

Summer Undergraduate Research Program

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Gerstner Sloan-Kettering
Graduate School of Biomedical Sciences

Overall structure of the oxoG•C complex. OxoG(*anti*) at the (−1) position forms a base pair with the 3′-terminal C14 base of the primer strand. The next template base C5 is paired with an incoming dGTP at the active site. [Image courtesy of the Patel laboratory]

Preface

The mission of Gerstner Sloan-Kettering Graduate School of Biomedical Sciences is to advance the frontiers of knowledge by providing to gifted and creative students in an interactive, innovative, and collegial environment the education and training they need to make new discoveries in the biological sciences.

PhD Program

The goal of the Gerstner Sloan-Kettering graduate program is to train a cadre of outstanding scientists who will exploit new advances and developing fields in biomedical sciences and apply their training directly to advancing the understanding of human disease. The School offers the next generation of basic scientists a program to study the biological sciences through the lens of cancer -- while giving students the tools they will need to put them in the vanguard of research that can be applied in any area of human disease.

Summer Undergraduate Research Program

The Gerstner Sloan-Kettering Graduate School sponsors a ten-week research program for outstanding undergraduate students who are interested in pursuing a career in biomedically related sciences. The explosion in knowledge that has driven recent progress in the diagnosis and treatment of cancer reflects the vitality of laboratory science at Sloan-Kettering. Students learn from scientists who are conducting research in areas such as developmental biology, genetics, structural biology, computational biology, cellular and molecular sciences, immunology, molecular pharmacology and chemistry, among others. Students have the opportunity to:

- Obtain hands-on research experience in cutting-edge laboratories
- Interact with faculty, postdoctoral fellows, and graduate students
- Attend a weekly seminar series, with presentations by Sloan-Kettering faculty
- Attend workshops such as presentation skills, interview skills, and others
- Attend and present at works-in-progress sessions with the cohort of summer students
- Present their research at a special poster session at the end of the program

Further information about the school and its programs can be found at www.sloankettering.edu or by contacting us at gradstudies@sloankettering.edu.

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Aligning Pathways: Towards an Integrated Map of Cellular Processes

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Biological pathways are valuable resources for a wide spectrum of computational methods in system biology, ranging from analysis of high-throughput profiles to simulation. Unfortunately, most pathway information is represented in the form of diagrams and textual descriptions that are not computationally accessible. To address this problem, different groups started to curate literature and create pathway data in computable formats. Within the last two years, the number of publicly available pathway databases have increased from 123 to 300.

Syntax and semantics of these databases, however, can be very diverse, since they are often based on different ontologies and organisms. BioPAX, a standard pathway exchange language, provides a common format and semantics is gaining support among leading data providers. Although this is a big step towards an integrated cell map, it is not sufficient. Pathways, similar to a jigsaw puzzle, are still highly fragmented. We need to find similar portions of pathways across different data sources to align and merge them. Loosely speaking, this is analogous to genome assembly from sequence fragments.

Patch is such an algorithm for pathway integration. It first finds similar reactions across heterogeneous data sources. When matching reactions, it maximally uses rich BioPAX representation and considers the state of the molecules (e.g. phosphorylation), subcellular locations and molecular complexes. These reaction-similarity results are then extended to the reaction networks to match portions of pathways. We have successfully tested our algorithm on data from several pathway databases. By comparing matched pathways, we were able to detect curation errors and representation differences. Patch is very useful for searching pathway databases for similar pathways, cross-validating results and ultimately pathway integration. Patch will be released as a free and publicly accessible web service.

