

NIEHS

Weaver, Jeremy

Postdoctoral Fellow

Biochemistry - General and Lipids

Kinetic evaluation of an inositol pyrophosphate kinase reveals its signaling credentials.

IP7 and IP8, the pyrophosphorylated members of the inositol phosphate family, are synthesized by ATP-dependent kinases (IP6K and IP7K respectively). IP6K's catalytic activity impacts metabolic homeostasis by its regulation of both the secretion and the actions of insulin. In contrast, IP7K has been considered unfit to participate in rapid signaling events. Early reports that IP7K is catalytically feeble seemed to reflect the high-energy constraints impeding synthesis of IP8 - Nature's most crowded phosphate array. Moreover, a consensus belief that IP7K is fully reversible as an ATP-synthase has pictured the enzyme as being at the mercy of fluctuating cellular ATP/ADP ratios. The fact that no laboratory has shown IP8 synthesis is receptor-regulated in vivo has further diminished IP7K's signaling credentials. We now overhaul all of these opinions. We isolated highly pure recombinant IP7K from *E. coli* with a continuous-flow cell disrupter. Substrates that were not commercially available were prepared enzymatically, and then purified electrophoretically. We ascertained IP7K's fundamental biochemical parameters: reaction rates, substrate affinities, and equilibrium conditions. We determined that the IP7K V_{max} (190 +/- 10 nmol/mg/min) is actually comparable to that of IP6K. We further established the equilibrium point for IP7K favors 80-90% IP8 accumulation. Contrary to the paradigm with higher inositol phosphate kinases (IP5K and ITPK1), our data also show IP7K is not reversible in a physiologically-relevant bioenergetic environment (i.e. $[ATP] > [ADP]$). IP7K activity was insensitive to physiologically-relevant changes to either $[AMP]$, $[ATP]/[ADP]$ ratio, or to $[ATP]$ itself (since the ATP K_m = 20-40 μM). Thus we show for the first time, independent of the bioenergetic context, IP7K is kinetically well equipped to participate in rapid cell-signaling events. To interrogate this novel viewpoint, we selected a metabolically active cell model with high levels of expression of IP7K: the L6 skeletal myoblast cell-line. IP8 turnover was studied by HPLC analysis. We discovered that either insulin, IGF or PDGF each raised IP8 levels 2-3 fold. Thus, despite the size of the energetic investment, cells place IP8 synthesis under receptor regulation. Precedents suggest this is a valuable observation: demonstrations of receptor-regulation of levels of IP3 - and, later, PIP3 - were critical in fermenting widespread interest in these molecules as cell-signals.

NIEHS

Pali, Stela

Postdoctoral Fellow

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

Combined disruption of ATM and CHK1 functionalities reveals redundancies in the DNA damage response pathways and results in synthetic growth inhibition following gamma-irradiation

Exposure of proliferating cells to genotoxic stresses activates a cascade of signaling events termed the DNA damage response (DDR). The DDR preserves genetic stability by detecting DNA lesions, activating cell cycle checkpoints, and promoting DNA damage repair. Ataxia Telangiectasia-Mutated (ATM), ATM and Rad 3-related kinase (ATR), and DNA-dependent Protein Kinase (DNA-PK) are crucial for lesion sensing and signal transduction. The checkpoint kinase-1 (CHK1) is a canonical ATR substrate and a pharmacological target for anti-cancer regimens. We hypothesized that ATM-deficient cells depend on signaling through CHK1 for

survival after genotoxic exposures. Our goal was to develop isogenic cell lines that are stably depleted for CHK1, ATM, or both and examine cross-talk and compensatory effects on cellular growth and the G2/M checkpoint after treatment with ionizing radiation (IR). Individual depletion of CHK1 and ATM rendered cells radiosensitive without abrogating their IR-mediated G2/M checkpoint. CHK1-deficiency led to enhanced ATM phosphorylation post gamma-IR and conversely, ATM-deficiency resulted in enhanced CHK1 phosphorylation, thus revealing a signaling co-dependency. Our data indicates a feedback regulatory loop with the phosphatase PP2A as one of the underlying mechanisms: compared to wild-type cells, the nuclei of CHK1-depleted cells show a lower abundance of PP2A catalytic subunit in conjunction with increased inhibitory PP2A phosphorylation on tyrosine-307. Stable depletion of CHK1 in an ATM-deficient background showed only a 50% reduction from wild-type CHK1 protein expression levels, suggesting that loss of CHK1 by ATM-deficient cells may be synthetically lethal. Combined ATM depletion and CHK1-deficiency resulted in an additive attenuation of the G2/M checkpoint response compared to the individual knockdown, whereas pharmacological ATM inhibition in conjunction with 90% CHK1 depletion abrogated the early G2/M checkpoint. Furthermore, at 48 hours post-IR the individually-depleted cell lines continued to proliferate, whereas the double-deficient cell line ATM-CHK1 halted proliferation. In conclusion, dual targeting of ATM and CHK1 functionalities disrupts the compensatory response to DNA damage, abrogates the G2/M checkpoint, and leads to synthetic growth inhibition. Combined functional disruption of ATM and CHK1 may be a worthwhile approach for developing more efficient anti-neoplastic treatments.

