NP23-NHD13 mice showed a markedly enhanced ability to differentiate into myeloid lineage cells compared to WT (56% vs 1.4 %). The NP23-NHD13 cells lost expression of myeloid markers after 26 days; the immunophenotype now was CD4-, CD8-, CD25-, CD44+, Thy-1.2+ and cKit+, consistent with the emergence of a self-renewing DN1 thymocyte. These cells were transplanted; all recipients were anemic, and demonstrated engraftment of NP23-NHD13 myeloid cells as well as a less prominent (8-38%) population of erythroid cells in the BM and spleen. Taken together, these results demonstrate that NP23-NHD13 thymic progenitors retain myeloid and erythroid potential and are potently leukemogenic, leading to the intriguing hypothesis that some human AML might originate in the thymus.

National Cancer Institute - Cancer Prevention Fellowship Program
Megan Roberts
Cancer Prevention Fellow
Cultural Social and Behavioral Sciences
Factors associated with Oncotype DX, 21-gene assay receipt among women with lymph node positive breast cancer
The Oncotype DX, 21-gene Breast Recurrence Score™ (RS) assay predicts breast cancer (BC) recurrence and adjuvant chemotherapy benefit in select patients with lymph node-positive (LN+), hormone receptor-positive (HR+), HER2 negative BC. Evidence suggests that the 21-gene assay reduces the overuse of adjuvant chemotherapy among women who are at low risk of breast cancer recurrence. In 2015, the assay was added to the National Comprehensive Cancer Network clinical guidelines for select women with 1-3 positive lymph nodes. However, the literature demonstrates that less than half of women receive the assay, and this number is even lower among minority populations. Previous studies have not been powered to examine multiple clinical, demographic and socioeconomic factors that are associated with uptake of the 21-gene assay in clinical practice. This study examines factors associated with ordering the test among women with LN+ BC in SEER databases. In this population-based study, incident BC cases in SEER registries (2010-2013) were linked to RS results from assays performed by Genomic Health. Our study sample included women with non-metastatic, LN+ (=1 positive LN), HER2-, HR+, BC. We used logistic regression to identify demographic, SES, and tumor characteristics associated with having the 21-gene assay ordered. A total of 4428 (14.0%) of 31520 women with LN+, HR+, HER2-, BC had the assay ordered. Uni- and multi-variate analyses identified key factors that were significantly associated with the proportion of women tested. In the multivariable analysis, age (aOR: 2.23, p<0.001, 65-74 v <45 years) and year of BC diagnosis (aOR:1.75, P<0.001 2013 vs 2010) were positively associated with assay receipt; whereas number of positive LN (aOR: 0.14, p<0.001, 4+ positive LN vs 1 positive LN), tumor grade and size, low SES, being black, and being widowed were negatively associated with assay uptake (p<0.001). Having Medicaid was associated with lower odds of test receipt (p=0.01). Finally, we identified geographic variation in assay ordering. Important demographic and SES variables were associated with test receipt in LN+ disease, and differed from those previously reported in node negative disease. Moving forward, increased awareness of these disparities,

particularly among low SES, Medicaid, Black and widowed patients, along with targeted interventions may help to improve quality of care and equity in test receipt.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
Bryan Bassig
Research Fellow
Cancer Risk and Prevention
Serologic markers of viral infection and risk of non-Hodgkin lymphoma in a pooled prospective study of three Chinese cohorts
Background: Several viral agents are associated with non-Hodgkin lymphoma (NHL), and these contribute to lymphomagenesis by transforming lymphocytes and/or chronic immune stimulation. There is a compelling rationale to evaluate serologic markers of viral infection and risk of NHL in Asians, given differences in the descriptive epidemiology of NHL and viral characteristics between East Asian and Western countries. There are very limited population-based prospective studies with pre-diagnostic blood samples that have comprehensively evaluated infectious risk factors for NHL in East Asians. Methods: We conducted a nested case-control study of 214 NHL cases and 214 individually-matched controls from three population-based prospective cohorts in Shanghai and Singapore. Antibodies to 21 viral antigens (herpesviruses, Hepatitis B (HBV) and C (HCV), and polyomaviruses) were measured in plasma/serum using fluorescent bead-based multiplex serology. Conditional logistic regression was used to evaluate associations between antibody levels and NHL. Results: For herpesviruses, an increased risk of NHL was observed for higher compared to lower early antigen diffuse (EA-D) (OR = 2.2, 95% CI = 1.2-4.1) and BZLF1-encoded replication activator (ZEBRA) (OR = 2.2, 95% CI = 1.0-4.9) antibodies associated with Epstein-Barr virus (EBV). An increased risk of NHL was also observed among those seropositive for the intermediate-early 1A antigen (OR = 1.9, 95% CI = 1.0-3.3) associated with human herpesvirus-6 (HHV-6). For hepatitis viruses, a significant NHL risk was observed for higher compared to lower antibodies to the HBV-associated core (HBc) antigen (OR = 1.8, 95% CI = 1.1-3.1). Seropositivity to HCV was low (1.4% cases; 0.9% controls). No overall associations with NHL risk were observed for polyomaviruses. Discussion: Our study suggests a role of specific viral agents in lymphomagenesis in East Asians. The findings for EBV are consistent with some data in Western cohorts and indicate that EBV reactivation may also be associated with NHL risk in the Chinese general population. HHV-6 is a lymphotropic virus that has not previously been associated with NHL prospectively in the general population to our knowledge. HBV is endemic to regions of East Asia, including China, and higher levels of antibodies to the HBc antigen may be a marker for NHL risk. These data further suggest some similar risk factors for NHL in diverse populations with different patterns of NHL rates and subtypes.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
Catherine Lerro
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Cancer incidence and alachlor use in the Agricultural Health Study: an updated analysis
Background: Alachlor is a chloroacetanilide herbicide that has been registered for control of broadleaf weeds and grasses in the United States (US) since 1969. It has been widely used for agricultural purposes

on corn, soybeans, sorghum, peanuts, and beans. Alachlor is known to be carcinogenic in vivo, causing thyroid, nasal, and stomach tumors in rats, though few epidemiologic studies have examined associations with human cancer risk. We evaluated alachlor use and cancer incidence among licensed pesticide applicators in the Agricultural Health Study, updating a previous analysis with follow-up through 2000 which found increased risk for lymphohematopoietic cancers.

Methods: Pesticide applicators in Iowa and North Carolina reported lifetime alachlor use at enrollment (1993-1997) and additional use during follow-up (1998-2005). Use was measured as cumulative lifetime days and intensity-weighted days, which adjusted for factors that influence exposure. Both exposure metrics were categorized as no use (referent) and quartiles of use. We used Poisson regression to estimate relative risks (RR) and 95% confidence intervals (CI) for incident cancers from enrollment through 2012 (North Carolina)/ 2013 (Iowa). Models were adjusted for attained age, tobacco use, alcohol use, correlated pesticides, and other potential confounders. We evaluated all cancer sites with at least 20 alachlor-exposed cases.

Results: Among 49,732 applicators, 25,698 used alachlor with 3,542 alachlor-exposed cancers. Compared with non-users, RRs for laryngeal cancer (n=34 exposed) were elevated in the second (RR=5.62, 95%CI=2.24-14.1), third (RR=6.68, 95%CI=2.76-16.1), and fourth quartiles (RR=6.44, 95%CI=2.20-18.8) of lifetime days (p-trend<0.01); results for intensity-weighted days were similar. This finding was robust after adjusting for other occupational exposures (i.e. grain dusts, asbestos, engine exhaust, solvents) and stratifying by smoking status at enrollment. No significant association was seen for any other cancer site evaluated. Prior associations for lymphohematopoietic cancer were not confirmed, though risk of myeloid leukemia was non-significantly elevated in the fourth quartile of days and intensity-weighted days of use.

Discussion: Our study is the first to find that alachlor exposure is associated with elevated risk of laryngeal cancer. Additional studies are needed to confirm this association.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
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Cancer Risk and Prevention
A Prospective Investigation of Coffee Drinking and Bladder Cancer Incidence in the United States
Coffee is among the most popular beverages in the world, but many coffee drinkers fear that it leads to negative health outcomes, including cancer. In 1991, coffee was classified as a Group 2B carcinogen, possibly carcinogenic to humans, by the International Agency for Research on Cancer (IARC) based on epidemiologic evidence suggesting a positive association with bladder cancer. In 2016, IARC downgraded this classification due to methodological concerns about confounding by cigarette smoking, which is often highly correlated with coffee drinking, and a lack of subsequent evidence. The expert panel also stressed the need for rigorous prospective studies that assessed coffee drinking prior to bladder cancer occurrence, included substantial case numbers, and had comprehensively assessed cigarette smoking. Therefore, we assessed the association in 469,047 participants of the NIH-AARP Study who were cancer-free at baseline. During follow-up from 1995-2011, we identified 6012 incident bladder cancer cases, nearly 8-times more cases than the largest prior study. Multivariable-adjusted Cox proportional hazards models were used to estimate hazard ratios (HR) and 95% confidence intervals (CI)

for coffee drinking and bladder cancer, with coffee nondrinkers as the reference group. Detailed baseline information on cigarette smoking intensity, duration, and time since quitting permitted careful adjustment for smoking. In our study, coffee drinking was positively associated with bladder cancer in models adjusted for age and sex (HR for =4 cups/day=1.91, 95% CI=1.70-2.14; P-trend<.0001). This association was substantially attenuated after adjusting for baseline smoking status and cigarettes smoked per day (HR for =4 cups/day=1.18 95% CI=1.05-1.33; P-trend=.0007) and further attenuated after more detailed adjustment for cigarette smoking during each decade of life (HR for =4 cups/day=1.09, 95% CI=0.93-1.20; P-trend=.16). Finally, there was no evidence of an association among never smokers (HR for =4 cups/day=0.87, 95% CI=0.65-1.17; P-trend=.84). The absence of an association between coffee drinking and bladder cancer among never smokers coupled with the degree of attenuation following adjustment for baseline and lifetime smoking provide strong evidence that coffee drinking is not related to bladder cancer and speak to the importance of careful accounting for cigarette smoking in epidemiologic studies of chronic diseases, particularly those caused by cigarette smoking.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
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Cancer Risk and Prevention
A prospective study of cancer risk associated with use of organochlorine insecticides among Agricultural Health Study farm spouses
Organochlorine (OC) insecticides were once the most commonly used pesticides in the US, comprising 72% of pesticides used in agricultural and residential settings.Although banned in western countries, they are widely persistent in the environment and are still used in developing countries.Previous epidemiologic studies have linked OC use to cancer risk among male agricultural workers, but have lacked statistical power to examine risks in women.We evaluated the personal use of specific OCs and cancer incidence among the female spouses of pesticide applicators in the prospective Agricultural Health Study cohort. At study enrollment (1993-1997) women provided information on their lifetime use of specific pesticides, including 7 OCs (aldrin, chlordane, dieldrin, DDT, heptachlor, lindane, and toxaphene), as well as farming and pesticide application practices, demographic information, health histories, and other potential confounders.Incident cancer cases were obtained from the North Carolina and Iowa state registries through 2011 and 2012, respectively.We used Poisson regression to calculate relative risks (RRs) and 95% confidence intervals (CIs) for 15 cancer types and the use of any OC and individual OCs.We included 28,909 female spouses in our analysis, of whom 2,191 reported use of at least one OC, and 287 were diagnosed with incident cancer during the study period.Chlordane was associated with an elevated risk of multiple myeloma (RR=2.71,95%CI:1.12-6.55).Lindane was associated with an increased risk of glioma (RR=4.45,95%CI:1.36-14.55) and pancreatic cancer (RR=3.7,95%CI:1.15-12.0).Dieldrin was associated with an increased risk for endocrine receptor-negative breast cancer (RR=3.55,95%CI:1.12-11.18).We report significantly increased risks of multiple myeloma, glioma, pancreatic cancer, and endocrine receptor-negative breast cancer, with personal use of specific OCs among women.This study represents the first prospective evaluation of individual OC use and risk of multiple cancer sites in a population of women, and the first examination of associations between OC use and risk of glioma and pancreatic cancer.This unique cohort allowed us to examine female-specific cancers, which are understudied with respect to pesticide exposure.Our results suggest that relatively

modest OC exposure is associated with elevated risks of multiple cancers.Future epidemiologic research should attempt to replicate these findings with a greater number of exposed cancer cases.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Primary central nervous system lymphomas in solid organ transplant recipients
Primary central nervous system lymphomas (PCNSLs) are non-Hodgkin lymphomas (NHLs) that affect the brain, leptomeninges, or spinal cord without systemic involvement. The risk of PCNSL is greatly increased in immunosuppressed HIV-infected individuals. Solid organ transplant recipients (SOTRs) are also immunosuppressed due to drugs given to prevent graft rejection, and they have elevated NHL risk. As PCNSL is rare, studies in SOTRs have been descriptive case series. Herein, we evaluate the incidence, risk factors, and survival of PCNSL in SOTRs. The US transplant registry was linked to 17 cancer registries (1987-2014). We estimated risk of PCNSL in SOTRs relative to the general population as a standardized incidence ratio (SIR=observed/expected cases). We used Poisson regression to estimate adjusted incidence rate ratios (aIRR) of PCNSL across subgroups of SOTRs, and Cox regression to assess risk of death or graft failure/retransplantation (GF) associated with PCNSL. Among 288,029 SOTRs, there were 168 PCNSL cases (SIR=65.1; 95%CI=55.6-75.7). Compared to kidney SOTRs, PCNSL risk was lower in liver (aIRR=0.5; 95%CI=0.3-0.9), not different in heart and/or lung, and higher in other/multiple SOTRs (aIRR=2.5; 95%CI=1.5-3.9). Asians/Pacific Islanders had higher PCNSL risk than non-Hispanic whites (aIRR=2.1; 95%CI=1.2-3.5). Induction immunosuppression with alemtuzumab (aIRR=3.1; 95%CI=1.6-6.2) or polyclonal antibodies (aIRR=2.0; 95%CI=1.4-3.1) carried a higher PCNSL risk. Epstein-Barr virus (EBV) seronegativity at transplant had higher PCNSL risk (aIRR=2.0; 95%CI=1.1-3.5) than seropositive SOTRs. PCNSL risk was highest in the first 1.5 years after transplant and decreased over time (ptrend<0.0001). Risk did not differ by age at transplant, sex, or maintenance immunosuppression. SOTRs with PCNSLs had a higher risk of death (adjusted hazard ratio[aHR]=12.0; 95%CI=9.8-14.7) or GF (aHR=3.1; 95%CI=2.8-4.5) than other SOTRs. In conclusion, PCNSL risk is highly elevated among SOTRs. Since EBV seronegative SOTRs have risk of primary EBV infection after transplant, these results highlight the important contribution of EBV to PCNSL risk. Risk is highest within 1.5 years after transplant, in people who receive multiple non-thoracic organs, and is associated with induction therapy with alemtuzumab or polyclonal antibodies. High risk of death and GF may be because chemotherapy drugs may not pass the blood-brain barrier and dose of immunosuppression is reduced after PCNSL.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Association between Circulating Levels of Sex Steroid Hormones and Esophageal/Gastric Cardia Adenocarcinoma
Esophageal adenocarcinoma (EA) and gastric cardia adenocarcinoma (GCA) have one of the most skewed sex ratios of any malignancy, with a male-to-female ratio of approximately 4:1. The reasons

underlying this are unknown—established risk factors, such as smoking and obesity, cannot account for the male predominance. Sex steroid hormones have been hypothesized to potentially underlie this sex disparity. This hypothesis is supported by hormonal modulation of the inflammatory process, estrogen receptor beta expression in esophageal and gastric cancer tissue, and lower EA/GCA rates among men with prostate cancer, who are likely to receive anti-androgen therapies. Additionally, a small hospital-based study reported higher testosterone levels among cases, yet case-control studies of cancer are highly susceptible to reverse causation whereby tumor growth alters circulating hormone concentrations. Therefore, we designed a prospective study to assess the relationship between hormones and EA/GCA risk, leveraging statistical power through combining three prospective cohort studies: PLCO Screening Trial, ATBC Cancer Prevention Study, and CPS-II Nutrition Cohort. EA and GCA were considered as a single outcome, as these tumors have overlapping pathogeneses, they are both glandular epithelial cancers at the gastroesophageal junction, and they have similar 5-year survival rates of ~20%. We restricted the study population to males, as there was inadequate statistical power for a female-only analysis. Using gas chromatography-mass spectrometry (GC-MS), we quantitated sex steroid hormones - dehydroepiandrosterone (DHEA), androstenedione, testosterone, dihydrotestosterone, androsterone, estrone, and estradiol (E2) – in serum from 259 EA/GCA cases and 259 controls, matched on study, age, race, and blood draw year/time. Multivariable conditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for associations between hormones and EA/GCA. Higher levels of E2 and DHEA were associated with a 48–72% decreased risk of EA/GCA (quartile 4 v. 1: OR=0.52, 95%CI=0.29–0.93; Ptrend=0.03 and OR=0.28, 95%CI=0.13–0.64; Ptrend=0.001, respectively). All other hormones examined were not associated with EA/GCA. This study provides the first evidence that higher levels of circulating E2 and DHEA may be associated with lower EA/GCA risk. Lower levels of E2 in men, compared to women, may partially explain the male predominance of this lethal malignancy.

National Cancer Institute - Division of Cancer Epidemiology and Genetics
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Exercise patterns over the life-course and all-cause mortality
BACKGROUND: Evidence documenting the health benefits of exercise during midlife is substantial however, less is known about life-course exercise participation (i.e., adolescence throughout adulthood) impact on mortality. This study modelled patterns in exercise participation between ages 15 through ~45yrs and their association with all-cause mortality.

METHODS: A total of 315,059 adults (58.2% males) aged 50-71yrs enrolled in the NIH-AARP cohort study completed a questionnaire at baseline that included a question about previous exercise participation (hours/week) at approximately 15yrs, 25yrs, 35yrs, and ~45yrs of age. Exercise patterns over the 4 life periods were modelled using semi-parametric group-based mixture models and merged with death records obtained from the National Death Index available through 2011. Associations between life-course exercise patterns and risk for mortality were modelled using Cox proportional Hazard models [(Hazard Ratios (HR) and 95% CI)] while adjusting for baseline covariates (i.e., age, sex, race, education, smoking status/dose, body mass index, and diet, all at the age of 50-71yrs). The inactive exercise pattern

over time (i.e., group that remained inactive throughout all of the life periods) was defined as the referent group.

RESULTS: There were 71,377 deaths recorded over an average follow-up period of 13.6 years. We identified 7 different patterns over time of exercise participation that overall, were characterized by either increases, decreases, or stable, exercise participation between ages 15 and 45yrs. The most noticeable risk reductions in mortality were associated with participants that were consistently active over time (i.e., active in their 15's and remained active throughout their 45's) (HR=0.74, 95% CI: 0.73, 0.78) and those that only became active in their midlife (i.e., inactive in their 15's but increased exercise participation when they reached 45's) (HR=0.75, 95% CI:0.73, 0.78). Being active during their 15's but becoming inactive later in their 45's was associated with the same risk for mortality as those that were consistently inactive over time (HR=0.97, 95% CI: 0.94, 1.01).

CONCLUSIONS: Participants that were active in their ~45's had the lowest risk for mortality, independent of previous exercise participation record. Exercise participation during midlife (i.e., ~45yrs) and not exercise participation early in life (i.e., 15's through 35's) seems to dictate the risk for all-cause mortality.

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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
An individualized absolute risk model for oropharyngeal cancer in the U.S. population
Background: Human papillomavirus (HPV) is now established as a major cause of oropharyngeal cancer (OPC), yet tools for risk prediction/stratification for the identification of high-risk individuals do not currently exist. Despite the rapid rise in OPC attributed to HPV among white men in the United States, OPC remains relatively rare at <10 cases per 100,000 adult individuals. We aimed to develop an individualized 1-year absolute risk model for oropharynx cancers in the U.S. population.

Methods: To estimate the 1-year absolute risk of HPV+ OPC, we used information from a case-series of OPC at Ohio State University (OSU; 2010-2015, n=241), SEER18 cancer registry data (2009-2013, n=16,846 OPC cases), and NHANES (2009-2014, n=9,327 participants). Weighted logistic regression was used to estimate OPC risk according to age, gender, race, smoking, alcohol use, lifetime number of sexual partners, and oral HPV status (OSU participants=cases, NHANES participants=controls). From this model, we estimated the attributable fraction (AF) for smoking, alcohol, and lifetime sexual partners

(within subgroups of age, gender, and race; n=48 subgroups) and calculated the baseline OPC rate for each subgroup by multiplying the annual SEER rate by (1-AF%). To estimate the 1-year absolute risk of OPC, we multiplied SEER baseline rates by odds ratios for smoking, alcohol, number of lifetime sexual partners, and HPV status.

Results: The highest risk of OPC in the US population is observed in white men aged 65-69 years, who are current/former smokers, heavy alcohol drinkers, have had 11 or more lifetime sexual partners, and are HPV positive (1-year absolute risk of OPC=1.29%). In comparison, individuals with the same risk profile but HPV negative have a much lower 1-year absolute risk of OPC (0.02%), demonstrating the importance of HPV for risk stratification.

Conclusions: Our models provide individualized estimates for risk of OPC. Identification of individuals at high absolute risk of OPC could enable efficient design of future natural history and prevention studies.

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Epidemiology/Biostatistics - Prognosis and Response Predictions
Risk of second malignancies after ductal carcinoma in situ: A population-based study
Purpose: The incidence of ductal carcinoma in situ (DCIS), a non-invasive breast cancer precursor, has increased nearly 6-fold since the introduction of mammography. Optimal treatment for DCIS, including the role of radiotherapy (RT), is currently debated. One of the primary potential harms of RT is the induction of second cancers. Randomized trials have been under-powered to measure risk of second cancers, but there is a clear clinical need to understand the risk of these significant adverse events. In the largest study to explore this question to date, we measure the risk of second cancers among survivors of DCIS and the extent to which radiotherapy contributes to this risk.

Methods: Eligible women were diagnosed with DCIS during 1992-2008 in 12 US Surveillance, Epidemiology and End Results cancer registries, and followed until 2013. Analyses of second breast cancer and non-breast cancer were restricted to 1- and 5-year survivors (n=61,083 and n=51,106) respectively. Standardized incidence ratios (SIR) compared cancer risk among DCIS survivors to general population risk. Poisson regression models and parametric survival models were used to estimate relative risks (RR) for second cancers associated with RT.

Results: During follow-up, 3,655 invasive breast cancers and 2,184 second non-breast malignancies were diagnosed in DCIS survivors. The SIR for invasive breast cancer was 2.45 (95% confidence interval (CI):2.35-2.54). An inverse association between RT and risk of invasive disease in the same breast was observed in the 5 years following diagnosis but not after (RR:0.67, 95%CI:0.58-0.78, p-value for interaction with time since diagnosis<0.001). The risk of all second non-breast cancers combined was lower in DCIS survivors than in the general population (SIR:0.87, 95%CI:0.84-0.91). RT was associated

with significantly increased risk of cancers within the field of breast irradiation (RR:1.34, 95%CI:1.12-1.60), including lung cancer (RR:1.31, 95%CI:1.07-1.60).

Conclusions: The lower risk of all non-breast cancers combined among DCIS survivors is likely attributable to a healthy screener effect. RT is associated with lower risk of invasive disease in the same breast in the first 5 years post-diagnosis. After 5 years, RT increases the risk of in-field second cancers. These findings can help to inform treatment decisions for DCIS patients and the intensity of medical surveillance and screening among survivors.

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Genetic Profiling of Sensory Neurons – Determination of S- and M-Cone Photoreceptor Identity and Synapse Formation
Mammalians have two major types of sensory neurons in the retina: rods, specialized for vision in dim-light, and cones for vision in well-lit conditions and the perception of color. Most mammals have two cone types, namely S- and M-cones. They diverge in their sensitivity to different wavelengths of light, based on their expression of different light-sensitive proteins: S-opsin for blue light and M-opsin for

green light. The purpose of this project is to identify genetic differences in cones and, more specifically, to find genes that are involved in cone synapse formation. Until now, cones have been classified mostly by the opsin they express. Recent findings in our laboratory point to the existence of additional genetic differences. Normally, the dendrites of the S-cone bipolar cell (SCBC), an interneuron that relays cone-signals to downstream neurons, exclusively contact S-cones. However, genetic disruption of the normal S- and M-opsin expression pattern in different knockout mice fails to alter that specific connection. Can we identify genes other than the opsins that are uniquely expressed in each cone type? And, can we identify molecules that facilitate the formation of the specific S-cone/SCBC synapse?

To identify such molecular signatures, we have turned to single cell RNA-seq to obtain complete genetic profiles of S- and M-cones. For this study, we used the 13-lined ground squirrel that, in contrast to mouse, is diurnal and has a cone-dominated retina. The two cone types are morphologically indistinguishable, so we developed a protocol to dissociate and label live cells with an antibody targeting the extracellular domain of S-opsin. We then manually collected single cells for next-generation sequencing. The analysis reveals differentially expressed genes that define cone identity beyond their expression of S- or M-opsins. We show immunohistochemical evidence for the applicability of our data and, additionally, we identified two S-cone specific cell-adhesion molecules that are known to play roles in synapse assembly in the brain. Additional experiments in CRISPR/Cas9 animal models will show if genetic ablation of these genes leads to changes in cone connectivity. The outcome of this study can help to understand circuit formation in the retina and synapse assembly in the nervous system. For clinical applications, this may be an important starting point for future studies that aim to replace or rewire photoreceptors in retinal degenerative diseases.

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Immunology - Autoimmune
Gut microbiota as a source of signals that trigger spontaneous ocular autoimmunity
Autoimmune uveitis is a major cause of blindness and is driven by T cells reactive to unique retinal antigens that have become activated and acquired the ability to cross the blood-retinal barrier (BRB). However, it is unknown where and how the autoreactive T cells become activated, as their specific antigens are sequestered behind the BRB. Our lab developed R161H transgenic mice that express a T cell receptor (TCR) specific for a retinal protein, interphotoreceptor retinoid binding protein (IRBP), and develop spontaneous uveitis, allowing to study natural triggers of disease. We demonstrated that elimination of gut commensals by oral broad-spectrum antibiotic treatment (ABX) or by rearing under germ-free (GF) conditions attenuated uveitis and reduced Th17 cells in the gut. R161H T cells were

activated in the intestine of specific-pathogen-free (SPF) mice through their clonotypic TCR. Upon exposure to bacteria-rich extracts of intestinal contents from SPF mice, but not from GF or ABX mice, retina-specific T cells upregulated activation markers several-fold more than nonspecific T cells. These results suggest a role for gut microbiota as a source of antigen for stimulating retina-specific T cells, but do not identify a specific commensal or antigen mimic, nor do they distinguish the relative importance of adaptive vs. innate microbial signals. To dissect these signals and search for culprit commensals, we performed 16S rRNA gene sequencing and gnotobiotic studies. Metagenomic analysis revealed significant alteration of the gut microbiome in ABX vs. SPF mice. Co-housing of GF-R161H mice with SPF mice restored full development of uveitis, but monocolonization with segmented filamentous bacteria (SFB, a Th17-inducing organism) or Turicibacter strain H121 (T.H121, from mono-contaminated GF-R161H mice that had uveitis) only partially restored disease. Unlike intestinal content extracts from SPF mice, extracts from mice monocolonized with SFB or T.H121 failed to activate R161H T cells in vitro, indicating a lack of “antigen” activity. Monocolonization with SFB, but not T.H121, restored gut Th17 cells, indicating the presence of innate “adjuvant” activity. We conclude that microbial-derived adaptive “antigen” and innate “adjuvant” activities are both required for the development of uveitis, and further work is needed to identify microbes involved in triggering uveitis.

National Human Genome Research Institute
Bonnie Huang
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Immunology - Lymphocyte Development and Activation

An in vivo CRISPR-based functional genetic screen reveals genes affecting T follicular helper cell differentiation and function

T follicular helper (Tfh) cells specialize in helping B cells produce high affinity antibodies and become memory B cells. These processes are crucial for long-term protective immunity, and Tfh cells are dysregulated in many human autoimmune diseases. Identifying genes governing Tfh function could provide therapeutic targets, but the differentiation of T cells into Tfh cells is only partially understood. To help evaluate potential novel Tfh-regulating genes, we developed a CRISPR-based functional genetic system to knock out genes in primary mouse T cells in vitro, and then measure Tfh cell differentiation in vivo.

We generated a retroviral construct to introduce guide RNAs (sgRNA) in cultured transgenic CD4 T cells expressing Cas9. Using test guides for Tcf7 and Ptprc (CD45), we observed up to 90% loss of targeted proteins within 3 days post-transduction. We then transferred the cells in vivo and infected mice with LCMV. T cells transduced with sgRNAs against Bcl6, a master regulatory Tfh gene, were severely impaired in Tfh differentiation. Analogously, sgRNAs disrupting Prdm1, which antagonizes Bcl6, yielded excess Tfh differentiation. We then scaled up, by generating a multiplex library including ~400 guides covering 80 genes associated with primary immunodeficiencies (PID) with suspected defective Tfh differentiation or help. T cells were transduced with pooled constructs encoding sgRNAs targeting PID genes, non-targeting controls, and positive controls including Bcl6 and Prdm1, and transferred in vivo. Post infection, Tfh and non-Tfh cells were sorted, retroviral inserts amplified and evaluated by deep sequencing to quantify the abundance of integrated viral constructs in the two populations. Bcl6 guides

were severely depleted in Tfh cells, while non-targeting control guides were 1:1. Guides targeting Icos and Sh2d1a, two genes required for Tfh cells, were also reduced in Tfh relative to non-Tfh cells. However, most PID genes affected overall T cell expansion in vivo, not specifically Tfh differentiation. We further screened an additional ~400 sgRNAs targeting ~80 druggable genes expressed more highly in Tfh than non-Tfh cells based on RNAseq . Hits were found for several genes in PI3K pathways, which we have now validated using single guides. We are currently evaluating phenotypes affected by these genes. Our data suggest this approach is a powerful technique for simultaneous rapid functional interrogation of many genes in T cells in vivo.

National Human Genome Research Institute
Ryan Johnson
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Genomics

Assessing the Genetic Diversity of Carbapenem-Resistant Enterobacteriaceae through Whole-Genome Sequencing

The mobilization of genetic determinants conferring carbapenem resistance has resulted in a serious public health issue, especially within hospital settings. Indeed, contaminated sinks, drains, and high-touch surfaces not only serve as a means for bacterial dissemination, but also as a microbial melting pot where bacteria freely modify and share genetic material. For example, the Klebsiella pneumoniae carbapenemase (blaKPC) gene has been associated with multiple transposable elements within different-sized plasmids belonging to various incompatibility (Inc) groups. The variable genetic context of blaKPC is concerning as it can result in an increased host tropism and subsequent spread. Thus, to better understand the diversity of blaKPC-containing genetic elements within the hospital environment, we isolated more than 70 blaKPC-containing organisms from hospital wastewater sources (effluent, sinks, and drains) and high-touch surfaces over a 5 year span. All isolates were subjected to whole-genome sequencing using the Illumina MiSeq and/or PacBio platforms. We additionally collected over 40 blaKPC-containing patient isolates to further examine blaKPC genetic diversity and discern possible patient-environment transmission. Environmental sampling identified over 15 blaKPC-positive bacterial species from multiple genera including Klebsiella, Escherichia, Enterobacter, Citrobacter, Pantoea, Aeromonas, and Leclercia. While most blaKPC genes were found predominately within the Tn4401 and IS26 transposable elements, there was significant diversity among blaKPC-positive plasmids. By using a kmer-containing approach, we found that the IncN family of blaKPC-positive plasmids (e.g. pKPC-47e) were highly prevalent in both environmental and patient isolates (~30% of isolates). However, we also identified over 10 different new blaKPC-positive plasmids via PacBio sequencing, including instances of the blaKPC-containing transposon moving into a previously characterized blaKPC-negative plasmid. Together, this study demonstrates the genetic promiscuity of the blaKPC gene and emphasizes the need for continued surveillance of carbapenemase producing organisms within the hospital environment.

National Human Genome Research Institute
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Genomics

Whole genome sequence reveals selection for cardiac function and muscle development genes in sporting dog breeds

Modern dogs are unique among domesticated species in that human selection has imposed strong and consistent selective pressure for specific characteristics. The resulting breeds reflect the development of closed populations with well-defined physical and behavioral attributes. Given the wide variation in appearance, personality or functional behaviors, the modern dog has emerged as an ideal system for disentangling phenotype -genotype associations. The Sporting dog group is unique as it includes several breeds whose primary functions relate to hunting, reflecting strong behavioral pressure in the presence of only modest morphologic selection. A genome-wide comparison of sporting to terrier breeds, a group that represents the end of a continuum in both form and function, reveals that genes underlying cardiac function and muscular development are under greatest selection in sporting breeds, including TRPM3, ADRB1, RYR3 and ASIC3. The positively selected allele of TRPM3 is significantly associated with the increased racing speed in an independent population of whippets, thereby accounting for an additional 12.5% of variance in racing performance beyond the effect of a previously identified myostatin (MSTN) gene. In addition, we find strong selection for a high impact mutation in a widely conserved site in CDH23, a gene associated with hearing loss, possibly as a mechanism to control startle reflex and response to loud noise. Together these results provide strong evidence that sporting dogs have adapted to their roles in field hunting by improving endurance, cardiac function, blood fluidity, and low startle reflex, demonstrating how strong behavioral selection alters physiology, and creates breeds with distinct capabilities.

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Immunology - Lymphocyte Development and Activation

Hyperactivated Phosphotidylinositol 3-Kinase (PI3K) d alters germinal centers and antibody responses

Phosphoinositol 3-kinase delta (PI3Kd) is expressed in lymphocytes where it is activated by a variety of cellular receptors and drives the generation of the second messenger PIP3 that activates signaling molecules including AKT. PI3Kd regulates many cellular events including differentiation, proliferation, survival and metabolism. Patients with heterozygous gain of function mutations in the catalytic subunit of PI3Kd (p110d encoded by Pik3cd) exhibit a primary immunodeficiency characterized by lymphopenia, lymphadenopathy, recurrent and chronic infections, and occasionally lymphoma. Patients have reduced circulating naïve and increased effector T cell numbers, and fewer class switched memory B cells, with inefficient responses to vaccination. However, the cellular and molecular mechanisms causing these phenotypes are unclear. The generation of good antibody responses to vaccination and infections requires the orchestration of T follicular helper (Tfh) cells and B cells in secondary lymphoid organs where the germinal center (GC) reaction takes place. Intriguingly, we have found increased circulating Tfh cells in activated PI3Kd patients. To provide insight into inefficient patient humoral responses, we generated a mouse model of constitutively active p110d (Pik3cd-Mut). Pik3cd-Mut mice recapitulate many features of the human disease including increased activated T and Tfh cells in steady state conditions. Despite increased GCs, antigen specific B cell responses to immunization are reduced. Moreover, mutant GCs are poorly organized as detected by multicolor immunofluorescence histology. Generation of mixed bone marrow chimeras and adoptive transfer of WT or mutant T and B cells into

WT mice demonstrate defects are both T and B cell-intrinsic. Interestingly, despite decreased antigen-specific responses, we found increased polyclonal antibodies that react against self-antigens and commensal bacteria. Moreover, Pik3cd-Mut mice treated with antibiotics display reduced Tfh cells, autoantibodies and spleen cellularity, suggesting that commensal antigenic stimulation is required for phenotypes associated with mutant PI3Kd. Our findings demonstrate that hyperactivated PI3Kd leads to increased generation of Tfh cells and germinal centers, yet with defective in vivo functions. These results underscore the importance of dynamic regulation of T and B cell responses by PI3Kd, and provide insight into humoral defects associated with this disease.

National Human Genome Research Institute
Osnat Tirosh
Postdoctoral Fellow
Genomics

The unusual skin microbiome of DOCK8-deficient patients

Viruses are the most abundant microorganism on earth and in the human body, and yet characterizing this viral diversity is challenging because of both detection and classification. Shtogun metagenomics sequencing direct from clinical or environmental samples provides a new and less biased fashion to explore the microbiome (bacteria, fungi and viruses).

On the skin of healthy volunteers, eukaryotic viruses represent less than 4% of the microbiome. This low representation is an obstacle when trying to discover novel viruses that colonize human skin. We have studied 27 children and young adults with DOCK8 immunodeficiency - a rare, combined primary immunodeficiency with tremendous skin viral involvement as one of the clinical manifestations. By applying shotgun metagenomics to skin swabs taken from DOCK8-deficient patients, we discovered that human viruses represent over 90% of the total skin microbial population.

Our analysis, using read alignment, showed that human papillomaviruses (HPVs) are the most common dsDNA virus on DOCK8 skin, with more than 60 different known types and variants of HPVs detected. In addition to the known HPVs, we identified over 40 new HPV types, 7 new HPV genera and 3 new papillomavirus species, defined as <90%, <70% and <60% respectively, in sequence identity in the most conserved HPV protein, the L1 capsid protein. Read mapping against known reference databases and reference free assembly further revealed a diverse DNA virus community of polyomaviruses, molluscum contagiosum, anelloviruses and herpes virus family members. To explore RNA viral diversity, we peformed RNA-sequencing to DOCK8 patients skin swabs and were able to detect the presence of several human RNA viruses such as rubella, coronavirus and parainfluenza virus. Using reference-free assembly methods we could assemble multiple contigs (parts of genomes) that do not have any corresponding gene or protein in the existing databases and could represent completely novel viruses. We are currently exploring this option by multiple alignments to conserved viral sequences.

Our study will shed new light on the viral involvement in DOCK8-related immunodeficiency and develop datasets to explore more broadly the viral diversity that can colonize and infect human skin.