Characterizing the Cytotoxic Effects of Single-Walled Carbon Nanotubes

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Single-walled carbon nanotubes (SWNTs) are pure carbon cylinders of 1.0 nanometer in diameter and up to several microns in length. SWNTs have become a major interest in the biomedical field, in particular as a vehicle for radioimmunotherapy or chemotherapy, and as a carrier for vaccines. Chemical functionalization of SWNTs allows for the coupling of multiple copies of antibodies, radioisotope chelates, DNA, or immunogenic peptides to a single tube for an increase in potency and to add multifunctionality. However, the cytotoxic effects of the various modified nanomolecular scaffolds are unknown. We investigated the effects of SWNT treatment on human promyelocytic leukemia cells (HL60s), a model cell line for tumor radioimmunotherapy, and human dendritic cells (DCs), immune cells primarily involved in antigen presentation. Cell viability was measured using the Alamar Blue and ATPlite assays, both of which measure overall *in vitro* cell metabolic activity, while apoptotic cells were identified through annexin-V and propidium iodide staining. Preliminary data suggests that neither aldehyde nor amine functionalized SWNTs have toxic effects on HL60s after 24 or 48 hours of treatment up to a concentration of 100 μ g/mL, while DOTA-functionalized SWNT treatment results in a slight loss in cell viability at the highest doses (10-100 μ g/mL) after 24-96 hours of treatment. This coincides with tritiated thymidine data suggesting a drop in cell proliferation at the same doses. Treatment of DCs with aldehyde functionalized SWNTs, the scaffold for immunogenic peptide attachment in vaccine studies, exhibits no effect on cell viability at any dose up to 100 μ g/mL after 24, 48, and 72 hours. Confocal microscopy of DCs treated with aldehyde functionalized SWNTs at similar concentrations shows rapid and robust uptake of the modified SWNTs. Overall, these data provide evidence that SWNTs alone do not cause severe *in vitro* cytotoxicity and therefore may be useful in biomedical applications.

Probing the Temporal Regulation of the Initiation of Meiotic Recombination

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DNA double-strand breaks (DSBs) catalyzed by Spo11 initiate meiotic recombination. The formation of meiotic DSBs exhibits temporal coupling to DNA replication. This mechanism operates on a regional basis as opposed to cell-wide basis, since delaying DNA replication by deleting all active replication origins from the left arm of chromosome III (chr *III-L*) causes a delay of approximately equal length in DSB formation specifically on that chromosome. By what molecular mechanism might DSB formation and DNA replication be linked? Sister chromatid cohesion is established soon after DNA replication, and a meiosis specific component of the cohesin complex, Rec8, has been previously implicated in key meiotic processes including sister chromatid cohesion, synaptonemal complex assembly, homologous recombination, and specification of Spo11 distribution. We thus hypothesize that the establishment of sister chromatid cohesion is necessary for the observed temporal coupling between DNA replication and DSB formation. Previous attempts at characterizing the role of Rec8 in the temporal regulation of meiotic DSB formation were inconclusive because *rec8Δ* exhibits a partial DSB repair phenotype that confounds assessment of DSB timing. We therefore combined the *rec8Δ* mutation with repair-defective *dmc1Δ* mutation. A *rec8Δ dmc1Δ* strain showed no delay in DSB formation in the origin-deleted chr *III-L*, suggesting that the coupling between DNA replication and DSB formation was abolished in *rec8Δ*. We also tested this hypothesis in *rec8Δ sae2Δ*. The *sae2Δ* mutation, which prevents DSB resection and leaves Spo11 covalently attached to DSB ends, causes a 4-fold reduction in DSB formation on the origin-deleted chr *III-L*. We found that a *rec8Δ sae2Δ* strain demonstrated less DSB repression (only 2-fold) in the origin-deleted chr *III-L*. Taken together, these results suggest that Rec8 and/or cohesion is involved in the temporal coupling between DNA replication and DSB formation in meiosis.