NIEHS

Fromm, George

Postdoctoral Fellow

Developmental Biology

Pausing of RNA Polymerase II Regulates Mammalian Developmental Potential

The regulation of transcription is vital in the coordination of events during development, and gene misregulation is often associated with developmental disorders and disease states. Thus, there is great interest in elucidating the mechanisms that underlie transcription regulation to identify novel targets and therapies. A critical point of gene regulation occurs during early transcription elongation, when pause-inducing factors such as the NELF complex cause RNA polymerase II (Pol II) to halt just downstream of the promoter. Release of Pol II into the gene and dissociation of NELF are triggered by recruitment of the kinase P-TEFb, which mediates productive elongation and subsequent gene activation. Pausing is widespread in metazoans and is associated with genes involved in development and the response to stimuli. This finding led to an appealing model; that pausing poises genes for rapid activation pending a signal, and consequently when pausing is lost, developmental genes could be activated resulting in a phenotypic change such as differentiation. The dynamics of pausing and pause release have therefore been proposed to play key roles in many cellular processes; however our understanding of these events is lacking. To address these questions, we knocked out (KO) the NELF subunit NELF-B in the mouse. Loss of NELF-B is embryonic lethal emphasizing an essential developmental role. To gain further insight, we generated induced pluripotent stem cells (iPSCs) from conditional NELF KO mice. Contrary to the model proposed above, we find that NELF-B KO iPSCs resist differentiation due to dysregulation and dampening of Mapk/ERK signaling pathway activity. We observe that many genes in the Mapk/ERK pathway are directly bound by NELF and harbor paused Pol II, and that the loss of NELF-mediated pausing results in a redistribution of Pol II at these genes and aberrant gene expression. Strikingly, we find that the majority of genes

misregulated upon NELF loss are signaling pathway components as opposed to the downstream inducible target genes. Inhibition of Mapk/ERK signaling has been shown to prevent primitive endoderm specification in the embryo. Remarkably, we find that NELF-B KO embryos share a similar fate. Thus, the lack of lineage differentiation during embryonic development contributes to lethality, and provides evidence that NELF-mediated pausing plays a critical role in establishing Mapk/ERK signaling pathway activity during development.

NIEHS

Lichti-Kaiser, Kristin

Postdoctoral Fellow

Developmental Biology

Transcription Factor Glis3 Plays a Critical Role in the Development of Functional Pancreatic beta-cells and Diabetes

Gli-similar (GLIS) 3 belongs to a subfamily of Krüppel-like zinc transcription factors related to members of the Gli and Zic family. Several genes including GLIS3 have been associated with risk for type-1 and type-2 diabetes and genetic aberrations in the GLIS3 gene have been associated with a syndrome characterized by neonatal diabetes and congenital hypothyroidism. We previously showed that Glis3 KO mice die at post-natal day 3 from neonatal diabetes as evidenced by hyperglycemia and hypoinsulinemia. This phenotype is due to a dramatic loss of insulin-secreting beta-cells in contrast to a smaller relative loss of other endocrine cell types. In addition, Glis3 regulates insulin gene expression in mature beta-cells, indicating that Glis3 plays a key role in both the development and function of mature pancreatic beta-cells. However, the spatial and temporal expression of Glis3 during pancreas development and the mechanism by which Glis3 contributes to the development and maintenance of functional beta-cells are unknown. In order to further study the precise role of Glis3 in pancreas development and function, we have generated a pancreas-specific Glis3 KO mouse model. Glis3^{fx/fx} mice were crossed with mice expressing cre-recombinase under the control of the Pdx1 gene promoter, which is activated at an early time-point during pancreas development. Glis3^{fx/fx};Pdx-cre KO (PGLis3KO) mice develop a delayed-onset diabetic phenotype at 2-3 months of age as evidenced by hyperglycemia, hypoinsulinemia, and loss of white adipose tissue. Gene expression profiling and immunofluorescence analysis demonstrated that the expression of insulin was significantly decreased at 2 months, but not at 2 weeks, of age in PGLis3KO mice. The expression of other pancreatic hormones and transcription factors important for endocrine cell development, including Ngn3 and Pdx1, was not significantly different than that of WT littermates, indicating that, unlike in the whole-body KO, there may not be a significant loss of beta-cells in the PGLis3KO mice. However, the expression of several genes that are critical for beta-cell function, including Glut2, MafA, and G6pc2, is decreased in the PGLis3KO mice compared to WT controls. The PGLis3KO mice provide an excellent model to examine various therapeutic strategies for diabetes. Our study shows that Glis3 has multiple critical functions in the pancreas and suggests that Glis3 may provide a new therapeutic target to intervene in diabetes.