National Human Genome Research Institute
Erin Turbitt
Visiting Fellow
Cultural Social and Behavioral Sciences
A randomized controlled trial comparing two consent interventions for enrollment into a genome sequencing study
Despite the widespread use of genome sequencing in research, evidence-based procedures for obtaining participants' consent to enter such studies are lacking. Prior research has found wide variation in length and reading level of genome sequencing consent material. Increasingly, these studies include return of secondary findings, which are results unrelated to the primary reason the sequencing was obtained. While the likelihood for detecting secondary findings among any one individual is low, participants should be made aware of this possibility during the consent process. This study evaluated the efficacy of a novel, evidence-based consent among women affected with primary ovarian insufficiency eligible to participate in an NIH sequencing study. Participants were randomized to receive either the novel or the standard consent document. A mixed methods approach involved data collection with questionnaires at baseline, immediate, and six-month follow up. Differences in quantitative outcomes were assessed using independent samples t-tests; thematic content analysis was used to analyze qualitative data. Of the 387 women contacted, 212 were recruited and randomized (response rate=55%), with complete data available for 188 participants. At six months, there were no differences between the two consent type groups in genome sequencing benefits knowledge (d=0.15, 95%CI: -0.18,0.32), genome sequencing limitations knowledge (d=0.01, 95%CI: -0.21,0.23), expected personal benefits (d=-0.01, 95%CI: -0.26,0.23), or decisional conflict regarding the choice to enroll (d=0.04, 95%CI: -0.14,0.21). Overall, participants had high expectations to learn information of personal benefit because of being in the study, and had positive attitudes and intentions toward receipt of secondary findings. These analyses demonstrate a lack of difference in outcomes between the longer, standard consent and the shorter, novel consent suggesting that a more concise, evidence-based consent is as effective to use when enrolling patients in research using genome sequencing. Participants have high expectations for receiving sequencing results of personal benefit, and may be at risk of misunderstanding the intent of the research (to create generalizable knowledge). Future research should including evaluation of interventions to ensure appropriateness of participants' expectations. Such progress is vital to ethical implementation of such technologies given the rapidity of the field of genomics.

National Heart, Lung, and Blood Institute
Madeleine Davison
Visiting Fellow
Protein Structure/Structural Biology
Competitive encounter complexes enhance specific binding through a novel funneling mechanism
Just as the classical lock-and-key binding model made way for more complex models including induced fit and conformational selection, it has now become apparent that simple binding interactions observed in vitro are not always replicated in a crowded cellular environment. Non-specific transient interactions, also known as encounter complexes, can occur outside of the normal binding site of two biomolecules, increasing binding affinity by reducing the search space prior to a productive interaction, from a search through solution to a simple search along the biomolecule's surface. However, until now, the study of

encounter complexes has been limited to proteins or nucleic acids that are known to interact specifically with each other. Here, we investigated whether non-specific binding partners can form encounter complexes, which is directly relevant in the context of crowded cells. Paramagnetic relaxation enhancement (PRE) is a nuclear magnetic resonance (NMR) spectroscopy technique that is highly sensitive to encounter complexes and remains the only method by which to study them. Using a nitroxide radical cysteine tag, we measured PRE for the specific interaction between NPr and EINNtr, two proteins involved in bacterial nitrogen regulation. Using three NPr mutants we could triangulate the location of NPr on the surface of EINNtr, which we found formed a specific complex for the majority of the time, but also sampled encounter complexes around the specific binding site. Next, we measured PRE for two proteins that are known not to interact – HPr, which is homologous to NPr but opposite in charge, and EINNtr. Despite the absence of a specific interaction, as assayed using NMR chemical shift perturbation experiments, surprisingly, we observed transient interactions between the two proteins. Furthermore, in direct competition experiments between NPr and HPr, we found that the HPr-EINNtr encounter complexes could block those of NPr and EINNtr, while simultaneously increasing the population of NPr found in the specific binding site. Since HPr is known to be at least 70-fold more abundant than NPr in bacterial cells, this represents a novel mechanism whereby competitive encounter complexes could funnel ligands like NPr towards their specific binding site. This mechanism is important for addressing the discrepancy between binding studies performed in vitro and in crowded cells and is therefore highly relevant to the fields of structural biology and drug discovery.

National Heart, Lung, and Blood Institute
Natasha Fillmore
Postdoctoral Fellow
Physiology

A role for PPAR alpha in regulating sex differences in cardiac hypertrophy

Heart failure remains a leading cause of death worldwide and treatment is complicated by sex differences in the development of this disease. Males are more likely than females to develop heart failure and cardiac hypertrophy, which contributes to the development of heart failure. While sex differences in cardiac hypertrophy are well documented, the mechanisms involved are poorly understood. The purpose of this study is to better understand the mechanisms that contribute to sex differences in cardiac hypertrophy. Male and female mice were treated with vehicle or Angiotensin II (1.5 mg•kg-1•day-1) to induce cardiac hypertrophy and a systems biology analysis was performed on RNAseq data to identify pathways central to sex differences in cardiac hypertrophy. Cardiac hypertrophy was observed after 2 weeks of Angiotensin II and sex differences became apparent after 3 weeks. After 3 weeks of treatment ejection fraction (EF) in females was not different from control values (54% after Angiotensin II treatment vs 56% at baseline). In males, however, EF dropped from 55% at baseline to 37%, which was significantly lower than EF in females after 3 weeks of treatment. RNA sequencing was performed on hearts and sex differences in mRNA expression at baseline and following hypertrophy were observed along with differences between baseline and hypertrophy within a sex. Sex differences in mRNA were substantial at baseline and reduced somewhat with hypertrophy, as the hypertrophic differences tended to overwhelm the sex differences. We selected genes that were significant for the sex-disease interaction, and mapped them to the protein-protein interaction network constructed using STRING data. This identified a network centered on peroxisome proliferator activated receptor (PPAR)

alpha. This transcription factor regulates fatty acid oxidation and is known to contribute to the development of cardiac hypertrophy. To examine the role of PPAR alpha in sex differences in cardiac hypertrophy, we treated male and female mice with a PPAR alpha inhibitor (GW6471; (4 mg•kg-1•day-1)) along with vehicle or Angiotensin II for 3 weeks. The PPAR alpha inhibitor blunted the development of hypertrophy in male hearts (5% increase after Angiotensin II +GW6471 treatment vs 20% increase after Angiotensin II treatment), blocking sex differences in cardiac hypertrophy. These results suggest that PPAR alpha contributes to sex differences in the development of cardiac hypertrophy.

National Heart, Lung, and Blood Institute
Thirupugal Govindarajan
Visiting Fellow
Developmental Biology

Functional characterization of Rbfox mediated protein interaction in skeletal muscle differentiation

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

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

National Heart, Lung, and Blood Institute
Ji-Hoon Park
Postdoctoral Fellow
Clinical and Translational Research - Cancer
Modeling a prevalent TP53 mutation that causes a Li-Fraumeni-like syndrome
Rationale: p53 plays an essential role in tumor suppression. The inheritance of germline TP53 mutations is rare (~1:5,000–20,000) but can cause the malignant cancer susceptibility disorder, Li-Fraumeni syndrome (LFS). In Brazil, a founder TP53 mutation (R337H in the oligomerization domain) has been reported to increase the risk of specific cancers such as adrenocortical carcinomas and to cause a Li-Fraumeni-like syndrome (LFLS). However, its role in tumorigenesis is still unclear, and its in vivo properties have not been studied. Because this mutation occurs at a high frequency (~0.3% in southern Brazil, affecting ~300,000 people), further understanding its biology could potentially benefit large numbers of patients.

Objective: We set out to gain more pathogenetic insights into the p53 R337H mutation by creating a mouse model and examining its in vivo and de novo tumorigenesis characteristics.

Methods and Results: We generated a p53 R334H knockin mouse (R337H in human). Compared to wild-type (WT) mice, R334H mice did not show increased tumor incidence, consistent with the mild cancer phenotype of patients. To further examine the effect of this mutation on tumorigenesis, mice were treated with the hepatic carcinogen diethylnitrosamine (DEN). More tumors developed on R334H liver in a mutant allele dose-dependent manner: WT, 5+/-0.4; heterozygous and homozygous R334H, 5.7+/-0.4 and 7.5+/-0.9 (% liver/body weight, p<0.05), respectively. In parallel, we observed decreased cell cycle arrest by FACS in R334H liver after DEN treatment along with decreased expression of p53 target genes (26 out of 84 were decreased >1.5-fold). R334H liver also showed increased DNA damage and decreased cell cycle arrest/apoptosis related protein expression. Crosslinking/immunoblotting showed decreased p53 oligomerization in R334H compared to WT DEN-treated livers, providing a mechanistic explanation for its compromised activity.

Conclusions: In a mouse with knockin of the p53 R334H LFSL mutation, we demonstrate its pro-tumorigenic effect in the DEN carcinogenesis model which may be applicable to other cancer models such as that for adrenocortical carcinoma. We also demonstrate an oligomerization defect of p53 R334H in vivo as suggested by prior in vitro studies. The p53 R334H mouse provides a vehicle for advancing our understanding of the biology of the Brazilian LFLS patient population.

National Heart, Lung, and Blood Institute
Todd Schoborg
Postdoctoral Fellow

Developmental Biology

Investigating molecular mechanisms of microcephaly through mitotic spindle-independent pathways

Autosomal recessive primary microcephaly (MCPH) is a neurodevelopmental disorder characterized by reduced brain size and life span. While the clinical aspects of the disorder are well characterized, the molecular mechanism remains poorly understood. The currently accepted hypothesis favors cell division defects induced by mitotic spindle errors as the cause of the disorder, as mistakes in chromosome segregation can lead to abnormal differentiation and apoptosis. Either of these scenarios can reduce neuron/glia numbers, which in turn results in a smaller brain. The most commonly mutated gene in human MCPH patients, Abnormal Spindle-Like, Microcephaly Associated (ASPM) is known to be important for proper centrosome and mitotic spindle function during mitosis. However, our recent analysis of the Drosophila melanogaster ortholog, Abnormal Spindle (Asp), showed that mitotic spindle & cell division defects are not the primary cause of MCPH in Asp mutant animals, suggesting the current model needs to be revised. To do so, we are establishing a set of criteria that defines MCPH using novel imaging methods such as microcomputed tomography (micro-CT) and optical sectioning of intact adult heads and brains, coupled with sophisticated image segmentation and registration algorithms. We are also performing single neuron labeling in adult brains to identify affected populations of neurons/glia in Asp mutant animals. Our data has revealed that a null mutation of Asp disrupts proper development of the adult optic lobes, including a severe disorganization of the lobula complex (LOX) neuropils. Central brain neuropils retain their morphology in the Asp mutant, similar to wild-type animals, suggesting MCPH is restricted to specific areas of the brain in asp mutants. Our structure-function analysis of the protein shows that a 573 amino acid region of Asp's N-terminus is sufficient to rescue the LOX complex defects in the Asp null mutant. We are currently investigating multiple pathways through which this N-terminal fragment suppresses MCPH, including a regulatory role in the interphase nucleus and a role in radial migration of optic lobe neurons during metamorphosis.

National Heart, Lung, and Blood Institute

Agila Somasundaram

Research Fellow

Intracellular Trafficking

Investigating Protein Dynamics at Sites of Exocytosis in Live PC12 Cells Using Total Internal Reflection Fluorescence Microscopy

Exocytosis is the cellular process in which cytoplasmic membrane-bound vesicles fuse with the plasma membrane and release their contents into the extracellular space. Calcium triggered exocytosis is critical for many physiological functions, including neurotransmitter release by neurons, and hormone secretion by endocrine glands. Dozens of proteins regulate this process, however, their temporal and spatial dynamics during exocytosis remain unclear. We hypothesize that elucidating the dynamics of these proteins in live cells will offer insights into their function. We use total internal reflection fluorescence (TIRF) microscopy to image protein dynamics during exocytosis in PC12 cells (rat adrenal chromaffin-derived cell line that contains synaptic-like microvesicles). TIRF microscopy enables visualization of cellular phenomena at or near the plasma membrane, thus ideal for studying exocytosis. To label vesicles, we over-expressed the vesicle protein, vesicular acetylcholine transporter (VAChT), fused to the green fluorescent protein pHluorin. We co-expressed exocytic protein of interest fused to the red fluorescent protein mCherry. We stimulated exocytosis by depolarizing cells with buffer containing high

KCl, and imaged fusion events in the green channel and dynamics of exocytic proteins in the red channel. Control experiments with just the mCherry tag did not reveal any changes in dynamics during fusion. Our key findings are (1) SNARE proteins (crucial for vesicle fusion) and Rab proteins (important for vesicle trafficking) are present at exocytic sites before fusion and diffuse away following fusion. (2) Tomosyn, known to modulate SNAREs, is also present at exocytic sites and diffuses away after fusion. (3) Certain endocytic proteins are recruited to exocytic sites. The proteins amphiphysin1, syndapin2 and endophilinA1, that contain the BAR domain (membrane curvature sensing/inducing domain), are transiently recruited to exocytic sites, and slow down fusion kinetics. Mutant proteins lacking the BAR domain were not recruited, and did not alter fusion kinetics. These results suggest that BAR domain proteins regulate exocytosis, likely by modulating the curved neck of the fusion pore. Our findings provide insights into the dynamics and regulatory roles of key mediators of exocytosis and endocytosis during fusion in live cells. Future work will involve examining in detail the precise molecular mechanisms by which endocytic proteins regulate exocytosis.

National Institute on Aging
Rachel Abbotts
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
XRCC1 maintains mitochondrial health via a PARP1/NAD+-dependent nuclear-to-mitochondrial signaling mechanism
Single strand break repair (SSBR) is a major mechanism for resolution of endogenous DNA damage. The nuclear-specific scaffold protein XRCC1 complexes with PARP1 to recruit SSBR proteins to damage sites. XRCC1 deficiency, recently linked to a neurodegenerative syndrome, results in inefficient SSBR with persistent DNA damage and PARP1 hyperactivation. In similar neurodegenerative disorders associated with DNA repair defects, such as ataxia telangiectasia, hyperPARylation depletes the PARP1 substrate NAD+ and consequently depresses the NAD+-consuming SIRT1/PGC1 axis that regulates mitophagy, the mechanism which removes damaged mitochondria. Elevated levels of ROS produced by damaged mitochondria modify genomic DNA and impair cell function and viability. My work explores the hypothesis that mitochondrial dysfunction, arising through a nuclear-to-mitochondrial signaling mechanism, contributes to the XRCC1 disease phenotype.

In vitro differentiation of the C2C12 mouse myoblast cell line allows comparison of replicating and non-replicating cells. Lentiviral XRCC1 knockdown generated a stable C2C12 population (KD) expressing XRCC1 at +/-15% of the vector-scramble control. Compared to control cells, KD cells exhibit a two-fold increase in cellular PAR, associated with a six-fold reduction in NAD+. In line with a perturbation of the SIRT1/PGC1 axis, KD cells exhibit increased mitochondrial DNA damage, mitochondrial membrane hyperpolarization, defective mitophagy, and increased mitochondrial content relative to control cells. Differentiation of KD cells further increases PARylation, NAD+ consumption, and mitochondrial dysfunction, reflecting inactivity of back-up DNA repair pathways in non-replicating cells. The electron transport chain inhibitor rotenone potentiates these outcomes in KD cells, while elevating mitochondrial and cellular ROS, inducing nuclear DNA damage, and activating apoptotic responses. Our results indicate a model in which inefficient repair of endogenous nuclear DNA damage upon XRCC1 deficiency causes

PARP1 hyperactivation. The subsequent NAD+ depletion depresses the SIRT1/PGC1 axis and impairs the mitophagy response to mitochondrial damage, leading to increased ROS production and genomic damage that impacts cell viability. This study presents novel evidence that the nuclear-specific DNA repair protein XRCC1 can impact mitochondrial health, and provides a mechanism for the neuropathology observed in XRCC1 and SSBR genetic disorders.

National Institute on Aging
SANKET AWATE
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
RNAi Screen to Identify Synthetic Lethal Interactions of FANCJ Helicase
FANCJ mutations are linked to Fanconi Anemia characterized by bone marrow failure, and are also associated with breast/ovarian cancer. FANCJ helicase was first identified by its binding to the tumor suppressor BRCA1, and their interaction is required for efficient repair of double-strand breaks (DSB) by homologous recombination (HR). FANCJ also preserves genomic stability by repairing processed interstrand cross-links to facilitate replication fork progression. To better understand FANCJ's molecular and genetic interactions in DNA repair and the replication stress response, we performed an RNA interference (RNAi) screen in an isogenic pair of FANCJ CRISPR knockout and wild-type human bone osteosarcoma U2OS cells. These studies were built upon the premise that FANCJ plays an important role in DNA repair to maintain cellular homeostasis and that back-up pathways exist to tolerate endogenous or exogenously induced DNA damage. To begin, we used a DNA damage response/DNA repair RNAi library (240 candidates) and co-treated the transfected FANCJ-deficient or FANCJ-proficient U2OS cells with a low dose of the chemotherapeutic DNA cross-linker drug mitomycin C (MMC). We reproducibly identified 6 gene targets which upon their depletion exerted a marked sensitization of FANCJ-deficient cells to MMC (6 nM) as measured by mitochondrial dehydrogenase activity indicative of reduced proliferation. Three of the 6 genes are implicated in DSB repair (GEN1, RAP80, PCNA), and 3 in the DNA damage response (ATM, EYA1, SSB1). Colony formation assays demonstrated a strong synthetic lethal interaction of FANCJ with RAP80, which is known to recruit a BRCA1 protein complex that inhibits DNA end-resection. Consistent with this, cells deficient in both FANCJ and RAP80 displayed reduced foci of 53BP1, a factor that also inhibits end-resection. Using a green fluorescence protein HR reporter cell line, we found that RAP80 depletion in FANCJ-deficient cells caused a significant decrease in HR accompanied by extensive end-resection marked by elevated staining of phosphorylated single-stranded binding protein RPA. Thus, absence of FANCJ in RAP80-deficient cells leads to aberrant DSB processing, which underlies gross chromosomal rearrangements and synthetic lethality. This is the first synthetic lethal interaction screen in a helicase-deficient background, and the results suggest that tumors deficient in FANCJ or RAP80 may be vulnerable to therapies that target the compensatory pathway.

National Institute on Aging
Cristina Banuelos
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Neuroscience - Integrative, Functional, and Cognitive
GABAergic Basal Forebrain Integrity is Compromised in Aged Monkeys with Cognitive Impairment

The search for strategies to promote optimally healthy cognitive aging has taken on increased urgency as people live longer. Basal forebrain projections to the cortex are anatomically positioned to influence a broad range of cognitive capacities including attention, executive function and memory. Although a long history of research on neurocognitive aging has focused on the role of the cholinergic basal forebrain, intermingled GABAergic cells are numerically as prominent and potently regulate the activity of their cortical projection targets, including the hippocampus and prefrontal cortex. The effects of aging on the non-cholinergic basal forebrain in primates, however, are largely unknown. In this study, we conducted quantitative morphometric analyses in brains from young rhesus monkeys (n=8, 10.1 years) and aged animals (n=16, 32.1 years) that displayed significant impairments on standard tests that require the prefrontal cortex and hippocampus (i.e., delayed response and delayed nonmatching-to-sample). Immunocytochemical techniques were used to visualize cholinergic (ChAT+) and GABAergic (GAD67+) neurons in evenly spaced histological sections through the medial septal nucleus (MS), nucleus of the diagonal band (nDB), and the nucleus basalis of Meynert (nBM). Distinct GABAergic cell groups were co-extensive and partially intermingled among cholinergic neurons throughout the basal forebrain, spanning over 14 mm in the rostrocaudal axis, emerging in the MS and nDB and continuing through the caudal portion of the nDB. Whereas cholinergic neurons tended to be clustered, GABAergic neurons were more homogeneously distributed throughout the regions of interest. Morphometric quantification revealed a significant decrease in GAD67+ cell number in the basal forebrain of aged monkeys compared to young (p=.044). Notably, parallel counts in adjacent sections demonstrated that ChAT+ cell number is preserved in the aged monkey basal forebrain. These findings raise the possibility that GABAergic basal forebrain integrity represents a novel target for efforts to promote healthy trajectories of cognitive aging.

National Institute on Aging
Tyler Demarest
Postdoctoral Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Identifying the Source of Reactive Oxygen Species following subcellular NAD+ depletion
Hallmarks of aging include metabolic decline, increased inflammation, and oxidative stress. These processes are inextricably linked but the mechanism that initiates them is not well understood. Nicotinamide adenine dinucleotide (NAD+) decreases in normal aging, Alzheimer's disease and premature aging disorders. These disorders also display increased levels of ROS and inflammation. We have shown that NAD+ supplementation can suppress ROS and inflammation. Therefore, metabolic alterations in NAD+ are likely involved in the regulation of these processes but exactly how decreased NAD+ results in ROS generation and inflammation requires further study. Here, we propose that decreases in NAD+ cause a redox shift that activates ROS via the upregulation of NADPH oxidase 4.

The terminal enzymes responsible for NAD+ synthesis are the nicotinamide mononucleotide adenyltransferases (NMNATs). To investigate the importance of NMNATs we performed knockdown experiments using control or NMNAT1/2/3 siRNA. Knockdown of NMNATs decreased NAD+ in human HEK293T cells and resulted in a 2-fold increase in cellular and mitochondrial ROS, with no change in mitochondrial membrane potential (MMP) as compared to control siRNA treated cells. These data

indicate that the source of ROS generation is cytoplasmic and mitochondrial, however, due to the absence of increased MMP, they further suggest that the electron transport chain is not the source of ROS. We therefore investigated another potential source of ROS, the NADPH oxidase (NOX) enzymes. There are several isozymes of NOX that generate ROS and are crucial for redox-signaling and immune functions. NOX expression is upregulated in age-associated inflammatory disorders such as atherosclerosis, stroke, Alzheimer's, and Parkinson's diseases. Indeed, administration of NOX inhibitor apocynin prevented both cellular and mitochondrial ROS following NMNAT knockdown, indicating that NOX is the source of ROS after NAD+ depletion. Interestingly, we observed increases in NOX4, specifically, which is the only NOX family member to localize to the cytosol and the mitochondria. These results suggest that following NAD+ decline, a redox shift activates ROS production by NOX4. Our results suggest that the inhibition of NOX4 may represent an important therapeutic target to mitigate oxidative stress and inflammation associated with aging and neurodegeneration.

National Institute on Aging
Yuki Kishimoto
Postdoctoral Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Chronic gut inflammation hastens the onset of motor dysfunction and pathology in a mouse model of Parkinson's disease
In addition to the motor symptoms related to degeneration of midbrain dopaminergic neurons, many patients with Parkinson's disease (PD) have symptoms of gastrointestinal dysfunction. Recent findings suggest that the alpha-synuclein pathology that typifies neurons affected in PD may occur first in the enteric nervous system and then propagate retrogradely to the brainstem via the vagus nerve. We previously reported that a pro-inflammatory diet exacerbated vagus nerve dysfunction, whereas an anti-inflammatory diet ameliorated this dysfunction in transgenic mice overexpressing mutant alpha-synuclein (A53T) in neurons (PD mice). Here, we tested the hypothesis that chronic low-level intestinal inflammation can accelerate PD symptoms and pathology in PD mice. Beginning at 3 months of age, wild type (WT) and PD mice were given either normal drinking water or water containing 0.5% (w/v) dextran sodium sulphate (DSS), which induces gut inflammation and cannot cross the blood brain barrier. Mice were monitored every 2 weeks for motor dysfunction and at the end of 12 weeks of treatment animals were sacrificed for pathological investigation. PD mice treated with DSS had symptoms of motor dysfunction 2 months earlier and had more severe PD pathology (alpha-synuclein accumulation in gut and brain and dopaminergic cell loss at substantia nigra) than PD mice treated with water alone. DSS treatment resulted in chronic low level ulcerative colitis-like gut histopathological changes in both WT and PD mice. Proinflammatory cytokines were increased in the colons of both DSS treated WT and PD mice. However, only PD mice treated with DSS had increased levels of proinflammatory cytokines in the brain. This finding was validated by the presence of microglia activation in the brains of PD mice treated with DSS. Interestingly, in serum there was no change in the levels of proinflammatory cytokines and the presence of alpha-synuclein could not be detected. Together, this suggests that chronic gut inflammation accelerates PD pathogenesis and that this may occur via retrograde propagation of alpha-synuclein pathology from the gut to the brain via the enteric nervous system.

National Institute on Aging
Roger Mullins
Postdoctoral Fellow
Informatics/Computational Biology
Validation of a data-driven neuroinformatics approach to identifying novel candidate genes for Alzheimer's disease
Data-driven methods for identifying genes involved in Alzheimer's disease (AD) pathogenesis aim to complement hypothesis-driven approaches that reflect current theories about the disease. We present a novel, unbiased, data-driven approach based on inter-correlated features among multi-modal 3D images. Using a custom MATLAB script, we correlated the spatial expression of 20,786 unique gene microarray probes from the six-specimen Allen Human Brain Atlas (AHBA) with a contrast image reflecting AD-related hypometabolism derived from brain fluorodeoxyglucose (FDG)-positron emission tomographies (PET) of 288 age-matched controls and 241 AD participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI). To validate the approach, we selected twenty-six novel candidate genes from the highest significant results and five canonical AD-related genes. These 31 genes were then tested using qPCR on human inferior parietal cortex and cerebellar tissue from 8 AD and 8 age-matched controls, with 21 of these candidate genes showing significantly different expression in the cortex, but not cerebellum, of AD subjects compared to controls. The candidate genes were also tested for Amyloid-beta mediated cellular toxicity using the C. elegans functional assay. Six of the 28 genes tested by RNAi knockdown showed significant effects in at least 2 of 3 independent trials. This consistency of the association of these genes with FDG hypometabolism, expression in AD-affected brain areas, and toxicity in an animal model suggests that this data-driven methodology is an effective method for identifying promising novel candidate genes for AD.

National Institute on Aging
Maja Mustapic
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Neuroscience - Neurodegeneration and Neurological disorders
Extracellular vesicle-based biomarkers for Alzheimer's disease in the Baltimore Longitudinal Study of Aging
Identifying patients at the preclinical stage of Alzheimer's disease (AD) is important for improving clinical care and clinical trial design. Currently existing CSF and PET-based biomarkers have limited preclinical diagnostic accuracy, are expensive, invasive, and only available in specialized centers. We have developed a set of blood biomarkers based on plasma extracellular vesicles (EVs) enriched for neuronal origin by expressing neural cell adhesion molecule L1CAM. In smaller case-control studies, we showed that Aß42, pTau181, and phospho-insulin receptor substrate-1 (pSer312-IRS-1 and p-panY-IRS-1) in plasma L1CAM+ EVs may be diagnostic for AD. In the present study, we are analyzing samples from the Baltimore Longitudinal Study of Aging (BLSA) to replicate previous findings and assess whether candidate EV biomarkers predict AD diagnosis at the clinical and preclinical stage.

We studied 969 samples from 373 BLSA participants, 128 diagnosed with AD during a given visit, 128 age and sex-matched controls followed over the same time, and 117 control subjects followed prospectively. Besides samples from the visit when AD diagnosis was made, we studied samples from 2 to 8 visits before. The average times between diagnosis and the two preceding visits were 2.3 and 4.5 years respectively.

We isolated L1CAM+ EVs and blindly quantified total-tau, pTau181, pSer312-IRS-1, p-panY-IRS-1, and the EV marker TSG101. EV concentration and size were determined by Nanoparticle tracking analysis (NTA). Statistical analysis is based on mixed linear models adjusting for NTA parameters, age and sex to assess differences; and logistic regression to assess diagnostic performance. We found that AD patients compared to controls have higher pTau181 both preclinically (F=9.37, df=348, p=0.002) and at time of diagnosis (F=13.98, df=345, p=0.0002); p-panY IRS-1 did not differ preclinically (F=3.54, df=352, p=0.061) and at the time of diagnosis it was significantly higher in AD patients (F=4.21, df=350, p=0.041). Model-adjusted pTau181 achieved area-under-curve (AUC) scores of 0.747 preclinically and 0.736 at the time of diagnosis. When combined with p-panY-IRS-1, preclinical AUC increased to 0.763 and clinical AUC to 0.747. These results confirm previous findings and suggest that L1CAM +EV biomarkers may predict AD 5 years prior to diagnosis.

National Institute on Aging
Yusuke Osawa
Visiting Fellow
Clinical and Translational Research - General
Does knee extension rate of force development add to peak torque in associations with physical function?
The ability to generate muscle force quickly decreases with aging and is important for daily activities such as walking, but most studies measure muscle performance using peak torque (PT), which does not account for speed of force development. Most equipment records the time course of force development and thus could be used to assess the rate of force development (RFD). RFD during isometric contraction is used extensively in sports research and has potential to inform studies of aging because it captures the speed-related, dynamic component of muscle performance. Using data from the Baltimore Longitudinal Study of Aging (BLSA), we assessed whether RFD adds to PT in associations with lower extremity performance. If RFD independently contributes to physical function, it may help identify weak older adults for screening and intervention. In 1089 BLSA participants (49.7% women; aged 26 to 96 years; women, 64.0 +/-13.8 years; men, 68.4 +/-14.4 years, mean +/- SD), we assessed the individual and combined associations of RFD and PT with physical performance, adjusted for demographics, BMI and body composition by DXA. PT was assessed by 30 deg/sec knee extension isokinetic dynamometry. Peak RFD was assessed during the 3 sec of a knee extension isometric strength test, with the knee joint positioned at 120 degrees flexion, as the maximum force-time slope in successive 50 msec epochs. Lower extremity performance was assessed as time to complete a 6m walk at usual and fast pace (6m-usual and fast), a 400m walk at fast pace (400m), distance covered in a 2.5min walk at normal pace (2.5min), time for 5 and 10 chair stands, and two overall tests of lower extremity performance (SPPB and HABCPPB). Analyses were sex-stratified and used generalized linear regression models adjusted for

age, race, BMI, appendicular lean mass and whole body fat mass. In men, independent of PT and cofactors, RFD was a significant (p< 0.05) predictor of all lower extremity performance tests except the 400m and 2.5min walks. In women, independent of PT, RFD was a significant independent correlate of the 6m-fast walk only (p<0.001). In conclusion, RFD independently contributes to physical functions in men, but less in women. The mechanisms underlying the sex difference are unclear and require further study.

National Institute on Aging
Jian Sima
Postdoctoral Fellow
Cell Biology - General
Eda ligand triggers a novel "partner recruitment" signaling mechanism: membrane insertion of its receptor Edar from an intracellular pool via PKA activation and STX6/VAMP7 trafficking machinery
Ectodysplasin (Eda) is a skin-specific TNF ligand. Eda signaling is mediated by Eda interaction with its membrane receptor Edars. Gene mutations in Eda signaling cause anhidrotic/hypohidrotic ectodermal dysplasia (EDA), which affects formation of human skin appendages including hair, teeth, and several exocrine glands. Previous studies mainly focused on signal transduction downstream of the Eda-Edar complex. However, the molecular mechanism of synthesis and membrane trafficking of Edar is unknown. In an ongoing study, we surprisingly found Eda promoting recruitment of its own receptor Edar from an intracellular pool into the plasma membrane. To elucidate the mechanism of this "partner-recruitment" trafficking of Edar, we utilized live-cell imaging, protein affinity purification, skin tissue culture, and transgenic mouse models. Live-cell imaging in keratinocyte cultures quantified the insertion rates and docking frequency of Edar membrane insertion as a function of Eda dosage. To identify possible proteins associated with Edar after Eda stimulation, we used protein affinity purification to show that Edar combined with STX6/VAMP7, a vesicle trafficking complex. Further cell culture experiments confirmed that complex formation between STX6/VAMP7 and Edar was induced by Eda stimulation. By analyzing mass spectral data, we further found evidence for PKA pathway activation mediated by Eda-Edar binding, and cell-based assays confirmed Eda-dependent PKA activation that was critical for phosphorylation of cytosolic Edar and its membrane insertion. Consistent with cell culture data, the relative level of Edar in plasma membranes in skin was markedly reduced in Tabby (Eda deficient) compared to WT mice. Also, application of recombinant Eda for 2 hr in skin tissue cultures increased the membrane Edar protein level and augmented Eda-induced downstream NF-KB activation in both WT and Tabby. Previous human genetic studies have identified several mutations of Edar in EDA patients, and we are currently examining the effects of each reported mutation to assess possible effects on Edar trafficking. Overall, current data reveal a regulatory mechanism of Eda signaling: Eda ligand binds to a small amount of membrane Edar and triggers PKA activation, which phosphorylates cytosol Edar and promotes further membrane insertion of more Edar via the STX6/VAMP7 vesicle trafficking complex.

National Institute on Aging
Vijay Varma
Postdoctoral Fellow

Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Brain and blood metabolite signatures of pathology and progression in Alzheimer's disease: a targeted metabolomics study

Background

We developed a novel approach to the discovery of blood metabolites related to AD progression by first identifying metabolites that accurately differentiated brain tissue samples from neuropathologically confirmed AD and control subjects. We then assayed these same metabolites in blood serum to test their associations with clinical, cognitive, neuroimaging, and CSF endophenotypes of AD. We then developed an integrated blood and brain endophenotype score (EASE-AD) summarizing the relationship of these metabolites to severity of AD pathology and disease progression. We finally applied a network biology approach to map the metabolite classes identified to key biologic pathways implicated in AD.

Methods

Quantitative metabolomics (BIOCRATES P180) were performed on AD and control brain samples from autopsy participants in the Baltimore Longitudinal Study of Aging (BLSA; N=43). Machine learning methods identified 26 metabolites that discriminated between clinical groups. These same signature metabolites were assayed in late (ADNI; N = 767) and early (BLSA; N = 207) preclinical blood samples to test associations AD-related brain atrophy, CSF measures of AD pathology, risk of conversion to incident AD, and trajectories of cognitive performance.

Results

Accuracy, sensitivity, and specificity of the assayed metabolites to discriminate AD from control samples from the ITG were 83.33%; 86.67%; and 80% respectively. Among the 26 top ranking metabolites, sphingolipids were highly represented. Specifically, sphingomyelin with acyl residue sums C16:0, C18:1, and C16:1 (SM C16:0, SM C18:1, SM C16:1,) and hydroxysphingomyelin with acyl residue sum C14:1 (SM (OH) C14:1) had the highest EASE-AD scores and were consistently associated with AD across early and late preclinical stages as well as with AD pathology at autopsy (p<0.05). Significant metabolites mapped to several biologically relevant pathways implicated in AD including tau phosphorylation, Aß metabolism, and acetylcholine biosynthesis.

Conclusions

This study proposes a new framework to identify biologically relevant blood markers of disease progression during the preclinical stages of AD. Results suggest that perturbations in sphingolipid metabolism are integral to the evolution of AD neuropathology as well as to the eventual expression of AD symptoms. These findings have important implications both for early detection as well as effective interventions in AD.

National Institute on Aging
Shi Zhang
Postdoctoral Fellow
Neuroscience - Neurodegeneration and Neurological disorders
The Lipid Peroxidation Product 4-Hydroxynonenal Initiates and Propagates alpha-Synuclein Pathology by Impairing Lysosome Function in Neurons
Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by intraneuronal accumulation of a-synuclein in the brainstem, substantia nigra and cerebral cortex resulting in autonomic and motor dysfunction, and cognitive impairment. Recent studies suggest that a-synuclein pathology can be propagated transneuronally, but the underlying molecular mechanisms are unknown. During aging, free radical–mediated membrane lipid peroxidation may contribute to neuronal dysfunction and death. It has been reported that 4-hydroxynonenal (HNE), an aldehyde produced during lipid peroxidation, associates with a-synuclein inclusions in PD, binds to a-synuclein, and can cause a-synuclein self-aggregation. Here we tested the hypothesis that HNE impairs the cellular system that removes damaged proteins and organelles, the autophagy – lysosome pathway, in rat primary cortical neurons. We found that HNE increases protein levels and immunoreactive puncta of LC3 II and p62 in the cytoplasm of HNE-treated neurons within 6 hours. Furthermore, we found that neurons exposed to HNE exhibit elevated pH levels, decreased protein substrate hydrolysis and cathepsin B activity, and accumulation of K63-linked polyubiquitinated proteins, which are the substrates targeted for lysosomal degradation. Our findings suggest that HNE causes early impairment of lysosomes, which plays a major role in degradation of misfolded protein such as a-synuclein. The amount of aggregated/oligomeric and S129-phosphorylated a-synuclein, the most toxic species, was increased in HNE-treated neurons. More intriguingly, we observed that HNE caused neurons to secrete extracellular vesicles (EVs) containing oligomeric and S129-phospho a-synuclein. EVs released from HNE-treated neurons were internalized by healthy neurons, which consequently degenerated. Additionally, we found increased HNE levels in cultured cells overexpressing human a-synuclein, and EVs released from those cells were more toxic to naïve neurons than were EVs from cells not overexpressing a-synuclein. Injection of EVs containing a-synuclein into the striatum of wild type mice resulted in spread of synuclein pathology to other anatomically connected brain regions. These results suggest that lipid peroxidation/HNE can cause lysosome dysfunction and the release of EVs containing aggregating a-synuclein which, in turn, mediate transneuronal propagation of a-synuclein pathology and neuronal degeneration in PD.

National Institute on Alcohol Abuse and Alcoholism
Mehdi Farokhnia
Postdoctoral Fellow
Clinical and Translational Research - General
Exogenous ghrelin administration affects alcohol self-administration and brain activity in heavy-drinking alcohol-dependent individuals: A human laboratory study
The gut communicates with the brain through neural, immune, and endocrine pathways. Ghrelin, a hormone synthesized by the stomach, increases appetite by acting on the arcuate nucleus of hypothalamus. In addition, ghrelin modulates central reward and stress pathways that contribute to drug-seeking behaviors. Ghrelin administration in rodents enhances alcohol motivation and intake. Studies in humans suggest a positive relationship between endogenous ghrelin levels and alcohol

craving and relapse. Also, intravenous (IV) ghrelin administration was shown to increase cue-induced craving in alcohol-dependent individuals. The present double-blind, placebo-controlled, crossover study investigated the effects of IV ghrelin on alcohol self-administration (ASA) and brain activity in heavy-drinking alcohol-dependent individuals. Participants underwent two experiments: 1) IV-ASA via the computerized alcohol infusion system (CAIS), 2) task-based and resting state brain functional magnetic resonance imaging (fMRI). Each experiment included two sessions during which a loading dose (3 mcg/kg) of IV ghrelin or placebo followed by a continuous ghrelin or placebo infusion (16.9 ng/kg/min) were administered. Results showed that participants self-administered higher number of alcohol infusions during the ghrelin than placebo session (mean increase: 24.97%, p=0.04). They also started pressing the button sooner (p=0.01) and received the first infusion earlier (p=0.03) during the ghrelin session. Participants reported higher food craving (p=0.01) during the ghrelin session; craving for alcohol was also increased, but did not reach statistical significance. As expected, IV ghrelin compared to placebo significantly decreased systolic (p=0.006) and diastolic (p=0.001) blood pressures. In regards to fMRI blood oxygen level dependent (BOLD) signal, IV ghrelin increased the alcohol- and food-related BOLD signal, respectively in left amygdala (p=0.01) and left nucleus accumbens (p=0.08). In addition, IV ghrelin decreased food-related BOLD signal in right medial orbitofrontal cortex (p=0.01). Finally, the resting state connectivity between caudate and anterior insula was bilaterally decreased by ghrelin. In conclusion, ghrelin administration increased alcohol self-administration and modulated brain activity in this sample. These data suggest that ghrelin signaling influences the neural correlates of alcohol consumption and can be considered as a therapeutic target for alcohol use disorder.