Characterization of the AdnAB Gene, a DSB-Resecting Enzyme Essential to Homologous Recombination, in a *Mycobacterium Smegmatis* Model

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DNA double strand breaks (DSBs) are a potentially lethal threat to cell survival. Bacteria repair broken DNA primarily through homologous recombination (HR). AdnAB is a mycobacterial enzyme recently identified as a helicase-nuclease heterodimer involved in the resection of DSBs, the first step in the repair pathway. It is homologous to the *Bacillus* AddAB enzyme, and is thought to perform a similar function as RecBCD, an *E. coli* DSB-resecting enzyme also present in the mycobacterial genome.¹ AdnAB is currently known to be an ATP-dependent nuclease which uses a molecular ruler to measure from the 5' end to the cleavage site. It is also known that the dual nucleases function distinctly, as do the helicases.¹ Here we detail an ongoing effort to further characterize the role of AdnAB in DNA repair in vivo. Survival assays performed against *Mycobacterium smegmatis* $\Delta recA$, $\Delta adnAB$, $\Delta recBCD$, and $\Delta adnAB/recBCD$ double mutants show no phenotype in the RecBCD deletion, supporting prior research which has found a similar lack. The $\Delta adnAB$ and $\Delta adnAB/recBCD$ double deletion mutants show a distinct but similar phenotype, with less cell survival when treated with the DNA damaging agents methyl methanesulfonate (which stalls replication forks)¹ and mitomycin C (a DNA crosslinker). A control experiment using the $\Delta recA$ strain (RecA is a necessary protein in the HR pathway) showed higher sensitivity to the damaging agents, indicating that HR is the pathway of DNA repair being used, and that sensitivity in our test deletions can be attributed to HR deficiency. We conclude that AdnAB is necessary for the HR pathway to function. We are currently working to create four distinct mutants, each with one of the four major domains of AdnAB disabled, in order to further explore the function of each individual enzyme component.

A Dynamic Role for *Engrailed* in Fissure Formation and Patterning of the Developing Murine Cerebellum

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The cerebellum begins to develop from dorsal rhombomere 1 of the hindbrain during embryonic day 9 (e9.0) in the mouse. By e15.5, the cerebellum is a saddle-shaped structure that has distinct molecular domains, and between e17.0 and e18.5 four primary fissures form which divide the cerebellum into lobules. The main cell types involved in the process of fissure formation, granule and Purkinje cells, originate from two main regions of neurogenesis in the cerebellar primordium, the rhombic lip and ventricular zone, respectively. The *engrailed* (*en*) gene, best known for setting up segmental compartments in *Drosophila* through regulation of the Hedgehog signaling pathway, has two mouse orthologs that play integral roles in three-dimensionally patterning the cerebellum. In the complete absence of *En1* the cerebellar anlage is never specified, resulting in the absence of any cerebellar structures. However, in the absence of *En2* the cerebellum forms but contains significantly fewer cells and fails to correctly pattern two of the fissures. To date, the specific mechanisms by which *En* acts during mouse cerebellar development are not known. We show that between e15.5 and e18.5, *En1* and *En2* are expressed in dynamic anterior-posterior bands as well as in one medial-lateral stripe. Using cell lineage-specific gene reactivation of *En2* in an *En2* null background, we demonstrate that *En2* has an important role in regulating cells derived from both the rhombic lip and the ventricular zone during early cerebellar patterning. Conditional reactivation of *En2* in all cerebellar precursor cells at e10.0 rescues the *En2* null phenotype, allowing a wild-type cerebellum to form. We propose that *En* plays a crucial role in the early specification and/or organization of cerebellar precursor cells derived from both the rhombic lip and the ventricular zone, as well as in providing temporal and spatial cues necessary for fissure formation and foliation during development of the murine cerebellum.