NIEHS

Ungewitter, Erica

Postdoctoral Fellow

Developmental Biology

GLI-similar 3 Maintains Sexually Dimorphic Germ Cell Development in Mouse Embryos

Germ cells are a uniquely important cell type because they give rise to gametes, the cells responsible for sexual reproduction and the propagation of all higher species. In the mouse,

programs of germ cell development are identical in male and female embryos until around embryonic day (E)13.5. At this time, germ cells in the ovary first express Stra8 and initiate meiosis. Germ cells in the testis, by contrast, arrest at the G0/G1 phase of mitosis and do not enter meiosis until early postnatal life. This dimorphic pattern of meiotic entry is mediated by the meiosis-inducing agent retinoic acid (RA). In the fetal testis, the RA-degrading enzyme Cyp26b1 inhibits RA action, thereby preventing male germ cells from entering meiosis. Fetal ovaries do not express Cyp26b1, and consequently, RA is able to induce Stra8 expression and subsequent meiosis in female germ cells. In this study, we set out to uncover new players involved in the dimorphic development of germ cells using mice as a model organism. We found that Gli-similar 3 (Glis3), a zinc finger transcription factor essential for lineage specification in endocrine organs such as thyroid and pancreas, has a testis-specific role in germ cell survival and meiosis. Glis3 expression is low in fetal ovaries at all stages of development, but high in fetal testes for a brief window of time around E13.5, coincident with the normal dimorphic initiation of meiosis. To identify the functions of Glis3, we examined the gonads of global Glis3 knockout animals. Germ cells in ovaries developed normally in the absence of Glis3; however, knockout testes had reduced numbers of germ cells and decreased expression of germ cell markers (Oct3/4 and Vasa). Defects in germ cell development are not the result of impaired somatic cell differentiation as somatic cell markers in knockout testes (Sox9 and Cyp11a) remain normal. Intriguingly, Stra8, the critical regulator of meiotic entry in germ cells, was ectopically elevated in knockout testes despite the fact that neither of the known regulators of Stra8 (Cyp26b1 and Nanos2) were changed. These findings reveal a novel role for Glis3 in germ cell survival and meiosis, whereby Glis3 prevents male germ cells from entering meiosis prematurely through a pathway independent of RA. We are currently investigating factors upstream and downstream of Glis3 with the goal of identifying the mechanisms underlying sexually dimorphic establishment of the germlines in mammals.

NIEHS

Dang, Huaixin

Visiting Fellow

Endocrinology

TAK1/TR4 regulates cold induced thermogenesis by inhibiting CREB-PGC1a pathway

Brown adipose tissue (BAT) can disperse stored energy as heat. Promoting BAT function is an attractive, if elusive, therapeutic approach to staunch the current obesity epidemic. Our previous study showed that nuclear receptor TAK1/TR4/NR2C2 $-/-$ mice are resistant to the development of obesity, glucose intolerance and insulin resistance. In this study, we found that TAK1 $-/-$ mice consume more oxygen and produce more carbon dioxide than wild type mice fed a normal diet suggesting a higher metabolic rate. This is consistent with our observations that exposed to 4 degree temperature, TAK1 $-/-$ mice are more tolerant to cold stress compared to wild type littermate. Microarray analysis showed that PGC1a, DIO2, adenylate cyclase 3(Adcy3) and CEBPa are expressed at significantly higher levels in TAK1 $-/-$ mice brown adipose tissue than WT tissue. Q-RTPCR analysis showed that this difference is significantly enhanced when mice are challenged with cold exposure. Knockdown of TAK1 expression in brown fat progenitor cells induced increased PGC1a, DIO2 and UCP1 expression and a better response to cAMP. Oil Red O staining suggests that BAT differentiation is enhanced in cells in which TAK1 is knocked down. Although over-expression of TAK1 in BAT progenitor cells has no significant impact on lipid accumulation, the cAMP-dependent induction of PGC1a, DIO2 and UCP1 was significantly reduced. Further studies will determine whether TAK1 directly inhibits the binding of CREB to the PGC1a and DIO2 promoter. Given the important role of PGC1a and DIO2 in metabolism,

TAK1 may provide a new target in the management and prevention of obesity and related pathologies, including diabetes.

NIEHS

Englert, Neal

Postdoctoral Fellow

Epigenetics

Epigenetic Modification of Histone (H3) and CYP2C9 Regulation: Involvement of Med25 as the Key Regulator

Cytochrome P450 (CYP450) enzymes are important for the hepatic metabolism of exogenous chemicals including common pharmaceutical drugs such as Warfarin and Ibuprofen, as well as the metabolism of endogenous substrates. Some CYPs have been found to be regulated by DNA methylation, however, little is known about the epigenetic regulation in human tissues. The CYP2C9 gene is regulated by liver-specific nuclear receptor HNF4alpha, which has been shown to recruit Med25, a subunit of the Mediator complex. Mediator complex is involved in the transcriptional regulation of most genes by recruiting Polymerase II, thus revealing its importance in controlling cellular function. Mediator has also displayed an ability to recruit histone modifiers such as G9a, a histone methyltransferase, which leads to gene repression by methylating H3K9. Polycomb repressive complex (PRC2), which represses expression by methylating H3K27, may also be recruited by Mediator complex. Here, we examined whether Med25 is involved with epigenetically regulating the expression of CYP2C9, through modifying histone markers in the chromatin of the CYP2C9 promoter. We hypothesize that Med25 is important not only for Polymerase II recruitment, but for controlling the chromatin architecture, and thus the expression, of target genes such as the CYP450s. We performed chromatin immunoprecipitation in HepG2 cells to identify histone modifications at the HNF4alpha binding site in relation to Med25 protein levels. Results indicated that altering Med25 expression modified H3K27 status; in the presence of Med25, the HNF4alpha binding site was enriched for activating markers H3K27ac and H3K27me1, with progressive demethylation. When Med25 expression was silenced, the H3K27 residue was highly methylated (H3K27me3), which is a prototypical gene-silencing marker. CYP2C9 transcript levels were reduced after silencing Med25 expression. These results suggest that Med25 induces a permissive chromatin state at the CYP2C9 proximal promoter. Additionally, confocal microscopy revealed that Med25 colocalized with activating histone markers (H3K27ac) in the nucleus of HepG2 cells while it did not colocalize with repressive markers (H3K27me2, H3K27me3). Our data indicate that Med25 is important for changing the epigenetic landscape to allow for increased levels of transcriptional activation of highly inducible genes such as the hepatic cytochrome P450s.