National Institute on Alcohol Abuse and Alcoholism
Ayesha Sengupta
Visiting Fellow
Neuroscience - General
5-HT neurons regulate fear learning by modulating basal amygdala circuits
The neurotransmitter serotonin (5-HT) is targeted by drugs (e.g. SSRIs) widely used to treat mood disorders such as anxiety and depression, but mechanisms of 5-HT involvement still remain unclear. Fear learning, which is important for avoiding environmental threats and crucial for survival, can become maladaptive in mood disorders. Dysfunctional neural fear circuits may contribute to emotional dysregulation in the etiology of such disorders. Lesion studies demonstrate intact 5-HT fibers in the amygdala are necessary for normal fear behavior. Basal amygdala (BA) circuits are a locus of fear learning and receive dense 5-HT input from the dorsal raphe (DR). To understand the involvement of 5-HT signaling and to optimize the efficacy of 5-HT-targeting drugs, characterizing the mechanisms of 5-HT modulation during fear behavior is essential. Here, we investigate the regulation of BA circuits by 5-HT projections during fear learning. We virally delivered light-gated opsins, or a control inert protein, in mice to selectively control 5-HT inputs to the BA. First, we manipulated 5-HT fiber activity during a fear learning task to establish the role of 5-HT projections to the BA. We provide bidirectional evidence for the involvement of 5-HT by showing 5-HT fiber excitation or inhibition enhances or reduces fearful behavior, respectively. We then conducted in vivo electrophysiology recordings that suggest 5-HT fiber excitation alters the responsiveness of BA neurons to sensory stimuli during fear learning. To study the mechanisms of this modulation, we excited 5-HT fibers in ex vivo brain slices and recorded light-evoked postsynaptic potentials in BA neurons that were abolished by 5-HT or glutamate receptor antagonists.

Thus, we demonstrate the co-release of 5-HT and glutamate from 5-HT fibers in the amygdala. 5-HT and glutamate signals were targeted to distinct BA cell types. Combining retrograde tracing with immunohistochemistry, we found a majority of DR neurons projecting to the BA co-express 5-HT and glutamate markers. C-fos staining indicated the co-expressing neurons are preferentially recruited in fear-learned mice compared to context-control mice. Collectively, our study provides new insight into serotonergic mechanisms of amygdala function and in emotional behaviors that are aberrant in psychiatric illnesses. Specifically, we find 5-HT inputs to the BA modulate distinct cell types through 5-HT and glutamate co-release, thereby regulating fear learning.

National Institute on Alcohol Abuse and Alcoholism
Shana Silverstein
Doctoral Candidate
Neuroscience - Integrative, Functional, and Cognitive
Prefrontal regulation underlying observational fear learning
Utilizing social information gained from observing others is a salient way to acquire necessary cues about safety or potential threats. While dysfunction of brain regions including the dorsomedial prefrontal cortex (dmPFC), amygdala and hippocampus are associated with risk for disorders such as psychopathy and anxiety, the neural circuitry mediating the effects of witnessed trauma remains poorly understood. To define these circuits, we first validated a mouse model of observational fear learning (OFL), in which the subject observes another mouse undergoing Pavlovian fear conditioning (30 tone+footshock pairings) and, at a later point in time, exhibits a fear reaction (freezing) to presentation of the tone. To functionally map the circuits involved in OFL, we used a retrograde tracer (Cholera Toxin B) to label inputs to the dmPFC in conjunction with co-labeling activated neurons (using c-fos as an analog of neural activation) in regions projecting to dmPFC in observing mice, demonstrators receiving a foot-shock and controls. We found a similar amount of dmPFC-projecting neurons activated in areas associated with either fear or social learning in observers and demonstrators, as compared to controls; significant overlap was found in insula, amygdala, ventral hippocampus, claustrum, and cingulate cortex. This suggests observing a traumatic event can result in equal activation of these areas as those physically experiencing the trauma. To establish a causal role of the dmPFC in OFL, we transfected this region with a light-sensitive opsin (adenoassociated virus, ArchT3.0) to silence neuronal activity during acquisition or retrieval of OFL. In another cohort we selectively silenced inhibitory parvalbumin-interneurons using PV-Cre transgenic mice in combination with transfusion of ArchT into dmPFC. We found that silencing the dmPFC neurons during acquisition, but not retrieval, prevented the acquisition and expression of OFL. Moreover, selectively silencing interneurons produced an attenuation of fear expression upon retrieval, suggesting parvalbumin-interneurons in this region may be responsible for forming an observational fear memory. Ongoing studies are probing the neural inputs from the amygdala and ventral hippocampus in a similar manner. Collectively, this data provide a novel insight into the neural circuits mediating OFL, with implications for understanding the pathophysiology of anxiety and psychological disorders resulting from witnessing trauma.

National Institute on Alcohol Abuse and Alcoholism
Mingjiang Xu

Research Fellow
Pharmacology and Toxicology/Environmental Health
ER stress-dependent release of mitochondrial DNA enriched microparticles contributes to acute-on-chronic alcoholic neutrophilia and hepatotoxicity
Over the last several years, one of the major advances in the field of alcoholic liver disease research was the discovery that binge alcohol consumption induced neutrophilia and hepatic neutrophil infiltration in chronically ethanol-fed mice and human subjects with excessive alcohol use (EAU); but the underlying mechanisms remain obscure. Here we demonstrated that chronic EAU patients with history of recent excessive drinking (EAU+RD) had higher serum levels of mitochondrial DNA (mtDNA)-enriched microparticles (MPs) than EAU without recent drinking (EAU-RD) and healthy controls, which correlated positively with circulating neutrophils. Similarly, mice with chronic-plus-binge ethanol feeding also had markedly elevated serum levels of mtDNA-enriched MPs with activation of hepatic endoplasmic reticulum (ER) stress and inflammatory responses, as demonstrated by the increased hepatic mRNA expression of various ER stress and inflammasome related genes, and increased protein levels of phospho-JNK, phospho-eiF2a, CHOP and cleaved caspase-1. Inhibition of ER stress by gene knockout and inhibitors or caspase-1 activity by inhibitors attenuated ethanol-induced elevation of mtDNA-enriched MPs, neutrophilia, and liver injury. Hepatic specific Perk gene knockout and cultured hepatocytes demonstrated that hepatocytes were the main source of ethanol-induced elevation of mtDNA-enriched MPs. Finally, administration of mtDNA-enriched MPs isolated from chronic-plus-binge ethanol feeding mice caused neutrophilia in chronically ethanol-fed wild-type mice but not in TLR9 knockout mice. In conclusion, chronic-plus-binge ethanol consumption activates hepatic ER stress-dependent mtDNA enriched MP release leading to neutrophilia and liver injury.

National Institute of Allergy and Infectious Diseases
BILLUR AKKAYA
Visiting Fellow
Immunology - General
Antigen-specific T regulatory (Treg) cells suppress dendritic cell (DC) function by forming unique physical interactions and capturing peptide-MHC class II (p-MHCII) complexes from the DC surface
Tregs constitute a small fraction of CD4+ T cells that have prominent function in preventing autoimmunity and limiting immunopathology. Transcription factor Foxp3 controls Treg lineage and mutations in Foxp3 locus lead to IPEX (Immunodysregulation polyendocrinopathy Enteropathy X-linked) Syndrome in humans indicating the critical role of Tregs in immune homeostasis. Tregs can employ numerous inhibitory mechanisms that target antigen presentation and effector T cell priming. Once activated through their antigen receptor, Tregs are able to initiate a number of immunosuppressive mechanisms that inhibit bystander effector T cells regardless of their antigen specificity. However, it has been documented that antigen specific Tregs exhibit enhanced capacity to suppress compared to polyclonal Tregs raising the possibility that antigen-specific Tregs use suppressor mechanisms that have not been yet characterized. To elucidate such mechanisms, we first compared the interaction of antigen-specific induced Tregs (iTregs) and antigen-specific T effector cells with DC using scanning electron microscopy and intravital two-photon microscopy. Antigen-specific iTregs uniquely and rapidly formed dense clusters around DC suggesting that they may prevent the access of antigen-specific CD4+ T cells to the DC surface. In an adoptive transfer model, this rapid interaction was accompanied by a decreased

ability of co-transferred naïve T cells to interact with the DCs as indicated by their failure to decrease their motility. More importantly, antigen-specific iTregs were found to specifically transendocytose cognate peptide-MHCII complexes from the DC surface to a greater extent than T effector cells resulting in diminished levels of antigen on the DC surface. Transmission electron microscopy of DC-Treg co-cultures showed that Tregs engulfed parts of DC processes as membrane invaginations within two hours and then internalized the p-MHCII complexes into endosomal vesicles. Taken together, these results indicate a two-step process by which antigen-specific Tregs inhibit antigen presentation. They rapidly and efficiently adhere to the DC surface and then deplete the pMHC II complexes from the DC resulting in potent suppression of the capacity of the DC to activate antigen-specific T cells.

National Institute of Allergy and Infectious Diseases
Davide Angeletti
Visiting Fellow
Virology - RNA and Retroviruses
Defining B cell and Antibody immunodominance to Influenza A virus
Influenza A virus (IAV) remains a major human pathogen despite seasonal vaccination. Intense interest has emerged in recent years to improve current influenza vaccination with the hope of developing a life-long universal influenza vaccine. In order to do so it is of fundamental importance to better understand basic immunological rules that govern B cell and antibody responses and their immunodominance (ID). ID is the phenomenon of unequal immunogenicity between antigens or epitopes on the same complex antigen. In this research project we investigated, understood and ultimately manipulated immunodominance.

As a model for a complex antigen we used IAV Hemagglutinin (HA). The protective Ab response is primarily directed to one of HA 5 non-overlapping antigenic sites on its globular head. Using sequential mAb selection we engineered a panel of drifted viruses which maintained only one intact antigenic site on HA globular head (named D4-viruses). By infecting mice with wt IAV and assaying the response using D4 we were able to dissect ID profiles. We defined ID in sera using ELISA and in germinal center B cells and plasma cells using flow cytometry and ELISPOT with recombinant HA probes based on the D4-viruses. We further compared ID profiles in sera and B cells upon immunization. With the D4 virus tool we could assess the influence of genetic background (by testing different mice strains) and CD4+ T cells, by depleting them. Using virus neutralization and hemgglutination assay we could define differences in antigenic site-specific polyclonal sera. Finally, by transferring in vivo antigenic site-specific Abs we could assess effects on ID.

Here, we described for the first time 1) the highly dynamic nature of the ID hierarchy with time after infection/immunization, 2) the radically different responses induced by infection vs. immunization, even in the same lymphoid organs, 3) the high correlation between GC B cell frequency and antibody responses, establishing GC B cells as the source of most serum antibodies, 4) the influence of genetic background on the ID hierarchy, 5) the lack of effect of CD4 T cells on early antibody responses, 5) the lack of immunodomination between Abs for different antigenic sites. Altogether these results will lay a foundation for deeper understanding of basic B cell biology and they can readily be applied for rational vaccine design.

National Institute of Allergy and Infectious Diseases
Timothy Break
Postdoctoral Fellow
Immunology - Infectious Disease
IFNg-producing T cells, not autoantibodies, drive susceptibility to mucosal candidiasis in Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED)
APECED, caused by mutations in autoimmune regulator (AIRE), leads to multisystem autoimmunity and susceptibility to chronic mucocutaneous candidiasis (CMC). AIRE deficiency impairs the thymic negative selection of self-reactive T-cells, which is sufficient to promote autoimmunity, as shown by adoptive transfer experiments of Aire-/- CD4 T cells. How AIRE-deficiency causes CMC, however, is elusive. It has been postulated that autoantibodies (AAbs) against IL-17/IL-22 explain CMC, but correlation between CMC and these AAbs is only seen in ~70% of patients, suggesting that other factors may also play a role. Here, we established the first model of mucosal candidiasis in Aire-/- mice, which have significantly elevated fungal load and mucosal injury post-oral infection, despite the absence of IL-17/IL-22 AAbs; in agreement, transfer of Aire-/- serum into WT mice does not promote Candida susceptibility. Strikingly, induction of IL-17A/F, IL-22 and IL-17-dependent antimicrobial peptides was not impaired in the oral mucosa of Aire-/- mice and APECED patients. Furthermore, neutralization of IL-17A/F or IL-22 in Aire-/- mice further increased mucosal fungal burden, indicating that Aire deficiency does not cause mucosal IL-17/IL-22 defects. Remarkably, Aire-/-Tcra-/- double-knockout mice control candidiasis, indicating that pathogenic T-cells drive infection susceptibility. Indeed, significantly greater numbers of activated CD44hiCD69+ CD4 and CD8 T-cells that over-produce IFNg accumulate in the Aire-/- oral mucosa. In agreement, IFNg but not Th2 or Th17 cytokine expression is highly elevated in the tongues of mice, and the IFNg-inducible chemokine CXCL9 is highly increased in Aire-/- mouse oral mucosa and APECED patient saliva. In agreement, microarray analysis revealed a very strong interferon signature in the Aire-/- oral mucosa, with 2 of the top 3 up-regulated canonical pathways being those of interferon signaling and interferon regulatory factors. Importantly, IFNg neutralization in Aire-/- mice led to control of fungal infection, directly revealing a pathogenic role of IFNg in promoting CMC. The repertoire and function of T-cells in the Aire-/- oral mucosa and the mechanisms by which they mediate Candida susceptibility are under ongoing investigation. Together, our data show that candidiasis in APECED is caused by an exaggerated T cell-dependent IFNg response, and reveal a novel IL-17/IL-22-independent axis that is critical for CMC host defense in humans.

National Institute of Allergy and Infectious Diseases
Nicholas Collins
Visiting Fellow
Immunology - General
Memory T cells localize to bone marrow during malnutrition.
Memory T cells are critical in providing protection against secondary infections. These cells require lipids and utilize fatty oxidation for their survival, and have been shown to preferentially localize to visceral fat for prolonged periods of time following infection. In states of malnutrition, visceral fat is mobilized and used as an energy source, resulting in a reduction in total fat mass throughout the body. How this affects the survival of pathogen-specific memory T cells is currently unclear. Intriguingly during caloric restriction, a model of malnutrition, fat in bone marrow is increased. In this study, we aimed to address

whether this counterintuitive response to malnutrition in bone marrow is important for the maintenance of memory T cells. Mice that had resolved infection with the gastrointestinal bacterium Yersinia pseudotuberculosis were placed on a 50% caloric restricted diet for 6 weeks. This lead to a reduction in visceral fat mass, while the total cellularity of the spleen was also diminished. Accordingly, the number of memory T cells that can recognize and respond to Y. pseudotuberculosis was reduced in these tissues. However, this population was found to be increased in number at several different bone marrow sites, including the femur and thoracic vertebrate. This increase was selective for memory T cells, as other populations including B cells, NK cells and hematopoietic precursors were either unaltered or reduced. Given that memory T cells are critical in providing life-long protection against secondary infections, these cells cannot afford to be lost when nutrients are limited. The results presented here suggest that the bone marrow provides a niche for memory T cells that promotes their survival during malnutrition, potentially involving an increase in lipids at this site. Thus, the bone marrow is a critical site involved in the preservation of immunological memory, allowing for life-long protection against pathogens.

National Institute of Allergy and Infectious Diseases
Michael Constantinides
Postdoctoral Fellow
Immunology - Innate and Cell-mediated Host Defenses
Mucosal-associated invariant T cells respond to cutaneous microbiota
The microbiome consists of a diverse array of commensal microorganisms that reside at barrier sites of the body and promote immune homeostasis through the release of microbial products, including derivatives of vitamin synthesis. Since many pathogenic bacteria and fungi also produce vitamins, the immune system has a specialized subset of T cells to detect the microbe-specific vitamin intermediates that are released, termed mucosal-associated invariant T (MAIT) cells. MAIT cells represent ~5% of T cells in human blood and they are further enriched in the intestines and lungs, where they are a major population of interleukin 17 (IL17)-producing lymphocytes. Though IL17 is critical for mounting immune responses to extracellular bacteria and fungi, little is known about the development MAIT cells. Furthermore, the contribution of MAIT cells to immunity in other barrier tissues has not been fully explored. Since the skin is constantly exposed to pathogens, we hypothesized that MAIT cells would be necessary for cutaneous immunity. We determined that MAIT cells represent 20-25% of T cells within murine skin and an even greater proportion of IL17-producing T cells than T helper 17 (Th17) cells. Analysis of human and non-human primate skin also revealed a high frequency of MAIT cells. To further characterize cutaneous MAIT cells, we compared the gene expression of these cells to conventional CD4 T cells by RNA-sequencing. Cutaneous MAIT cells express a Th17-like transcriptional program, including receptors for the interleukins IL1 and IL23, suggesting that these signaling pathways may be necessary for their homeostasis. By analyzing mice deficient in these receptors, we determined that IL23 is required for the maintenance of MAIT cells in the skin. Since both IL1 and IL23 are produced in response to the microbiota, we also assessed germ-free mice. In the absence of commensal bacteria and fungi, MAIT cells were dramatically decreased in the skin and other barrier tissues, indicating that the microbiota is necessary for the development of the MAIT cell population. To test whether MAIT cells are capable of responding specifically to skin commensals, we applied cultures of commensal bacteria

isolated from the skin of healthy human volunteers to the skin of mice and found that MAIT cells proliferated and a greater percentage were capable of producing IL17. This work indicates that MAIT cells contribute substantially to skin immunity and are dependent on the microbiota.

National Institute of Allergy and Infectious Diseases
Jigar Desai
Visiting Fellow
Immunology - Infectious Disease

C5ar1-dependent phagocyte effector functions protect against systemic candidiasis

The commensal yeast Candida albicans causes systemic candidiasis, the most common nosocomial human fungal infection, which leads to renal failure and mortality of >40% despite antifungal therapy. Myeloid phagocytes, but not lymphocytes, are critical for protection against systemic candidiasis in mice and humans; however, the molecular basis of myeloid cell-dependent protective immune responses remains poorly defined. Since patients with inherited C5 deficiency and patients treated with the anti-C5 humanized monoclonal antibody eculizumab have been reported to occasionally develop candidiasis, we sought to examine the role of C5a signaling in host defense against systemic candidiasis. We found that C5ar1 and C5l2 transcripts and their ligand C5a were significantly induced post-Candida infection in wild-type (WT) Balb/cJ mice. C5ar1-/- mice exhibited dramatically increased susceptibility with 100% mortality by day 5 while WT mice had 40% mortality at day 14 post-infection. C5ar1-/- mice had significantly increased fungal burden in the kidney and renal tissue injury; instead, C5l2 was dispensable as tissue fungal burden in C5l2-deficient animals were similar to that of WT animals. Since C5ar1 is expressed in both myeloid and stromal cells including renal tubular cells, we generated bone marrow radiation chimeras and found that hematopoietic C5ar1 expression promotes protection. Using C5ar1-reporter mice, we found that neutrophils, monocytes and macrophages, but not dendritic cells, express C5ar1 in the Candida-infected kidney. Indeed, phagocyte C5ar1 expression is essential for survival, as LysM-Cre[tg]GFP-C5ar1flox/flox mice universally succumbed to the infection similar to C5ar1-/- mice. Mechanistically, C5ar1 does not mediate phagocyte recruitment in the Candida-infected kidney as shown by flow cytometric immunophenotyping and mixed chimera experiments. Using a novel reporter Candida strain that simultaneously reports phagocytic uptake and fungal viability in vivo, our data indicate that Candida phagocytosis and killing by neutrophils and mononuclear phagocytes are impaired in the C5ar1-/- infected kidney. Ongoing studies in S100a8-Cre[tg]GFP-C5ar1flox/flox and Cx3cr1-Cre[tg]GFP-C5ar1flox/flox are aimed to determine the relative contributions of neutrophils versus mononuclear phagocytes in C5ar1-dependent protection as are studies in WT and C5ar1-/- mice aimed at defining the molecular mechanisms of C5a-C5ar1-dependent phagocyte effector functions.

National Institute of Allergy and Infectious Diseases
Rebecca Drummond
Visiting Fellow
Immunology - Infectious Disease

A CARD9-CXCR2-C5AR1 Axis Controls Neutrophil Recruitment to the Fungal Infected Central Nervous System

Candida albicans is the most common human fungal pathogen and causes systemic infections that are

associated with an unacceptably high mortality rate, despite the availability of antifungal therapy. C-type lectin receptors (CLRs), expressed on myeloid cells, are crucial for innate Candida recognition and initiation of antifungal immune responses. Many CLRs utilize a common signaling pathway, which uses the adaptor protein CARD9. Humans with autosomal recessive CARD9 deficiency are susceptible to fungal infections that have a unique tropism for the brain and meninges. We recently showed that CARD9 is required for the production of CXC chemokines from recruited neutrophils and glial cells in the brain following fungal infection that subsequently drove the protective accumulation of neutrophils in the brain, which was needed to control fungal growth in this tissue. Here, we have used several mouse lines deficient in various chemokine receptors, neutrophil chemoattractants and CARD9-coupled CLRs to define the immune pathways governing neutrophil recruitment to the central nervous system (CNS) following C. albicans infection. We show that neutrophil recruitment is dependent on the chemokine receptor CXCR2, in line with our earlier work indicating at defective production of CXCR2 ligands in CARD9-deficient mice and humans. We also found that C5AR1, a complement receptor for the anaphylatoxin C5a, was also required for neutrophil recruitment and control of brain infection in mice. In line with this, we found that Card9-deficient mice had reduced production of C5a in the brain following fungal infection. We found no role for other well-known neutrophil chemoattractant axes, including CCR1, CXCR1 and LTB4R1, as animals deficient in each of these chemotactic axes controlled fungal brain burdens and recruited neutrophils normally. Finally, we found no individual role for any of the well-known CARD9-coupled CLRs, including Dectin-1, Dectin-2, Dectin-3 and Mincle, indicating at high functional redundancy upstream of CARD9; studies in FcgR-/- and Dectin-1-/- FcgR-/- double knockout mice are ongoing to further examine this redundancy. In summary, our data details the molecular pathway controlling neutrophil recruitment to the fungal-infected brain, thus further increasing our understanding of the unique manifestation of systemic candidiasis in CARD9-deficient patients and indicating at several potential new therapeutic targets for controlling fungal meningoencephalitis.

National Institute of Allergy and Infectious Diseases
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Visiting Fellow
Microbiology and Antimicrobials
Discovery of Novel Anti-Tuberculosis Drugs from Sphagnum Bog Bacteria
Globally, tuberculosis (TB) causes around 1.4 million deaths annually. It is estimated that one-third of the world's population is infected with Mycobacterium tuberculosis (Mtb), the pathogen that causes TB. Due to the growing threat of anti-TB drug resistance, we are in urgent need of new drugs to treat the disease. Sphagnum peat bogs are acidic and hypoxic environments, with similar conditions to those encountered in tuberculous granulomas. Moreover, these habitats are also major natural reservoirs of mycobacterial species. Natural selection has endowed other microorganisms living in these nutrient-bare sphagnum areas with the ability to compete with endogenous slow-growing mycobacteria for these scarce nutrients by secreting secondary metabolites. These factors support sphagnum peat bogs as ideal sources for discovery of novel anti-TB drugs.

Sphagnum bog samples were collected across Northeastern US. Bog bacteria, including Chryseobacterium, Variovorax, Pedobacter, and Pantoea were found to produce secondary metabolites against Mtb. These positive hits were selected for whole genome sequencing based on scarce prior

reports of their natural products and their exclusive, potent activity against Mtb (MIC <6.25 umg/ml; other species MICs >100 umg/ml). No cytotoxicity against HepG2 cells was observed for these metabolites (IC50 >100 umg/ml). Novel biosynthetic gene clusters, including non-ribosomal peptide (NRP), polyketide (PK) and/or hybrid NRP/PK among others, were identified using automated bioinformatics software PRISM and anti-SMASH 3.0. Based on the in silico predict structure, bioactivity-guided fractionation of bacterial biomass samples and LC-MS analysis, novel compounds were identified. Subsequent transcriptional profiling of Mtb treated with these metabolites along with reporter and macromolecular incorporation assays yielded signatures indicating novel mechanisms of action beyond DNA damage and cell wall disruption (current pipeline of anti-TB drugs).

Currently, compounds with unique scaffolds and novel mechanisms of action are being fully isolated and characterized. These compounds have the potential to form a basis for new anti-TB classes addressing bacterial targets that are currently underexploited. Our findings highlight the benefits of searching bog environments for new species that make natural products with potent, exclusive anti-TB activity. These metabolites could bypass resistance and form the foundation of new therapies.

National Institute of Allergy and Infectious Diseases
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Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
TGF-Beta Activation by Proliferating Cholangiocytes Drives Biliary Fibrosis
Injuries that disrupt normal tissue architecture require a rapid and controlled repair process, which, if uncoordinated, can result in local scarring, also known as fibrosis. Dysregulated repair can permanently distort tissues, predisposing individuals to organ dysfunction and increased risk of death. Fibrosis of the biliary tree, the network of ducts that drain bile and toxins from liver, is particularly devastating, as scarring can result in backup of bile and liver necrosis. The pathogenesis of biliary fibrosis remains enigmatic; however, the proliferation of biliary epithelial cells, also known as cholangiocytes, and TGF-beta signaling have both been extensively observed in patients with biliary fibrosis.

TGF-beta is a known fibrogenic protein secreted as an inactive propeptide. Recent studies have unveiled that integrin alpha-v (ITGAV), a trans-membrane receptor that aids in adhesion to the surrounding matrix, can bind pro-TGF-beta and mechanically cleave the protein into its active form. Given the extensive epithelial proliferation observed during the pathogenesis of biliary fibrosis, we hypothesized that ITGAV expressed on proliferating cholangiocytes may activate latent TGF-beta, thus contributing to fibrosis.

To test this hypothesis, we engineered a novel strain of mice that delete ITGAV specifically in cholangiocytes (ITGAV-cKO mice). We subjected these mice and wild type Cre-negative litter mates to bile duct ligation (BDL), an experimental model that closely mimics the pathophysiology observed in patients with biliary fibrosis. Two weeks after BDL, wild type mice exhibited extensive TGF-beta activation as assessed by downstream SMAD3 phosphorylation and aSMA expression, a marker of

fibroblast activation. Additionally, we observed significant increases in collagen deposition by peribiliary myofibroblasts compared to sham-operated controls. In contrast, ITGAV-cKO mice exhibited an 81.2% reduction in SMAD3 phosphorylation and a 33.7% reduction in aSMA expression compared to wildtype litter mates. These data corresponded with a 31.7% reduction in fibrosis as assessed by peribiliary collagen deposition.

These data reveal that proliferating cholangiocytes are responsible for a significant proportion of the TGF-beta activation and subsequent fibrosis observed after BDL. Future studies using alternate models of biliary fibrosis may extend and solidify the importance of ITGAV-mediated TGF-beta activation as a key driver of biliary fibrosis.

National Institute of Allergy and Infectious Diseases
Jeffrey Grabowski
Postdoctoral Fellow
Virology - RNA and Retroviruses
Flavivirus replication in Ixodes scapularis (black-legged tick) ex vivo organotypic cultures and application for control
The Lyme disease tick vector, Ixodes scapularis, transmits a number of pathogens, including tick-borne flaviviruses (TBFVs). TBFV infections cause thousands of human encephalitis cases worldwide each year. In the US, confirmed human infections with TBFV Powassan virus (POWV) are increasing and have a fatality rate of 10-30%. In addition, Langat virus (LGTV) is an attenuated TBFV and often used as an experimental model TBFV. Currently, the detailed characteristics of TBFV replication and dissemination within tick organs are poorly characterized, and are important due to the relevance of organs being barriers to infection that affect downstream transmission. A deeper understanding of virus biology in the tick vector may inform effective countermeasures to reduce TBFV transmission. The goals of this work were 1) to develop short-term, ex vivo organ cultures of the midgut, salivary glands, and synganglion (brain) from the female I. scapularis tick, 2) to examine initial TBFV replication and spread in individual organs, and 3) to utilize the organ cultures for dsRNA-mediated RNA interference (RNAi) assays to identify impact on TBFV replication. Organs were dissected from unfed female ticks and cultured ex vivo. The organotypic cultures were metabolically active for up to 9-10 days and supported TBFV growth. Specifically, live videography of organs infected with green fluorescent protein-tagged LGTV demonstrated LGTV replication and spread. Furthermore, immunohistochemistry confirmed LGTV envelope and POWV protein expression and spread within the infected organ cultures. Infectious LGTV and POWV were produced from infected organ cultures, with greater LGTV replication than POWV. Thus, the ex vivo organ cultures were a suitable system for study of virus replication in individual organs. Transfection of TBFV-infected midgut and salivary glands with dsRNA specific to the LGTV 3’UTR reduced infectious LGTV/POWV replication, providing a proof-of-concept use of RNAi in I. scapularis organ cultures. The results of this study provided novel information on TBFV replication and spread in specific cell types using ex vivo I. scapularis tick organs. This system may prove useful as a translational tool for identifying potential tick transcripts/proteins that can be used as targets for TBFV therapeutics.

National Institute of Allergy and Infectious Diseases
Kevin Hart
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Clinical and Translational Research - General
Failure of Liver Regeneration in the Progression of Non-Alcoholic Fatty Liver Disease
Associated with the unabated increases in obesity and type II diabetes, non-alcoholic fatty liver disease (NAFLD) is now the most common progressive liver disease in developed countries and is quickly becoming the top indication for liver transplantation in the US. Its pathophysiologic spectrum including non-alcoholic steatohepatitis (NASH) and cirrhosis is marked by abnormal fat accumulation in hepatocytes (steatosis), immune infiltration, and untreatable scarring, or fibrosis, of the liver. The liver has a remarkable ability to regenerate to maintain critical liver functions in response to a variety of injuries. Recent work has elucidated important pathways regulating hepatocyte proliferation, but it is unclear if these are important in NAFLD and progression to NASH. In previous NAFLD studies, we detected hepatomegaly beyond the increases due to lipid accumulation. Thus, to test if liver regeneration compensates for hepatocyte damage and stress during NAFLD, we quantified hepatocyte proliferation by Ki67 staining in livers of mice challenged with a high fat diet. Significant proliferation of hepatocytes was detected 4-6 weeks after the initiation of NAFLD, but was significantly reduced by 10 weeks, and almost undetectable at 40-weeks. This was despite continuing increases in hepatic expression of pro-regenerative mediators including il6, tnfa, tnfrsf1a, tgfa, osmr, and hgf, suggesting a block in regenerative capacity during the progressing of NAFLD. This was due in part to the deregulation of epidermal growth factor receptor (egfr), a known regenerative pathway. Indeed, decreased expression of Egfr correlated with a loss of hepatocyte proliferation at all time points following the development of NASH. Furthermore, there was a strong negative correlation between egfr and markers of fibrosis in murine NASH. Analysis of microarray data from human liver biopsies revealed similar increases in the regenerative signals il6, tgfa, and hgf, but a significant decrease in egfr-signaling components in NASH livers. These data demonstrate that liver regenerative pathways are induced in NAFLD, but are quickly silenced as disease progresses. Consequently, they are incapable of compensating for the accumulating hepatocyte damage, which can accelerate the progression of liver fibrosis. Thus, EGFR expression in the liver may serve as a useful biomarker of regenerative capacity in NAFLD, and therapeutic restoration of this pathway may inhibit disease progression.

National Institute of Allergy and Infectious Diseases
Sharat Jacob Vayttaden
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Signal Transduction - General
IRAK1 Containing Supramolecular Organizing Centers Regulate Multi-TLR Signaling
Macrophages sense microbes through a diverse receptor family including toll like receptors (TLR). Immune signaling is organized through higher-order complexes called supramolecular organizing centres (SMOCs). These facilitate efficient protein interactions. Known SMOCs of immune response associate only with their cognate pathways and don't include proteins of other pathways. My work establishes an Interleukin-1 receptor-associated kinase 1 (IRAK1) SMOC that links multiple pathways. This diversifies the immune response to multi-stimuli as seen during infection.

How do multiple immune pathways work together? To address this I asked if single/dual TLR pathway stimuli would lead to different spatial organization of signaling proteins. I expressed fluorescent-tagged proteins in murine macrophages and stimulated single/dual TLRs. The screen used widefield and TIRF imaging, and hits were validated through confocal and high content imaging analysis.

I discovered that IRAK1 forms large cytoplasmic clusters on dual TLR stimulation. Cells with IRAK1 clusters show normal NFkB but reduced MAPK activity. IRAK1 clusters colocalized with inhibitors of NFkB (allowing normal signaling) and activators of MAPK pathway (depressing MAPK signaling). TLR pathway proteins were also sequestered in these clusters. In IRAK1KO cells, phosphoprotein and cytokine responses to dual-TLR ligands were higher than WT cells. Surprisingly, the IRAK1 clusters were enriched for non-TLR pathways like the inflammasome components - ASC and Caspase 11. Cells that were treated with dual TLR stimulation showed stronger inflammasome readouts like IL1alpha/beta than single TLR stimulation, and IRAK1KO cells showed decreased inflammasome response under dual TLR stimulation. Together, these data suggest a role for IRAK1 in regulating signal flux through the TLR pathways while facilitating inflammasome activation.

In order to test if IRAK1 was involved in a facilitating a more effective immune response to a microbe where the IL-1 response is critical for host protection, we infected IRAK1KO and WT mice with Yersinia pseudotuberculosis. Infection doses that were not lethal for WT mice lead to 100% mortality in IRAK1KO mice. In conclusion, we have identified a novel role for murine IRAK1 SMOCs in diversification of immune pathway activation, and we demonstrate that the loss of this IRAK1 function leads to a previously unappreciated severe susceptibility of IRAK1 KO mice to Yersinia infection.

National Institute of Allergy and Infectious Diseases
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Immunology - Innate and Cell-mediated Host Defenses

The O-antigen capsular polysaccharide of Francisella tularensis subspecies tularensis alters host cell mitochondrial metabolism to improve virulence

Francisella tularensis is a highly pathogenic intracellular bacterium, and the causative agent of tularemia. F. tularensis subspecies tularensis (Ftt) is the most virulent strain, having an ID50 < 10 cfu and can cause up to 60% mortality if left untreated. Typically, macrophages will shift their metabolic state from oxidative phosphorylation to glycolysis when bacterial ligands are sensed. This metabolic shift is necessary for host cell defense, including cell activation and production of pro-inflammatory cytokines. We have previously established that the O-antigen capsular polysaccharide of Ftt rapidly inhibits inflammatory responses in macrophages through impairment of the metabolic shift from oxidative phosphorylation to glycolysis. However, the specific host cell metabolic processes targeted by Ftt capsule were not elucidated. Here, we identified mechanisms by which Ftt and its capsule alters host metabolism. Murine bone marrow derived macrophages were treated with capsule isolated from Ftt,

and host cell bioenergetics were determined using a Seahorse XFe96 analyzer. Exposure to capsule increased mitochondrial respiration in macrophages, which was associated with greater substrate utilization through complex I of the electron transport chain. Infection with live Ftt resulted in a similar increase in mitochondrial respiration through complex I substrate utilization early during the course of the infection. The role of capsule in modulation of mitochondrial function was confirmed utilizing a capsule mutant, which failed to similarly impact mitochondrial function. Exposure of infected macrophages to inhibitors of mitochondrial function resulted in decreased intracellular bacterial loads, confirming Ftt dependence on host cell mitochondrial respiration. These are the first studies demonstrating the capability of bacterial capsule to alter host cell mitochondrial function, resulting in impairment of host cell activation and a productive inflammatory response. Furthermore, our data underscore the importance of early manipulation of host cell metabolism by Ftt capsule as a mechanism of virulence to provide a metabolic niche advantageous for infection.

National Institute of Allergy and Infectious Diseases
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HIV and AIDS Research

Both brain macrophages and memory CD4+ T-cells may serve as potential reservoirs in SIV-infected rhesus macaques

Human immunodeficiency virus (HIV) infects the central nervous system (CNS) early in the course of infection, which can lead to HIV-induced encephalitis (HIVE/neuroAIDS). This can result in HIV-associated neurocognitive disorder (HAND) due to associated impairment of cognitive and motor functions. Highly active antiretroviral therapy (HAART) can improve cognitive function, however, neurocognitive disorders can still arise either due to persistent HIV replication and/or inflammation in the brain. Simian-immunodeficiency virus (SIV) infected non-human primates can serve as a relevant model for AIDS neuropathogenesis with pathologic and clinical features reminiscent of HIVE in humans. However, the current SIVE/neuroAIDS models have limitations, such as the requirement for modalities to quickly deplete T cells, in order to enhance disease progression. In a recent study, we isolated a neuropathogenic clone SIVsm804E-CL757 (CL757) from rhesus macaques following four in vivo serial passages. This virus induces SIVE in 50% of the animals infected where high CSF viral loads and the formation of characteristic lesions in the brain are observed. The aim of this current study was to isolate and identify cellular subsets in the brain that can serve as potential viral reservoirs. Isolation of mononuclear cells from the brains of SIV infected macaques showed that CSF viral load correlated with an increase in the number of mononuclear cells, more specifically brain CD4+ memory T cells (mCD4) and macrophages (MF), indicating cell infiltration in the brain of animals with SIVE. Using cell sorting and SIV qPCR we show that both mCD4 and MF harbor viral DNA and corroborated in situ using DNAscope. Virus isolation by co-culturing techniques confirmed productive infection of both cell types. Only in animals exhibiting SIVE/neuroAIDS was SIV DNA detected in MF. Phylogenetic analysis using gp120 sequences show that SIV variants in MF and mCD4 are compartmentalized with sequences from the CSF and distinct from sequences in the blood mCD4 cells indicating viral replication within each cellular subset and indicate a previously unanticipated role of CD4 T cells as a potential reservoir in the brain.