Role of Vascular Cell Adhesion Molecule-1 Expressed in Breast Cancer Cells During Lung Metastasis

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The high mortality rate of breast cancer is primarily due to metastasis, and the lung is one of the most common sites for metastasis. We have previously isolated discrete breast cancer populations that metastasize to the lung. Genetic analysis from these metastatic variants identified a gene set that can further predict lung relapse in breast cancer patients and vascular cell adhesion molecule-1 (VCAM-1) is one of the lung metastasis signature genes. VCAM-1 is an adhesion molecule predominantly expressed on endothelial cells and immune cells, but the function of VCAM-1 expressed on tumor cells is unknown. In tumor microenvironments, multiple stromal cells express integrins, the specific counter-receptors of VCAM-1. Moreover, the binding of VCAM-1 by integrins on the cell surface can initiate outside-in signaling via the intracellular domain of VCAM-1. Therefore, we hypothesize that VCAM-1 serves as a mediator in tumor-stromal interactions to facilitate lung metastasis. To address this hypothesis, we generated tumor cells overexpressing two different forms of VCAM-1: the full-length form and a truncated form of VCAM-1 lacking the intracellular domain. The level of mRNA and protein were detected through RT-PCR and western blots respectively to ensure that VCAM-1 had been successfully overexpressed. We then used an adhesion assay to determine the binding functionality of VCAM-1 on the surface of tumor cells when its intracellular domain was absent. Our data displayed that the truncated form of VCAM-1, when compared to the full length form, supported macrophage adhesion at similar level. Finally, tumor cells were then intravenously injected into immune deficient mice and these mice were imaged weekly to observe lung metastasis. The results indicated that truncated form of VCAM-1 delayed the lung metastasis in vivo. Based on our results, we plan to further delineate the signaling pathways activated via the intracellular domain of VCAM-1 to facilitate lung metastasis.

Effect of Circulating MCP-1 on Monocyte Emigration from the Bone Marrow

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Circulating blood monocytes supply peripheral tissues with macrophage and dendritic cell precursors. Chemokine receptor-mediated recruitment of monocytes is essential for defense against microbial infections. Recruitment of Ly6C^{high} inflammatory monocytes from bone marrow to the circulation depends on CCR2, a chemokine receptor that responds to monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a protein that is secreted upon microbial infection or during inflammatory responses. MCP-1 and CCR2 mediated signals trigger inflammatory monocyte emigration from bone marrow, and defects in these signals render mice highly susceptible to *L. monocytogenes*, *M. tuberculosis*, *T. gondii*, and *C. neoformans* infections. The focus of our research was to determine whether MCP-1 circulating in the bloodstream mediates monocyte emigration from the bone marrow. To investigate this, our first step was to generate recombinant, LPS-free MCP-1 for administration to mice. Our strategy was to engineer a His-tag on the C-terminus of MCP-1. We designed an MCP-1-His construct and used overlapping PCR to generate the cDNA flanked by EcoR1 and XhoI sites. The PCR product was digested with restriction enzymes and ligated into expression vector pcDNA5.3. The ligated plasmid was introduced into DH5 α and purified by Maxiprep. The pcDNA5.3 MCP-1-His construct was then transfected into 293 cells using Lipofectamine 2000 and 48 hours post transfection, media from transfected cells was harvested and the MCP-1 concentration was measured using ELISA. We are currently determining the concentration of MCP-1 obtained from transfected cells and we plan to purify and concentrate MCP-1-His protein using a Nickel column. Upon obtaining sufficient quantities of recombinant protein, we will intravenously administer MCP-1 protein to MCP-1^{-/-} mice and measure the number of monocytes in the bloodstream by flow cytometry. This study will improve our understanding of the chemokine-mediated monocyte recruitment process.