NIEHS

Joubert, Bonnie

Research Fellow

Epigenetics

Maternal smoking and DNA methylation in newborns: An in utero effect or epigenetic inheritance?

Maternal smoking in pregnancy is related to multiple adverse health outcomes in children and underlying mechanisms may include epigenetic modifications. We previously identified differential DNA methylation related to maternal smoking during pregnancy at 26 CpG sites (CpGs) in 10 genes including those involved in the metabolism of the components of tobacco smoke (AHRR, CYP1A1) or developmental processes (e.g., GFI1 and MYO1G). However, it is

unclear whether these methylation signals in the infant epigenome reflect in utero exposure only or possibly epigenetic inheritance of smoking-related modifications from the mother. To investigate this, we evaluated the relationship between the timing of mother's smoking (before or during pregnancy) as well as the combined effect of grandmother's and mother's smoking in pregnancy on methylation at the 26 previously identified CpGs. We measured DNA methylation with Illumina's Infinium HumanMethylation450 Beadchip in 1,042 newborn cord bloods from the Norwegian Mother and Child Cohort Study (MoBa). Both self-report and cotinine measured in pregnancy were used to categorize the timing of mother's smoking (never smoked, formerly smoked, smoked in pregnancy but quit by 18 weeks, and actively smoked throughout pregnancy). Grandmother's smoking was assessed by mother's response to "Did your mother smoke when she was pregnant with you?" Grandmother and mother smoking information were combined (neither smoked, only grandmother smoked in her pregnancy, only mother smoked in her pregnancy, and both grandmother and mother smoked in their pregnancies). The association between smoking category and methylation was assessed using robust linear regression, adjusting for covariates. Bonferroni correction was used to adjust the level of statistical significance to $p < 0.0019$. Only active smoking throughout pregnancy was statistically significantly associated with differential cord blood DNA methylation ($p < 1.6 \times 10^{-5}$ for all 26 CpGs). We did not observe statistically significant associations for former smoking or quitting early in pregnancy (minimum $p = 0.010$). There was no statistically significant effect of grandmother smoking alone and if mother smoked, the additional effect of grandmother smoking was not significant (minimum $p = 0.078$). Our findings suggest that DNA methylation at these locations in the infant epigenome reflects in utero exposure rather than epigenetic inheritance of smoking-related modifications.

NIEHS

Kadmiel, Mahita

Visiting Fellow

Gene Expression

Glucocorticoid receptor action at the interface with the environment

The cornea is the transparent outermost layer of the eye. It forms the first barrier of the eye, and as such it is exposed to the environment. Allergies and infections of the eye can result in corneal diseases that may lead to blindness if left untreated. Glucocorticoids have been used to treat eye diseases pertaining to the cornea. However, the use of glucocorticoids sometimes comes with adverse effects. For example, chronic use of glucocorticoids is associated with the risk of development of cataract and glaucoma. Thus, further understanding of the role of glucocorticoid receptor (GR) signaling in the eye and specifically the cornea is required to improve therapeutic strategies. In this study, we utilized an immortalized human corneal epithelial cell line (HCE) to examine glucocorticoid function. By qRT-PCR and immunoblotting techniques, we found that GR is expressed in HCE cells. Confocal imaging revealed that stimulation of GR with the synthetic glucocorticoid dexamethasone was able to induce nuclear translocation of the receptor, indicating that corneal epithelial cells express a functional GR. Genome-wide expression profiling in HCE cells revealed that glucocorticoids significantly regulated 8399 probes, of which 97% of the probes were repressed, suggesting that transrepression is a predominant function of glucocorticoids in corneal epithelial cells. Ingenuity Pathway Analysis identified G-protein coupled receptor pathway as a highly-ranked pathway altered in HCE cells by dexamethasone treatment. Moreover, cell development, movement, morphology and cell-to-cell signaling were ranked as the top significant glucocorticoid-regulated

biological functions. Consistently, in vitro wound healing assay in HCE cells exhibited a remarkable delay in wound healing when treated with dexamethasone. This effect was reversible by treating with RU486, an inhibitor of GR, indicating that dexamethasone-induced delay in wound healing is mediated via GR. Taken together, our results demonstrate that glucocorticoids significantly alter the gene expression profile of corneal epithelial cells, which may be manifested in biological outcomes such as delayed wound healing.