National Institute of Allergy and Infectious Diseases
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Immunology - Autoimmune
Loss-of-Function Mutations in GIMAP5 Lead to a Novel Mendelian Disease of Autoimmunity
The incidence and prevalence of autoimmune diseases have increased in developed countries to the point of being among the top ten causes of death among young and middle aged women. Lymphocyte quiescence and regulation are recognized as being critical in the prevention of autoimmunity, however, these pathways have been difficult to study in vivo in humans. The use of whole-genome sequencing to identify Mendelian autoimmune disorders shows great promise for addressing this need and identifying novel drug targets. We have identified a cohort of eight human patients from three pedigrees suffering from a novel recessive Mendelian disease of autoimmunity characterized by lymphopenia, thrombocytopenia, autoantibodies and liver disease. Whole-genome sequencing revealed mutations in a gene called GIMAP5 whose expression is nearly exclusive to lymphocytes in humans. Mice deficient for Gimap5 are phenotypically very similar to our human patients and polymorphisms in human GIMAP5 have previously been linked to SLE and diabetes highlighting it's role in immune regulation. Western blots reveal that the GIMAP5 mutations in our patients lead to a near complete loss of mature protein and defective protein folding has been confirmed via in vitro structural studies.

Very little is known about the biochemical role of GIMAP5 at a cellular level, which is a crucial obstacle to exploiting this gene or the cellular processes it regulates as a therapeutic target. Due to the loss of lymphocyte quiescence in the absence of GIMAP5 and localization to the lysosome we hypothesized that GIMAP5 may be a negative regulator of the mTORC1. The mTORC1 and the associated regulatory Ragulator complex are known to be critical loci for regulation of cell growth and metabolism and play a key role in the regulation and quiescence of lymphocytes. Consistent with this, we observe robust association between GIMAP5 and components of mTORC1 and the ragulator complex. This association was enhanced upon ligation of the T cell receptor suggesting that it may represent a novel pathway of mTORC1 regulation specifically in T lymphocytes. Finally, we treated Gimap5 deficient mice and one human patient with rapamycin and observe robust clinical improvement as well as decreased lymphocyte activation in both. In conclusion we have described a new Mendelian disease in humans caused by mutations in GIMAP5 and go on to identify a novel, tissue specific role for this protein in the regulation of mTORC1.

National Institute of Allergy and Infectious Diseases
Qingbo Liu
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HIV and AIDS Research
Quaternary Configuration of the Functional CD4-binding Site in the HIV-1 Env Trimer
The native HIV-1 envelope (Env) spike on the surface of mature virions is a heavily glycosylated trimer of gp120–gp41 heterodimers, which mediates viral attachment and entry. Binding of the gp120 Env trimer to the host CD4 receptor is the first step in the HIV-1 infectious cycle. Although the CD4-binding site of

gp120 has been extensively characterized by mutagenesis and co-crystallization with soluble CD4 (sCD4), most of these studies were performed with monomeric gp120 subunits, thus hindering the evaluation of the role of quaternary elements that may be involved in the initial CD4. Moreover, the initial receptor interaction has been difficult to study as gp120 Env trimer, upon CD4 binding, undergoes major structural rearrangements, transitioning to a state that is competent for interaction with the CCR5 or CXCR4 coreceptor.

Here we present the cryo-EM structure of a conformationally constrained HIV-1 Env soluble trimer (DS-SOSIP.664) complexed with sCD4 and a trimer-specific broadly neutralizing antibody, PGT145. DS-SOSIP.664 trimer remains in a pre-fusion, closed conformation after interaction with CD4, permitted to visualize the initial contact with CD4. We found that the initial CD4-contact site in the HIV-1 Env trimer is constituted by a quaternary surface formed by coalescence of the previously defined CD4-binding region in the outer domain of one gp120 protomer with a second CD4-binding site (CD4-BS2) that encompasses discontinuous elements from the inner domain of a neighboring gp120 protomer. Disruption of CD4-BS2 destabilized CD4-trimer interaction and abrogated HIV-1 infectivity by preventing acquisition of coreceptor-binding competence. A corresponding reduction in HIV-1 infectivity occurred upon mutation of CD4 residues that interact with CD4-BS2. We also documented quaternary interactions for selected neutralizing antibodies targeting the CD4 supersite, which suggested an immunogenic nature of CD4-BS2 region in vivo. Thus the CD4-BS2 region may provide a new molecular target for the development of HIV-1 entry inhibitors.

National Institute of Allergy and Infectious Diseases
Dustin McCraw
Postdoctoral Fellow
Protein Structure/Structural Biology

Structural characterization of influenza virus-like particles displaying hemagglutinin have structural variances from native virus with potential for immunological impact.

Despite the availability of seasonal vaccines and antiviral medications, influenza virus continues to be a major health concern and pandemic threat due to the continually changing antigenic regions of the major surface antigen, hemagglutinin (HA). Highly conserved epitopes reside within the less-accessible stem domain of HA and are the targets of a number of broadly neutralizing antibodies, thereby holding promise for the design of a universal influenza vaccine.

Recombinant virus-like particles (VLPs) containing HA are a promising vaccine platform that have been shown to protect mice against heterosubtypic challenge in animal models. However, the structure of influenza VLPs and the organization of HA molecules on the VLP surface is not fully understood. This inhibits our ability to optimize the vaccine and understand its capacity to elicit cross-reactive stem antibodies.

Here, we used cryo-electron microscopy and image analysis to characterize the structure and morphology of influenza VLPs that display HA from the 1918 H1N1 pandemic. Multimodal immunoassays were used to further characterize VLP reactivity with heterosubtypic stem antibodies that bind to conserved regions of HA. Finally, we used cryo-EM density map segmentation to computationally isolate the various structural features of the VLP.

We found that the VLPs were predominantly spherical in shape with a median width of 110 nm. HA was uniformly distributed on the VLP surface with an average spacing of 8 nm, which is a tighter packing than the 14 nm reported for influenza virus. Despite the tighter packing, heterosubtypic antibodies still bound strongly to conserved epitopes on the HA stem domain. Segmentation identified two major VLP structural features, including HA displayed on the surface and HA contained internally within the VLP—which we termed 'molecular cargo'.

Our observations suggest that influenza H1 VLPs share a similar morphology to H1N1 viruses and that the tightly packed arrangement of HA molecules on the VLP surface does not ablate binding by broadly neutralizing stem antibodies. HA was confirmed to be both externally exposed and internally contained as 'molecular cargo', which may suggest the promotion of multiple immunological pathways. Further studies of additional influenza VLP subtypes would aid in optimizing the display of conserved epitopes for designed immunogens—and lay the groundwork for a universal influenza vaccine eliciting broadly neutralizing antibodies.

National Institute of Allergy and Infectious Diseases
Eugene Oteng
Other
Molecular Biology - Eukaryotic
High-efficiency enrichment and identification of aptamers to Plasmodium falciparum infected erythrocytes
Aptamers are synthetic oligonucleotides with the ability to recognize a specific molecule with antibody-like affinity and specificity. Despite its broad and promising diagnostic and therapeutic potential, adoption of aptamer technology has been hindered by the high failure rate of systematic evolution of ligands by exponential enrichment (SELEX), the process by which aptamers are discovered. Here we performed parallel SELEX experiments to compare the effect of two different methods for the single strand recovery step of SELEX on the efficiency of aptamer enrichment. Our results indicate, for the first time, that the commonly used alkaline denaturation-based method fails to effectively enrich aptamers whereas an alternative strand separation method, lambda exonuclease digestion, succeeds. To further support our experimental findings, we analyzed SELEX publications spanning 13 years and uncovered a highly significant association between studies that required prolonged selection rounds and use of alkaline denaturation as the single strand recovery method. These results implicate the alkaline denaturation step as a significant cause for inefficient aptamer selection and SELEX failure.

We confirmed the efficiency of lambda-based SELEX by isolating aptamers against determinants unique to the surface of erythrocytes infected with Plasmodium falciparum, the causative agent of the deadliest of the human malarias. We validated the binding specificity using both laboratory strains and geographically distinct clinical isolates, an important first. Taken together, our work demonstrates that employing alternative methods for single strand recovery can greatly improve the success of SELEX. Our finding remove a significant barrier to the broad adoption of aptamer technology, for example, opening new approaches for monitoring important global disease such as malaria.

National Institute of Allergy and Infectious Diseases
Eduardo Pinheiro Amaral
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Immunology - Innate and Cell-mediated Host Defenses
Involvement of ferroptosis in necrotic host cell death induction during severe tuberculosis
Tuberculosis (TB) remains a major global public health problem, causing 1.4 million deaths annually. The pathogenesis of TB is closely associated with necrotic tissue damage in lungs and other affected organs. Nevertheless, the mechanism by which cellular necrosis is induced following Mycobacterium tuberculosis (Mtb) infection remains poorly understood. Ferroptosis is a type of programmed necrotic cell death modulated by free iron, reactive oxygen species (ROS) and lipid peroxide accumulation. Importantly, increased levels of both ROS and heme oxygenase-1 (an enzyme that contributes to generation of free iron) have been observed in active disease in Mtb infected humans. Based on these findings, we hypothesized that the induction of necrosis during Mtb infection is regulated by ferroptosis. To test this concept we infected bone-marrow derived macrophages with virulent (H37Rv) Mtb and measured lipid peroxide, LDH release and glutathione (GSH) levels. We observed increased lipid peroxidation, and LDH release along with reduced GSH levels, three hallmarks of ferroptotic cell death. Moreover, treatment of the macrophage cultures with ferrostatin-1 (Fer-1), an inhibitor of ferroptosis, abrogated the necrosis induced by Mtb and resulted in reduced lipid peroxide levels and LDH release as well as elevated GSH levels. To investigate the role of ferroptosis in vivo, we infected mice with H37Rv by either intrapharyngeal or aerosol inoculation and beginning at 15 days post-infection (p.i.) treated them daily with Fer-1 or vehicle by intraperitoneal injection. On day 28 p.i. disease progression was evaluated by CFU burden, lipid peroxide levels, and induction of pulmonary necrosis as assessed by intravenous injection of Sytox dye which stains DNA in necrotic cells. We found that Fer-1 treatment completely restored GSH and lipid peroxide to pre-infection levels and this in turn was reflected in a reduction in the DNA stained necrotic areas in the infected lung tissue. Importantly, the Fer-1 treated animals exhibited a one log10 lower pulmonary CFU burden compared to untreated animals indicating improved control of infection. These results suggest that ferroptosis plays a major role in the regulation of necrosis in Mtb infection and identify iron-regulated cell death as a possible target for therapeutic intervention in tuberculosis.

National Institute of Allergy and Infectious Diseases
Lydia Roberts
Postdoctoral Fellow

Immunology - Infectious Disease

Pulmonary tissue-resident CD4+ T cells are associated with vaccine-mediated protection against tularemia

Pulmonary infection with the highly virulent intracellular bacterium, Francisella tularensis (Ftt) results in an acute, lethal disease called tularemia. Depletion of CD4+ T cells in Ftt immune animals results in death within 5 days of infection, underscoring the importance of a rapid T response. In other models of pulmonary infection, the contribution of specific T cell subsets can be discriminated via their location in the tissue. For example, effective control of pathogens in the lung is often dependent on the presence of tissue resident T cells rather than the circulating pool of T cells. Thus, we hypothesized that optimal protection against Ftt would require T cells poised to respond at the site of infection rather than those circulating through this compartment. We analyzed three pools of anatomically distinct lung T cells: airway, circulating, and tissue resident among mice vaccinated with two different strains of F. tularensis Live Vaccine Strain (LVS) that provoke varying levels of protection 28 days after vaccination. Airway T cells were isolated by brocheoalveolar lavage. Circulating T cells were discriminated via an intravenous injection of antibody to label all T cells in the blood immediately prior to euthanasia. This technique does not label tissue resident T cells. Surprisingly, ATCC LVS vaccinated mice, which had sub-par immunity against Ftt infection, had significantly more airway CD4+ T cells compared to RML LVS vaccinated mice which were solidly protected against Ftt challenge. In contrast, RML LVS immune mice had significantly more tissue-resident, polyfunctional, effector CD4+ T cells. ATCC LVS and RML LVS immune mice had similar numbers of circulating CD4+ T cells. Upon challenge with Ftt, the differences observed after vaccination were maintained. Together, these data suggest that expansion and maintenance of a pool of polyfunctional, tissue resident CD4+ T cells, and not airway or circulating CD4+ T cells, is required for survival of Ftt infection. Moreover, our data suggest that establishment of this pool of CD4+ T cells occurred early after immunization, i.e. days 3-7, and was minimally influenced by infection 28 days later. Thus, these data provide important insights into the nature of the protective CD4+ T cell response that must be provoked by future vaccine candidates.

National Institute of Allergy and Infectious Diseases

Benjamin Schwarz

Postdoctoral Fellow

Biochemistry - General and Lipids

Manipulation of host metabolism by unique Francisella tularensis phospholipids

Intracellular bacterial pathogens must interface with host metabolism to meet biosynthetic and energetic needs while avoiding an effective immune response. The molecular tools used by pathogens to manipulate host cells may also offer potential bioinspired avenues toward novel immunomodulatory therapeutics. Francisella tularensis subsp. tularensis (Ftt) is an intracellular pathogen with the ability to cause rapid, lethal disease in humans at very low infectious doses (1-10 organisms). Ftt and Ftt-derived molecules actively suppress inflammatory responses as a mechanism of virulence. In particular, crude lipid preparations from Ftt are potently immunosuppressive. Using LC-MS we have identified unique phosphatidylethanolamine (PE) and phosphatidylcholine (PC) species with saturated very long chain acyl (VLCA) tails (=C22) as active Ftt lipid species mediating anti-inflammatory responses. Activation of the inflammatory response in macrophages, a preferred host cells of Ftt, is mediated by a shift to glycolysis that can be accompanied by a decrease in mitochondrial respiration. We hypothesized that Ftt lipids

may manipulate host metabolism and alter metabolic shifts that lead to inflammatory activation. Real-time measurements of extracellular oxygen and proton flux demonstrated that Ftt lipids increased both glycolysis and oxidative phosphorylation in primary macrophages in a dose-dependent manner. Respiratory increases were reflected across multiple mitochondrial substrates including pyruvate, succinate and fatty acids. These results indicate enhanced ATP production without a clear nutrient preference. Under these conditions excess ATP production may drive or be driven by biosynthetic pathways as evidenced by accumulation of both phospholipids and neutral lipids in host cells treated with Ftt-derived PE measured by immunofluorescent microscopy. Taken together these results suggest that Ftt-derived lipids actively control host metabolism potentially as a mechanism of immune suppression though the exact connection remains unclear. Continuing studies are utilizing qPCR transcriptional analysis and LC-MS lipidomic analysis to identify specific pathways affected by these lipids. Further mechanistic understanding of the metabolic and immune effects of Ftt phospholipids could lead to strategies for metabolic control of Ftt and other intracellular pathogens as well as the development of novel phospholipid-based metabolic therapeutics.

National Institute of Allergy and Infectious Diseases
Alix Warburton
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Virology - DNA
Characterization of HPV16 Integration in Cervical Cancer
Persistent infection with high oncogenic risk human papillomaviruses (HPV) accounts for ~99% of cervical cancers, and is a leading cause of other anogenital and head and neck cancers, making it a significant global health burden. In most HPV-associated cancers the viral genome is found integrated into host chromatin. Integration is not part of the normal PV life cycle and usually results in dysregulation of the viral E6/E7 oncogenes, which promotes cellular proliferation, abolishes cell cycle checkpoints and causes progressive genetic instability. Each integration event is unique depending on how and where the virus integrates and any associated rearrangements and/or amplifications of cellular flanking sequences. We have recently demonstrated that tandemly integrated HPV16 in the cervical cell line 20861 can form a Brd4-dependent super-enhancer-like element; a novel mechanism driving viral oncogene expression from the integrated locus. Super-enhancers are large clusters of enhancer elements that are highly occupied by the transcriptional machinery and have been identified as regions frequently perturbed in cancer. Transcription from this integration site can be targeted by BET-inhibitors which disrupt Brd4 binding, resulting in downregulation of E6/E7 and reduced cell growth. This has great therapeutic potential against HPV-positive tumors. We therefore wanted to characterize the architecture of the integration site in 20861 cells to elucidate the genetic and epigenetic mechanisms operating at this locus, which may act to promote de novo super-enhancer formation and oncogenic progression. Through next-generation sequencing and FISH-based techniques, we show that around 30 tandem copies of HPV16 are interspersed with ~25 Kb of amplified cellular sequence at the integration site. A highly expressed fusion transcript encoding E6/E7 spliced into an acceptor site within chromosome 2 was identified from RNA-seq, which correlated with enrichment of super-enhancer markers at both viral and cellular sequences throughout the integrated locus from ChIP-seq. Interestingly, a strong peak of activity over the amplified cellular sequence aligned to a keratinocyte-specific enhancer identified from ENCODE data, suggesting that the viral integration event hijacked and

amplified a latent cellular enhancer to form a super-enhancer that drives high levels of viral oncogene expression. This is a novel mechanism by which HPV integration can promote oncogenesis.

National Institute of Allergy and Infectious Diseases
Yingda Xie
Clinical Fellow
Clinical and Translational Research - General
Evaluation of a new rapid molecular drug susceptibility test for tuberculosis
Background: Diagnosis of Mycobacterium tuberculosis (Mtb) drug resistance using conventional culture-based phenotypic drug-susceptibility testing (DST) is slow and requires significant laboratory infrastructure and training. These challenges perpetuate inadequate diagnosis and treatment of drug-resistant tuberculosis associated with morbidity, mortality, and ongoing transmission. Since its WHO endorsement and rollout, Xpert MTB/RIF (Cepheid, Sunnyvale, CA) has contributed to a global increase in the rapid molecular detection of rifampin-resistant tuberculosis using GeneXpert instrumentation. However, Xpert MTB/RIF provides no information regarding resistance to fluoroquinolones and second-line injectable drugs (SLIDs), which form the backbone of treatment regimens for multidrug-resistant tuberculosis. We conducted the first prospective diagnostic accuracy study of a new GeneXpert cartridge-based molecular beacon assay that detects mutations associated with Mtb resistance to fluoroquinolones, SLIDs, and isoniazid within 2 hours.

Methods: Sputum samples were collected from adults with tuberculosis symptoms in China and South Korea. Xpert MTB/RIF assay, the investigational assay, and culture were performed. Mtb isolates underwent two reference tests: phenotypic DST using liquid culture and DNA sequencing of katG, gyrA, gyrB, rrs genes and eis and inhA promoter regions.

Results: Among 308 Mtb culture-positive participants, compared to DNA sequencing, investigational assay sensitivities for detecting mutations associated with resistance were isoniazid 98.1% (95% CI 94.4, 99.6), fluoroquinolones 95.8% (89.6, 98.8), kanamycin 92.7% (80.1, 98.5), and amikacin 96.8% (83.3, 99.9); specificity was 99.6% (97.9, 100) or greater. Compared to phenotypic DST, investigational assay sensitivities for detecting resistance were isoniazid 83.3% (77.1, 88.5); ofloxacin 88.4% (80.2, 94.1); 0.5 µg/mL moxifloxacin 87.6% (79.0, 93.7); 2.0 µg/mL moxifloxacin 96.2% (87.0, 99.5); kanamycin 71.4% (56.7, 83.4); and amikacin 70.7% (54.5, 83.9). Specificity for detection of phenotypic resistance was 94.3% or greater except for 2.0 ug/mL moxifloxacin (specificity 84.0% [78.9, 88.3]).

Conclusion: The investigational assay accurately detected Mtb mutations associated with isoniazid, fluoroquinolone, and SLID resistance, and holds promise as a rapid near-patient test to guide therapeutic decisions for tuberculosis patients within a single patient-provider encounter.

National Institute of Allergy and Infectious Diseases
Serena Yan
Visiting Fellow
Immunology - General
Visualizing naive lymphocyte extravasation in vivo with high-resolution
The trafficking of immune cells in lymph nodes plays a critical role in immunity. Lymphocytes recirculate from the blood, through lymph nodes (LNs), into lymphatics and back to the blood in search of foreign antigens. In the non-inflamed state, millions of naïve lymphocytes enter mammalian LNs daily via high endothelial venules (HEVs) and exit via lymphatics. Yet the number of lymphocytes present in a resting LN remains stable over time, indicating the existence of tightly regulated control mechanisms. Various studies showed that lymphocytes extravasate through HEVs via a multistep adhesion cascade. However, the route of lymphocyte transmigration through venular walls composed of HEV endothelial cells, and the mechanisms regulating lymphocyte morphology and movement are still undefined. Here we established a novel four-dimensional imaging platform to precisely determine the profile and dynamics of naïve lymphocyte transmigration in vivo that allows live-imaging by laser scanning microscopy over prolonged periods time (>4 h). This 4D imaging system allows for advanced spatial and temporal resolution. Actin dynamics are essential for various cellular processes, including the control of cell shape and movement. By utilizing this high-resolution confocal intravital imaging technique with LifeAct-GFP bone-marrow reconstituted mice, we observed that actin accumulation at the leading edge of the lymphocytes are required for lymphocyte extravasation. Various reports suggest the migration of lymphocytes through endothelial cells can occur via junctions between adjacent endothelial cells (paracellular), or through the body of endothelial cells (transcellular). Here we labeled the LN vasculature with fluorescently-labeled antibody against PECAM-1 and found that naïve lymphocytes preferentially cross the HEVs via the paracellular route. Furthermore, we were able to observe real-time HEV pocket formation. In conclusion, the remarkable spatial and temporal resolution of this imaging technique allowed us to confirm the importance of actin polymerization during lymphocyte extravasation in vivo, and determine the routes of lymphocyte transmigration. The confocal intravital imaging technique established here can be adapted to address many peripheral LN-related biological questions, including the dynamics of cell migration, cell-cell interactions and changes in HEV morphology.

National Institute of Allergy and Infectious Diseases - Vaccine Research Center
Ke Bai
Postdoctoral Fellow
Virology - RNA and Retroviruses
Identification of a set of promiscuous CD8+ T cell epitopes in Ebola glycoprotein
Ebolaviruses cause hemorrhagic fever that is often fatal in human and macaques. Of the five species of Ebolavirus, the Zaire species is associated with the majority of Ebola outbreaks in humans while Bundibugyo (Bundi), identified in late 2007, is the most recently discovered species. Since there is no way to predict the potential species of the next outbreak, there is an urgent need to develop a vaccine that is efficacious against multiple Ebolavirus species. Furthermore, for a vaccine to be potent across a broad population, it must be capable of generating a response in diverse MHC genotypes. We have previously shown that CD8+ T cells are required for rAd5-Zaire vaccine-induced immune protection

against Zaire Ebola virus challenge. We have also shown that Zaire DNA/rAd5 vaccination provides heterologous protection in macaques against Bundi challenge in the absence of a Bundi-specific antibody response, suggesting cellular immune responses played an important role. We wondered if the heterologous protection observed was mediated through a set of restricted MHC I alleles. To test this hypothesis, we vaccinated a cohort of 6 outbred macaques and performed MHC class I genotyping to identify the major haplotypes of Mafa-A and Mafa-B for each animal. Based on this genotyping, subsets of the cohort were found to have one or more alleles in common, but no single allele was shared by the entire group. By using intracellular staining with Bundi glycoprotein (GP) peptide stimulation, we identified 6 epitopes in GP targeted by CD8+ T cells from these macaques. Surprisingly, although there is no common haplotype shared by all the macaques, all 6 epitopes clustered within a 52-residue stretch located in a conserved region. We were also able to associate specific Mafa-A and B haplotypes to their epitope presentation. 2 out of 6 epitopes were related to the Mafa-A036 haplotype. Each of the remaining 4 epitopes showed association with more than one MHC I allele. Interestingly, published computational predictions suggest the same conserved region of GP harbors a promiscuous human CD8+ T cell epitope that is predicted to interact with MHC I alleles found in more than 50% of the population. In summary, we have defined a set of epitopes that is presented broadly across MHC I haplotypes, and these promiscuous epitopes provide promising candidates for the development of a vaccine capable of protecting against multiple Ebolavirus species in a genetically diverse population.

National Institute of Arthritis and Musculoskeletal and Skin Diseases
Sarah Geiger
Doctoral Candidate
Immunology - General
Feeding immunity: The regulation of circadian immune responses by mealtimes
Circadian rhythms are daily oscillations in behavior and physiology that prepare organisms to better react to and anticipate daily changes in the environment. Both, mice and humans have a striking circadian variability in the responsiveness to innate immune stimuli, and in mice, susceptibility to sepsis induced by acute stimulation of the immune system with the bacterial component LPS is time-of-day dependent. The intrinsic cellular clock, the building block of the organism's whole body clock, exists in immune cells and can control some aspects of innate immunity, but what extrinsic signals, termed 'zeitgebers' or time-givers, entrain the circadian behavior of immune response are not known.

We have investigated the relative contribution of light and the feeding cycle to innate immune responses by dissociating the light cycle and access to food through time restricted feeding (TRF), allowing access to food either during the dark - and natural feeding time of mice - or the light phase. Using NanoString, an RNA profiling technique, we found that the circadian clock in the liver is entirely programmed by food, whereas in the spleen, mainly light influences the expression of clock genes. Strikingly, in the setting of TRF, susceptibility to LPS-induced morbidity was high when animals were fed during their natural feeding time (93% mortality) and mice appeared highly protected (38% mortality) when food was restricted to the light phase. This suggests that morbidity is regulated by the food, not the light cycle, and that food is the 'zeitgeber' for circadian susceptibility to LPS-induced sepsis. Daily mortality patterns following LPS stimulation could not be attributed to corticosteroids or ketone bodies, both of which possess anti-inflammatory properties and exhibit strong food dependency. Despite their important role in sensing of innate immune stimuli, myeloid cells are also not responsible for food-

entrained innate immunity, as LysM-Cre x BMAL1-flox/flox mice continued to exhibit food, not light entrained LPS sensitivity. We are currently investigating liver-specific BMAL1 deficient mice to further locate the source of food regulated mortality, and we will also systematically measure serum metabolites and proteins to identify the molecular mediators of food controlled immunity. This may provide new insights with which to design strategies to prevent and improve metabolic and inflammatory diseases in the continuously growing population of individuals at risk.

National Institute of Arthritis and Musculoskeletal and Skin Diseases
Yaima Lightfoot
Postdoctoral Fellow
Immunology - Autoimmune
Peptidylarginine deiminases contribute to the pathogenesis of systemic autoimmunity
Neutrophils represent a crucial first-line defense against invading pathogens. In addition to their phagocytic function, neutrophils can release neutrophil extracellular traps (NETs), structures of decondensed chromatin decorated with cytosolic and granular components, which allow neutrophils to capture and immobilize microbes. The process of NET production and release, known as NETosis, has not been fully delineated and may be stimuli-specific. However, histone citrullination and subsequent chromatin decondensation are believed to be required for NETosis. In neutrophils, the conversion of arginine to citrulline on histones is catalyzed by peptidylarginine deiminases, specifically peptidylarginine deiminase 4 (PAD4). Despite the importance of NETs in limiting systemic pathogen dissemination, recent studies have implicated NETs in noninfectious diseases, including systemic lupus erythematosus (SLE). Previously, chemical inhibition of PAD activity demonstrated therapeutic potential in reducing disease severity and morbidity in animal models of lupus; however, the specificity of these compounds in vivo remained to be confirmed. Here, we employed mice lacking PAD4 or PAD2 (another PAD expressed in myeloid cells) to test the hypothesis that enhanced PAD activity and/or NETosis promote sterile systemic inflammation and can precipitate autoimmunity. For these studies, mice were treated topically with the Toll-like receptor 7 (TLR7)-agonist Imiquimod, which induces systemic autoimmunity with lupus-like characteristics in non-autoimmune-prone mice. In line with our hypothesis, PAD4 deficiency protected mice against SLE disease features, including splenomegaly, anti-dsDNA autoantibody production, aberrant immune cell activation, kidney damage, and vascular disease. In contrast, an intermediate phenotype was observed in mice lacking PAD2, where disease symptoms were notably reduced compared to wild-type mice, but to a lesser extent than the PAD4 knockout mice. These data highlight PAD4 as a promising novel target in the pathogenesis of SLE, as well as in other autoimmune disorders where NETs have been shown to be involved.

National Institute of Arthritis and Musculoskeletal and Skin Diseases
Franziska Petermann
Postdoctoral Fellow
Gene Expression
Deletion of NeST reduces IFN-g expression and chromosomal looping at the Ifng locus
The cytokine Interferon gamma (IFN-g) has crucial functions for both the innate and the adaptive

immune system. Defects in IFN-g production or sensing result in a highly reduced ability of the host to fight infections elicited by intracellular pathogens. Therefore, the expression of IFN-g needs to be tightly regulated to ensure proper pathogen clearance while suppressing harmful, overboarding immune responses. Previous studies have identified and functionally characterized multiple regulatory elements at the mouse Ifng locus controlling its chromatin structure and transcriptional activity. These regulatory elements that include enhancers, silencers and insulators, are localized in an approximately 100 kilobases (kb) large region surrounding Ifng. Recently, a non-coding antisense transcript, LincRNA-Ifng-3' (also termed Tmevgp1 or NeST), beginning 120 kb downstream of Ifng, has been found to promote Ifng transcription. A study in transgenic mice demonstrates that NeST RNA enhances, in trans, the IFN-g production in CD8+ T cells and modulates the susceptibility to Theiler's virus persistence and Salmonella enterica-induced pathogenesis.

I found that the NeST RNA is highly expressed at late time points of helper T 1 cell differentiation. To gain deeper insight into the mechanisms of how the NeST locus regulates Ifng gene transcription, I analyzed the Ifng expression in a NeST knockout mouse. Consistent with previous results, in-vitro experiments revealed a reduced expression of IFN-g in knock-out helper T 1 cells, gamma-delta T cells, natural killer cells and cytotoxic T cells. However, a LNA-mediated knock-down of the NeST RNA did not result in reduced IFN-g expression which argued against an involvement of the RNA molecule itself in regulating the transcription of Ifng. Performing chromosome conformation capture assays I observed that the intrachromosomal looping of the extended Ifng-NeST locus was perturbed in the absence of NeST. This change in locus architecture was accompanied by reduced binding of the looping-inducing protein CTCF. Furthermore, NeST knockout mice infected with Toxoplasma gondii showed increased brain cyst burden compared to WT.

These results indicate that the NeST locus, as a cis-acting enhancer, might be involved in the regulation and/or maintenance of the IFN-g expression in effector lymphocytes by inducing chromatin looping and, in turn, increasing the availability of molecules of the transcription machinery at the Ifng locus.

National Institute of Child Health and Human Development
Vasilisa Aksenova
Visiting Fellow
Intracellular Trafficking

Distinct roles of nucleoporins in nuclear function

The nuclear envelope (NE) separates chromosomes from the cytoplasm and helps to maintain genome integrity. To exchange molecules between the cytoplasm and the nucleus, proteins and RNAs are transported through channels in the NE, called nuclear pore complexes (NPCs). NPCs consist of roughly 30 distinct proteins (nucleoporins), forming a central channel with filaments extending into the nucleus and cytoplasm. Besides macromolecular trafficking, nucleoporins participate in the control of gene expression, chromatin maintenance and mitotic progression. In particular, Nup153, Tpr, and Nup50 localize to nucleoplasmic filaments, and they are collectively called the basket nucleoporins. The nucleoplasmic filaments/basket structure has been proposed to serve as a platform for RNA modification and export, as well as for chromatin remodeling. Understanding the roles of individual

basket nucleoporins in these processes is difficult, however, because depletion by RNAi requires an extended incubation, during which nuclear trafficking becomes increasingly disrupted.

As an alternative approach, we used CRISPR/Cas9 to add an Auxin-induced degron (AID) tag to each nucleoporins in DLD-1 cells that have also been engineered to express a plant SCF ubiquitin ligase subunit, TIR1. Upon addition of the plant hormone Auxin, the nucleoporins are ubiquitinated by the TIR1/SCF complex and degraded by proteasomes in less than one hour. Immediately after degradation, we examined changes to the nucleoplasmic basket structure as well as nuclear localization sequence (NLS)-dependent protein import and gene expression patterns. We found that basket nucleoporins were co-dependent on each other during different stages of the cell cycle for their association to NPCs. For example, Nup153 is essential for Nup50 association with the basket but dispensable for Tpr attachment. Interestingly, protein import was specifically inhibited upon Auxin addition in both Nup153- and Nup50-AID lines, but not in the TPR-AID. Likewise, RNA seq showed similar patterns of gene expression in the Nup153- and Nup50-AID lines that were distinct pattern after TPR-AID degradation. Together, these findings show that NPC subunits play distinct, individual roles in nuclear function and gene expression. This system will allow us to dissect these roles at a molecular level, in order to understand the important and complex regulatory roles of individual nucleoporins.

National Institute of Child Health and Human Development
Griffith Bell
Postdoctoral Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Maternal Polycystic Ovary Syndrome and offspring development: The Upstate KIDS Study
Backgound: Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism - elevated levels of circulating testosterone in women - and is the most common cause of female infertility. Mothers with PCOS can be at increased risk for perinatal complications, and there is growing evidence that offspring of mothers with PCOS may also be at higher risk for developmental disorders. Few studies exist regarding maternal PCOS and early childhood development in the United States. Our objective is to examine the relationship between maternal PCOS and offspring development through 36 months of age. Methods: The Upstate KIDS Study is a population-based prospective cohort study of infants born between 2008 and 2010 in New York State (excluding New York City), originally designed to - and finding no impact of - infertility treatment exposure on child development. In all, 4,453 mothers completed one or more developmental screening instruments for 5,388 children (35.5% twins). Mothers self-reported any physician's diagnosis of PCOS, as well as what type of related treatment received on baseline study questionnaires. Parents completed the Ages and Stages Questionnaires (ASQ) on their child's development at 4, 8, 12, 18, 24, 30, and 36 months of age to assess development in the fine motor, gross motor, communication, personal-social functioning, and problem-solving domains. We used generalized linear mixed models to estimate odds ratios (OR) between PCOS diagnosis and failures in the ASQ adjusted for maternal age, race, BMI, education, marital status, smoking, alcohol consumption, diabetes, insurance, and plurality. Results: In total, 457 mothers (10.3%) reported a diagnosis of PCOS. Diagnosis of maternal PCOS was associated with increased risk of offspring failing the fine motor domain, (aOR 1.77; 95% CI: 1.09-2.89), largely driven by higher risk in female singletons (aOR 2.23; 95% CI: 1.16-4.29). Twins of mothers with PCOS had higher risk of failing the communication (aOR 1.94; 95% CI: 1.19-3.18) and personal-social (aOR 1.76; 95% CI: 1.12-2.77) domains compared to twins born to

mothers without PCOS. Conclusions: Maternal PCOS was associated with higher risk of fine motor developmental delays in offspring, particularly among females. This is consistent with prior literature and represents the first study of PCOS and early childhood development in a prospective US cohort.

National Institute of Child Health and Human Development
Dan Benjamini
Visiting Fellow
Radiology/Imaging/PET and Neuroimaging

Magnetic Resonance Microdynamic Imaging of biological tissue components

Purpose: One of the greatest challenges in the field of quantitative magnetic resonance imaging (MRI) is inferring salient mesoscopic and microscopic features of tissue within a macroscopic imaging voxel. The majority of currently used quantitative MRI methods in brain research employ idealized models to describe specific structures and regions. However, the brain is microscopically heterogeneous. A typical brain volume contains different components, such as axons, glia, myelin, and a mixture of extracellular matrix molecules, neurofilaments, etc. The high level of complexity and variability of brain tissue requires rethinking the use of parametric model-based methods.

Methods: Here we propose and vet an MRI method - which we term magnetic resonance microdynamic imaging (MRMI) - that reveals such distinct microanatomical components on the basis of their various relaxation and diffusion properties, specifically, measurements of their multidimensional joint distribution obtained via relaxation-diffusion correlation (REDCO) experiments. To date, such measurements have been relegated only to nuclear magnetic resonance spectroscopy applications but have not been combined with MRI owing to the vast amount of required data, leading to infeasible scan time. Here we introduce an efficient experimental design and a powerful mathematical processing pipeline that considerably reduces these data requirements, leading to reasonable scanning times with excellent prospects for preclinical and clinical applications.

Results: We use this method to identify and quantify specific tissue components in fixed spinal cord (axons, neurons, glia, interstitial spaces, and myelin-associated water content) on the basis of their multispectral REDCO signatures, and then correlate them with immunohistochemistry findings, which resulted in excellent agreement.

Conclusion: The spatially resolved MRMI images allow us, for the first time, to detect and distinguish between various intra- and extracellular components. These have the potential to become a new family of microdynamic biomarkers for neuroinflammation, characterization of traumatic brain injury, and more. MRMI delivers unprecedented imaging data, which could have only been obtained by using histological procedures on fixed specimen. This method is not limited to nervous tissue application and can be used on any type of tissue or material, providing opportunities for investigators in a range of disciplines.

National Institute of Child Health and Human Development
Razvan Chereji
Research Fellow
Chromatin and Chromosomes

MNase-Sensitive Complexes in Yeast: Nucleosomes and Non-histone Barriers

Nucleosomes are the basic units of DNA packaging in eukaryotes. Most yeast genes and many genes in higher eukaryotes have a nucleosome depleted region (NDR) just upstream of the transcription start site (TSS), flanked by arrays of regularly spaced nucleosomes. This organization has been attributed to the presence of non-histone complexes at promoters, which create potential barriers that prevent nucleosome formation and organize nucleosomes into phased arrays. Additional contributions are made by poly(A) sequences and by multiple chromatin remodelers. Micrococcal nuclease (MNase) is commonly used to map nucleosomes genome-wide, but nucleosome maps are affected by the degree of digestion. It has been proposed that many yeast promoters are not nucleosome-free but occupied by unstable, “fragile” nucleosomes.

The presence of fragile nucleosomes at promoters presents some interesting problems: How can transcription complexes form if the promoter is occupied by a fragile nucleosome? How could a fragile nucleosome be positioned specifically to act as a nucleosome phasing barrier? Intrigued by these questions, we performed a comprehensive analysis of chromatin accessibility and MNase sensitivity in S. cerevisiae.