Understanding the Mechanisms that Cause Resistance to BCL2 Family Inhibition

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Programmed cell death, or apoptosis, plays an integral role in development, growth, and disease. Mechanisms that control apoptosis are altered in cancer. B-cell lymphoma-2 (BCL2) family proteins, defined by the presence of a small amino acid sequence the BCL2 homology (BH) domain, are essential regulators of apoptosis. There are more than 20 BH domain containing proteins encoded in mammalian genomes and they can be divided into two distinct classes depending on their ability to antagonize or protagonize cell death. There are six anti-apoptotic family members (BCL2, BCLxl, BCLw, MCL1, BFL1, BCLb) and over-expression of any one of these can drive tumorigenesis. An effort to control the function of BCL2 proteins has lead to the production of multiple small molecule inhibitors, of which many are currently in clinical trials. One such small molecule inhibitor, ABT737 (ABT), is an antagonist of anti-apoptotic proteins BCL2, BCLxl, and BCLw, but not MCL1, BFL1, and BCLb. Here, ten leukemia and lymphoma cell lines with various BCL2 family protein expression patterns were analyzed. In order to determine their relative sensitivity to ABT, cell lines were treated with drug and cell survival was assessed. We show that sensitivity or resistance to the drug is not easily defined simply by the expression pattern of anti-apoptotic BCL2 proteins. Therefore, we performed further experiments to identify the mechanisms that determine sensitivity to ABT. These experiments included assessing whether loss of function of the BCL2 family members by shRNA-mediated knockdown could confer sensitivity in ABT resistant cell lines. Conversely, we evaluated whether over-expression of BCL2 family members would lead to ABT resistance in otherwise sensitive cells. This research could provide a more complete understanding of this biochemical pathway and help determine if a cancer patient could be aided by BCL2 family antagonist therapies.

The Role of CtIP in Microhomology-Mediated End Joining in Class Switch Recombination

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B lymphocytes are essential components of the mammalian immune system as they make immunoglobins (Ig), or antibodies, that can recognize and bind antigens derived from pathogens. For an efficient immune response, B cells somatically modify their genome in a reaction termed class switch recombination (CSR). During CSR, a B cell switches from producing IgM to one expressing a secondary Ig isotype such as IgG, IgE or IgA, each having a distinct effector function. CSR is a deletional-recombination reaction that proceeds via the generation of DNA double strand breaks (DSBs) into defined regions of the Ig locus termed switch (S) regions. The distal S region DSBs subsequently join and the intervening chromosomal fragment is lost. It is now clearly established that the B cell specific DNA cytidine deaminase AID is required for the generation of the DSBs; however, the mechanism that joins the DSBs during the completion phase of CSR is poorly understood. Two pathways have been proposed to participate in the end-joining phase of CSR- non-homologous end-joining (NHEJ) that does not rely on any homology between the DSBs been ligated and Microhomology-Mediated End Joining (MMEJ) that requires between 4-25 nucleotides of homology between the participating DSBs. While the NHEJ pathway has been well-defined, we have very limited knowledge of the components of the MMEJ reaction other than a requirement of the DNA end-processing factor CtIP. To directly determine the requirement of and interplay between the two DNA repair pathways in CSR, we have used shRNA techniques to knock down individually, or in combination, components of the NHEJ (Ku70, DNA ligase IV) and MMEJ (CtIP) pathways in a B cell line. The modified cell lines were then assayed for CSR and analyzed for the extent of microhomology at the recombined S sequences. Our studies provide novel insights into the end-joining mechanism operational during CSR.

Investigating the Role of Heparanase-1 in Metastasis of Human Breast Cancer Cells to the Brain

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The vast majority of fatalities from breast cancer are due to metastases, particularly to the brain; yet the mechanisms underlying this organotropism remain largely unknown. Several studies have shown a positive correlation between elevated expression levels of the matrix-degrading enzyme Heparanase-1 (HPSE) and poor patient survival. Mechanistically, HPSE is likely to play two key roles in local invasion and colonization of distant sites due to its role as the obligate heparan sulfate proteoglycan (HSPG)-degrading enzyme. First, matrix-degrading enzymes are required to break down the extracellular matrix (ECM) and basement membrane both locally and at the secondary site. Specifically for brain metastases, tumor cells must cross the blood-brain barrier, and the high concentration of HSPGs here make HPSE a likely candidate for promoting this process. Second, HPSE can establish or strengthen growth signaling loops by releasing HS-bound growth factors and other small bioactive molecules from the ECM to contribute to the pro-tumorigenic microenvironment. Previous work from this lab using a xenograft mouse model of metastasis has shown that HPSE expression levels are four-fold higher in brain-homing derivatives relative to the parental human breast cancer cell line (MDA-MB-231) whereas lung-homing fractions remain the same and bone-homing HPSE levels drop. Additionally, inhibiting HPSE in the RIP1-Tag2 model of pancreatic cancer results in a dramatic decrease in invasion, while overexpression of HPSE produces a more invasive phenotype. To define the role of HPSE in the metastatic process, we created a sequence-matching shRNA to knockdown HPSE in the brain-homing cell line. We will present data from analysis of this HPSE-knockdown line, including invasion, proliferation, and apoptosis *in vitro* assays, as well as *in vivo* incidence of metastatic secondary tumors following intracardiac injection of these cells into athymic nude mice.