NIEHS

kazgan, nevzat

Postdoctoral Fellow

Gene Expression

Intestine-specific deletion of SIRT1 alters systemic lipid and bile acid homeostasis in mice
SIRT1, the most conserved mammalian NAD⁺-dependent protein/histone deacetylase and the orthologue of yeast anti-aging protein Sir2, is a key metabolic sensor in various tissues in mammalian species. However, the role of SIRT1 in small intestine, an essential metabolic organ that is primarily involved in nutrient absorption and sensing, is still largely unknown. To elucidate the function of SIRT1 in intestinal metabolism, we generated a novel intestine-specific SIRT1 KO mouse model, SIRT1 IKO mice, by breeding SIRT1 floxed mice with Villin-Cre mice. Utilizing this mouse model, we demonstrate that intestinal SIRT1 is an important regulator of intestinal lipid and (ileal) bile acid absorption that feedback modulates systemic lipid and bile acid homeostasis. Deletion of SIRT1 only in the mouse intestinal epithelial cells results in decreased expression levels of lipid transporters SRB1 and CD36, proteins that are essential for prechylomicron complex formation. As a result, SIRT1 IKO mice have reduced total body fat percentage. Moreover, loss of SIRT1 in the intestinal epithelial cells leads to decreased intestinal HNF1a/FXR signaling pathway, transcription factors that play an important role in systemic bile acid metabolism, resulting in reduced expression of ileal bile acid transporter genes, Asbt and Osta/Ostβ. This defect decreases the ileal absorption of bile acids, leading to increased fecal bile acid output. We provide evidence that SIRT1 regulates the HNF1a/FXR signaling pathway through deacetylation of DCoH2, a dimerization cofactor of HNF1a. Deacetylation of DCoH2 facilitates dimerization and DNA binding function of HNF1a. Furthermore, in contrast to deletion of hepatic SIRT1, intestinal SIRT1 deficiency stimulates hepatic bile acid synthesis, leading to decreased contents of cholesterol and triglyceride in the liver and protecting against liver damage upon atherogenic diet feeding. Taken together, our findings uncover novel functions of SIRT1 in intestinal lipid and bile acid absorption and reveal a previously unknown molecular mechanism by which SIRT1 regulates the HNF1a/FXR signaling pathway. Our studies further point out that the same molecular mechanism can yield distinct pathophysiologies in the same metabolic pathway in different tissues, and suggest that tissue specificity should be considered when applying SIRT1 small molecule modulators-based therapeutic strategies to bile acid and cholesterol diseases.

NIEHS

ZeRuth, Gary

Postdoctoral Fellow

Gene Expression

The Krüppel-like protein Gli-similar 3 (Glis3) functions as a key regulator of insulin transcription
Transcriptional regulation of insulin in pancreatic beta cells is mediated primarily through enhancer elements located within the 5' upstream regulatory region of the preproinsulin (INS) gene. Recently, we demonstrated the Krüppel-like transcription factor, Glis3, can bind the

insulin promoter and positively influence insulin transcription while genome-wide association studies (GWAS) have identified GLIS3 as a risk locus for both type 1 and 2 diabetes. In this study, we examined in detail the activation of insulin transcription by Glis3 with the co-regulators, CBP/p300, Pdx1, NeuroD1, and MafA. Using quantitative RT-PCR and luciferase reporter assays, we demonstrate that Glis3 acts synergistically with Pdx1, NeuroD1, and MafA to activate the insulin promoter. We further show by means of co-immunoprecipitation assays that both the Glis3 N-terminus and C-terminal transactivation domain associate with CBP/p300 and that the observed synergism between the different transcription factors relies in large part on the recruitment of CBP/p300 by Glis3 to the insulin promoter. Our data show that Glis3 expression, the binding of Glis3 to GlisBS, and its recruitment of CBP are required for optimal activation of the insulin promoter not only by Glis3, but also by Pdx1, MafA, and NeuroD1 in pancreatic beta cells. Mutations in the GlisBS or siRNA-directed knockdown of GLIS3 diminished insulin promoter activation by Pdx1, NeuroD1, and MafA and chromatin immunoprecipitation (ChIP) analysis indicated that neither Pdx1 nor MafA were able to stably associate with the insulin promoter when the GlisBS were mutated. In addition, we showed that a single nucleotide mutation within the human INS promoter implicated in the development of neonatal diabetes similarly abated activation by Pdx1, NeuroD1, and MafA in the absence of exogenously expressed Glis3, which likely overcomes the reduced affinity for the mutated binding site to restore INS promoter activation. We therefore propose a model whereby recruitment of CBP/p300 by Glis3 provides a scaffold for the formation of a larger transcriptional regulatory complex that stabilizes the binding of Pdx1, NeuroD1, and MafA complexes to their respective binding sites within the insulin promoter. Taken together, these results indicate that Glis3 plays a pivotal role in the transcriptional regulation of insulin and may serve as a potential therapeutic target for the treatment of diabetes.

NIEHS

Cinghu, Senthilkumar

Postdoctoral Fellow

Informatics/Computational Biology

Meta-analysis identifies key determinants of embryonic stem cell identity and homeostasis
Embryonic stem cells (ESCs) can self-renew indefinitely and can differentiate into all derivatives of the 3 germ layers, making them an attractive model for regenerative medicine and disease modeling. Successful development of ESC-based therapies, however, largely depends on understanding the genetic network governing ESC self-renewal and differentiation. To characterize the genetic network controlling the ESC identity, we developed a novel bioinformatics pipeline for a systematic meta-analysis of 68 published gene expression datasets, and have rank-ordered all mouse genes based on their likelihood to have a role in ESC maintenance. Not surprisingly, master ESC regulator Oct4 is ranked number one, followed by Nanog and Sox2. Moreover, several other known regulators of ESC maintenance were ranked atop our gene list, along with a number of genes that have not been previously implicated in ESC biology. To identify novel regulators of mESC identity, we used RNAi-mediated loss-of-function experiments and phenotypic assays to test 49 candidate genes ranked within the top 2%. Depletion of 17 candidates showed cellular and molecular changes consistent with mESC differentiation, suggesting that these genes are critical for ESCs to maintain their self-renewal characteristics. To understand the roles of these potential regulators of ESCs, we studied our top-hit Nucleolin (Ncl). Ncl codes for a highly conserved protein abundant in stem and cancer cells. We show that Ncl depletion elevates endogenous reactive oxygen species (ROS) levels and p53 activity, resulting in p53-mediated suppression of Nanog and subsequent ESC