We analyzed the histone content of all MNase-sensitive complexes by MNase-ChIP-seq and Sonication-ChIP-seq, and we find that yeast promoters are predominantly bound by non-histone protein complexes, with little evidence for fragile nucleosomes. We do detect MNase-sensitive nucleosomes elsewhere in the genome, including transcription termination sites. However, they have high A/T-content, suggesting that MNase sensitivity does not indicate instability, but the preference of MNase for A/T-rich DNA. We also found that tRNA genes are occupied by transcription factor complexes that are less accessible to MNase compared to nucleosomes. We confirmed our observations by analyzing previously published nucleosome maps, obtained by alternative methods: ChIP-exo, chemical mapping, and ATAC-seq. Thus, histone ChIP-seq experiments are essential to distinguish nucleosomes from other DNA-binding proteins that protect against MNase.

Our study has created a paradigm shift in our understanding of nucleosome organization of promoters, challenging recent publications from high impact journals. A reviewer appreciated our study as “the most systematic integration of approaches to date”. Our study was published as a “Matters Arising” article in Molecular Cell in 2017.

National Institute of Child Health and Human Development
Junho Cho
Visiting Fellow
Genetics
Hepatic glucose-6-phosphatase-a restoration corrects autophagy impairment and prevents hepatocellular adenoma in glycogen storage disease type Ia
Background:

Hepatocellular adenoma (HCA) and carcinoma (HCC) of unknown etiology is a long-term liver complication of glycogen storage disease type Ia (GSD-Ia), a metabolic disorder caused by a deficiency in the enzyme glucose-6-phosphatase-a (G6Pase-a). With strong compliance, the dietary therapy manages the life-threatening hallmark of the disease, fasting hypoglycemia, and GSD-Ia patients can attain near normal growth and pubertal development. However, no current therapy addresses the long-term liver complications. Mice lacking G6Pase-a mimic the phenotype of human GSD-Ia but die early, before HCA or HCC develops, making mechanism and treatment studies of liver complication difficult.

Results:

We have generated and shown that liver-specific G6Pase-a-knockout (L-G6pc-/-) mice have a more normal lifespan and develop HCA/HCC. Using this model, we have shown that in adult liver there are sustained autophagy impairment and persistent accumulation of p62, a specific autophagy substrate that contributes to HCA development. Moreover, the HCA/HCC lesions display p62 aggregates accumulation, marked mitochondrial damage, oxidative DNA damage, all leading to cancer development along with activation of oncogenic pathways including ß-catenin and Yes-associated protein signaling. Importantly, a recombinant adeno-associated virus (rAAV) vector-mediated gene therapy that restores G6Pase-a expression in the livers of adult L-G6pc-/- mice corrected the hallmark metabolic abnormalities, normalized defective autophagy and prevented the development of HCA/HCC. However, any tumors present before gene therapy remained and did not resolve.

Impacts:

Our study reveals a previously unrecognized role of autophagy in HCA development in GSD-Ia. Moreover, we demonstrate that in adult murine GSD-Ia model, G6Pase-a gene therapy can treat the metabolic disease as well as the long-term liver complications. Thus, even when administered to adult GSD-Ia patients exhibiting a pre-malignant liver phenotype, no liver adenoma or carcinoma will develop. This is a promising observation for human gene therapy which is currently undergoing investigational new drug (IND)-enabling studies.

National Institute of Child Health and Human Development
Eric Christenson
Postdoctoral Fellow

Biochemistry - Proteins

Mitoferrin-1 is a promiscuous metal transporter to effect iron uptake by mitochondria

Iron is integral to cellular metabolism - all known life comprises machinery to acquire, utilize, and sequester the metal to exploit its redox properties. Iron is, however, a double-edged sword in that it can generate deleterious reactive oxygen species via Fenton chemistry. Thus to suppress iron toxicity, the metal is reined in by cellular chaperones. Two of the most abundant chaperones are heme and iron-sulfur clusters, prosthetic groups employed for oxygen transport, energy consumption, and numerous other enzymatic processes. Heme and most Fe-S clusters are assembled inside the mitochondrial matrix, necessitating an iron import mechanism.

Genetic experiments have demonstrated that mitoferrin-1 (Mfrn1) is essential for mitochondrial uptake of iron. Mfrn1 is also a member of the mitochondrial carrier family which links the mitochondrial matrix with the cytosol by transporting nucleotides, amino acids, and other metabolites. Accordingly, Mfrn1 has been proposed to be an iron transporter but this function has not been unambiguously demonstrated. Hence, we sought to reconstitute and directly interrogate Mfrn1's putative iron transport activity in vitro using purified protein and defined liposomes.

Using isothermal titration calorimetry, we ascertained that detergent-solubilized Mfrn1 can bind iron, manganese, cobalt, and nickel with micromolar affinities. We developed a novel iron transport assay, incorporating purified Mfrn1 into liposomes and demonstrated that the protein is a bona fide iron transporter with a Km of 4 micromolar. Notably, Mfrn1 transports free rather than chelated iron – potential iron ligands like glutathione, cysteine, or pyruvate had no effect on the iron transport rate. Mitochondria also employ zinc and copper as enzymatic cofactors and we found that Mfrn1 can transport those metals as well. Guided by sequence conservation and structural homology modeling, we performed extensive mutagenesis on Mfrn1 to identify residues important for substrate recognition and transport. Cysteines are common metal-coordinating residues and ablating the three conserved in Mfrn1 severely impairs transport but surprisingly has no effect on metal binding. Instead, five other conserved residues - three histidines and two methionines - mediate Mfrn1-metal interactions and subsequent transport. We conclude that Mfrn1 is a genuine transporter for supplying iron, copper, and zinc to the mitochondrial matrix.

National Institute of Child Health and Human Development

antony cougnoux

Postdoctoral Fellow

Immunology - General

Characterization of neuroinflammation in Niemann-Pick type C1 disease mice.

Niemann-Pick type C1 (NPC1) is a rare neurodegenerative disease characterized by progressive neuronal loss leading to loss of motor function and early death. One of the earliest pathological findings in NPC1 is neuroinflammation in affected regions of the brain. However, the role played by inflammation in the disease progression remains unknown. The potential clinical utility of suppressing neuroinflammation in NPC1 is not known. To explore this further, we analyzed the immune cells in the brains of NPC1 mutant mice using flow cytometry and found an increasing number of peripheral immune cells present as the disease progresses. The disease was not corrected by bone marrow transplant or treatment of the mice by Fasudil-HA, a drug previously used in amyotrophic lateral sclerosis (ALS) to prevent the recruitment

of peripheral immune cells. However, from our analysis we found that microglia, the central nervous system resident immune cells, undergo major modifications characteristic of their activation. To understand the molecular mechanism related to the activation of NPC1 microglia, we isolated and characterized these cells. Transcriptomic, biochemical and immunohistochemical analyses provided insight into the molecular mechanisms altered in the NPC1 microglia. Comparing microglia isolated from NPC1 mutant mice to microglia isolated from mouse models of Alzheimer's (AD), ALS, Pompe, Fabry, Mucopolysaccharidosis type IV, aging and lipopolysaccharide stimulation, has provided insight into common biochemical pathways modified in these diseases. One of those pathways is lysosome storage and the highest similarities were between NPC1 and an AD model. It has previously been shown that treatment with b-cyclodextrins decreases disease pathology in both of these mouse models, thus validating the relevance of our findings. We also defined a second pathway linking energy metabolism and morphological changes affected in all the neurodegenerative diseases analyzed. Based on these findings, preliminary results in NPC1 using specific inhibitors show a delay in the loss of Purkinje cell neurons and an extended lifespan of the mice, concomitant with a reduced activation of the microglia. These results suggest a significant role of the neuroinflammation signaling pathway in NPC1 disease progression that is shared with Alzheimer's and other neurodegenerative diseases, allowing possible intervention of this pathway with specific inhibitors to reduce disease progression.

National Institute of Child Health and Human Development
Hadis Dashtestani
Visiting Fellow
Radiology/Imaging/PET and Neuroimaging
A neuroimaging approach to determine functional biomarkers associated with psychopathic personality traits in a moral judgment task
Antisocial personality disorder (ASPD) is characterized by a violation of the rights of others and lack of conformity to social norms. ASPD is prevalent in incarcerated populations and often goes undiagnosed. This poses a high cost on society, which indicates the critical need for early detection of ASPD to implement treatments. While ASPD is traditionally diagnosed through psychiatric evaluation in accordance with the symptoms outlined in DSMV, ASPD patients can be extremely manipulative, resulting in controversial diagnoses by the subjective measures. However, understanding the neural basis behind ASPD can greatly enhance traditional diagnostic methods. Scientists have also found correlations between psychopathic personality traits and responses to moral judgment (MJ) tasks. Our study is the first neuroimaging study that has implemented the MJ task with personality assessments of psychopathic traits in a cost-effective and patient-friendly environment. We utilize functional near infrared spectroscopy (fNIRS), which is portable and tolerable to patient movements. fNIRS measures brain activation by monitoring changes of oxygenated hemoglobin in the brain similar to fMRI-BOLD. The task used in our study of MJ is based on series of questions which examines personal verses impersonal dilemmas, defined as emotionally salient scenarios versus more distant ones. We hypothesized that the brain exhibits distinguishable hemodynamic patterns for each category. We also investigated the correlation between these patterns and psychopathic traits. Using the hemodynamic responses of 20 typical subjects, we analyzed the fNIRS data using a non-linear classification method called cubic SVM. We specifically chose SVM because it determines the separating hyperplane (high dimensional analog to the plane separating the two groups) only from signals located close to the

interface between personal and impersonal hemodynamic responses. Our results show that we can differentiate between the personal and impersonal hemodynamic responses with mean accuracy of 83%. This confirms our hypothesis of distinguishable hemodynamic patterns by category and suggests that it is possible to classify degrees of psychopathy based on neural activity. Consequently, we offer a novel approach to provide functional biomarker for ASPD using fNIRS, combined with advanced machine learning techniques. We plan to apply our method on incarcerated populations in the future to assess the degree of ASPD.

National Institute of Child Health and Human Development
Raffaella De Pace
Postdoctoral Fellow
Intracellular Trafficking
Abstract removed at request of author
Abstract removed at request of author

National Institute of Child Health and Human Development
Tyler Ekins
Other
Neuroscience - Cellular and Molecular
Hippocampal interneuron migration and inhibitory network organization in Lis1 mutant mice
Type I lissencephaly is a neuronal migration disorder frequently caused by mutations in the lis1 gene and results in brain malformation, epilepsy, and cognitive deficits. Our goal of this study is to investigate how lis1 influences hippocampal neuron migration and inhibitory circuit formation, and to discover the source of epileptiform activity. To this end, we utilized both global lis1 hemizygous (lis1+/-) mice as well as a lis1 conditional knock-out mouse line (lis1fl/+), which we crossed to several cre lines to delete one copy of lis1 exclusively from pyramidal cells (Emx-Cre), all interneurons (Dlx5/6-Cre), or a subset of interneurons (Nkx2.1-Cre). We observed that by late adolescence, the same number of parvalbumin+ interneurons (PV-INTs), somatostatin+ (SOM) INTs, and reelin+ (Rln) INTs are present in CA1 for all of the previously listed genetic lines. However, within CA1, all of these INT populations were shifted away from stratum oriens and towards stratum radiatum across all genetic lines. These results suggest that if by late adolescence, a similar number of INTs reach the hippocampus but are mislocated along the radial axis, the second phase of INT migration is disrupted in lis1 mutant mice; radial as opposed to tangential migration may be influencing INT final somatic position. To further study this phenomenon, we will image hippocampal neuron migration in live slices from neonatal mice. Furthermore, to investigate how migration deficits may affect hippocampal connectivity and contribute to epilepsy, we will record PV-INTs to examine spontaneous inhibitory and excitatory events. We will also conduct paired recordings between PV-INTs and synaptically coupled pyramidal cells (PCs) to assess how PV-INT somatic position influences connection probability and examine if release probability, short-term plasticity and/or kinetics change in lis1 mutant mice . If we discover that PV-INTs in the lis1 mutant connect to PCs at a lower rate or if their efficacy of synaptic transmission is reduced, it would suggest that decreased inhibition from PV-INTs is at least partially responsible for the observed epilepsy in lis1 mutant mice, a

question that could be further studied by 2-photon calcium imaging using a lis1 mutant with fluorescent PV+ cells.

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Neuroscience - Cellular and Molecular
Cav2.3, a calcium channel dysregulated in Fragile X Syndrome, contributes to hippocampal excitability through calcium-dependent potassium channels

Fragile X syndrome (FXS) is an inherited form of intellectual disability with a high co-morbidity with autism and epilepsy. FXS results from a mutation in a single gene, Fragile X Mental Retardation Protein (FMRP), on the X chromosome. FMRP is an mRNA regulating protein, thus its loss leads to the perturbation of a wide range of cellular functions and endows FXS with a challenging, multi-facetted disease presentation. One of the prominent features of FXS is neuronal hyperexcitability and propensity for epilepsy, suggesting the potential dysregulation of voltage-gated ion channels.

High-throughput sequencing of mRNAs regulated by FMRP, and thus potentially disrupted in FXS, has recently identified voltage-gated Ca2+ (Cav2.3) and K+ (Kv4.2) channels to be among the affected proteins in FXS. We investigated the effect of Cav2.3 loss on the electrophysiological properties and neuronal excitability in the mouse hippocampus, a brain area important for learning, and prone to epileptic activity. Using whole-cell patch-clamp recordings from hippocampal CA1 pyramidal neurons, we found that these cells exhibited higher firing frequencies, larger frequency-dependent action potential broadening, and reduced after-hyperpolarization in Cav2.3-KO mice compared to WT. Using pharmacological blockade of different channels involved in action potential generation, we show that the hyperexcitability is caused by a reduced function of Ca2+-dependent K+ channels. Specifically, using whole-cell and single-channel recordings, we find that both Ca2+-gated (BK) and Ca2+-modulated (Kv4.2) K+ currents are significantly reduced in Cav2.3-KO neurons.

To elucidate how the altered membrane properties of CA1 neurons affect their output function, we analyzed the short-term plasticity between CA1 and the subiculum, a brain region downstream of CA1. Paired pulse facilitation experiments demonstrated a much larger facilitation effect in Cav2.3-KO animals compared to WT, in line with altered action potentials observed in CA1.

In summary, our data shows for the first time that Cav2.3 is the Ca2+ source for Ca2+-dependent K+ channels regulating action potential repolarization. This leads to increased excitability and altered

synaptic plasticity. Cav2.3 emerges therefore as a promising target for addressing circuit level hyperexcitability in FXS mouse models.

National Institute of Child Health and Human Development
Eric Horstick
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Physiology
Motivated state transition requires somatostatin signaling and deep-brain photoreceptors
Motivated states allow plasticity of an animal's behavior, facilitating adaptive responses to fluctuating internal homeostatic states and external challenges. A nearly universal motivated drive exists for finding resources; whether food, shelter, or mates. These goals are achieved by active modulation of sensory responsiveness and locomotor patterns. However, in most environments, navigatable cues are not immediately available. This challenge is overcome using sophisticated and conserved behavioral patterns broadly observed across species. Despite the importance of these goal-directed behaviors for survival, the underlying neural mechanisms are poorly understood, mostly due to a lack of vertebrate genetic models. Light is a potent behavioral driver for zebrafish, as well as for many organisms. We first identified a previously uncharacterized light-search state in larval zebrafish, performed in the absence of salient navigation cues. Using spatial analysis we found that after loss of illumination, larvae first show movement patterns consistent with a local search behavior that gradually transitions to an outward roaming phase. Each phase of the search process exhibited distinct patterns of sensory responsiveness and movement trajectories that allowed efficient identification and navigation to local or remote sources of illumination respectively. We identified that the initiation of the local search pattern, yet not the roaming search required retinal input. Conversely, zebrafish otpa mutants, null for the transcription factor orthopediaA, failed to transition out of the local-search response. OrthopediaA is required for mid-brain dopaminergic (DA) neurons, thyrotropin (trh) and somatatostatin (sst) neurosecretory neurons, as well as melanopsin (opn4a) expressing deep-brain photoreceptors in the anterior hypothalamus. Using CRISPR generated mutants we found that the otpa phenotype was not due to DA or trh neuron loss. However, loss of opn4a deep-brain photoreceptors and sst signaling recapitulated the transition defect observed in otpa. Thus, in response to loss of illumination, zebrafish perform classic search strategies observed in numerous species including C. elegans, Drosphilia, and mammals – including humans. These results demonstrate dynamic regulation of search state transition. By using this novel model we can dissect the circuitry of goal-mediated behavior and explore the mechanisms for how organisms respond to complex and challenging environments.

National Institute of Child Health and Human Development
Joo Yun Jun
Postdoctoral Fellow
Physiology
Melanocortin 3 receptor (MC3R), a novel regulator of hepatic autophagy in obesity
Autophagy plays a pivotal role in cell metabolism, quality control, and stress homeostasis. Defective cellular autophagic response has been reported in diverse human diseases, including obesity. We

previously have reported that humans with MC3R hypoactive mutations (C17A + G241A) had increased BMI and body weight, producing more adipose tissue expansion than humans without MC3R mutations. However, the precise signaling mechanisms that MC3R modulates to alter metabolic homeostasis remain unclear. Based on the evidence of the essential role of hepatic autophagy in metabolic regulation, we hypothesized that the peripheral MC3R signaling pathway modulates autophagic lipid degradation in obesity. To investigate the role of MC3R in autophagy regulation, we generated humanized knock-in mouse models that carry either wild-type (MC3RhWT/hWT) or hypoactive human double mutant (C17A+G241A, MC3RhDM/hDM) MC3R. MC3RhDM/hDM mice exhibit an obese phenotype with significantly greater fat mass and hepatic triglycerides, which are similar in phenotype to MC3R-/- compared to MC3RhWT/hWT or C57/BL6 mice. First, we demonstrated that primary hepatocytes treated with the MC3R specific agonist D-trp-gamma-MSH had significantly induced LC3II, the marker of autophagosome formation, suggesting the possible role of MC3R in autophagy. Second, we found overnight fasting induced hepatic LC3 II in MC3RhWT/hWT and C57/BL6, but not in MC3RhDM/hDM or MC3R-/-; rather there was significant upregulation of basal LC3 II in the fed state. Next, although primary cultured hepatocytes from MC3RhWT/hWT had substantial induction of autophagic flux after chloroquine treatment with or without starvation, cells from MC3RhDM/hDM and MC3R-/- failed to show induction of autophagic flux, suggesting MC3R may play a role in the regulation of autolysosomal degradation in hepatic autophagy processes. Additionally electron microscopy analysis demonstrated liver in MC3RhDM/hDM mice had less lipid droplet-associated autophagosomes after fasting vs. MC3RhWT/hWT mice, indicating MC3R mediated autophagy is targeted for lipid droplet degradation to regulate lipid metabolism in the liver. In conclusion, hypoactive MC3R mutation or the absence of peripheral MC3R appears to cause dysregulation of hepatic autophagy, thus contributing to the accumulation of hepatic lipid content. Altered autophagy may be part of the explanation for the increased prevalence of childhood obesity among humans with hypoactive MC3R.

National Institute of Child Health and Human Development
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Developmental Biology

Live imaging of the lymphatic vascular network using transgenic zebrafish

The lymphatic vascular system is essential for tissue fluid homeostasis, immune trafficking, and absorption of dietary fats. It is also critically involved in a wide range of diseases including lymphedema, cancer metastasis, inflammatory and immunological disorders, and metabolic disease. Despite its importance, we still know very little about this system or its pathologies - there is no cure for lymphedema, and we don't know how tumor cells use lymphatic vessels for metastasis. The difficulty in visualizing and experimentally manipulating lymphatic vessels in vivo has been one of the key roadblocks to increasing our understanding of this crucial system.

In order to overcome this problem, we developed a powerful new arsenal of transgenic tools enabling detailed visualization and functional manipulation of lymphatic vessels in the optically clear, experimentally accessible zebrafish model. These tools use an mrc1a promoter that we recently cloned to drive lymphatic-specific expression. We developed an mrc1a:egfp reporter for high-resolution optical

imaging of lymphatic vessel growth and development in real time in living animals. We also developed an mrc1a:ert2gal4 driver for lymphatic-specific, inducible expression of transgenes from UAS reporters, and an mrc1a:ERT2creERT2 driver for lymphatic-specific, inducible genome manipulation. Together, these transgenic tools provide an unparalleled opportunity for detailed anatomical and functional characterization of lymphatic development.

We used our mrc1a:egfp transgenic line to visualize and completely characterize the assembly of the lymphatic vascular network in the developing zebrafish trunk. Our findings reveal a highly stereotyped system that forms in a stepwise, carefully choreographed manner. These developing zebrafish lymphatics strongly resemble the developing lymphatics of humans, affirming the usefulness of the fish as an experimental model for lymphangiogenesis. We also show for the first time that immune cells traffic through zebrafish lymphatics, one of the key functional features of mammalian lymphatics.

With the help of our newly developed transgenic tools, we are carrying out genetic screens for lymphatic-specific mutants and functional characterization of lymphatic-specific genes. We expect these to yield important insights into lymphangiogenesis and identify new targets for therapies directed against lymphatic-associated disease.

National Institute of Child Health and Human Development
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Immunology - Innate and Cell-mediated Host Defenses

A pathogenic bacterium targets the host cell Hippo pathway during infection

Phagocytosis of invading bacteria by immune cells is the first line of host defense. The immune cells typically deliver bacteria to lysosomes for degradation. Pathogens like Legionella pneumophila, the bacterium that causes a severe form of pneumonia called Legionnaires' disease, translocate effectors into the host cell to prevent lysosome-mediated killing and to establish a niche for intracellular replication. Despite intensive research over the past decade, the host signaling pathways that are manipulated by L. pneumophila effectors have remained largely unknown. Since protein phosphorylation is one of the most important post-translational modifications in living cells, we focused our efforts on identifying human protein targets for L. pneumophila effectors that possess kinase activity. We recently developed a screening platform for kinase substrates by combining a non-radioactive phosphorylation assay with a microarray containing thousands of human proteins. Using this novel approach, we discovered that LegK7, a kinase effector present in many Legionella species, phosphorylates human MOB kinase activator 1A (Mob1A), a component of the Hippo pathway. The Hippo pathway is highly conserved from yeasts to humans and controls important functions, including cell death, cell proliferation and differentiation. Using purified proteins in an in vitro kinase assay, we validated that LegK7 efficiently phosphorylates human Mob1A at several key residues. In addition, we observed increased phosphorylation of Mob1A in transiently transfected human HEK293T cells

producing wild type LegK7 but not the catalytically inactive protein variant. Furthermore, we found that phosphorylation of Mob1A was elevated in macrophages infected with wild type L. pneumophila but not in cells challenged with an avirulent L. pneumophila strain. These results demonstrated that Mob1A is indeed a novel target of L. pneumophila during infection. Interestingly, production of LegK7 in human HEK293T cells induced apoptosis, a form of programmed cell death, and a growth arrest in yeasts cells. Together, our findings reveal an unexpected link between the human Hippo pathway and microbial infection, and show that L. pneumophila uses effectors, such as LegK7, to manipulate the Hippo pathway during infection.

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Exploring family- and peer-level mediators of sexual orientation disparity in depressive symptoms from late adolescence into young adulthood
BACKGROUND: Compared to heterosexual youth, sexual minority youth are more likely to report depressive symptoms. According to the minority stress theory, sexual minority youth experience more depressive symptoms due to greater parental rejection and increased peer victimization. Few studies have tested both negative and positive aspects of family and peer influence as mediators. This study examined (1) if sexual minority adolescents reported more depressive symptoms and experienced a more rapid increase in depressive symptoms during the transition into young adulthood, (2) if significant differences were explained by four novel mediators (family satisfaction, parental acceptance of friends, peer support, and cyberbullying victimization), and (3) possible sex differences.

METHOD: Data were drawn from waves 2-6 of the NEXT longitudinal survey (n=2396; mean age=17.1; 56.2% female; 8.6% sexual minority, defined as non-heterosexual sexual attraction). Latent growth modeling with mediation was used to model possible change in depressive symptoms over time and test mediated effects. Multiple-group analyses were conducted to test for sex differences. Analyses controlled for the complex survey design and covariates (race/ethnicity and family affluence).

RESULTS: Sexual minority youth reported more depressive symptoms across all 5 waves, but sexual minority status did not predict change in depressive symptoms. Mediation tests indicate that sexual minority status was associated with lower family satisfaction, lower parental acceptance of friends, and greater cyberbullying victimization, which in turn were associated with more depression symptoms which pre-existed at study baseline. Higher peer support was associated with fewer depressive

symptoms, but it was not a significant mediator because it was unrelated to sexual orientation status. Multiple-group analyses revealed that the indirect effect of sexual orientation on depressive symptoms through cyberbullying victimization was stronger among males than females. All other significant paths did not differ by sex.

CONCLUSIONS: Sexual minority adolescents reported more depressive symptoms but did not experience a more rapid increase in depressive symptoms. Lower levels of positive aspects of family influence partially explain why sexual minority youth report more depressive symptoms. Sexual minority males may be particularly prone to negative peer influence which contributes to more adolescent depressive symptoms.

National Institute of Child Health and Human Development
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Cell Biology - General
Large-Scale Immunofluorescence Imaging of Human Subcutaneous Adipose Tissue Reveals Insulin Refractive and Responsive Plasma Membrane Segments
Peripheral insulin resistance (IR), as exemplified by the lack of response of tissues to an insulin challenge, is a precursor to prediabetes, metabolic syndrome, and type II diabetes. It is unclear how IR manifests structurally in adipose tissue. Traditional metrics such as glucose uptake assays are a tissue average, and thus cannot represent architectural variability. We hypothesized IR causes a shift in the mosaic of membrane response. We developed imaging assays both broad (greater than 4 mm x 4 mm) and detailed (less than 1 micron resolution) of adipose tissue to provide functional, individual membrane measurements of the activation of ex vivo human adipose tissue. We tested if AKT (Thr308) phosphorylation (pAKT), a signaling node of the insulin-signaling pathway, was a biomarker reflecting the number and distribution of insulin-responsive or refractory membranes in human adipose tissue. Adipose was obtained by subcutaneous biopsy from adult obese subjects at the NIH Clinical Center and was fixed within one hour. Tissue regions revealed two membrane populations: low pAKT-expressing, primarily seen in the absence of insulin stimulation, and high pAKT-expressing, primarily seen in tissue from healthy subjects following insulin stimulation, as fit by a mixture model (basal-like and insulin-responsive). We included nonspecific binding controls to ensure reproducibility among separate subjects. In response to maximal insulin stimulation we found the membrane fraction with high pAKT is significantly correlated (Pearson Rho = 0.7 p = 0.0001) with the fraction of tissue that translocated GLUT4, the insulin-stimulated glucose transporter, to the adipocyte plasma membrane. Additionally, the more insulin-sensitive a subject was, as calculated by the insulin-modified frequently sampled intravenous glucose tolerance test, the larger the fraction of insulin-responsive membrane within adipose tissue, in agreement with previous coarse-grained work from isolated adipocytes. Our validation of a two state mosaic membrane also showed large contiguous membrane regions, (i.e. refractory regions adjacent to other refractory regions) potentially indicative of paracrine signaling. Understanding the spatial nature of IR in native tissue will lead to a better mechanistic understanding of why some membranes still respond to insulin in prediabetic subjects and ultimately how to modulate paracrine signaling to increase the number of insulin-responsive regions in such individuals.

National Institute of Child Health and Human Development
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Developmental Biology
Profiling the in vivo endothelial translatome using AngioTag zebrafish
Understanding the signaling pathways and gene regulation involved in vascular development is critical to the generation of new therapeutics for patients suffering from two of the leading causes of death in the US - stroke and cancer. Existing methods for global analysis of vascular-specific gene expression rely on either in vitro culture or post-mortem dissection and isolation of vascular cells. However, vascular endothelial cells in vivo are exquisitely regulated by their local environment, which is absent or disrupted using existing methods to determine signaling pathways in these cells, resulting in distorted and misleading gene expression information.

In order to profile the gene expression patterns of undisturbed endothelial cells in living animals, we generated a novel "AngioTag" zebrafish transgenic line that allows for the isolation of actively translating mRNAs from endothelial cells in their native environment. This transgenic line uses the endothelial cell-specific Kdrl promoter to drive expression of an epitope-tagged rpl10a 60S ribosomal subunit protein, allowing for easy and rapid Translating Ribosome Affinity Purification (TRAP) of endothelial-cell translated mRNAs. By performing RNAseq on TRAP-isolated mRNAs from AngioTag animals, we have demonstrated strong enrichment of endothelial-specific genes and depletion of genes specific for other tissues, confirming the usefulness of our new AngioTag transgenic line and the TRAP-RNAseq method.

We are currently utilizing this powerful new profiling tool to dissect endothelial signaling pathways activated during vascular development and disease, including the key Vascular Endothelial Growth Factor (VEGF) signaling pathway. Together with a wide variety of genetic and experimental models affecting the VEGF pathway already available in the lab, we will use AngioTag TRAP-RNAseq to determine the changes that occur in the absence of key VEGF signaling genes and uncover the full in vivo regulatory network downstream from VEGF signaling. Completion of this project will not only yield new and important genes involved in vascular development, but offer an unparalleled resource to study cause and effect relationships in the context of gene loss of function in vivo.

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Molecular Biology - Prokaryotic
A dual-acting small protein regulates intracellular magnesium concentration
Small proteins have historically been missed by bioinformatic and biochemical analyses. Although recent work has demonstrated that many small proteins are made and have biological effects, very few mechanisms have been elucidated. Characterized small proteins are generally membrane associated and modulate larger membrane proteins. Here, we report that a 31-amino acid small transmembrane protein from Escherichia coli, MgtS, acts differentially on two distinct membrane proteins to increase intracellular Mg2+ levels in low Mg2+ conditions. Maintenance of Mg2+ homeostasis is essential for

optimal E. coli growth and processes mediating Mg2+ uptake are highly regulated. MgtS is induced when Mg2+ is limited to increase intracellular Mg2+. A mutant of mgtS accrues less Mg2+ and has slow growth in low Mg2+ media. Although only 31 amino acids long, MgtA appears to have two structurally different binding partners and has opposing effects on their function upon binding. Co-purification shows that MgtS directly binds to both the Mg2+ importer MgtA and the phosphate transporter PitA. MgtS appears to stabilize MgtA, as deletion of mgtS results in reduced MgtA and overexpression increases MtgA. This appears to occur via prevention of MgtA proteolysis since MgtS also interacts with a membrane-localized protease and overexpression of a protease substrate as a competitor rescues MgtA levels in an mgtS deletion. Thus, MgtS increases intracellular Mg2+ by stabilizing the MgtA Mg2+ importer. In addition, MgtS prevents PitA activity. Overexpression of MgtS inhibits phosphate import by PitA, which likely increases intracellular Mg2+ by preventing Mg2+ leakage out of the cell. MgtA and PitA belong to different protein families and it is thus surprising that they are both binding partners of the same small protein. Mutational analysis aimed at determining important residues for MgtS function indicate that different residues affect MgtS interaction with its two target proteins. We conclude that MgtS increases intracellular Mg2+ in Mg2+ limiting conditions by promoting MgtA stability to increase Mg2+ import while inhibiting PitA activity to prevents Mg2+ leakage. These results add another layer of complexity to Mg2+ homeostasis, contribute to the understanding of small protein-mediated regulation, and provide two novel small protein-large protein interacting partners to tease out the structural features important for small protein-large protein binding interactions.

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Stress, Aging, and Oxidative Stress/Free Radical Research
IRBIT links nucleotide metabolism to aging in the gut
Aging leads to decline in organ function and tissue organization, resulting in increased morbidity. Gastrointestinal disorders associated with aging are a major contributor to this increase in morbidity. Within the gut, there are age-related changes in the microbiota, immune signaling and inflammation response. At the cellular level, aging in the gut is coupled with decreased epithelial integrity. Using the genetically tractable model organism Drosophila melanogaster, we have identified IRBIT as a key protein in maintaining gut homeostasis and epithelial integrity in aging.

We recently reported a conserved role for IRBIT in inhibiting ribonucleotide reductase (RNR), an enzyme that produces dNTPs within the cell for DNA synthesis. During embryogenesis, in situ hybridization

reveals localization of IRBIT to regions that will become the midgut, and it is expressed highly in the adult midgut. WT flies show a marked decrease in IRBIT protein level as they age, hinting at a function for IRBIT in aging. Therefore, we probed IRBIT's potential role in regulating gut aging by generating an IRBIT null fly (IRBIT-/-). One day old WT and IRBIT-/- flies are indistinguishable at the tissue architecture level. However, rapid progressive aging occurs in the IRBIT-/- flies, whereby one week old flies resemble dramatically older WT flies. IRBIT -/- flies show an increase in undifferentiated enteroblast progenitor cells, that are RNR positive. This increase in RNR positive progenitor cells contributes to tissue dysplasia. IRBIT-/- flies also show decreased cell-cell contacts when stained for junctional proteins in the posterior midgut epithelium. Consistent with these findings, RNA seq analysis of gene expression in IRBIT-/- midgut also suggests premature induction of age-related gene expression patterns. These phenotypes are fully rescued with full length IRBIT; suppressing RNR by hydroxyurea treatment also rescues the IRBIT-/- phenotype, which reveals IRBIT's role in aging is through RNR inhibition. Finally, artificial elevation of dNTP levels extensively phenocopies the IRBIT deletion.

Altogether, these data suggest that IRBIT plays a key role in aging and tissue homeostasis in the fly gut via its role antagonizing RNR in nucleotide metabolism. We are currently examining the interplay of IRBIT with inflammatory pathways and microbiota changes that are characteristic features of aging in flies.

National Institute of Child Health and Human Development
Shristi Rawal
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Epidemiology/Biostatistics - Prognosis and Response Predictions
Does Thyroid Function in Early Pregnancy Matter? A Prospective and Longitudinal Study of Thyroid Markers in Pregnancy and Risk of Gestational Diabetes
Background: Thyroid hormones play pivotal roles in glucose homeostasis, with free triiodothyronine (FT3) being the primary biologically active hormone involved in glucose metabolism. Previous studies report that FT3:FT4 ratio, a marker of deiodinase activity indicating conversion of free thyroxine (FT4) to FT3, is associated with clinical measures of glucose metabolism in non-pregnant individuals. Yet, studies examining association between FT3:FT4 ratio and risk of gestational diabetes (GDM) are lacking.

Methods: In a case-control study including 107 GDM cases and 214 controls (matched 1:2 on age, race, and gestational age at blood draw) from the NICHD Fetal Growth Studies-Singleton Cohort (2009-2013), we prospectively and longitudinally examined associations of FT3:FT4 ratio and other markers of thyroid function with subsequent GDM. Plasma thyroid-stimulating hormone (TSH), FT3, FT4, thyroid peroxidase antibody (TPO-Ab), thyroglobulin antibody (TG-Ab) were measured, and FT3:FT4 ratio was derived, from blood samples collected twice before GDM diagnosis (i.e. gestational weeks 10-14 and 15-26), and at weeks 23-31 and 33-39. GDM diagnosis was ascertained from medical records. Adjusted odds ratios (aORs) for GDM were estimated using conditional logistic regression adjusting for demographics, pre-pregnancy body mass index, thyroid autoimmunity status, and other major GDM risk factors. Results: FT3 and FT3:FT4 ratio were positively associated with GDM risk at both visits before GDM diagnosis; aOR

(95% CI) comparing the highest vs. lowest quartile of FT3 was 4.2 (1.7, 10.6) at weeks 10-14 (Ptrend=0.001) and 2.8 (1.2, 6.5) at weeks 15-26 (Ptrend=0.007). Similarly, the corresponding risk estimates for FT3:FT4 ratio were 7.6 (2.6, 22.1) and 10.3 (3.4, 31.9) at weeks 10-14 (Ptrend =0.001) and 15-26 (Ptrend =0.0001), respectively. TSH or FT4 levels were not independently associated with GDM risk. Isolated hypothyroxinemia at weeks 15-26, but not 10-14, was significantly related to increased GDM risk; aOR (95% CI) comparing women with hypothyroxinemia to euthyroid was 4.9 (1.5, 16.0). Subclinical hypothyroidism in either visit was not related to GDM risk.

Conclusion: In this prospective longitudinal study, we identified FT3:FT4 ratio as a novel risk factor for GDM. Our findings suggest that higher FT3 levels, resulting from de novo synthesis or increased deiodinase activity, may be involved in the development of GDM starting as early as the first trimester.

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Neuroscience - Cellular and Molecular

Protective role of olesoxime in alpha-synuclein-induced mitochondrial dysfunction.

Parkinson's disease (PD) is a neurodegenerative disease associated with loss of dopaminergic neurons and presence of Lewy bodies, whose main protein component is alpha-synuclein (a-syn). The molecular determinants underlying a-syn secretion, aggregation, and intracellular toxicity are still unclear. Yet, in vitro and in vivo studies of the effects of overexpression of either wild type or mutant forms of a-syn have reported mitochondrial abnormalities and ability of a-syn to bind to the outer and inner mitochondrial membranes. Recently, using a channel reconstitution assay, our lab showed that a-syn interacts with the outer mitochondrial membrane voltage-dependent anion channel (VDAC). Moreover, under certain conditions a-syn can either partially block this channel or translocate through it. Olesoxime is a drug candidate which has been shown to provide significant pro-survival benefits in a human neuronal model of a-syn-mediated toxicity. The dual goal of this work is to address a tentative mechanism of olesoxime protection against a-syn-induced mitochondrial dysfunctions and to clarify the role of VDAC in a-syn toxicity and olesoxime protection. Since VDAC permeability and mitochondrial membrane potential are closely related, we first assayed the effect of olesoxime treatment (10 micromolar, 24 h) on mitochondrial membrane potential in human neuroblastoma cells overexpressing a-syn. First, compared to control cells (empty vector), a-syn expression resulted in a significant reduction of membrane potential probed by the mitotracker dye. Second, olesoxime treatment significantly attenuated this loss of membrane potential while it did not affect this parameter by itself, that is, without a-syn expression. We then assessed the a-syn and olesoxime putatively common target, VDAC, using multi-channel VDAC voltage-gating protocol. We observed that olesoxime promotes VDAC closure. In single-channel experiments we found that olesoxime prevented a-syn translocation through VDAC. To interact with VDAC, a-syn first binds to membrane lipids, thus we wanted to see whether olesoxime effect could result from a lower a-syn membrane binding. Fluorescence correlation spectroscopy revealed that olesoxime did not affect a-syn binding to liposomes with the membrane lipid composition matching channel experiments. Current efforts are dedicated to confirm our in vitro results in a-syn overexpressing cells to gain insights into VDAC's role in a-syn- and olesoxime-mediated effects.