Development of a Systematic Synthesis Toward Spiroaminals

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Spiroketals are structural motifs that are commonly found in biologically active natural products and have attracted much attention in organic synthesis. Their wide range of stereochemical diversity and ability to bind to a variety of different biological targets have made spiroketals an interesting scaffold for library synthesis. Successful development of a systematic synthesis of spiroketals in our lab has in turn led to the synthesis of spiroaminals, another member of the spirocycle family. Spiroaminals are also prominently featured in natural products and are found in azaspiracids—natural products that are common in shellfish and which are known to possess many toxic and harmful qualities. Herein, we report our efforts to develop a stereocontrolled synthesis of spiroaminals using kinetic spirocyclization reactions.

Characterization of Gag Perinuclear Clusters with Fluorescence Recovery after Photobleaching

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Retroviral Gag polyproteins are sufficient for the formation of virus like particles (VLPs). Gag proteins are synthesized in the cytosol and then transit via an unknown trafficking pathway to the plasma membrane, where virions bud and are released from the cell. Earlier studies on the trafficking of HIV-1 Gag have found that Gag accumulates in perinuclear clusters before moving to its final location on the plasma membrane. The nature of these perinuclear clusters is not yet known. We used fluorescence recovery after photobleaching (FRAP) to analyze the mobility of Gag-GFP fusion proteins into perinuclear clusters. After photobleaching, the perinuclear clusters fully regained their fluorescence rapidly (97% recovery with a $t_{1/2}$ of 3.69 sec), and retained their original size, shape and position. Similar results were obtained with a GagGFP mutant that cannot be myristoylated as well as a GagGFP mutant that cannot multimerize, implying that membrane binding and Gag multimerization are not required for transit of Gag into and out of the perinuclear clusters. By contrast, Gag at the plasma membrane was stable and exhibited little to no recovery after photobleaching. Three hours after treatment cycloheximide, COS-1 cells lacked any perinuclear clusters although the population of cytoplasmic Gag was still present. This suggests that maintenance of the perinuclear clusters may require the synthesis of either new Gag or another protein. To follow the fate of the Gag molecules in the perinuclear clusters, we used a photoactivatable (PA) version of Gag-GFP. After activation of PA-GagGFP, Gag exited the perinuclear clusters and spread to other nearby perinuclear clusters. Repeated bleaching of one perinuclear cluster eliminated fluorescence in all other clusters and in the cytoplasm. These results suggest that a dynamic pool of cytoplasmic HIV-1 Gag continually enters and exits the perinuclear clusters prior to trafficking to the plasma membrane.

Efforts Toward the Total Synthesis of Aconitine Alkaloids

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For many generations the roots of *Aconitum* plants have been used in herbal preparations for their analgesic effects, in the treatment of certain heart conditions, and as poisons. The primary chemicals of interest in these traditional medicines are the aconitine alkaloids. Several compounds within this class show potent Na⁺ ion channel activity, ranging from the ion channel activation of aconitine to the ion channel blocking of lappaconitine. The biological effects exhibited by the aconitine alkaloids demonstrate the diverse utility of these compounds, and suggests that a wide variety of drugs could be developed from small changes in their molecular architecture. The promise of unforeseen medical applications, as well as the inherent structural complexity of the aconitine family makes them ideal targets for a total synthesis. Recently, our lab has undertaken a total synthesis of this class of compounds; a key step of which is a Diels-Alder reaction between a cyclopropene and a substituted cyclopentadiene. The product of this initial cycloaddition reaction dictates the stereochemistry of all subsequent steps, and thus the enantiopurity of the final product. Therefore, in order to develop an enantioselective synthesis of the aconitine family an asymmetric cycloaddition strategy must be devised. Here we report progress toward achieving a total synthesis of the aconitine alkaloids which begins with an unprecedented Diels-Alder reaction of a chiral cyclopentadiene.