differentiation. We demonstrate that p53 depletion, Nanog overexpression, or antioxidant treatment restores the phenotype due to Ncl depletion. Furthermore, we show that Nanog's positive regulation of Ncl provides the means for Nanog to suppress p53 activity in a Ncl-dependent manner to maintain ESCs. Together, these findings support a conceptually novel mechanism involving a Ncl-dependent Nanog-p53 bistable switch regulating the homeostatic balance between self-renewal and differentiation in ESCs. Given that many cancers have elevated levels of Ncl and Nanog in addition to impaired p53 signaling, our findings have profound implications for understanding tumorigenesis. Altogether, our studies unearth a wealth of novel ESC regulators and reveal a previously unknown regulatory circuitry involving genes associated with traits in both ESCs and cancer.

NIEHS

Li, YuanYuan

Postdoctoral Fellow

Informatics/Computational Biology

T-KDE: A method for analyzing genome-wide protein binding patterns from ChIP-seq data

A protein may bind to its target DNA sites constitutively, i.e., in more than 90% of cell lines regardless of cell type. Intuitively, constitutive sites should be biologically functional. For many proteins, however, what proportion of their binding sites are constitutively bound remains unknown. The lack of both ChIP-seq data across multiple cell types and a robust analytic method made addressing this question difficult. Recently, the ENCODE consortium has profiled genome-wide binding patterns of many transcription factors (TF) in multiple cell lines. A conventional approach to identify constitutive binding sites would divide the genome into fixed width bins and count the number of peak centers within each bin. However, this binning method suffers from several problems including a boundary effect. We propose a method, T-KDE, which combines a binary range tree, a kernel density estimator and a mode finding algorithm to identify the locations of constitutive peak centers from multiple cell lines. The binary tree algorithm partitions the locations of all peak centers throughout the genome into smaller subgroups. Then, within each subgroup, T-KDE estimates a density curve for the peak-center locations and finds its mode. We define a mode as a constitutive site when its neighborhood contains peak centers from more than 90% of the cell lines. We applied our method to all ENCODE human TF binding site datasets with six or more cell lines. We found that the number of constitutive sites for different TFs varied from hundreds to tens of thousands. Rad21 had the highest proportion of constitutive sites (8.0%). Not surprisingly, the proportion of constitutive sites for the transcriptional co-activator protein p300 was negligible because many p300 sites are tissue-specific enhancers. Interestingly, many Pol II sites (4,733) were also constitutive. Gene ontology (GO) analysis showed that genes with constitutive Pol II binding sites in their promoters are highly enriched with GO terms such as cellular metabolic process ($p=8e-177$) and cell cycle ($p=1e-46$), suggesting that these constitutive sites are biologically meaningful. In conclusion, T-KDE is general and can be applied directly to ChIP-seq peak data to identify constitutive sites that are bound by a protein either directly or indirectly across multiple cell lines. Besides the constitutive sites for a given TF, T-KDE can be used to identify genomic "hot spots" that were bound by different proteins.

NIEHS

Robertson, Sabrina

Postdoctoral Fellow

Neuroscience - Cellular and Molecular

Developmental origins of central norepinephrine neuron diversity

Central norepinephrine (NE)-producing neurons comprise a diverse population of cells that modulate many disparate behaviors and physiological processes such as attention, stress and appetite. Reflected in this diversity of function, central NE neurons differ in their anatomical location, connectivity, and response to disease and environmental insult. At present, the mechanisms that generate this diversity are unknown, as are molecular markers capable of distinguishing individual NE subtypes. Such knowledge is fundamental to understanding the basis of selective NE neuron subtype vulnerability to disease and environmental insult, and for gaining selective access to individual subpopulations of NE neurons for experimental study. To begin filling this gap, we used an intersectional recombinase-based genetic approach to elucidate the lineal relationship between molecularly distinct NE progenitor populations in the developing hindbrain and mature NE neuron subtype identity. We have identified four genetically separable subpopulations of NE neurons that differ in their anatomical distribution and have revealed previously unappreciated lineal relationships between NE neurons located in distant regions of the brainstem. We have also found that these subpopulations differ in their axon morphology and that each projects to a unique set of targets. Our ability to visualize these subpopulations in complete isolation has uncovered provocative details of the structure and organization of the NE efferent system, including the identification of a projection to the prefrontal cortex that challenges current dogma that the locus coeruleus is the sole source of NE projections to the cortex. This novel molecular classification of the NE system provides, for the first time, multiple molecular points of entry for functional manipulation of individual NE circuits with unprecedented precision, at all stages of development. Phenotypes resulting from such manipulation promise unique insights into complex NE-related behavioral and physiological processes including arousal, attention, mood, memory, appetite, and homeostasis.