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Protein Structure/Structural Biology
STRUCTURAL BASIS FOR SUBSTRATE RECOGNITION BY HUMAN DHHC17 PALMITOYLTRANSFERASE
Protein palmitoylation refers to post-translational attachment of a saturated 16-carbon lipid to a cysteine via a thioester bond. This modification plays critical roles in a myriad of physiological processes including signaling by oncogenic Ras, localization of synaptic signaling receptors and modulation of protein aggregation in neurons. Currenlty, more than 500 human proteins are known to be palmitoylated. Protein palmitoylation is carried out by integral membrane enzymes that are characterized by a highly conserved Asp-His-His-Cys motif (DHHC) within a cysteine rich domain. There are several members of the so-called DHHC family of palmitoyltransferases, ranging from 7 in yeast to 24 in mammals. Of all the DHHCs, DHHC17 is of special medical interest. DHHC17 has been implicated in the regulation of several neuronal proteins, among which Huntingtin and Snap25 are major targets. Additionally, DHHC17 knockout significantly affects hippocampal memory and synaptic plasticity in mice. A substrate-recognition motif has been identified for DHHC17. However, a molecular understanding of DHHC17 activity has been seriously hampered by the absence of detailed structural information and biochemical dissection of its interactions with protein substrates. We have used a combination of X-ray crystallography, in-vitro binding assays and cell-based activity assays in order to address these questions. We solved the crystal structure of the binding domain of DHHC17 in complex with a peptide fragment of Snap25b, at 2.1Å resolution. The structure reveals atomic details of the binding interface between DHHC17 and Snap25b, representing the first high-resolution structure for any DHHC/substrate complex. Subsequently, we validated the structural model by alanine scanning mutagenesis of residues involved in the interaction and characterized the thermodynamics of binding via isothermal titration calorimetry. We then showed that mutations of critically important residues identified in the structure, impair substrate palmitoylation in a cell-based assay. Finally, we demonstrated that the same molecular signature in DHHC17 is important for interaction with Huntingtin, the most prominent substrate of DHHC17. These data provide the first atomic level insights into substrate recognition by DHHC17 palmitoyltransferase. They will be useful for studying DHHC17 involvement in neuronal pathologies and for the development of specific small-molecule inhibitors of DHHC17 activity.

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Neuroscience - General
The assembly of local neocortical microcircuits depends on pyramidal cell subtype identity and interneuron embryonic lineage
In the cerebral cortex, excitatory pyramidal cells (PCs) can be segregated based on the target of their long-range axonal projection: intratelencephalic (IT-type) PCs target cortex/striatum while pyramidal tract (PT-type) PCs target the brainstem, midbrain, and spinal cord. The output of these PCs is regulated by a diverse group of local inhibitory interneurons (INs). Importantly, INs can be also segregated into two non-overlapping subgroups based on their embryonic lineage from either the caudal or medial ganglionic eminences (CGE and MGE). In deep cortical layers, IT and PT PCs form local overlapping

microcircuits, and recent evidence suggests they are differentially regulated by CGE- and MGE-derived INs. Here, we show that IT PCs form preferential synaptic connections with CGE INs and influence their radial migration during development. We used the Htr3a-GFP mouse line to target CGE INs, and retrograde tracer injections into the contralateral visual cortex or ipsilateral superior colliculus to target IT or PT PCs, respectively. In dual whole-cell patch clamp recordings we found that IT PCs form synaptic connections to and from CGE INs with higher probability than PT PCs. Strikingly, IT PCs made excitatory connections on to CGE INs at high rates, while no connections from PT PCs to CGE INs were found. To confirm this connectivity bias we used combinations of transgenic mouse lines and viral vectors to express channelrhodopsin selectively in populations of PCs of either class. Indeed, population input from IT PCs drove robust network responses in CGE INs while input from PT PCs was rare and weak. Finally, PC type is determined during development by specific transcription factors. In particular, Satb2 is necessary for IT-type specification, and its loss results in disruption of the corpus callosum and ectopic projections to subcerebral targets. We conditionally knocked out Satb2 in PCs in order to induce IT PCs to adopt a PT-type identity (induced PT, iPT). We found that iPT PCs formed synaptic connections onto CGE INs at much lower rates than control IT PCs. Furthermore, loss of IT PCs disrupted the radial migration of CGE INs such that they were no longer preferentially found in superficial layers (where the majority of IT PCs reside). This was consistent across both sensory and motor regions. Our data show that PC projection identity and interneuron embryonic lineage play key roles directing the assembly and organization of cortical circuits.

National Institute of Child Health and Human Development
Ashley Xiao
Postdoctoral Fellow
Developmental Biology

Neurotrophic factor-a1 (NF-a1): A key regulator of proliferation and differentiation during embryonic neurodevelopment

Embryonic neurodevelopment requires a precise regulation of neuronal proliferation and differentiation. Neurotrophic factor-a1 (NF-a1), which is also a carboxypeptidase, has been identified as a critical factor that promotes neuronal survival. qRT-PCR and in situ hybridization show that NF-a1 mRNA was detectable as early as E5.5 in mouse embryos and increased gradually in the brain from E10.5 to P1, indicating a possible role in embryonic neurodevelopment. We have studied the function of NF-a1 on neural stem cell (NSC) proliferation and differentiation, by applying NF-a1 to E13.5 mouse cortex-derived neurospheres as an in vitro model system. We found that NF-a1 markedly decreased neurosphere proliferation compared to control. Concomitantly, ß-Catenin, the downstream target of Wnt3a ligand receptor complex which interacts with NF-a1, was also reduced, suggesting Wnt-ß-Catenin pathway is involved in the NF-a1-mediated downregulation of proliferation. Further, immunocytochemical analysis showed that NF-a1 significantly increased differentiation of NSCs to GFAP+ (glial marker) cells, and decreased Tuj1+ (neuronal marker) cells. The non-enzymatically active form of NF-a1 also showed similar effects, suggesting NF-a1 promotes the differentiation of NSCs into astrocytes independent of enzymatic activity. To confirm the function of NF-a1 in astrocyte differentiation in vivo, neurosphere-derived stem cells from NF-a1-KO and WT mice were examined. NCS from cortex of E13.5 NF-a1-KO mice had 30% fewer GFAP+ cells and 34% more Tuj1+ cells than WT mice. Interestingly, addition of NF-a1 to cultured NSCs from KO mice increased GFAP+ cells and decreased

Tuj1+ cells. In vivo, both NF-a1 and GFAP are expressed in brain during E14.5 to P1. Importantly, there was a significantly (49%) fewer GFAP+ cells, but no difference in MAP2+ neuronal cells in the neocortex of NF-a1 KO versus WT mice. Mechanistically, NF-a1 mediated-astrocyte differentiation from NCSs by increasing phosphorylation of ERK1/2 was blocked by ERK inhibitor. ERK-targeted downstream transcription factor Sox9 showed similar changes. qRT-PCR showed that Sox9 and GFAP mRNA levels were elevated by NF-a1, and abolished by si-Sox9 RNA transfection, indicating NF-a1 promotes NSCs differentiation via MAPK/MEK-Sox9 signaling pathway. This study has uncovered a unique cell fate determinant, NF-a1, that performs the dual role of switching off proliferation and activating differentiation of embryonic NSCs to astrocytes.

National Institute of Child Health and Human Development
Sara Young-Baird
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Molecular Biology - Eukaryotic
Abstract removed at request of author
Abstract removed at request of author

National Institute on Drug Abuse
Alessandro Bonifazi
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Chemistry
Novel bivalent ligands based on the sumanirole pharmacophore reveal dopamine D2 receptor (D2R) biased agonism and potential allosteric modulation
Several neuropsychiatric disorders have been associated with hypoactivation or hyperactivation of specific dopamine transmission pathways involving the D2R subtype. G-protein coupled receptors (GPCRs) can adopt several active conformations stabilized by ligands interacting with spatially distinct binding sites. Although classical approaches to GPCR drug design have targeted the orthosteric binding site (OBS), the canonical binding site recognized by the endogenous neurotransmitters (e.g., dopamine), a secondary binding pocket (SBP) is an alternative target. Sumanirole is a full agonist showing high D2R affinity (Ki=46 nM) and modest D2R selectivity (D3R/D2R=12). Despite failing in clinical trials for the treatment of restless legs syndrome, sumanirole is a valuable template for structure-activity relationship (SAR) investigation. In this study, a bivalent molecular approach has been applied to combine the primary pharmacophore of sumanirole with secondary pharmacophores via linkers of different length, rigidity and lipophilicity. The resulting bivalent ligands were designed to explore the molecular requirements of the SBP and determine effects on D2R affinity, selectivity, biased agonism and allosteric properties. The new compounds have provided SARs that reveal how affinity and selectivity for the D2R subtype change when: i) linkers and secondary aromatic pharmacophores are substituted in N-1 and/or N-5 of the sumanirole scaffold; ii) different secondary pharmacophores, varying in their physiochemical properties and chirality are inserted to recognize a specific secondary binding site; iii) linkers vary in length, rigidity and chirality or hydrophilicity. All newly synthesized compounds were evaluated in radioligand binding assays, using the agonist [3H]7-OH-DPAT to label hD2R or hD3R, and in 4 different functional BRET (bioluminescence energy transfer) assays: i) Gi protein activation, ii) Go protein

activation, iii) adenylyl cyclase inhibition, and iv) ß-arrestin2 recruitment. These studies led to the discovery of novel full agonists with highly selective D2R G-protein bias profiles. To further validate the observed G-protein functional selectivity, two independent mathematical models (the operational model and the EC50-Emax model) have been used to calculate bias factors. Additional in vivo studies are underway in an attempt to relate the activation of specific signaling pathways with their behavioral effects in models of substance use disorders.

National Institute on Drug Abuse
Yi He
Visiting Fellow
Neuroscience - General

GPR55 receptors modulate nicotine's action in rodents

Cannabinoid CB1 receptors (CB1R) and CB2 receptors (CB2R) are involved in drug reward and addiction, and therefore, have been considered as important potential therapeutic targets for treatment of substance dependence. Interestingly, growing evidence indicates that many CB1R and CB2R agonists or antagonists, such as ?9-THC, AM251, rimonabant and anandamide exhibit affinity for the GPR55 receptor (GPR55R), a potential cannabinoid-related orphan receptor. However, little is known as to whether the GPR55R is also involved in drug reward and addiction. Recent studies suggest that the brain GPR55R may modulate excitatory synaptic transmission, in a manner opposite to the CB1R. Thus, we hypothesized that the GPR55R might be involved in drug reward in a manner similar to the CB2R. To test this hypothesis, we used the pharmacological and genetic approaches for evaluating the role of the GPR55R in a rodent self-administration paradigm. Specifically, O-1602, a potent GPR55R-selective agonist, and CID 16020046, a selective GPR55R antagonist, were used as pharmacological tools. GPR55 wildtype (WT) and knockout (KO) mice were used as genetic tools. We found that: 1) O-1602 (10, 20mg/kg, i.p.) dose-dependently reduced intravenous nicotine self-administration in alcohol-preferring P rats under fixed-ratio 1 reinforcement schedules, and that these effects can be blocked by CID 16020046; 2) GPR55-WT mice, but not GPR55-KO mice, receiving the same doses of O-1602 displayed a similar reduction in nicotine self-administration; 3) administration of O-1602 in GPR55-WT mice does not alter oral sucrose self-administration and locomotor behavior, suggesting a specific effect on nicotine self-administration, not due to non-specific sedation or locomotor impairment after O-1602 administration; 4) in vivo microdialysis demonstrated that systemic or local administration of O-1602 into the nucleus accumbens (NAc) had no effect on extracellular dopamine in the NAc, suggesting that non-dopaminergic mechanisms involved; finally, 5) immunohistochemistry assay detected significant GRP55R expression in the prefrontal cortex (PFC) in WT mice, but not in GPR55-KO mice, suggesting that the GPR55R in the PFC may be the target of O-1602. Taken together, the present findings suggest that the GPR55R may be involved in nicotine reward and addiction, and therefore deserves further studies as a new promising therapeutic target for treatment of nicotine dependence.

National Institute on Drug Abuse
Andrew Kesner
Doctoral Candidate

Neuroscience - Integrative, Functional, and Cognitive

Elucidating the role of the supramammillary nucleus in motivational processes

Motivational capacity to interact with the environment is fundamental for everyday healthy living. Motivated behaviors are ultimately manifested through reward and aversion processes, where animals must approach positive 'rewarding' stimuli and avoid negative 'aversive' stimuli in order to survive. The neural mechanisms mediating these basic processes are not yet fully understood, but when they go awry, individuals may suffer from motivational disorders such as addiction, obesity, anxiety, and depression. Optogenetics – where neurons are genetically modified to express light-sensitive ion channels and are influenced via implanted optic fibers – allows investigation of previously understudied brain regions involved with motivational processes. One such understudied region, the supramammillary nucleus (SuM), is a small nucleus that provides dense projections throughout the cerebrum. Past research on SuM has focused on its role in arousal, learning, and memory. Our lab previously found that pharmacological stimulation of SuM neurons can reinforce behavior, i.e. it is rewarding. In this study, we first confirmed that excitation of SuM neurons is rewarding using a self-stimulation procedure where mice were asked to press a lever to receive optogenetic stimulation of SuM neurons. Mice with optic fibers and viral-vector mediated expression of channelrhodopsin-2 (ChR2) in SuM, but not in areas adjacent to SuM, quickly learned to press on a lever to earn optogenetic stimulation, and switch responding when lever assignments are reversed. Next, using a Cre-dependent ChR2 vector we found the rewarding effects of stimulating SuM neurons is mediated by glutamatergic neurons, but not dopaminergic or GABAergic neurons. Then, using optogenetics to dissect which SuM glutamatergic projections mediate reward, we found stimulation of glutamatergic neurons projecting to the septal area, but not other sites, is highly rewarding. Lastly, to study the natural role of SuM neurons in reward-seeking behavior, we performed electrophysiological recordings from single neurons in freely moving mice earning sucrose rewards. Most SuM neurons changed their firing rates as a function of sucrose seeking, taking, or both. Our results implicate the SuM and its afferent targets in motivational processes. As this brain circuitry is non-canonical in terms of classical reward circuits, it particularly warrants future research into its role in motivationally relevant psychiatric disorders.

National Institute on Drug Abuse

Alexandre Kisner

Postdoctoral Fellow

Biophysics

A Novel Cluster of Fast-spiking, Glutamatergic Parvalbumin-Positive Neurons in the Lateral Hypothalamus Orchestrates Feeding

The cytoarchitecture and synaptic organization of neuronal circuits that control feeding behaviors play fundamental roles in regulating homeostatic control of the body. Beginning with early lesion and electrical stimulation studies, the lateral hypothalamus (LH) has long been considered essential in regulating feeding. In addition, the exquisite repertoire of genetically distinct cell types in the LH make it an ideal circuit to study the neuronal basis of survival behaviors. However, very little is known about which cell types and relevant projections are important for orchestrating feeding. In this work, we characterized and determined the role of lateral hypothalamic parvalbumin-expressing neurons (LHPV) in regulating feeding behaviors. These neurons are distributed as a compact and small cluster in the very lateral part of the LH. Using a combination of electrophysiology, optogenetics, and in situ hybridization

assays, we determined that LHPV neurons are fast-spiking, release the excitatory neurotransmitter glutamate, and co-express vesicular glutamate transporter 2 (Vglut2) mRNA. Thus, clearly demonstrating that these cells are glutamatergic, in contrast to parvalbumin neurons in the neocortex and hippocampus. Furthermore, we manipulated the activity of LHPV neurons using chemogenetic techniques to determine whether these neurons are necessary and sufficient to drive feeding behaviors. We found that chemogenetic inhibition of LHPV neurons evoked food intake in sated mice. In contrast, chemogenetic activation of these neurons did not affect food intake. Thus, our results indicate that inhibitory inputs onto LHPV neurons orchestrate feeding behaviors. This study sheds light on the importance of understanding the functional roles of specific cell types within the LH in coordinating complex behaviors such as feeding. Additionally, understanding the mechanisms regulating food intake will allow the identification of novel targets for therapies of metabolic disorders.

National Institute on Drug Abuse
Xuan Li
Research Fellow
Neuroscience - Neurodegeneration and Neurological disorders
A novel role of thalamostriatal projections in incubation of methamphetamine craving
A key feature of methamphetamine addiction is high relapse rates. In a rodent model of drug relapse, cue-induced methamphetamine seeking in rats progressively increases after prolonged withdrawal from intravenous methamphetamine self-administration. We recently demonstrated that dorsomedial striatum (DMS) plays a critical role in this "incubation of methamphetamine craving". Here, we examined the role of neural projections into DMS in incubation of methamphetamine craving. First, we identified which projections into DMS are activated during "incubated" methamphetamine seeking. We injected retrograde tracer CTb (cholera toxin b) into DMS on withdrawal day 15 and tested half of rats for methamphetamine seeking on withdrawal day 30. The remaining rats served as our control group (left in home cage on test day). After the seeking test, we perfused the rats and performed immunohistochemistry to double-label CTb and Fos (a neuronal activity marker) in different brain areas. We found that "incubated" cue-induced methamphetamine craving was associated with increased CTb+Fos neurons in anterior intralaminar nuclei of thalamus (AIT), a brain region that has been involved in arousal and consciousness. To demonstrate a causal role of AIT in this incubation, we injected the respective GABAa and GABAb agonists muscimol and baclofen bilaterally into AIT prior to the methamphetamine seeking test on withdrawal day 30. We found that pharmacological inactivation of AIT decreased "incubated" methamphetamine seeking. Lastly, we used an anatomical disconnection procedure to determine whether this projection is functionally involved in this incubation. Injections of the dopamine D1-family antagonist SCH23390 into DMS in one hemisphere combined with injections of muscimol and baclofen into AIT in the contralateral hemisphere decreased methamphetamine seeking test on withdrawal day 30. Taken together, our data demonstrate that AIT, a brain area that has not been previously studied in drug relapse, and its projection to DMS play a critical role in incubation of methamphetamine craving.

National Institute on Drug Abuse
Rajtarun Madangopal

Research Fellow
Neuroscience - Integrative, Functional, and Cognitive
In vivo labeling of active neurons in the prelimbic cortex during a cocaine discrimination task using a novel photo-activatable genetically encoded calcium integrator
It has been shown previously that environmental stimuli paired with a single cocaine self-administration experience can elicit cocaine-seeking behavior when presented in the absence of drug for up to one year after the initial drug-cue pairing. The identification of in vivo neural activity and circuits that mediate such learned associations are essential to addiction research. However, we currently lack the temporal precision necessary to dissociate neural activity during such pairing from other events that are part of a self-administration session. Our lab and others have shown that specific patterns of neurons identified using the activity marker Fos (Fos-expressing ensembles) mediate distinct long-term memories and learned behaviors involving complex sets of cues and rewards. However, the expression of immediate early genes such as Fos can only be detected several hours after behavioral tests and may be weakly correlated with neural activity. As a result, the temporal resolution for labeling activated neurons using Fos as a biomarker spans several hours, making it impossible to use for dissociating individual behavioral epochs such as discriminative stimulus presentation and operant responding. To address this problem, we use a calcium integrator protein named CaMPARI (calcium modulated photo-activatable ratiometric integrator) that can be permanently converted from its native green state to an 'active' red state only in strongly activated cells (high calcium influx) by excitation using UV light. We have developed a procedure for achieving CaMPARI photoconversion in the prefrontal cortex of freely behaving rats. We use an AAV packaged construct to express the reporter protein and an optical fiber to deliver UV excitation light into the prelimbic cortex during specific behavioral epochs. Using this procedure, we have successfully labelled active neurons in the prelimbic cortex during simple behaviors such as novel context exposure, and foot-shock presentation. We are currently applying this procedure to label neurons that are activated during discriminative cues that predict cocaine reward availability/omission in a trial-based operant discrimination task. Using this technique, we can label separately, within the same animal, neurons activated by cues predicting reward availability and omission.

National Institute on Drug Abuse
Ernesto Solis, Jr.
Postdoctoral Fellow
Pharmacology and Toxicology/Environmental Health
Heroin and fentanyl induce hypoxia and hyperglycemia in the rat nucleus accumbens
Opiates are used clinically as analgesic agents but are also highly addictive substances that are abused and can result in overdose. Since opioids can induce respiratory depression leading to hypoxia, we sought to understand the effect of opioids on the brain. Thus, we employed Pt-Ir sensors coupled with fixed-potential amperometry in freely moving rats and examined how two opioid drugs—heroin and fentanyl—affect oxygen levels in the nucleus accumbens (NAc). We observed that when injected intravenously at self-administration doses (100 μg/kg for heroin and 10 μg/kg for fentanyl) both opioids induced a rapid and strong drop in oxygen levels that returned to baseline within minutes. To determine if oxygen in arterial blood contributed to this oxygen decrease, we measured oxygen in response to the opioids in subcutaneous tissue and observed a similar drop in oxygen, which suggests that the decrease

in blood oxygen levels could be viewed as the cause of opioid-induced brain hypoxia. In additional work, we employed enzyme-based glucose biosensors to study the effect of heroin and fentanyl on levels of NAc glucose, a critical substrate for neuronal activity. With self-administration doses, both iv heroin and fentanyl increased glucose levels dose-dependently. Glucose levels increased starting at doses of 100 µg/kg for heroin and 3 µg/kg for fentanyl. At higher doses, 300 µg/kg for heroin and 40 µg/kg for fentanyl, we observed an immediate transient fall in glucose (within 2 min post-injection) associated with a period of behavioral freezing and inhibition of respiration. The rapid decreases in oxygen levels Induced by both heroin and fentanyl preceded the increases in glucose. Although it is generally believed that brain entry of oxygen and glucose is governed via a common neurovascular mechanism, the finding that opioids induce hypoxia and hyperglycemia at different times implies distinct regulation of glucose and oxygen entry into the brain in relation to brain neuronal activity.

National Institute on Drug Abuse
Marco Venniro
Postdoctoral Fellow
Neuroscience - General

An excitatory insula-amygdala pathway mediates relapse after contingency management

Despite decades of research on neurobiological mechanisms of psychostimulant addiction, the only effective treatment for many addicts is contingency management, a behavioral treatment that uses alternative non-drug rewards to maintain abstinence. However, when contingency management is discontinued, most addicts relapse to drug use. The brain mechanisms underlying relapse after cessation of contingency management are largely unknown, and until recently, an animal model of this human condition did not exist. We recently developed a choice-based rat model of relapse after voluntary abstinence (contingency management). In this model, we first train rats to self-administer palatable food (the alternative non-drug reward) and then to self-administer a drug for several weeks. We then assessed relapse to drug seeking after voluntary abstinence, achieved via a discrete choice procedure between drug and palatable food. Under these 'contingency management' conditions, like human addicts, rats choose to abstain from drugs, and relapse to drug seeking when the alternative food reward is removed. Here, we studied the role of central amygdala (CeA) and its afferent projections in this form of relapse.

Relapse to methamphetamine seeking after voluntary abstinence was associated with increased expression of the activity marker Fos in CeA, but not basolateral amygdala (BLA). Systemic injections of the dopamine D1-family receptor antagonist SCH39166 decreased relapse and CeA Fos expression; in situ hybridization showed higher co-labeling of Fos with Drd1 (dopamine receptor type 1) than with Drd2 (dopamine receptor type 2). CeA SCH39166 injections decreased relapse after voluntary abstinence; in contrast, BLA SCH39166 injections or CeA injections of the dopamine D2-family receptor antagonist raclopride were ineffective. Double-labeling of Fos with the retrograde tracer cholera toxin subunit-B (CTb, injected in CeA) demonstrated that relapse after voluntary abstinence was associated with selective activation of the ventral anterior insula (AIV)?CeA projection. AIV inactivation with GABA receptor agonists and chemogenetic inactivation of the AIV?CeA projection decreased relapse after voluntary abstinence. Electron microscopy data showed that AIV-vGluT1-expressing projection-neurons form excitatory synapses on CeA cells.

Our results identify the AIV?CeA projection as a new addiction- and motivation-related projection and a potential new target for relapse prevention.

National Institute on Drug Abuse
Wendy Xin
Doctoral Candidate
Neuroscience - Cellular and Molecular

A novel role for oligodendrocytes in CNS glutamate homeostasis

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Glutamate uptake and degradation are critical for neuronal signaling and prevention of excitotoxicity. Perturbations in glutamate processing and transport accompany many pathological states, including epilepsy, ALS, and Alzheimer's disease (AD), highlighting the importance of identifying novel factors that contribute to CNS glutamate homeostasis. Astrocytes are ubiquitous CNS glial cells that express high levels of glutamate transporters as well as the glutamate metabolizing enzyme glutamine synthetase (GS), and are believed to be the only glial cell type contributing to glutamate homeostasis. However, using immunohistochemistry in mice expressing eGFP under the promoter of the myelin associated protein MOBP (MOBP-eGFP mice), I made the surprising discovery that there is widespread GS expression in oligodendrocytes (OLs). Mature OLs are the myelinating cells of the CNS; they produce myelin and ensheath axons to provide electrical insulation and trophic support. Although a few studies observed GS expression in cells morphologically identified as OLs, the longstanding assumption that OLs do not express glutamate transporters has thus far discouraged follow-up of these observations. I therefore aimed to confirm whether mature OLs indeed express GS and have the capacity to take up extracellular glutamate. Both qPCR and fluorescent in situ hybridization revealed high levels of GS mRNA in OLs and immunostaining with multiple GS antibodies confirmed expression at the protein level. To test whether OLs can take up extracellular glutamate, we performed patch-clamp recordings from OLs in acute brain slices from MOBP-eGFP mice and detected electrogenic transporter currents sensitive to the glutamate transporter blocker TBOA in 100% of recorded cells. To determine whether OLs are spatially positioned to access glutamate released from synaptic terminals, we performed electron microscopy on brain tissue from MOBP-eGFP mice and observed numerous examples of OL processes directly adjacent to glutamatergic synapses. We therefore conclude that OLs are positioned and equipped to take up and metabolize glutamate. These results represent a profound departure from the canonical view of glutamate metabolism in the CNS, and suggest novel functional roles for OLs. As such, OLs may represent a new target for treatment in diseases that involve glutamate dysregulation, including epilepsy and AD.

National Institute on Deafness and Other Communication Disorders
Andrew Breglio
Doctoral Candidate
Clinical and Translational Research - General

Cisplatin-Induced Hearing Loss is Associated with Long-Term Drug Accumulation in the Inner Ear

Cisplatin chemotherapy has been a mainstay of adult and pediatric cancer treatment for nearly four decades. Unfortunately, this cisplatin treatment results in significant permanent hearing loss for around

half a million Americans annually. Despite many years of study, it is still unclear why the cochlea—the organ of hearing—is uniquely susceptible to cisplatin-induced damage. Previous studies have focused on the loss of cochlear sensory cells known as hair cells. To understand and address cisplatin ototoxicity, we have developed and now leveraged a clinically accurate mouse model of cisplatin-induced hearing loss. Physiological testing of cochlear function in these mice revealed multiple types of deficit, including impaired maintenance of the electrochemical potential within the cochlea (91.2 +/- 6.6mV in saline controls, 59.7 +/- 23.5mV in cisplatin-treated, p<0.05). The unusual molecular structure of cisplatin, which incorporates the rare metal platinum, allowed us to employ inductively coupled plasma mass spectrometry (ICP-MS) to directly measure cisplatin concentrations in tissue. By ICP-MS we observed that cisplatin progressively accumulates in the cochlea during treatment in mice, and is highly retained there for months afterwards. This contrasts with most other organs, where cisplatin levels progressively decline in the days to weeks following treatment. Similarly, in post-mortem cochlear tissue obtained from cisplatin-treated human patients we measured elevated cisplatin levels, as compared to age- and sex-matched controls, as late as 18 months after last infusion (p<0.05 for all matched pairs). We then adapted the technique of laser ablation ICP-MS—traditionally used for non-biological analyses—to construct 2D images of the cochlear distribution of cisplatin in thin sections from both mouse and human patient cochleae. We measured the highest cisplatin levels in the stria vascularis, the cochlear region responsible for maintenance of the electrochemical potential. These results demonstrate that cisplatin-induced hearing loss is associated with long-term accumulation of the drug in the cochlea. They also point to the stria vascularis as an important new target for prevention of this hearing loss. Coupling of our mouse model to ICP-MS is allowing us to move forward with screening interventions aimed at preventing cisplatin accumulation in the cochlea and ultimately preventing the debilitating hearing loss often associated with cancer treatment.

National Institute of Dental and Craniofacial Research
Loreto Abusleme
Visiting Fellow
Immunology - Infectious Disease
The bacterial and fungal oral microbiome in patients with impaired T helper 17 immunity
There is increasing interest in defining the role of microbial communities in disease susceptibility. Similarly, it is essential to better understand how different components of the immune response are shaping the microbiome, determining its relationship with the host. At mucosal surfaces, T helper 17 (Th17) cells are key regulators involved in epithelial surveillance, mediating mucosal defense against extracellular bacteria and fungi. To date, the role of Th17 immunity in shaping human microbial communities at barrier surfaces remains minimally explored. To understand how Th17 immunity affects the oral microbiome establishment, we are studying a patient population with Autosomal-Dominant Hyper IgE Syndrome (AD-HIES) that exhibits impaired Th17 differentiation due to loss-of-function mutations in the transcription factor STAT3. This Th17 defect renders AD-HIES patients susceptible to mucocutaneous candidiasis, among other infections. Therefore, we evaluated the prevalence and severity of oral fungal disease in AD-HIES patients and characterized their bacterial and fungal mucosal oral communities with high-throughput sequencing of 16SrRNA and ITS1 libraries, respectively. A cohort of age/gender-matched healthy volunteers was included as controls. Detailed oral phenotyping in a cohort of adult AD-HIES patients (n=36) revealed significant susceptibility to oral candidiasis with 83% of

patients reporting recurrent oral candidiasis and 50% of them exhibiting active lesions at the time of sampling, despite administration of antifungal prophylaxis. Analyses of the oral bacteriome/mycobiome from a patient subset (n=18) showed that AD-HIES patients harbor unique bacterial and fungal communities that separate clearly from those of controls (n=25), possibly due to reduced fungal/bacterial diversity found in AD-HIES subjects. A clear predominance of Candida species (mostly C. albicans) was observed in AD-HIES communities, particularly in patients with active lesions, alongside a generalized depletion of health-associated bacteria with the exception of Streptococcus spp., which were overrepresented in AD-HIES subjects with lesions. Our data show that defective oral mucosal Th17 immunity, in the context of AD-HIES, predisposes to oral fungal infections and significant microbial community shifts. It also suggests a potential inter-kingdom synergistic interaction between Candida and Streptococcus which we hypothesize may contribute to fungal disease susceptibility.

National Institute of Dental and Craniofacial Research
Nicolas Dutzan
Visiting Fellow
Immunology - Lymphocyte Development and Activation
Targeting STAT3 in inflammatory bone loss
Periodontitis is a very common human disease characterized by inflammatory bone destruction in the oral cavity. It affects more than 64 million adults in the United States and is often linked to systemic or distant co-morbidities. T helper (Th) cells and specifically Th17 have been identified as major constituents in the inflammatory lesion of pathology in periodontitis. However, the specific role of Th17 cells in periodontitis and whether they drive inflammatory pathology is not fully understood. We find that Th17 are amplified in the lesions of human periodontitis and that they represent the major source of IL17 in animal models of periodontitis. Th17 differentiation and IL-17A expression are tightly regulated by signal transducer and activator of transcription-3 (STAT3). To analyze the role of Th17/STAT3 in humans with periodontitis we have evaluated a large cohort of patients with autosomal dominant mutations in STAT3 (AD-HIES). AD-HIES patients have a defect in Th17 differentiation and lack Th17 cells in the circulation. We clinically characterized patients with AD-HIES and evaluated Th17 responses in their oral tissues. We find that AD-HIES patients have reduced susceptibility to periodontitis and present minimal oral inflammation, consistent with blunted Th17 tissue responses. To mechanistically dissect the role of Th17cells and STAT3 in periodontitis, we performed periodontitis induction in mouse models specifically lacking Th17 cells. Cd4creStat3floxed mice have radically reduced (almost absent Th17 cells) and are resistant to inflammatory bone loss. These results reveal a pathogenic role for Th17 cells in periodontitis and suggest pharmacologic inhibition of Th17 through Stat3 in the treatment/prevention of disease. Indeed we performed preclinical studies of Stat3 inhibition (using C188-9 inhibitor, a small-molecule compound designed to prevent Stat3 activation) and demonstrate that pharmacologic inhibition of Stat3 will prevent inflammatory bone loss in periodontitis models. Our work uncovers the pathogenic potential of Th17 cells in periodontal inflammatory bone loss and suggests pharmacologic inhibition through Stat3 in the prevention of this common inflammatory disease.

National Institute of Dental and Craniofacial Research
Kuniyuki Nakamura
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Perlecan is essential to the functioning and repair of the blood-brain barrier through interacting with and activating pericytes in a mouse ischemic stroke model

Ischemic stroke causes severe symptoms and is the leading cause of disability in the U.S. However, there is no effective treatment promoting functional recovery after the acute phase treatment. The blood-brain barrier (BBB), a highly selective permeability barrier essential to maintaining homeostasis in the brain, is formed by specialized brain endothelial cells (ECs), basement membranes (BMs), pericytes, and astrocyte end-feet. Breaking of the BBB occurs when the integrity of the BBB components is lost under the ischemic conditions. In the process of repairing BBB function, pericytes are activated by platelet-derived growth factor receptor ß (PDGFRß).

Perlecan, a major heparan sulfate proteoglycan of the BM, is expressed by ECs and is adjacent to pericytes, suggesting functions in support of the BBB. However, the role of perlecan in relation to the BBB is unknown. We hypothesized that perlecan may play a protective role in the BBB maintenance and may act on pericytes during the process of repairing the BBB disruption caused by ischemic stroke.

We induced a 60-minute, middle cerebral artery occlusion (MCAO) in adult conditional perlecan-deficient (Perlecan-/--Tg) mice, which express the perlecan transgene only in the cartilage but not in the brain so as to rescue the perinatal lethality of Perlecan-/- mice.

In wild-type mice, MCAO induced increased expression of perlecan in the infarct lesion. On post-surgery day (PSD) 2 after MCAO, Perlecan-/--Tg mice demonstrated larger infarct volumes and more BBB leakage than the control mice. On PSD 3, Perlecan-/--Tg mice had decreased numbers of PDGFRß-positive pericytes in the ischemic lesion compared to control mice, suggesting that perlecan may contribute to pericyte activation. In wild-type mice, integrin a5 expression, a potential receptor for perlecan, was upregulated both in pericytes and ECs in the ischemic lesion. In addition, we found that pericytes attached to recombinant perlecan domain V (DV) through integrin a5ß1 and that perlecan DV promoted the migration of pericytes induced by PDGF-BB.

These results revealed that perlecan is necessary to maintain the BBB function and that the activation of pericytes by perlecan may contribute to the process of repairing the BBB after ischemic stroke. Perlecan DV may be useful as a potential therapeutic agent promoting the process of repairing BBB function in ischemic stroke.

National Institute of Diabetes and Digestive and Kidney Diseases
Luiz Barella
Postdoctoral Fellow
Endocrinology
Beta-arrestin-1 is required for the proper function of pancreatic beta-cells and whole body glucose homeostasis

A key pathophysiological feature of type 2 diabetes (T2D) is the inability of pancreatic beta-cells to release sufficient amounts of insulin to overcome peripheral insulin resistance and maintain normal

glucose homeostasis. Beta-cell function is regulated by hormones and neurotransmitters most of which act on specific G protein-coupled receptors (GPCR) that are expressed on the surface of pancreatic beta-cells. GPCR signaling is modulated by beta-arrestins, which regulate the activity of a very large number of physiological functions. The two members of the beta-arrestin family, beta-arrestin-1 and -2 (barr1 and barr2, respectively) are widely expressed throughout the body. Studies with whole body barr1 and barr2 KO mice have shown that beta-arrestins play important roles in several key metabolic functions including the maintenance of euglycemia. However, the potential role of beta-arrestins in modulating the function of pancreatic beta-cells in vivo remains unclear.

In the present study, we analyzed a mouse strain in which we inactivated the barr1 gene in a conditional fashion specifically in beta-cells of adult mice (beta-barr1-KO mice). In vivo studies showed that beta-barr1-KO mice exhibited a pronounced impairment in glucose tolerance and glucose-stimulated insulin secretion (GSIS), when mice were maintained on high-fat diet (HFD). Studies with isolated perifused pancreatic islets prepared from HFD beta-barr1-KO mice confirmed that the lack of barr1 in beta-cells led to a significant decrease in GSIS. We also noted that the ability of bethanechol (a muscarinic receptor agonist) and exendin-4 (a GLP1 receptor agonist) to facilitate insulin secretion was severely impaired in beta-barr1-KO mice.

To test the hypothesis that enhanced barr1 signaling in beta-cells might ameliorate the metabolic deficits associated with the consumption of a HFD, we also generated transgenic mice over-expressing barr1 in beta-cells (RIPII-barr1 mice). These transgenic mice displayed metabolic phenotypes that were opposite to those observed with the beta-barr1-KO mice, including improved glucose tolerance and GSIS in vivo.

This is the first study demonstrating that barr1 is critical for the proper function of pancreatic beta-cells and for maintaining whole body glucose homeostasis. Unveiling the cellular mechanisms through which beta-cell barr1 signaling promotes beta-cell function may lead to the development of novel drugs useful for the treatment of T2D.