Determining Components of the Loner Mediated Pathway during *Drosophila* Myoblast Fusion

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Cell-cell fusion is an integral part of several important biological processes. The formation and repair of muscle relies on cell-cell fusion. In *Drosophila*, each muscle originates from a single Founder Cell (FC) that contains all the necessary information for that specific muscle's formation. A FC undergoes multiple fusions with surrounding Fusion Competent Myoblasts (FCMs) to form a muscle. Genetic studies have identified *loner* as an essential fusion gene in *Drosophila*. *loner* encodes a guanine nucleotide exchange factor (GEF) that has been shown to have activity towards small GTPases called ADP-ribosylation factors (ARFs). Preliminary data suggest that *Loner* is involved in myoblast migration. However, the components of the *Loner* signaling pathway as well as the mechanism of its function during myoblast fusion remain unknown.

To gain insight to *Loner*'s functions during myoblast fusion, a genetic enhancer screen was performed. *loner* heterozygotes were crossed to heterozygous deficiency (Df) flies, and the resulting double heterozygous offspring were analyzed for lethality. A lethal interaction would suggest a genetic interaction between *loner* and a gene that is removed by the deficiency. We screened 300 Dfs on the second and third chromosomes, and eight interacting deficiencies were found. These regions were further analyzed by examining the muscle phenotypes in double heterozygote (Df; *loner*) embryos as well as homozygous deficiency embryos. Df DK3-68, which removes 60 known and annotated genes on the third chromosome, was found to be the most promising. To identify the gene(s) interacting with *loner*, smaller Dfs within DK3-68 were analyzed for both *loner* interactions and muscle phenotypes. A smaller Df, Df 7963, which removes 9 genes within a small region of chromosome 3, was found to have a similar phenotype to DK3-68. Further experiments are being conducted to determine the specific gene within this region responsible for the observed muscle phenotype.

Profiling Methyltransferase Targets with Engineered EuHMTase1

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Protein posttranslational modification epigenetically regulates gene transcription, influencing cellular development and disease processes. One type of modification, methylation, is carried out by protein lysine methyltransferases (PKMTs) which use the cofactor S-adenosyl-L-methionine (SAM) as the methyl donor. The upregulation of certain PKMTs has been found in tumors, and PKMT downregulation has been implicated for growth inhibition and chromosomal instability in cancer cells. PKMTs were known to methylate histone tails, however recent studies have shown that PKMTs also target many cancer-relevant non-histone proteins, such as the tumor suppressor protein p53. Identifying these cancer-specific non-histone PKMT targets is therefore crucial to elucidate the molecular mechanism of PKMT in cancer. Current methods to determine the non-histone substrates of PKMTs are insufficient for determining the specific profile of each of the >60 human PKMTs.

We plan to develop a bioorthogonal technology to profile PKMT targets with substantially increased sensitivity and specificity. The SAM binding pocket of a designated PKMT will be engineered to utilize synthetic SAM derivatives. In conjunction with the modified enzymes, the SAM derivatives are expected to transfer ethyl, allyl, propargyl and benzyl groups to the substrates in place of the methyl chemical moiety. The distinctly labeled substrates may be enriched by antibodies or affinity resins and characterized by mass spectrometry. We have engineered mutant forms of GLP1 (EuHMTase1), a PKMT implicated in parotid gland tumors, by replacing bulky residues in the SAM-binding pocket with smaller alanine and glycine residues, enlarging the cofactor binding pocket to permit the transfer of the larger derivative groups. The resultant 13 GLP1 variants were tested for the ability to exploit the synthetic SAM derivatives as alternative cofactors using an enzyme-coupled high throughput screening fluorescence assay.