NIEHS

Wang, Qingshan

Visiting Fellow

Neuroscience - Neurodegeneration and Neurological disorders

Endogenous substance P regulates microglial density in substantia nigra through neurokinin-1 receptor/NADPH oxidase axis-mediated chemotaxis

The distribution and density of microglia differ greatly among brain regions. We have previously reported the density of microglia in the substantia nigra (SN) is 5 fold higher than other brain regions, implicating why the SN is selectively more sensitive to the neuroinflammation-mediated pathogenesis of Parkinson's disease (PD). However, the reason for the high density of nigral microglia is not known. We hypothesized that substance P (SP), a major endogenous pro-inflammatory peptide stored at high concentrations in the SN, is a major regulator for the high density of nigral microglia. Developmental studies revealed that nigral microglial density peaked around postnatal 30 (P30). In contrast, SP was detected at high levels in SN as early as P1. Transgenic mice incapable of producing endogenous SP (TAC1^{-/-}) exhibited reduced nigral microglial density compared to wild type (WT) controls. This finding led us to speculate that SP may attract the migration of microglia toward to the SN. We confirmed the chemotactic potential of SP in vitro by demonstrating that SP induced the migration of microglia in a transwell culture system. In vivo studies further showed facilitated directional migration of transplanted enhance-green fluorescent protein (EGFP)-labeled microglia towards the brain region injected with SP in TAC1^{-/-} mice. Additional studies on the signaling pathways mediating chemotaxis by SP revealed that both neurokinin-1 receptor (NK1R), the G-protein coupled receptor for SP, and NADPH oxidase (NOX2, a key superoxide-producing enzyme on microglia)

are necessary for the chemoattractant properties of SP. Whereby SP-induced migration of microglia prepared from either NK1R^{-/-} or NOX2^{-/-} mice was greatly reduced. Furthermore, pharmacological inhibition of NK1R or NOX2 showed a similar inhibition. Evidence suggesting a cross talk between NK1R and NOX2 was provided by showing SP-stimulated NOX2 activation, as measured by membrane translocation of p47phox (a cytosolic subunit of NOX2) and release of superoxide were mediated through NK1R/ β -arrestin1-dependent pathways. In summary, these results strongly suggest that SP is capable of recruiting microglia to the SN through a novel NK1R-NOX2 axis-mediated pathway, accounting for the high microglial density in the SN. These findings further suggest that intimate interactions between SP and microglia in the SN play a critical role for the pathogenesis of PD.

NIEHS

Campos, Christopher

Postdoctoral Fellow

Pharmacology and Toxicology/Environmental Health

transient barrier disruption increases transport function at the blood-brain barrier

The blood-brain barrier (BBB) resides within the brain capillary endothelium and regulates the exchange of endogenous solutes and xenobiotics between blood and brain. The BBB is a physical barrier characterized by the presence of tight junctions (TJ) as well as a metabolic barrier consisting of ATP-driven drug efflux pumps including P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp). The molecular organization and intracellular communication required to maintain this dynamic barrier is complex and poorly understood. Characterizing this communication may reveal a novel therapeutic target allowing for improved brain drug delivery. Clinically, hyperosmolar agents including mannitol are used to transiently disrupt the TJ in patients with malignant glioma, facilitating the administration of chemotherapeutics. Additionally, hyperosmolar agents are used in the treatment of brain edema in subjects with acute ischemic stroke. We hypothesize that the transient disruption of the TJ by hyperosmotic stress will lead to compensatory increases in drug efflux transporter function at the BBB, thereby limiting the penetration of therapeutic drugs into the brain. To confirm hyperosmotic stress-induced transient disruption of TJ, rat brain capillaries were loaded with Texas Red (TR), a small molecule membrane impermeable dye and imaged during a one-minute exposure to 100mM mannitol. This treatment resulted in leakage of TR from the capillary lumen into the surrounding media, indicating a mechanical disruption of TJ. Next, we evaluated the consequence of this transient disruption of TJ on BBB transporter function. For these studies, capillaries were exposed to hyperosmotic mannitol media and after 30 minutes normal media was replaced. Protein expression and transport function were assayed 3 hours later. Here, we report that transient disruption of the TJ following hyperosmotic stress increased specific transport function of both P-gp and Bcrp and western blot analysis indicated increased protein expression of both transporters, indicating for the first time that transport function may be mechanistically connected to TJ disruption. These results suggest that hyperosmotic stress-induced increases in transport function and expression may limit the efficacy of therapies administered following transient barrier disruption.