National Institute of Diabetes and Digestive and Kidney Diseases
Jack Davison
Visiting Fellow
Microbiology and Antimicrobials

A New Natural Product Antibiotic Reveals Cellular Uptake Facilitated by the NorA Multidrug Efflux Protein

The spread of antibiotic resistance in pathogenic bacteria is a major global concern, and has outpaced our capacity to develop new antimicrobial drugs from traditional sources. During efforts to discover new broad-spectrum antibiotics from marine invertebrates, we identified the compound P10 from a sponge, Theonella swinhoei, collected in the Pacific Ocean off the coast of Palau. P10 potently inhibits drug-resistant clinical strains of Staphylococcus, Acinetobacter, and Pseudomonas, among others. P10 is a simple amide analog of the known antibiotic blasticidin S (BlaS), but is up to 16-fold more active than its parent compound. In this work, we aimed to uncover the mechanism behind the potency of P10 by examination of the pathways mediating its activity, and that of BlaS, in S. aureus and E. coli.

We generated mutants of S. aureus resistant to BlaS and P10, hypothesizing that the pathways conferring resistance would reveal aspects of the mechanism of action. Whole genome sequencing identified 58 mutations in 16 strains resistant to each compound. Complementing the S. aureus experiments, we characterized pathways important to BlaS and P10 activity in E. coli. A genome-wide knockout library was pooled and exposed to the antibiotics. Deep sequencing of barcode motifs associated with each strain allowed quantitation of the contribution of each gene to resistance by assessing relative strain populations after drug exposure. Genes associated with membrane transport and integrity were over-represented in both experiments, indicating that cell permeability is a key determinant of activity in this class of antibiotics. A single gene, norA, was mutated in 69% of S. aureus strains. The norA gene is a known mediator of resistance, encoding an efflux pump that exports diverse substrates. However, the mutations we observed caused loss of function, indicating that BlaS and P10 enter the cell via NorA in an inversion of its typical role. We heterologously expressed NorA in E. coli and developed an LC-MS assay to directly quantify antibiotic uptake, confirming higher cellular levels of BlaS and P10 associated with NorA expression. Additionally, P10 was more abundant in E. coli cells than BlaS, suggesting that improved membrane penetration accounts for its potency. Directional promiscuity in NorA could be exploited to select against resistance conferred by this protein family, by sequentially applying drugs with opposing selection pressure during a course of treatment.

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Endocrinology
Hypothalamic PVN neurons rapidly modify insulin secretion
Insulin is secreted from pancreatic ß-cells as a response to changes in levels of circulating nutrients and hormones. This process is tightly regulated by the autonomic nervous system that strongly innervates the pancreatic islets. Although the role of intra-islet effects of autonomic nerve terminals is well studied, little is known about the way preautonomic centers in the central nervous system regulate insulin

secretion. Retrograde tracing studies have shown that the brain region with the highest density of second order preautonomic neurons, for both sympathetic and parasympathetic innervation of pancreas, is the paraventricular nucleus of the hypothalamus (PVN). We are using chemogenetics to manipulate the activity of specific neuronal populations in the PVN and monitor the changes in circulating insulin levels. To do so, we stereotactically inject AAV viruses that induce the expression of DREADDs (Designer Receptor Exclusively Activated by Designer Drugs) in the PVN. These receptors (excitatory or inhibitory) can only be pharmacologically activated by administration of a synthetic ligand. We find that stimulation of Sim1 neurons in the PVN results in a rapid decrease in glucose stimulated insulin secretion (GSIS) which is accompanied by an increase in glucose levels. Notably, stimulation of these neurons also significantly suppresses basal fasting insulin levels. Chemogenetic inhibition of Sim1 neurons, on the other hand, significantly enhances GSIS and reduces glycemia. Further, stimulation of a smaller population of PVN neurons, the oxytocin neurons, leads to a rapid improvement of GSIS. To characterize this neuronal network, we are testing the role of autonomic preganglionic neurons downstream of the PVN in insulin secretion. To achieve this, we use an AAV-Cre virus than can travel anterogradely from the PVN to the brainstem (parasympathetic neurons) or the spinal cord (sympathetic neurons). Then a second AAV virus injected locally will induce a Cre-dependent expression of DREADDs, so that only neurons that receive projections from the PVN express these receptors. Also, it is important to know if Sim1 neurons can sense acute changes in blood glucose. To assess that, we are using in vivo photometry to monitor the activity of Sim1 neurons in hyper- or hypoglycemic states. Taken together, the findings of this study will lead to a functional characterization of the neurocircuits that are implicated in the central regulation of insulin secretion.

National Institute of Diabetes and Digestive and Kidney Diseases
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Molecular Biology - Prokaryotic
The bacteriophage T4 MotB protein, a DNA binding protein, boosts the level of T4 late gene expression
Condensation and organization of genomic DNA is crucial in all cells for orderly replication and gene expression. In bacteria, histone-like proteins such as the abundant H-NS and its less abundant homolog StpA, are DNA binding proteins that form higher-order nucleoprotein complexes needed for DNA condensation. H-NS targets AT-rich DNA sequences and condenses genomic DNA through a proposed looping mechanism that typically represses transcription at the affected region. As phage genomes and xenogeneic sequences acquired from horizontal gene transfer often display a high AT content, H-NS can protect bacteria from the expression of foreign genes by preferentially binding these sequences.

Bacteriophage T4 (65.5% AT) is a lytic virus that infects E. coli (45% AT) resulting in cell lysis after ~20 min. The T4 genome is expressed temporally from early, middle and late promoters. The T4 late promoter, TATAAATA, is strikingly similar to the H-NS binding motif, TCGATAAATT. Thus, it is not surprising that T4 may need to overcome H-NS repression. T4 Arn, a protein that structurally mimics DNA has been shown to bind H-NS, preventing its interaction with DNA and formation of higher order structures. However, Arn is not essential.

The T4 motB gene encodes a highly conserved early protein whose function has not been characterized previously. We find that expression of plasmid-borne motB is highly toxic to E. coli, resulting in

decondensation of host DNA, cell lengthening, significant reduction in actively dividing cells compared to a vector control, and cell lysis. MotB co-purifies with DNA, H-NS, and StpA. Electrophoresis mobility shift assays indicate that H-NS and MotB bind similar AT-rich sequences. However, MotB binds with 10-100-fold higher affinity than H-NS depending on the sequence. Although a T4 motB amber mutant has no noticeable phenotype in a plaque assay, RNA-seq indicates that the expression of several T4 late genes are significantly reduced. These findings are consistent with MotB relieving H-NS binding to and repression of late promoters and/or a direct activation of late gene expression by MotB. We hypothesize that the interaction of MotB with either H-NS (and StpA), DNA, or both is part of a mechanism used by T4 to disrupt H-NS dependent repression leading to optimal expression of its late genes. Determining why and how MotB is toxic can provide paths to the formation of anti-bacterials that work by a novel mechanism.

National Institute of Diabetes and Digestive and Kidney Diseases
Melissa Pinard
Postdoctoral Fellow
Biochemistry - Proteins
Crystal Structure of the TonB-dependent outer membrane transporters: IutA of hypervirulent Klebsiella pneumoniae
TonB dependent transporters (TBDTs) are beta barrel transmembrane proteins that bind and shuttle iron chelates (siderophores) across the outer membrane of some gram-negative bacteria. Bacteria require an internal iron concentration of at least 10-6 M for growth and survival. Under normal physiological conditions iron is insoluble (10-18 M) and to circumvent this problem bacteria secrete siderophores with high affinities to bind and transport iron into the cell. Hypervirulent Klebsiella pneumoniae (hvKP) is the highly invasive variant of the classical K. pneumoniae (cKP) and the causative agent of life- and organ-threatening community infections in young healthy hosts. These infections include: liver abscess, pneumonia, bacteremia, meningitis and endophthalmitis. Antimicrobial resistance presents a challenge in treatment and recent studies have shown that some strains have already acquired carbapenemases as is seen in the K. pneumoniae carbapenemase-producing strain responsible for the deadly 2011 outbreak at the National Institutes of Health Clinical Center. Therefore, there is a need for new antimicrobial targets. The hvKP strains and not cKP strains have acquired a virulence plasmid that contains genes encoding for a number of virulence factors including the hypermucoviscous phenotype, multiple siderophores and duplicate TBDTs which may be important for its increased virulence. Studies have shown that the siderophore aerobactin is responsible for growth and survival of hvKP and is a critical virulence factor. Therefore, inhibition of the aerobactin receptor-IutA in hvKP strains is of great interest. We have solved the apo-structures of both the chromosome and plasmid-encoded IutA to a resolution of 1.8 and 3.4 angstrom respectively using x-ray crystallography. We observed the plug domain (found in the N-terminal region), which is necessary for binding and transport, occludes the barrel pore in both structures. Using co-crystallization techniques we determined the chromosome encoded-IutA-aerobactin complex to a resolution of 1.9 angstrom. Aerobactin binding induces a conformational change in one of the extracellular loops and results in the closing of the top of the barrel. In addition the N-terminal domain is disordered in the complex.

Identification of the aerobactin binding site gives insight into the mode of binding and transport, as well as the structure-based design of small molecule drugs that are specific to hvKP-IutA.

National Institute of Diabetes and Digestive and Kidney Diseases
Stephan Rosshart
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Wild mouse gut microbiome protects laboratory mice against lethal influenza virus infection and colorectal cancer
Mouse models are paramount for understanding basic immunological mechanisms, but are limited in recapitulating human diseases. Studies that modify host-microbe interactions in laboratory mice clearly show the influence of the microbiome on host physiology. Differences in the gut microbiome in particular contribute to the variability of research results obtained with the same inbred mouse strains from different vendors and vivaria. In an effort to identify an external reference that better recapitulates physiologically important interactions found in the natural world outside of the laboratory environment, we asked how far removed the gut microbiome of barrier-bred laboratory mice is from that of their outbred, wild-living relative, Mus musculus domesticus. Through genetic analysis we identify the closely related wild relative of classical laboratory strains among 21 distinct populations from Europe, Asia and the Americas. We establish that the gut microbiomes of wild mice vs. barrier-raised C57BL/6 mice from the leading commercial vendors worldwide differ significantly, and that the wild mouse microbiome can be transferred to laboratory mice and maintained over several generations under vivarium conditions. Offspring of pregnant germ-free C57BL/6 mice reconstituted with the wild mouse gut microbiome exhibit reduced inflammation and increased survival following influenza A virus infection. This restoration of the natural 'microbial identity' of laboratory mice also improved their resistance against mutagen- and inflammation-induced colorectal tumorigenesis. Collectively, these data demonstrate the beneficial effects of a gut microbiome that evolved in the natural world in two diseases of global relevance to human health. As an external microbiome reference for classical laboratory mouse strains, it may facilitate the development of animal models of greater biological relevance by recapitulating complex physiological phenomena found in the natural world.

National Institute of Diabetes and Digestive and Kidney Diseases
Simona Rosu
Postdoctoral Fellow
Chromatin and Chromosomes
Histone dynamics during oocyte meiosis in C. elegans
During meiosis, which is essential for gamete formation, homologous chromosomes must pair, undergo recombination, and segregate from each other. Many questions remain about chromatin dynamics during meiosis in oocyte development. To gain new insights into this process, I am using live imaging in the model organism C. elegans to explore chromatin dynamics during various stages of meiotic prophase. I have constructed strains containing histone H2B fused to Dendra2, a fluorescent protein that irreversibly photoconverts from green to red fluorescence. This allows me to follow the fate of a selected pool of histone H2B in live animals over time. Unexpectedly, I have found widespread histone

H2B exchange in the late stages (diplotene to diakinesis) of oocyte meiotic prophase. This is surprising, as at this stage no replication is occurring and transcription is thought to be shut down. Thus the mechanism and role of histone exchange at this stage are unknown. I have confirmed this observation in three different ways: 1) by the complete recovery of GFP::H2B fluorescence after photobleaching in a strain carrying a GFP::H2B transgene (indicating new unbleached histones are loaded onto chromatin), 2) by the disappearance of converted Dendra2::H2B in a strain overexpressing the Dendra2::H2B transgene in the germline (indicating that converted histones are unloaded, and new unconverted histones are loaded onto chromatin), and finally, 3) by the redistribution of converted H2B::Dendra2 from a sub-region of chromatin to the entire chromatin in a strain where the Dendra2 tag was inserted at an endogenous H2B locus by CRISPR. This suggests that H2B histones are widely unloaded from chromatin at the diplotene to diakinesis stage in meiosis, and either reloaded when the nucleoplasmic pool is limited (as in the endogenously tagged strain), or degraded when the nucleoplasmic pool is large (as in the over-expressing transgenic strain). This exchange is specific to the diplotene/diakinesis stage of meiosis, as I have not observed this phenomenon in terminally differentiated somatic cells or in germ cells at earlier stages of meiotic prophase in the timeframe assayed. In future work, I plan to screen candidate histone chaperones and chromatin remodelers to find factors involved in this meiotic histone exchange. I will also examine the consequence of blocking histone exchange, which may lead to defects in meiosis or embryo development.

National Institute of Diabetes and Digestive and Kidney Diseases
Pengfei Tian
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Protein Structure/Structural Biology
Design of fold switching proteins using evolutionary information
Most foldable protein sequences adopt only a single native fold. Recent studies have, however, identified protein sequences which fold into different structures subject to changes in the environment or single point mutations, the best characterized example being the sequences which have been designed to “switch” between the folds of the GA and GB domains of streptococcal protein G. These “bridge” sequences have a propensity to fold into both structures: many can populate both folds in equilibrium and can bind to both GA and GB’s native partners. Such sequences could potentially be used as molecular switches. Moreover, they have been suggested as models for the emergence of new folds and functions in evolution. The nature of sequences which can switch fold, however, remains elusive.

In this work, we have developed a theoretical method to rationalize the experimentally observed conformational switching and guide the rational design of such proteins. First, we built a model for the fitness landscape of a given protein sequence to fold into a given structure, based on the evolutionary co-variation of residues extracted from the variation of protein sequences within a protein family. To validate the accuracy of our model, we have designed and tested five GA mutants guided by this fitness model using computer simulation. Circular dichroism and 2D nuclear magnetic resonance spectra, and isothermal titration calorimetry, have shown that our designed proteins, with sequence identity to the wild type are as low as 50%, can still fold to target (GA) structure and also bind to their native partners.

By appropriately combining two such models to describe the joint fitness landscape of the GA and GB folds, we are able to describe the relative propensity of a given sequence for the two folds. We have successfully validated the model against the known series of designed GA/GB mutants. Using Monte Carlo simulations in sequence space on this landscape, we are able to identify pathways of mutations connecting the two folds. We find that there are still many possible sequence pathways connecting the two folds, and a wide range of putative "fold switch" sequences close to the transition region. In conclusion, our model provides a sophisticated framework for the design of bi-stable (and bi-functional) protein sequences with potential applications to protein engineering and protein evolution.

National Institute of Diabetes and Digestive and Kidney Diseases
Lu Zhu
Postdoctoral Fellow
Endocrinology
Hepatic beta-arrestin-2 is essential for maintaining euglycemia
The type 2 diabetes (T2D) epidemic represents a major burden to human health in most parts of the world. One of the key pathophysiological features of T2D is an increase in hepatic glucose production (HGP). To facilitate the development of novel therapeutic agents that inhibit pathologically elevated HGP, it is essential to better understand the molecular pathways that regulate hepatic glucose fluxes. HGP is modulated by the activity of several G protein-coupled receptors (GPCRs) including hepatic glucagon receptors (GCGRs) which play a key role in maintaining euglycemia. GCGR activity is excessively high in T2D, leading to pathological increases in HGP. The activity of almost all GPCRs is regulated by a pair of proteins known as beta-arrestin-1 and -2 (alternative names: arrestin-2 and -3, respectively). Here, we demonstrate that selective inactivation of beta-arrestin-2 (barr2) in hepatocytes of adult mice (hep-barr2-KO mice) leads to striking deficits in glucose homeostasis associated with increased hepatic GCGR signaling. In contrast, mice lacking beta-arrestin-1 selectively in hepatocytes did not display any metabolic deficits. We show that barr2 interacts with the GCGR in an activity-dependent fashion, thus dampening GCGR signaling and promoting hepatic GCGR internalization. Importantly, mice that over-expressed barr2 in hepatocytes displayed greatly reduced hepatic GCGR signaling and were protected against the metabolic deficits caused by the consumption of a high-fat diet. In contrast to a previous report that examined whole body barr2 KO mice, we found that hepatic insulin receptor signaling was not affected by the lack of barr2. Our data support the novel concept that strategies aimed at enhancing hepatic barr2 activity could be potentially useful to suppress HGP and maintain euglycemia for therapeutic purposes.

National Institute of Environmental Health Sciences
Franziska Bollmann
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Decreasing Reactive Oxygen Species during Chronic Inflammation Increases Arthritis Severity
Chronic granulomatous disease (CGD) is characterized by recurring bacterial and fungal infections. 65% of patients suffer from the X-linked recessive form of CGD (X-CGD), caused by mutations in the CYBB gene of the NADPH oxidase 2 (Nox2) complex. Approximately 20% of males affected by X-CGD die

before reaching adulthood. In addition to recurrent infections, a small percentage of CGD patients exhibit increased inflammation, manifested as autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus.

Mice with mutations in the Cybb gene (Cybb KO) have diminished production of reactive oxygen species (ROS) and resemble human X-CGD. In a recent study, Cybb KO mice were shown to develop mild spontaneous arthritis, suggesting that ROS generated by this pathway play important roles in chronic inflammation.

A widely used mouse model of chronic inflammation involves tristetraprolin (TTP) deficiency. TTP, an mRNA binding protein, directly binds AU-rich elements in the 3'-untranslated region of target mRNAs, promoting their deadenylation and degradation. Loss of TTP (TTP KO) results in a severe inflammatory syndrome, including myeloid hyperplasia and arthritis, that is highly dependent on increased TNFalpha expression. However, the impaired vascular function in TTP KO mice is related to enhanced Nox2-dependent ROS production.

I hypothesized that the decreased ROS production seen in the Cybb KO mice would improve the vascular function in TTP KO mice. To this end, I generated Cybb-TTP double KO mice. Surprisingly, the phenotype in double KO mice was more severe than that seen in either single KO alone. A markedly decreased survival rate, increased myeloid hyperplasia, as well as increased peripheral blood platelets and monocytes were observed. I found a significant increase in arthritis severity, with arthritic involvement in joints that were not involved in either single KO model, such as the talocalcaneal joints. mRNA analyses of affected joints revealed an increased IL6 expression when compared to joints from either single KO. Ongoing RNASeq experiments hope to identify the mechanisms behind this apparently synergistic genetic combination.

Taken together, this study reveals surprising evidence that a decrease in ROS can actually increase the severity of an inflammatory syndrome, in contrast to the prevailing opinion about the harmfulness of ROS.

National Institute of Environmental Health Sciences
Rachel Carroll
Research Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Analyzing breast cancer-specific mortality in Louisiana SEER data via a spatial accelerated failure time model
Background: Breast cancer (BCa) is one of the most common cancers among US women. BCa survival time following diagnosis is associated with several demographic and clinical variables, such as race, marital status, age at diagnosis, cancer grade, tumor type, and treatment. There is also evidence suggesting that BCa survival rates differ by geographic location at diagnosis. Geographic location is related to many risk factors, e.g. socioeconomic status, and can be used as a surrogate for these unmeasured potential risk factors. However, this information is not well understood or widely used in BCa research.

Methods: In this study, we used a Bayesian accelerated failure time model with spatial frailty terms to investigate the spatial differences in BCa mortality using 2000-2013 Louisiana SEER data. This data had a reasonable number of patients and parishes with good representations of demographic risk factors. Additional explorations were executed to understand the potential causes for parishes to have shorter survival times.

Results: Model fits indicated that there were clear, meaningful spatial differences in BCa mortality across the parishes of Louisiana; specifically according to the spatial frailty estimate, the mean survival time of a woman in the best parish was 1.43 times longer than that of a woman in the worst parish. The consistency of estimates across a range of fitted models suggested effects of geographic location was independent of the clinical and demographic risk factors we adjusted for. Parishes in the first and fourth quartiles of the spatial frailty term had lower percent high education (13.79 vs. 19.91, p=.01), number of hospital per square mile (.004 vs. .02, p=.02), and median income (33.28 vs. 39.61, p=.03) as well as higher % Medicaid eligible (30.13 vs. 24.37, p=.04) and percent poverty (22.29 vs. 18.10, p=.07). This indicated that parishes with worse socio-economic status and/or less access to care had shorter survival times. Further, among the parishes along the Red and Mississippi Rivers with average income lower than the state median, 80% had shorter survival time than state average and 50% were in the lowest quartile of BCa survival times.

Conclusion: We believe it is important to incorporate spatial frailties to understand BCa survival. Our findings suggest that BCa survival could potentially be improved if care became more accessible in parishes that were identified as having shorter average survival times.

National Institute of Environmental Health Sciences
Kelly Carstens
Doctoral Candidate
Neuroscience - Neurodegeneration and Neurological disorders

Perineuronal nets: A critical regulator of developmental plasticity in the autism spectrum disorder Rett Syndrome

The proper wiring of neural circuits is dependent on early life experiences that occur during developmental windows known as ‘critical periods’. Disruption in the expression/timing of critical periods has been linked to severe learning deficits, such as those observed in autism spectrum disorders. A deeper understanding of critical period regulation will thus help us identify novel therapeutic targets for disorders in which critical period plasticity is impaired. One potential regulator of synaptic plasticity is perineuronal nets (PNNs), a specialized form of extracellular matrix proteins. PNNs are thought to function by stabilizing synaptic connections and inhibiting synaptic plasticity around postnatal day 30 (P30) in the mouse, coinciding with the end of several critical periods. Intriguingly, PNNs are increased in patients with a form of autism called Rett Syndrome, implicating them as a pathogenic mechanism altering critical periods in this disease. Therefore, we sought to characterize PNNs in the hippocampus of a mouse model of Rett Syndrome (MeCP2 KO). We focused on area CA2 of the hippocampus, an area implicated in social memory and aggressive behavior, where PNN staining

densely surrounds the pyramidal neurons. We found that PNNs develop abnormally early in area CA2 and other brain regions of Rett mice compared to control mice. We therefore investigated whether the premature development of PNNs is linked with a premature closure of plasticity at excitatory synapses in CA2 in the Rett hippocampus. We have previously shown that CA2 synapses become resistant to long-term potentiation (LTP) at P14 in acute brain slices and that we can enable LTP in CA2 by enzymatically removing PNNs. Indeed, when PNNs have yet to develop in control mice at P8-11, potentiation is inducible in CA2 (195 +/-0.1% of baseline, 10 minutes after the LTP protocol). However, in Rett mice, CA2 plasticity was prematurely lost at this age. Importantly, enzymatic degradation of PNNs was sufficient to restore CA2 plasticity in Rett mice (181 +/-0.2% of baseline), suggesting that PNNs indeed play a causal role in closing a window of synaptic plasticity in CA2 early in this disease. Overall, our findings identify a novel window of plasticity in the hippocampus and reveal PNNs as a possible mechanism mediating the severe learning impairments that emerge in Rett syndrome infants.

National Institute of Environmental Health Sciences
Qing Chen
Visiting Fellow
Stem Cells - General
Cnot3 is required for male fertility and germline stem cell maintenance
The Ccr4-Not complex is the main deadenylase in eukaryotic cells, and it regulates mRNA poly(A)-tail length to influence mRNA stability and/or translation. We have previously shown that Ccr4-Not is required for embryonic stem cell (ESC) maintenance and poly(A) tail-length regulation serves as a critical post-transcriptional mechanism in the control of the pluripotent state.

In addition to ESCs, multiple lines of evidence suggest that Ccr4-Not may also be involved in germ cell development. First, germ cells extensively rely on post-transcriptional gene regulation. Second, germ cells are considered to harbor latent pluripotency potential because they can re-acquire pluripotency via fertilization, teratocarcinogenesis, or spontaneous conversion during culture. Third, key germ cell factors (such as Nanos2) interact with Ccr4-Not in male germline stem cells. Therefore, we hypothesize that Ccr4-Not may play an important role in germ cell development, possibly in male germline stem cells. To test the hypothesis, we first examined the expression of Ccr4-Not subunits in adult testis. We found that the Cnot3 subunit is indeed highly enriched in male germline stem cells. We next generated a Cnot3 conditional knockout mice and derived the germline-specific deletion by breeding with the Vasa-CreERT2 strain. We found that germline-specific deletion of Cnot3 in males resulted in severe loss of germ cells and complete infertility. More importantly, careful examinations of neonatal males suggested that Cnot3 deletion led to specific loss of germline stem cells. Finally, to understand the underlying mechanism, we developed a novel technique to measure poly(A)-tail length of all detectable transcripts at the genomic scale. We are now using this technique to examine poly(A)-tail length changes after Cnot3 deletion in cultured germline stem cells. We are also carrying out RNA-seq and ribosome-profiling experiments to determine the impact of Cnot3 deletion on mRNA steady state level, stability, and translation efficiency. Together, our results will for the first time reveal the molecular mechanism of Ccr4-Not-dependent poly(A)-tail length regulation in germline stem cell maintenance and male germ cell development.

National Institute of Environmental Health Sciences

Amanda Conway

Postdoctoral Fellow

Gene Expression

Identification of nuclear export receptor CRM1 as a novel regulator of developmental genes in embryonic stem cells

Developmental genes mediate pattern formation and cell fate specification during the development of multicellular organisms. While precise regulation of developmental genes is critical for normal differentiation, deregulation of these genes can cause diseases such as cancer. Therefore, we sought to understand how developmental genes are dynamically and precisely regulated in embryonic stem cells (ESCs) and during differentiation. Here, we report a novel role for nuclear export receptor CRM1 in transcriptional regulation of developmental genes in ESCs. While CRM1 is known to mediate nucleocytoplasmic shuttling, its role on chromatin is less well understood. Using biochemical fractionations, we found that CRM1 localizes to chromatin in ESCs. To determine the genome-wide targets of CRM1, we performed ChIP-seq experiments using CRM1-specific antibodies. Analysis of ChIP-seq data identified 1,218 CRM1 binding sites corresponding to 563 genes. The majority of CRM1 peaks (80%) are located at the TSS or within gene bodies. Functional annotation of CRM1 target genes revealed strong association with developmental genes, specifically those encoding the homeodomain family. Correspondingly, CRM1 target genes are lowly expressed in ESCs and contain high levels of H3K27me3. Methylation of H3K27 is mediated by the Polycomb repressive complex (PRC2), and indeed, immunoprecipitation experiments confirm that CRM1 interacts with PRC2 members Jarid2, Suz12, Ezh2, and Eed. In addition to PRC2, we found that >85% of the CRM1 targets overlap with those of the Erk2 kinase. Treatment of ESCs with an inhibitor of the Erk pathway demonstrated that active Erk2 is necessary for CRM1 and PRC2 enrichment on chromatin. Next, we reactivated the Erk pathway with bFGF and Activin and assessed the dynamics of CRM1 and Erk2 on chromatin at various time points. Interestingly, we found that while Erk2 does not bind chromatin in the presence of inhibitors, it is reestablished at developmental genes within 6h of pathway stimulation. On the other hand, CRM1 is recruited to chromatin after 12h suggesting that active Erk2 directly recruits CRM1 to developmental genes. Finally, we found that PRC2 and H3K27me3 are established on chromatin 24-48h following CRM1 occupancy, leading to stable repression of target genes. In conclusion, we elucidated the temporal dynamics of developmental gene repression in ESCs following Erk activation and identified CRM1 as a novel regulator of PRC2-mediated gene silencing

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National Institute of Environmental Health Sciences

Brian Deskin

Postdoctoral Fellow

Gene Expression

GABP-alpha regulates transcriptional circuitry controlling ESC identity

Maintenance of the pluripotency network of gene expression in embryonic stem cells (ESCs) is an essential facet of metazoan development. Epigenetic modifications coupled with transcription factor binding to accessible chromatin domains dictate cell fate and maintain cell identity. Insights gained toward understanding transcriptional regulation of ESCs is critical for developing novel therapies for

regenerative medicine and understanding disease pathology. Our lab previously identified GABP-alpha as a high-rank candidate of likely regulators of ESC identity. The purpose of this study is to elucidate the role of GABP-alpha in ESCs. Knockdown (KD) of GABP-alpha with siRNA resulted in a loss of ESC morphology and downregulation of pluripotency genes Oct4 and Nanog. To identify the direct targets of GABP-alpha, ChIP-Seq analysis of GABP-alpha was performed. We found that GABP-alpha binds primarily proximal promoters within CpG islands of transcriptionally activated/poised genes. To further elucidate GABP-alpha's role in the regulation of the pluripotency network we integrated published genome-wide RNA Polymerase II (RNAPII) and epigenetic ChIP-seq data in ESCs and revealed that genes transcriptionally regulated by GABP-alpha are enriched for RNAPII and the active histone mark H3K4me3 while devoid of the repressive H3K27me3 mark. IP/Mass-spectrometry analysis of GABP-alpha revealed that GABP-alpha interacts with chromatin-associated proteins including KDM5A, Sin3b, and HDAC1. Comparison of our ChIP-Seq data with published ChIP-Seq data for KDM5A show a significant colocalization of GABP-alpha with KDM5A at transcriptionally active and poised genes. The occupancy of GABP-alpha with KDM5A at H3K4me3-containing promoters implies a potential role for GABP-alpha maintaining permissive chromatin at active and poised genes. Given GABP-alpha's interaction with key chromatin modifying enzymes we propose a role for GABP-alpha in maintaining a rapid turnover of transcription states at active genes and repression of aberrant transcription through the recruitment of KDM5A and other chromatin modifying enzymes. This work illuminates the role GABP-alpha in regulating the ESC pluripotency network.

National Institute of Environmental Health Sciences
Kerry Dorr
Postdoctoral Fellow
Genetics

Deletion of JAZF1 protects against high fat diet-induced obesity, glucose intolerance and insulin resistance

Obesity is a global epidemic associated with increased morbidity and mortality. Related to this epidemic is a substantial rise in obesity-related conditions, including type 2 diabetes, heart and cardiovascular disease, and various types of cancer. Homeostasis requires a balance between energy intake and energy expenditure. Imbalances in this system can give rise to obesity. There is substantial experimental evidence suggesting a genetic predisposition to the development of obesity and obesity-related illnesses. GWAS studies have indicated that single nucleotide polymorphisms in the JAZF1 gene are highly associated with metabolic syndrome and type 2 diabetes. JAZF1 encodes a nuclear zinc-finger protein involved in the regulation of gene transcription. JAZF1 is expressed in multiple metabolic tissues, including adipose tissue and the islet cells of the pancreas. However, its function in the regulation of glucose and lipid homeostasis and how it relates to metabolic syndrome is unknown. To further explore the role of JAZF1 in metabolism and disease, we generated JAZF1 knockout mice (JAZF1-KO). We demonstrated that when fed a high fat diet (HFD), JAZF1-KO mice exhibit lower body weight and fat mass with reduced adipocyte size compared to controls. In addition, HFD JAZF1-KO mice exhibited normal blood glucose concentrations, while obese WT littermates developed severe hyperglycemia. Concomitantly, JAZF1-KO mice maintained high insulin sensitivity, while HFD-treated obese control mice developed insulin resistance. Furthermore, energy expenditure analysis of both oxygen consumption

and carbon dioxide output revealed that HFD-treated JAZF1-KO mice have increased energy expenditure. Gene expression analysis identified a significant upregulation in B3 adrenergic receptor (ADRB3) expression in HFD-treated KO mice. ADRB3 is expressed in adipocytes and is involved in thermogenesis and lipolysis. Studies suggest that disturbances of thermogenesis-related pathways play a significant role in the development of obesity. Moreover, upregulation of ADRB3 is associated with increased lipolysis, the hydrolysis of triglycerides into glycerol and free fatty acids. Taken together, our results show that JAZF1-KO mice are resistant to HFD-induced obesity and suggest that JAZF1 plays a critical role in lipolysis by regulating ADRB3. Importantly, our findings provide a mechanism for the role for JAZF1 in regulating energy homeostasis and the development of obesity.

National Institute of Environmental Health Sciences
Chunfang Gu
Research Fellow
Signal Transduction - General
Programming of cancer cell metabolism by the inositol pyrophosphate IP7.
IP7 is an inositol pyrophosphate; a diffusible cellular signal comprising 5 monophosphates and 1 diphosphate, all crammed onto an inositol ring. IP7's highly ‘energetic’ nature empowers it to directly phosphorylate - and regulate - many proteins. Steady-state cellular IP7 levels reflect a dynamic balance between synthesis by ‘IP6-kinases’ (IP6Ks) versus metabolism by ‘IP7-kinases’ (PPIP5Ks). Previously, physiological functions of IP7 have been studied by either its elimination (by IP6K knockout) or by a 10-20 elevation in its levels (IP6K overexpression). Here, we only elevate IP7 levels 3-fold; this we accomplished by CRISPR-based PPIP5K knockout in a human colon cancer cell line, HCT116. This new approach shows IP7 regulates metabolic homeostasis and cellular proliferation. We obtained microarray data from four independent PPIP5K-/- clonal lines; Gene Set Enrichment Analysis scored highly significant activation of p53 pathways (Normalized Enrichment Score = 2.52; zero False Discovery Value). Western analysis showed p53 expression increased 2-fold. Since p53 modulates mitochondrial biomass, we assayed expression by qPCR of mitochondrial respiratory proteins, SCO2 and COX4; their expression increases 50-70% in PPIP5K-/- cells. Next, confocal analysis of PPIP5K-/- cells labeled with Mitotracker Green revealed 40% increase in mitochondrial mass. In situ respiratory analysis (Seahorse) showed 30% higher rates of oxidative phosphorylation. Glucose consumption and ATP levels are 35-40% higher in PPIP5K-/- cells. Significantly, both IP7 and ATP in PPIP5K-/- cells revert to wild-type levels upon stable transfection of PPIP5K, but a kinase-dead mutant PPIP5K has no rescuing effect. Unexpectedly, considering the elevated bioenergetic status of PPIP5K-/- cells, their proliferation rate is just 25% that of wild-type cells. This growth-restricted, hypermetabolic state of PPIP5K-/- cells echoes a specialized, senescent phenotype observed by other workers upon adding chemotherapeutics to tumor cells ('therapy-induced senescence'). This led us to discover prototypical markers of senescence in our PPIP5K-/- cells: induction of beta-galactosidase expression, and enhanced stress-dependent

phosphorylation of the H2AX histone variant. We conclude IP7 regulates mitochondrial mass and energy metabolism, and knockdown of PPIP5Ks converts a tumor cell line into a hypermetabolic, senescent-like phenotype; by regulating cancer metabolism, PPIP5Ks may be targets for tumor therapy.

National Institute of Environmental Health Sciences
Juhee Haam
Research Fellow
Neuroscience - General
Acetylcholine regulates the hippocampal output to the entorhinal cortex to gate memory consolidation
Neuromodulation of neural networks, whereby a selected circuit is regulated by a particular modulator, plays a critical role in learning and memory. Among the neuromodulators, acetylcholine (ACh) plays a unique role in memory formation in that it not only stimulates encoding but also inhibits consolidation while encoding actively occurs. In recent decades, numerous studies have shown that ACh facilitates long-term potentiation in pathways involved in memory encoding. Nonetheless, the underlying mechanism behind the cholinergic suppression of memory consolidation is largely unknown. In this study, using the fiber photometry, we recorded activity of entorhinal cortex (EC) neurons in freely moving mice and found that cholinergic tone, present during active waking, inhibits neuronal activity in deep layer of the EC. We further dissected the circuit using slice electrophysiology to examine the hippocampal area CA1 to EC circuit, which is the gateway to the memory consolidation pathway. Optogenetic stimulation of cholinergic neurons caused a decrease in CA1-evoked synaptic currents in layer V neurons of the EC via activating the negative feedback regulator oriens lacunosum moleculare (OLM) neurons. To test the physiological importance of the OLM-mediated negative feedback modulation, we injected the AAV virus that carries Cre-dependent diphtheria toxin A to the hippocampus of mice that express Cre recombinase selectively in OLM interneurons. We found that OLM-ablated mice present impairment in hippocampus-dependent learning but not in hippocampus-independent learning. Given that proper regulation of memory encoding and consolidation is critical for reliable memory formation, our data provide an important mechanism through which ACh suppresses memory consolidation for uninterrupted encoding.

National Institute of Environmental Health Sciences
Wan-Chi Lin
Visiting Fellow
Physiology
Epithelial Membrane Protein 2 Regulates Transepithelial Migration of Neutrophils into the Inflamed Airspace
The pulmonary epithelium serves as a physical barrier to inhaled pathogens and has recently been recognized to have immune functions. Although the epithelium has been proposed to regulate the terminal entry of neutrophils (PMNs) into the airspace lumen during infection, the underlying mechanisms remain poorly described. Epithelial membrane protein 2 (EMP2) is a tetraspan membrane protein of the GAS-3/PMP22 family that is thought to regulate trafficking of integrins and other signaling proteins to lipid raft membrane microdomains. EMP2 is highly expressed in the lung epithelium of rodents and humans; however, to date, no function has been assigned to EMP2 in lung biology. Given

the importance of lipid rafts and integrins to host defense, we hypothesized that EMP2 might regulate the pulmonary innate immune response. To address this, EMP2-/- mice were generated and exposed via the airway to LPS and bacterial pathogens. EMP2-/- mice exhibited a substantial deficit in intra-alveolar PMN accumulation in response to K. pneumoniae, S. pneumoniae, P. aeruginosa, and LPS. Deficient alveolar PMN accumulation was also observed after direct i.t. administration of the chemokine CXCL1. As bone marrow chimeric mice revealed the alveolar PMN deficit to track with EMP2 deletion in radioresistant (lung parenchymal) cells, and in vivo migration of PMNs to other sites (peritoneum) was intact in EMP2-/- mice, we speculated that pulmonary epithelial dysfunction was responsible for deficient transit of PMNs into the EMP2-/- airspace lumen. Immunofluorescence staining of live lung slices as well as flow cytometry confirmed increased accumulation of PMNs in the interstitium of EMP2-/- lungs, suggesting intact vascular egress but deficient transepithelial migration of PMNs. Several epithelial adhesion molecules that have been reported to promote PMN transepithelial transmigration, including ICAM-1, CD47 and beta3 integrins, were found to have abnormal display on the surface of alveolar epithelial cells of EMP2-/- mice. In ongoing studies, we have generated stable EMP2 knockdown in two human bronchial epithelial cell lines, Calu-3 and NCI-H292, and are investigating the impact on epithelial expression of adhesion molecules and transepithelial PMN migration. Taken together, these studies identify epithelial EMP2 as a novel master regulator of PMN traffic into the airspace in response to diverse environmental challenges.