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Pharmacology and Toxicology/Environmental Health

Activator Protein 1 Regulation of Human CYP2C9 Expression by Electrophilic Stress Involves MAPK Activation and DNA Looping

CYP2C9 is an important human enzyme that metabolizes both commonly used therapeutic drugs and physiologically important endogenous compounds. CYP2C9 expression is induced by drugs such as rifampicin, hyperforin, phenytoin and dexamethasone. Exposure to these drugs is partly responsible for the inter-individual variability in CYP2C9 expression and metabolism of CYP2C9 substrates. For example, studies in humans have reported enhanced clearance of drugs such as tolbutamide and phenytoin by treatment with rifampicin. Most drugs and foreign compounds are metabolized in the liver to electrophilic and reactive metabolites. Moreover, drugs capable of undergoing redox cycling induce oxidative stress by generation of semiquinone radicals and reactive oxygen species. It not yet known whether the induction of CYP2C9 expression by drugs and xenobiotic is due to secondary effects such as formation of the electrophilic metabolites and induction of oxidative stress. Both CYP2C9 and CYP2C19 expression were induced by tert-butylhydroquinone (tBHQ) in primary human hepatocytes. As a pro-oxidant, tBHQ regulates the expression of cytoprotective genes by activation of redox-sensing transcription factors, such as the nuclear factor E2-related factor 2 (Nrf2) and activator protein 1 (AP-1). The promoter region of CYP2C9 contains putative AP-1 sites at positions -2201 and -1930 which are highly conserved in CYP2C19. Ectopic expression of Nrf2 had no effect on CYP2C9 promoter activity. We demonstrate by promoter luciferase assay that CYP2C9 is transactivated by cFos and JunD heterodimer. Using inhibitors of mitogen activated protein kinases, we showed that ERK and JNK are essential for tBHQ-induced expression of CYP2C9 by increased expression of cFos and phosphorylation of JunD, respectively. Binding assays demonstrate that cFos distinctly interacts with the distal and JunD with the proximal AP-1 site. Because cFos regulates target genes as heterodimer with Jun proteins, we hypothesize that DNA looping is required to bring together the distal and proximal AP-1 sites. Chromosome conformation capture (3C) analyses confirmed the formation of DNA loop at the CYP2C9 promoter mediated by cFos bound to the distal and JunD on proximal site for activation of CYP2C9 transcription. These results suggest that oxidative stress generated by exposure to drugs and xenobiotic may induce the expression of CYP2C9 and alter the metabolic clearance of numerous CYP2C9 drug substrates.

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Pharmacology and Toxicology/Environmental Health

Glucocorticoid Receptor Regulation of P-glycoprotein at the Blood-Brain and Blood-Spinal Cord Barriers

The blood-brain (BBB) and the blood-spinal cord (BSCB) barriers, comprised of the brain and spinal cord capillary endothelia, constitute a primary obstacle to CNS drug delivery. An important element of barrier function is the ATP-driven drug efflux transporter, P-glycoprotein (P-gp), which exhibits broad substrate specificity and high luminal plasma membrane expression in brain and spinal cord capillaries. We previously demonstrated that several ligand-activated nuclear receptors regulate the expression and activity of P-gp. Here, we examined whether the glucocorticoid receptor (GR), a ligand-activated nuclear receptor targeted by both natural and synthetic glucocorticoids, regulates P-gp at CNS barriers. Naturally occurring glucocorticoids regulate a wide range of physiological effects including gluconeogenesis, homeostasis, and apoptosis. However, the role of these hormones in maintenance of CNS barriers remains unresolved. Furthermore, the effects of synthetic glucocorticoids, a broad class of widely prescribed anti-inflammatory drugs, on CNS barriers are poorly understood. However, these

potent anti-inflammatory synthetic glucocorticoids are a mainstay in the treatment of cerebral edema and spinal cord injury. We hypothesize that both natural and synthetic glucocorticoids alter the expression and activity of P-gp at CNS barriers, thereby modifying drug delivery to the CNS. We confirmed the expression of GR in both CNS barriers by qPCR and immunoblotting and demonstrated that depletion of corticosterone via adrenalectomy significantly decreased the expression and activity of P-gp in both the BBB and the BSCB. In-vivo treatment of both intact and adrenalectomized rats with the synthetic glucocorticoid dexamethasone significantly increased P-gp activity and protein expression in both CNS barriers. In-vitro exposure of brain and spinal cord capillaries to dexamethasone also increased P-gp expression and activity and the GR antagonist, RU-486, abolished these increases. These results demonstrate that the endogenous glucocorticoid, corticosterone, maintains P-gp expression and activity at the BBB and BSCB while the synthetic glucocorticoid, dexamethasone, in the presence or absence of endogenous glucocorticoids, increases the activity and expression of P-gp in a GR-dependent manner. Thus, natural glucocorticoids have a protective role in maintaining CNS barriers, while synthetic glucocorticoids may hinder delivery of therapeutic drugs to the CNS.

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Protein Structure/Structural Biology

The Polymerase Reaction Exposed: Observing a DNA Polymerase Choose Right from Wrong
DNA polymerase (pol) β is a model polymerase involved in gap-filling DNA synthesis utilizing two metals to facilitate nucleotidyl transfer. Previous structural studies have trapped catalytic intermediates by utilizing substrate analogues (dideoxy-terminated primer or non-hydrolysable incoming nucleotide). To identify novel intermediates during catalysis, we now employ natural substrates (correct and incorrect nucleotides) and follow product formation in real time with thirteen different crystal structures. We are able to observe molecular adjustments at the active site that hasten correct nucleotide insertion and deter incorrect insertion not appreciated previously. A new metal binding site is transiently formed during correct, but not incorrect, nucleotide insertion. Additionally, long incubations indicate that pyrophosphate more easily dissociates after incorrect, compared to correct, nucleotide insertion. This appears to be coupled to subdomain repositioning that is required for catalytic activation/deactivation. The structures provide insights into a fundamental chemical reaction that impacts polymerase fidelity and genome stability.