National Institute of Environmental Health Sciences
Yu-Hua Lo
Visiting Fellow
Biochemistry - Proteins
Structural Analysis Reveals the Features of Ribosome Assembly Factor WDR74 Important for Localization and Interaction with the AAA-ATPase NVL2
Ribosomes are large ribonucleoprotein machines that carry out the essential role of translating mRNA into proteins. The assembly of ribosomes is an intricate process that relies on the aid of hundreds of ribosomal assembly factors. Defects in ribosome assembly factors have been linked to a group of human diseases classified as ribosomopathies, and aberrant ribosome production has been associated with numerous cancers. Previous in vivo studies revealed that the type II AAA-ATPase, NVL2, is responsible for catalyzing the removal of the assembly factor WDR74 from pre-60S particles, however little is known about the function of either protein. NVL2 contains two tandem AAA domains (D1 and D2) and knockdown of NVL2 has been shown to inhibit cancer cell progression highlighting its potential as a therapeutic target. Our goals are to uncover the role of WDR74 and NVL2 during ribosome maturation and to determine how NVL2 recognizes and dissociates WDR74 from nucleolar pre-60S particles. Through multiple structural analyses we show the first structure of WDR74 composed of an N-terminal WD40 domain followed by a lysine rich C-terminus that extends away from the WD40 domain and is required for nucleolar localization. Co-immunoprecipitation assays with the mammalian homologues identified a well-conserved interface within WDR74 that is important for its association with NVL2 and that WDR74 associates with the D1 domain of NVL2. This represents a novel mode of interaction between a type II AAA-ATPase with its substrate. Moreover, WDR74 has a greater affinity for binding to a mutant of NVL2 that is deficient in ATP hydrolysis, by mutation of the Walker B motif in both the D1 and D2 AAA domains, suggesting that ATP hydrolysis drives release of WDR74 from both NVL2 and pre-

60S particles. We also mapped the interface between NVL2 and Mtr4, a large DExH RNA helicase whose primary function is to target RNA substrates including pre-rRNA to the exosome for degradation. Our data indicated that N terminus of NVL2 associates with Mtr4, and NVL2 is able to bind to both WDR74 and Mtr4 simultaneously, suggesting that WDR74 may also function to recruit the exosome cofactor Mtr4 to nucleolar pre-60S particles which could be important for pre-rRNA processing and/or a ribosome assembly surveillance mechanism. Taken together, our data suggest that Mtr4, NVL2, and WDR74 assemble into a larger complex with one another providing new links between the pre-60S ribosome and the exosome complex.

National Institute of Environmental Health Sciences
Oswaldo Lozoya
Research Fellow
Epigenetics

Mitochondrial dysfunction results in remodeling of the epigenome and transcriptome

Mitochondria are the primary organelles that manage ATP and ROS in cells, using reducing equivalents NADH and FADH2 generated in the tricarboxylic acid (TCA) cycle. The TCA cycle can run smoothly and generate acetyl-CoA so long as NADH and FADH2 are oxidized in the electron transport chain (ETC). Like other mitochondrial metabolites, acetyl-CoA is not only used in metabolic processes outside mitochondria like lipogenesis and protein acetylation, but also as a cofactor for epigenetic reactions in the nucleus. Although other cytosolic reactions also produce acetyl-CoA, mitochondria are the main source of acetyl-CoA in cells; yet, it is unclear whether mitochondria drive or respond to epigenetic remodeling.

Our hypothesis is that mitochondria regulate the epigenome through TCA output, which in turn drives nuclear gene expression. To test this, we used an in vitro model of mtDNA depletion by Tet-inducible expression of a dominant negative mitochondrial DNA polymerase gamma (DN-POLG) transgene. Cells were collected at days 0, 3, 6 and 9 of doxycycline treatment to profile changes in gene expression (RNA-seq and microarrays), histone marks (Western Blot and ChIPseq), metabolite levels (mass spectrometry) and DNA methylation (BeadChip arrays). We also profiled DN-POLG cells co-expressing two non-mammalian enzymes (NDI1 and the alternative oxidase AOX) that restore NADH oxidation in a pseudo mitochondrial ETC.

We found mtDNA content dropped upon DN-POLG expression to undetectable levels by day 9; also, global histone acetylation levels decreased. Integrative 'omics revealed differential expression of 1,282 genes, 90% of which had epigenetic marks changing at promoters (H3K9ac, DNA methylation or both). Changes in histone acetylation involved genes regulating one-carbon metabolism, acetyl-CoA levels and pyrimidine homeostasis, whereas altered DNA methylation was associated with genes controlling fatty acid oxidation and amino acid degradation; pathways identified by those genes were consistent with metabolomics-based profiling. Importantly, all these epigenetic and transcriptional effects were prevented by co-expressing NDI1 and AOX, indicating that mitochondrial NADH oxidation and TCA output suffice to regulate the epigenetic landscape without ATP production.

In sum, our results show mitochondrial dysfunction results in the remodeling of the epigenome and transcriptome in the nucleus, which are independent of mitochondrial ATP production.

National Institute of Environmental Health Sciences
Kathryn McClelland
Visiting Fellow
Genetics
Loss of COUP-TFII (NR2F2) Affects Fetal Testicular Development.
One of the most significant landmarks during testis morphogenesis is the formation of testis cords. Testis cords, known as seminiferous tubules in adult testes, are tubular structures of ensheathed Sertoli cells encasing germ cells. Testis cords provide a unique niche for sperm production later in life. After testis determination, testis cords aggregate, coil and form orderly loops throughout the interstitial space. Later in testis development, a wave proliferation causes increased coiling of the cords. Interstitial cells control the later morphogenic events, and are hypothesized to initiate initial cord formation. We sought to understand how interstitial cells participate in the process of testis cord morphogenesis. We discovered that the transcription factor COUP-TFII is expressed in a unique pattern during testis morphogenesis; it is restricted to non-steroidogenic interstitial cells. To determine its role in testis cord formation and expansion, we generated inducible conditional knockout models to ablate COUP-TFII in the testis before and after testis determination. Ablation of COUP-TFII before testis determination affects testis cord morphogenesis. 3-D modelling demonstrated that while testis size was not affected by ablation, testis cord morphogenesis was stunted, resulting in a decreased number of segmented testis cords. Despite this defect, the overall volume of the testis cords remained the same as in the properly segmented control testis, indicating the defect in cord morphogenesis is unlikely due to compromised cell proliferation. Unfortunately, this model is embryonic lethal before the onset of cord expansion. When COUP-TFII was ablated after testis determination, the morphology of the knock-out testis appeared unaffected, indicating that initially cord segmentation had taken place correctly. However, after from the onset of cord expansion the size of the knock-out testis was significantly reduced. Additionally, Sertoli cell and steroidogenic cell number was decreased in the knock-out, although these populations do not express COUP-TFII. These data indicate that COUP-TFII in the mesenchyme plays a dynamic role central to two phases of testis development. First, it is involved in the control of testis cord morphogenesis and segmentation. Second, it is involved in the expansion of somatic cell populations, which in turn contributes to the maturation and coiling of the testis cords.

National Institute of Environmental Health Sciences
Bart Phillips
Postdoctoral Fellow
Gene Expression
Identification of Testis Proteins That Positively and Negatively Regulate Tnp1 and Tnp2 Translation
Spermatogenesis is the tightly regulated process of germ cell differentiation required to maintain male fertility. One necessary component of the process is transformation of chromatin architecture, wherein histones are sequentially replaced with proteins that condense the germ cell DNA for packaging into the sperm head. Two evolutionarily conserved proteins involved in germ cell chromatin condensation are Transition Proteins 1 and 2 (TNP1 and TNP2). We confirm here that Tnp1 and Tnp2 transcripts are present at an earlier stage of spermatogenesis than their corresponding proteins, suggesting developmentally-prompted translational regulation. We hypothesized that specific RNA-binding proteins

are responsible for both the storage and activation of Tnp1 and Tnp2 mRNAs. To identify such proteins, we incubated biotinylated oligonucleotides complementary to either Tnp1 or Tnp2 mRNA with adult mouse testis lysate in order to isolate endogenous Tnp transcripts and their associated proteins. Coupling this sequence-specific RNA/protein capture strategy with mass spectrometry, we generated a list of 52 RNA-regulatory proteins associating with Tnp1 or Tnp2 mRNA, 42 of which were shared. Further, four of the proteins had been previously identified as binding Tnp1 or Tnp2, validating our results. We performed fluorescent staining in adult mouse testis for three of the new candidates: DAZL, ILF2 and MSI2. DAZL and MSI2 were expressed in more undifferentiated germ cells than ILF2, suggesting that they repress translation, while ILF2, expressed later, might promote translation. We confirmed these activities by using a luciferase reporter with Tnp1 or Tnp2 3' UTRs. Expression of DAZL or MSI2 revealed a decrease in reporter expression, while ILF2 increased expression. Further, mutational analysis of the 3' UTRs identified the unique sequences responsible for mediating the translational control by each protein. These results reveal a model for stepwise Tnp1 and Tnp2 translational regulation: Tnp mRNAs are first translationally repressed by DAZL and MSI2 and then later translationally activated by ILF2. Overall, these data provide a broad view of the proteins associating with Tnp1 and Tnp2 mRNA and provide a mechanism for their translational regulation. We propose that the identification of these RNA-regulatory molecules and sites of interaction will elucidate RNA biology and spermatogenesis and may have clinical relevance for male infertility.

National Institute of Environmental Health Sciences
Monica Pillon
Visiting Fellow
Biochemistry - Proteins
Grc3 Programs the Essential Endoribonuclease Las1 for Specific RNA Cleavage
Nucleases are found throughout all walks of life and support numerous biological processes. Despite their long history, new nucleases continue to be discovered including the Las1 endoribonuclease, which plays a vital role in eukaryotic ribosome assembly. Mutations in the Las1 gene have been linked to a congenital motor neuron disease and X-linked intellectual disability disorders, underscoring the importance of this enzyme. Ribosome assembly begins with the transcription of the pre-ribosomal RNA (rRNA) as a poly-cistronic transcript, which includes the 18S, 5.8S, and 25S rRNA as well as two external sequences (5'ETS and 3'ETS) and two internal sequences (ITS1 and ITS2). Removal of these four spacer sequences is a coordinated process requiring endo- and exo-nucleases. The first step in ITS2 removal is cleavage at the undefined C2 site by the endoribonuclease encoded within the Las1 HEPN domain. The Grc3 polynucleotide kinase subsequently targets the resulting 5'-hydroxyl for phosphorylation, thus providing the signal for Rat1-dependent ITS2 degradation. While the key players for ITS2 processing have been identified, the regulatory mechanism remains unknown. We asked 'What are the molecular determinants for specificity at the C2 site?' We reconstituted rRNA processing using RNA that maintains the secondary structure and sequence surrounding the C2 site. We determined that recombinant Las1 displays promiscuous RNase activity, analogous to the HEPN containing C2c2 nuclease from the type VI CRISPR-Cas system. Unexpectedly, we discovered that Grc3 reprograms Las1 cleavage exclusively to the C2 site. We unambiguously mapped the C2 site using mass spectrometry and determined that both the RNA structure and sequence are critical for C2 recognition by Grc3. Using electron microscopy and SEC-MALS, we revealed the higher-order assembly of the Las1/Grc3 complex and show that the

heterotetramer is essential for ribosome production in S. cerevisiae. Biochemical characterization of the tetramer supports functional cross-talk between the Las1 nuclease and Grc3 kinase domains; thus allowing for an exquisite level of control of enzymatic activity. In conclusion, this work uncovered the structural organization of the Las1/Grc3 tetramer which is required for ITS2 recognition by Grc3 and subsequent reprogramming of Las1 for the C2 site through functional cross-talk. This mechanism represents a new example of a "programmable endonuclease" guided by protein-RNA interactions.

National Institute of Environmental Health Sciences
Emmi Rotgers
Visiting Fellow
Endocrinology

Aberrant expression of Steroidogenic factor 1 (Sf1) in the ovarian somatic cells disrupts ovarian function and fertility

Sexual development and reproduction require a fine-tuned endocrine system that is controlled by hormones such as sex steroids. Steroidogenic factor 1 (SF1, encoded by Nr5a1) is a master regulator of steroidogenesis. It controls the expression of key steroidogenic enzymes, and promotes cholesterol metabolism in steroidogenic cells. Knockout of Sf1 in the mouse results in complete gonadal and adrenal agenesis. In humans, SF1 mutations are associated with a spectrum of disorders, ranging from gonadal dysgenesis and adrenal insufficiency to premature ovarian failure. Conversely, Sf1 overexpression in the mouse adrenal cortex induces adrenocortical hyperplasia, and SF1 levels are increased in human adrenocortical tumors. These observations highlight the importance of a properly tuned expression of Sf1 for function in reproductive organs and adrenals. In this study, we set out to study the consequence of aberrant Sf1 expression in the SF1-positive cell populations in the female. We developed a mouse model (Sf1-Cre; Sf1 LSL/+), where ectopic Sf1 expression is induced in all SF1-positive cell populations including ovarian somatic cells, adrenal cortex, anterior pituitary and hypothalamus. Soon after sexual maturity (8 weeks of age), the mutant ovaries showed an accumulation of corpora lutea, and hyperplasia of the steroidogenic interstitial tissue. When we followed the estrus cycle of the females for 21 days in young adulthood (3 months of age), the mutant females spent a longer time in metestrus than the controls. 87% of the mutant females were in metestrus for three or more consecutive days, compared to 22% of the controls. Finally, we assessed the fertility of mutant and control littermate females by breeding them with wildtype males until 40 weeks of age. The median age for delivering the last litter in the fertility study was 38 weeks for the controls and 28 weeks for the mutants, indicating premature cessation of fertility in the mutant females. Furthermore, the mutant females tended to have increased bodyweight, suggesting that, aberrant Sf1 expression led to a metabolic phenotype. In summary, aberrant SF1 in the steroidogenic cell lineage disrupted ovarian function, and caused premature infertility. A broad analysis of the endocrine function is necessary to identify the mechanisms that underlie the infertility and obesity, since Sf1 expression is altered not only in the ovarian somatic cells, but also in the adrenal, pituitary and hypothalamus.

National Institute of Environmental Health Sciences
David Scoville
Postdoctoral Fellow

Cell Biology - General

The role of the transcription factor Glis3 in directing pancreatic beta-cell development and function

Pancreatic beta-cells play a central role in regulating glucose homeostasis, through sensing glucose in the blood stream and responding to high levels of glucose via the secretion of insulin. Failure of the beta-cells to properly regulate glucose is referred to as Diabetes Mellitus, a condition affecting nearly 1 in 10 Americans. Given the beta-cells' central role in regulating blood glucose, understanding the genes that regulate beta-cell development and function remains a critical goal. One prominent regulator of both beta-cell fate and function is Glis3, a transcription factor with both co-activating and co-repressive functions. Both humans and mice lacking a functional copy of Glis3 are diabetic from birth, with a near complete absence of beta-cells. Furthermore, point mutations in Glis3 have been associated with Diabetes in adult humans, suggesting Glis3 as a critical regulator of beta-cell function. Yet, while much is known about the phenotype of Glis3 deletions, little is known about the molecular function of Glis3 within the beta-cell.

My research has focused on identifying how Glis3 regulates beta-cell development and function. First, to determine which genes are regulated by Glis3, a mouse model system with a pancreas-specific deletion of Glis3 was utilized. Microarray analysis of pancreatic islets from these mice revealed 516 genes upregulated and 160 genes downregulated upon deletion of Glis3, and ChIP-seq analysis of Glis3 is under investigation to identify direct targets. However, several of these genes regulated by Glis3 lack a known function. Prominent among these novel genes were a series of long non-coding RNAs (LncRNAs). LncRNAs are transcribed RNAs >200 nucleotides that are not translated into protein, but have a variety of functions within the cell. One particular lncRNA, which we refer to as Glis3 Regulated 1 (G3R1), is restricted in expression to the pancreatic beta-cells and the brain. Similarly, an apparent human homolog of G3R1 also exists, and displays the same expression pattern in human tissues. Both Luciferase assays and Chromatin Immunoprecipitation experiments indicate direct regulation of G3R1 by Glis3. We have therefore generated a G3R1 knockout mouse for further study. Taken together, our studies of Glis3 gene regulation and of novel lncRNAs within the pancreatic beta-cell will lead to a better understanding of how beta-cell fate is controlled and maintained, and which genes are essential for postnatal function.

National Institute of Mental Health

Amicia Elliott

Postdoctoral Fellow

Neuroscience - Cellular and Molecular

Using light-sheet microscopy to study the neural control of motor patterns in the fruitfly

This work explores how behavioral sequences are produced by central neural circuits in the fruitfly, D. melanogaster, and demonstrates that motor output activity patterns from the CNS resemble the muscle activity patterns during a developmentally critical behavior. To achieve a detailed cellular understanding of a behavioral neural circuit, we built a light-sheet microscope that is capable of imaging the whole CNS rapidly at high resolution. The calcium biosensor GCaMP6s, expressed in specific target neurons distributed throughout the brain, is used to monitor neuronal activity on this microscope. We are studying the pupal ecdysis sequence, which consists of three serially executed, stereotyped behavioral programs that are activated by peripheral release of Ecdysis Triggering Hormone (ETH). The G-protein coupled receptor for ETH, ETHR, is expressed in ~300 neurons. Existing data indicate that specific subpopulations of these neurons are required for each behavioral phase of ecdysis, yet the identities of

the individual neurons that control each behavioral phase and their regulatory mechanisms remain largely unknown. Importantly, the three behavioral phases correlate with motor neuron activity downstream of the ETHR neurons, during ecdysis. However, in excised brains we have found spontaneous, rhythmic activity in the pupal motor neurons that is tightly coordinated and descends from the brain to ventral nerve cord, until stimulated with ETH. This motor activity is then rapidly reorganized into patterns localized to the ventral nerve cord, with temporal characteristics that closely match the behavioral phases of the ecdysis sequence. Interestingly, calcium activity in the muscles of intact animals shows similar coordinated spontaneous activity before the onset of ecdysis behavior, in addition to the phase-specific patterns during ecdysis. Furthermore, targeted suppression of neurons in the ecdysis central pattern generator eliminates ecdysis-specific reorganization in the excised brain as well as patterned activity in the muscles, but the spontaneous activity remains intact. Ongoing studies include developing a detailed spatiotemporal map of the motor output before and during ETH stimulation with the goal of understanding the neural circuit that controls ecdysis sequence generation.

National Institute of Mental Health
Michael Gregory
Clinical Fellow
Neuroscience - Integrative, Functional, and Cognitive

Neanderthal-Derived Polygenic Score is Associated with Brain and Skull Shape in Living Humans

Background: Before their disappearance from the fossil record approximately 40,000 years ago, Neanderthals, the ancient hominin lineage most closely related to modern humans, interbred with ancestors of present-day humans. The legacy of this gene flow persists through Neanderthal-derived variants that survive in modern human DNA; however, the neural implications of this inheritance are uncertain. Here, using MRI in a large cohort of healthy individuals of European-descent, we investigated whether the amount of Neanderthal-originating polymorphism carried in living humans is related to cranial and brain morphology.

Methods: We collected T1-weighted MRI images and genome-wide SNP data on 221 Caucasian individuals. After imputation, we determined the load of Neanderthal-derived genetic variants (“NeanderScore”) in each individual. MRI data was used to create 3D-skull surfaces using an in-house pipeline, as well as gray-matter volume, white-matter volume, sulcal depth and gyrification index maps. NeanderScore was compared with each measure, determining brain and skull regions associated with Neanderthal-derived SNP load. Results were thresholded at p<0.05 FWE-corrected for multiple comparisons.

Results: Validating our approach, we found that greater NeanderScore was associated with skull shapes resembling those of Neanderthal cranial remains, particularly in occipital and parietal bones. Further, convergent NeanderScore-related findings were found in the brain (measured by gray-matter volume, white-matter volume, sulcal depth, and gyrification index), localizing to the visual cortex and intraparietal sulcus.

Conclusions: This work provides insights into ancestral human neurobiology and suggests that Neanderthal-derived genetic variation is neurologically functional in the contemporary population. The findings indicate that this inheritance is particularly implicated in the visual system.

National Institute of Mental Health
Jacob Nordman
Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive

Synaptic Potentiation of Glutamatergic Neurons at the MeA Primes Aggression

Aggression and violence are common symptoms of many psychiatric disorders, but the underlying mechanisms that lead to their expression remain largely unknown. The amygdala is a key region involved in the regulation of emotion and has been implicated as a primary locus of psychopathology. The medial amygdala (MeA) is a major subdivision of the amygdala and is an important modulator of aggression and violence. Previous studies have shown that high-frequency stimulation of neurons at the MeA can enhance aggression during future social encounters, producing a phenomenon called aggression priming. The precise mechanism by which the medial amygdala mediates aggression and its role in psychiatric illness, however, requires further elucidation. In this study we aim to define the role of the MeA in aggression. Using optogenetic and electrophysiological techniques, we find that high frequency photostimulation (HFPS, 100 Hz, 4 Trains) of glutamatergic neurons within the MeA induces short term plasticity lasting around 1-2 hours. When mice were given HFPS of glutamatergic neurons at the MeA 30 minutes before a social interaction test, we observed sustained rises in aggression and violence that lasted up to 1-2 hours. To confirm that the HFPS protocol was indeed potentiating glutamatergic neurons within the MeA, we delivered low frequency photostimulation (LFPS, 1 Hz, 15 min), an established protocol for inducing long term depression, immediately after HFPS, and found that aggression behavior returned to baseline. We next investigated the circuit level mechanism by which the MeA drives aggression priming by stimulating glutamatergic MeA axonal projections to the ventral medial hypothalamus (VmH) and the bed nucleus of the stria terminalis (BNST). We found that MeA-VmH synapses increase overall aggression while regulating attack duration, but not attack initiation, while MeA-BNST synapses were responsible for attack initiatiaion, but with insignificant changes in attack duration. These findings suggest that different synaptic targets might regulate different aggression features within the MeA circuit. Future research will be aimed at discerning the specific contribution of these downstream targets in MeA induced aggression priming. These findings will be useful in discovering preventative therapeutic strategies targeting the MeA, which can be used to curb excessive aggression and violence associated with psychiatric disorders.

National Institute of Mental Health
Jessica Reed
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging

Effects of Ketamine on Brain Activation during an Emotional Attention Bias Task in Depression
Current pharmacological treatments for major depressive disorder (MDD) are often only effective after several weeks of use, or may not be effective at all for some patients. There is tremendous need for rapidly acting medications, which could greatly improve treatment for patients with MDD. Ketamine, a glutamatergic modulator, has been shown to have rapid antidepressant effects. Since little is known about its mechanism of action, studying ketamine's effects on brain function affected by MDD could provide valuable insights. One such function is altered emotional processing, often resulting in attentional bias toward negative information. Emotion processing tasks show variations in brain activity during positive and negative stimuli that differ between MDD and healthy participants, particularly in frontal and anterior cingulate regions. In healthy volunteers, ketamine alters the processing of affective stimuli in prefrontal cortex. Here, we investigated the effects of ketamine on brain activation during an affective attentional bias task in MDD. BOLD signal was measured with 3T functional MRI while participants performed a dot probe task with emotional faces, as part of a double-blind placebo-controlled crossover study. Participants included 26 unmedicated treatment-resistant patients with MDD and 33 healthy volunteers, ages 18-65. Ketamine (0.5 mg/kg) and placebo infusions were given two weeks apart, with fMRI scans two days after each infusion. During the task, two faces appeared side-by-side, one neutral and the other angry, happy, or neutral. This was followed by a dot, to which the participant responded to indicate if it was on the left or right. Trials with the dot on the same side as the emotional face were considered congruent. Data were processed using AFNI, and a whole brain analysis was conducted using a linear mixed effect model with four factors: group, drug, emotion, and congruency (FWE-corrected p-value less than 0.05). We found a significant interaction between group, drug, and emotion in bilateral anterior cingulate, extending to areas of medial frontal and superior frontal gyri. This interaction indicated that during placebo, the pattern of activity in MDD patients was opposite that of healthy participants. Yet after ketamine, the MDD patients showed activation similar to healthy individuals on placebo. These findings suggest that ketamine's antidepressant effects may be related to a normalization of function during emotional processing.

National Institute of Neurological Disorders and Stroke
Ginger Hunter
Postdoctoral Fellow
Developmental Biology
Actomyosin contractility regulates cellular protrusion-mediated lateral inhibition in vivo
Here we investigate whether the retraction of cellular protrusions and/or cortical tension generated by the actomyosin cytoskeleton contributes to the mechanical forces required for Notch signaling, using bristle patterning in the Drosophila pupal notum as a model system. Dynamic, actin-based cellular protrusions (e.g., cytonemes) mediate cell-cell signaling across several species and signaling paradigms, including Notch signaling, a conserved mechanism that drives cell fate determination. Activation of Notch receptor by Delta ligand requires a pulling force in ligand expressing cells. The pulling force required for Notch activation is proposed to be generated through ligand endocytosis, but it is not obvious this could function within protrusions themselves, since membrane tension and the underlying actin network likely limit endocytosis to the base of actin-rich protrusions.

First, we address the role of actomyosin contractility in Notch signaling. Using a genetic approach, we find that contractility is required in ligand and receptor expressing cells for bristle patterning. Expression

of dominant negative (DN) non-muscle myosin II (NMM2) or unphosphorylatable regulatory light chain (RLC) leads to defects in bristle precursor cell spacing, indicative of defective protrusion-based Notch signaling between distant cells. Expression of either of these constructs with a transcriptional reporter of Notch signaling shows that loss of contractility in ligand expressing cells is sufficient to decrease Notch response in adjacent and distant cells compared to controls. Epistasis experiments suggest contractility drives Notch signaling in parallel with endocytosis. Next, we ask how loss of contractility affects protrusion dynamics and morphology. Expression of unphosphorylatable RLC decreases tissue tension but does not affect protrusion morphology, whereas DN-NMM2 expression leads to irregular shapes. We develop an ex vivo protocol to test acute effects of pharmacological inhibitors on protrusion behavior and interactions. Treatment of notum explants with Rho kinase inhibitor to decrease NMM2 activation did not affect protrusion formation, but inhibited movement of contractile foci of interacting protrusions. These results support a model in which actomyosin contractility in protrusions and at the cell cortex is required for Notch activation in vivo. Currently we are developing transgenes to visualize and quantify real-time pulling forces on Delta-ligand in vivo.

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Distinct Myeloid Cell Subsets Promote Angiogenesis and Damaged Tissue Clearance Following Mild Traumatic Brain Injury
Mild traumatic brain injury (mTBI) leads to a robust immune response featuring resident microglia/macrophages and recruitment of peripherally-derived monocytes/macrophages. We sought novel insights into the dynamics and function of innate myeloid cells following brain injury by studying a closed-skull focal contusion model of mTBI that permits real-time imaging of the response by intravital two-photon microscopy (TPM). We combined intravital imaging with meningeal whole-mount immunohistochemistry and cell-specific depletions to obtain a better understanding of how myeloid cells contribute to inflammation and tissue repair following focal brain injury. TPM studies of CX3CR1GFP/+ CCR2RFP/+ dual reporter mice revealed a substantial involvement of myeloid cells during mTBI. Resident microglia tended to the damaged glial limitans almost immediately after injury. This was followed by recruitment of inflammatory CCR2+ monocytes that swarmed the meninges and an accumulation of CX3CR1+ macrophages over the ensuing 4-7 days. This transition coincided with dynamic changes in gene expression that shifted from pro-inflammatory to wound-healing over time. Early cell death and vascular destruction led to an impressive re-vascularization of the lesioned meninges, with 80-100% wound closure observed within 7 days of injury. We used histo-cytometry to define the anatomical position of the different myeloid subpopulations within the injured meninges during the repair phase. This revealed the presence of non-classical and resident macrophages that highly expressed the remodeling enzyme matrix metalloproteinase 2 (Mmp2) around the lesion core. Cell-specific depletions revealed distinct contributions of myeloid cell subsets to the mTBI wound-healing response. While peripheral depletion of neutrophils and classical monocytes increased pathology due to inefficient dead cell clearance, re-vascularization of the wound was not affected. Interestingly, vascular regeneration was significantly impaired only when non-classical monocytes and resident meningeal macrophages were depleted, resulting in reduced numbers of CD206+ macrophages

in the lesion. These studies demonstrate that neutrophils, monocytes, and resident macrophages participate in coordinated yet divergent wound-healing responses to mTBI.

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Novel Repurposing of Propranolol as an Anti-Tumor Agent in Glioblastoma

Introduction

Glioblastoma (GBM) is the most common and uniformly fatal malignant brain tumor. Despite improved understanding of its pathogenesis, outcomes have changed little in recent decades. With a 14-month median survival, novel anti-tumor strategies are imperative. Propranolol is a beta-1 and beta-2 adrenergic receptor (BAR1, BAR2) antagonist used for treatment of hypertension and arrhythmias. Propranolol was serendipitously discovered to have anti-tumor effects in infantile hemangiomas, thought to be due to inverse agonism of BARs leading to decreased intracellular levels of cAMP. Preclinical studies suggest broad anti-tumor activity of propranolol against melanoma, prostate and breast cancer. We investigated whether propranolol may have a role in the treatment of GBM.

Methods

Three GBM cell lines (U251, S635 and 9L) were cultured and treated with propranolol or metoprolol (another BAR antagonist). Cell viability, quantitative RT-PCR, flow cytometry and migration assays were performed using clinically relevant concentrations of propranolol. Immunofluorescence was used to determine BAR1 and BAR2 expression. RNA interference (RNAi) with siRNA was used to determine the dependency of propranolol anti-tumor activity on BAR1 and BAR2 expression.

Results

Treatment with propranolol reduced GBM cell viability with an IC50 of 100uM 24 hours post-exposure. Metoprolol did not affect cell survival in doses up to 200uM. Treated cells demonstrated a 6-fold reduction in migratory capacity and increased rates of apoptosis. Propranolol led to a 10-fold upregulation of VEGF mRNA expression ($p = 0.02$) with no effect on other HIF inducible genes including GLUT1 and EPO. An increase in Bax vs Bcl-2 expression was consistent with observed pro-apoptotic effects. A receptor independent effect mechanism of propranolol was suggested by failure of 5mM 8-Br-cAMP to rescue treated cells. Similarly, siRNA against BAR1 and BAR2 did not reduce viability or affect treatment response to propranolol.

Conclusions

Propranolol induces apoptosis and decreases GBM cell viability in vitro. This finding appears to be specific to propranolol and is not observed with other BAR antagonists. Consistent with previous

findings in other cancers, VEGF transcription is paradoxically increased. Preliminary data suggest that propranolol may exert its anti-tumor effects independent of BAR expression, or its propranolol mediated antagonism.

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New Relevance Search Algorithm for PubMed
With more than 27 million articles in PubMed, retrieving and ranking the most relevant papers for a given query is increasingly challenging. Starting in the 2000s, the machine learning community have focused on document ranking and created learning-to-rank (L2R) algorithms, demonstrating that robust and accurate relevance models can be built by utilizing various relevance signals and large training datasets. Recently, this technology has matured enough to scale up to real-world applications.

In order for L2R to learn a ranking model, it first needs a gold standard to target. We created one from actual PubMed queries, using the anonymized queries stored in the logs, as well as any actions users subsequently took. There are two main user actions that we consider to indicate that the document is relevant. One is the abstract click, when a user clicks on a document in the list of results matching their query. The other is full text click, which occurs when a user requests the full text, after having clicked on an abstract. We collected about one year and a half worth of logs, and we assigned relevance scores to documents for each query, based on their number of abstract and full text clicks. The gold standard consists of the queries and the corresponding documents, ordered by descending relevance.

Next, we designed a set of more than 150 features that capture the relatedness between the query and the document (e.g., the number of matches), document specifications (e.g., its publication type) and query specifications (e.g., the query length). The objective for L2R is to correctly predict the relevance score of each document in the gold standard, based on this set of features only.

We observed a threefold improvement in ranking quality for the first 20 results, i.e. the first page of results. We also optimized the pipeline, named Solr-L2R, as it needed to comply with PubMed's load requirements. It is now able to process about a thousand queries per second in parallel at a speed of 100ms per query.

We compared Solr-L2R with PubMed's relevance search online by calculating the click through rates for each, that is, the proportion of queries where users click at least once on the first page of results. Solr-L2R showed a significant improvement in terms of click through rates of 10.8%. Solr-L2R has now been deployed in PubMed production system and is used when 'Best Match' sort order is selected in place of the previous relevance search.

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Genomics
Evolutionary origins of multi-tissue enhancers involve seeding by high information content "anchor" binding site
The vast majority of genes require the promoter acting together with distal enhancers in order to initiate transcription. While enhancers stereotypically are highly tissue-specific, the majority of enhancers are active in multiple distinct tissue types. Yet, there is currently limited understanding of structural or evolutionary features that distinguish tissue-specific and broadly active ("pleiotropic") enhancers. Here, we computationally dissected ~2m enhancers across 97 human tissues predicted by the Roadmap Initiative. Tissue-specific and pleiotropic enhancers exhibit sharp divergence in TF motif enrichment, preferencing developmental and housekeeping functions, respectively. Unexpectedly, however, high-resolution DNase footprint data reveals that, compared to tissue-specific enhancers, pleiotropic enhancers harbor up to three-fold higher concentrations of putatively-bound sites; most of these are unique or reused in very few tissues. This is surprising given that human enhancers are more pleiotropic than expected by chance, suggesting pervasive reuse of binding sites (BS) across tissues. To resolve this paradox, we hypothesize that pleiotropic enhancers evolve around an "anchor BS" with persistent binding across tissues, flanked by BS used in one or a few tissues. Supporting this, we observe that enhancer sites bound across multiple tissues exhibit two-fold higher evolutionary conservation than flanking sites. Moreover, their TF motif sequences have significantly higher information content, making them less prone to evolutionary 'turnover.' Finally, using multi-species alignment data to probe evolutionary origins, we find that the enhancers that expand their range of activity to additional tissues over time, becoming more pleiotropic, tend to be enriched for high-information content TF motifs. Taken together, this work uncovers signal differences between pleiotropic and tissue-specific enhancer logic, while tracing a novel dependency between enhancer logic and enhancer evolution.

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Predicting drug–drug interactions through drug structural similarities and interaction networks incorporating pharmacokinetics and pharmacodynamics knowledge
Drug-drug interactions (DDIs) occurs when a drug affects the efficacy of another drug that is co-administered. Although DDI may have beneficial effects, it can cause serious adverse effects and sometimes lead to drug withdrawal from the market. During drug development, the prediction of such DDI would help reduce the time and costs by prioritizing drug candidates by rigorous evaluation of drug candidates. The primary mechanisms of DDIs are based on pharmacokinetics (PK) and pharmacodynamics (PD). Many studies for predicting DDI have been reported based on various approaches such as physiologically based pharmacokinetic (PBPK) modeling, molecular structural similarity analysis, ontology and annotation based analysis, network modeling, QSAR modeling, and data mining from clinical data. This study examines the effects of 2D structural similarities of drugs on DDI.

We proposed models for predicting DDIs using the structural similarities of drugs from the PK and PD networks and investigated the factors influencing DDIs for further improvement of the predictions. Our assumption was that a query drug (Dq) and a drug to be examined (De) likely have DDI if the drugs in the interaction network of De are structurally similar to Dq. A network of De describes the associations between the drugs and the proteins relating to PK and PD for De. These include target proteins, proteins interacting with target proteins, enzymes, and transporters for De. We constructed logistic regression models for DDI prediction using only 2D structural similarities between each Dq and the drugs in the network of De. The results indicated that our models could effectively predict DDIs. Our work demonstrated: (1) structural similarities between Dq and the drugs in the network of De can be used for predicting DDIs between Dq and De; (2) the integration of both structural similarity scores relating to PK and PD was crucial for DDI prediction; (3) the inclusion of pharmacogenetically associated knowledge only made minor contribution to DDI predictions. Two case studies showed the ability of this approach for predicting DDI. Eight out of the top ten predicted DDIs with warfarin, and five out of the top ten predicted DDIs with simvastatin were supported by reports in literature and multiple databases.

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NetREX: Network Rewiring using EXpression - Towards Context Specifc Regulatory Networks
Network inference of gene regulatory networks (GRNs) has successfully uncovered the relationship between transcription factors (TFs) and their target genes. However, gene regulatory networks are typically constructed disregarding the fact that regulatory programs are conditioned on tissue type, developmental stage, sex, and other factors. Due to lack of the biological context specificity, these context-agnostic networks may not provide insight for revealing the precise actions of genes for a specific biological system under concern. Collecting multitude of features required for a reliable construction of GRNs such as physical and functional features for every context of interest is costly. Therefore, we need methods that are able to utilize the knowledge about a context-agnostic network for construction of a context specific regulatory network.

To address this challenge, we developed a computational approach that utilizes expression data obtained in a specific biological context and a GRN constructed in a different but related context to construct a context specific GRN. Our method, NetREX, is inspired by network component analysis that estimates TF activities and their influences on target genes given predetermined topology of a TF-gene regulatory network. To predict a network under a different condition, NetREX removes the restriction that the topology of the TF-gene regulatory network is fixed and allows for adding and removing edges to that network. Mathematically, we use l0 norm to directly handle the number of removed and added edges as well as induce sparse solutions in our formulation.

We tested our NetREX on simulated data and found that NetREX is able to dramatically improve the accuracy of the regulatory networks. Subsequently, we applied NetREX for constructing regulatory

networks for adult female flies. We used the network constructed in a recent study as the prior network, which was built by integrating diverse data. Starting with this network, we utilized a new expression data set that we collected for adult female flies where perturbations in expression were achieved by genetic deletions. We accessed the biological relevance of the predicted networks by using GO annotations and PPI interactions. The networks predicted by NetREX showed higher biological consistency than alternative approaches. In addition, we used the list of recently identified targets of the Doublesex (DSX) transcription factor to demonstrate the predictive power.