The Crystallization of Insensitive; a Vital Regulatory Protein in the Development of *Drosophila Melanogaster*

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Studies in *Drosophila Melanogaster* have found a novel protein responsible for inhibiting a Notch pathway that regulates division of Sensory Organ Precursor (SOP) cells. It has been established that in wild type *Drosophila*, the Notch pathway prevents SOP cells from dividing. Upregulation of the Insensitive protein inhibited the Notch pathway and increased the division of SOP cells. ChIP-on-chip arrays were used to determine that protein bound to something, but whether Insensitive bound to DNA or other proteins was unclear. The main goal of the determination of the 3-Dimensional (3-D) structure of Insensitive is to elucidate its binding partners, which would also explain its regulatory mechanism. Based on the full length Insensitive, six shorter constructs featuring various parts of the protein were designed and developed for further protein purification and crystallization. The full length protein, tagged with GST, was expressed and purified through a GST column. The elution of the protein via addition of glutathione introduced additional purification steps that were required to remove the peptide. Coupled with digestion by the protease Factor Xa, these purifications caused a significant loss in the quantity of remaining protein. Furthermore addition of Factor Xa and digestion for 24 hours yielded the free protein, GST, and severely degraded parts of the protein. The shorter constructs are currently being used for the expression and subsequent protein purification using a SUMO specific column and the PreScission protease. These steps have provided us with information about the effectiveness of the system in expressing Insensitive and optimization of its purification. After successful expression of the protein and its subparts, numerous crystallization trials will be held. These trials are aimed at obtaining crystals diffracting well enough to supply the necessary data for determination of 3-D structure of Insensitive or its fragments.

Construction and Validation of Canonical Wnt Signaling Reporters for High-Resolution Live Imaging and Fate Mapping in Embryonic Stem (ES) Cells and Mice

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The Canonical Wnt signaling pathway controls a variety of patterning and morphogenetic events during embryonic development, homeostasis and disease. It regulates gene expression mainly through the T cell factor (TCF)/Lef-1 transcription factor family. Our lab is interested in Wnt3a, which is expressed in the mouse primitive streak (PS), where it plays a key role in paraxial mesoderm morphogenesis and somitogenesis.

To visualize the dynamics and determine the fate of the Wnt responding cells in the PS, a series of Wnt signaling reporters were constructed. We tested two promoter combinations, each comprising different numbers of multimerized TCF/Lef sites placed adjacent to different minimal promoters. The two variants were: (1) *TCF/Lef₆/hsp* - hsp68 minimal promoter and 6 copies of TCF/Lef response elements (TCF/Lef RE), (2) *TCF/Lef₇/Siam* – the *Xenopus Siamois* promoter and 7 copies of TCF/Lef RE. Four reporter cassettes were used. Human Histone2B (H2B) protein fusions of the spectrally-distinct fluorescent proteins Venus and mCherry were chosen for imaging cell dynamics within the PS, while the green-to-red photoconvertible fluorescent protein (KikGR) and an inducible form of the Cre recombinase (CreER²) were chosen for fate mapping. Each of the two promoter variants was cloned in front of each of four reporter cassettes.

To validate constructs, those containing fluorescent proteins were transfected into Wnt responding cells, and fluorescence levels were determined between cultures propagated in Wnt3a vs. Wnt5a (non-canonical Wnt) conditional media. Constructs were then transfected into mouse ES cells and will be used to generate transgenic mice. The inducible Cre constructs were directly used to generate transgenic mice by pronuclear injection.

The availability of ES cells and mice strains in which Wnt responsive cells can be live imaged and tracked will facilitate future work on the roles of canonical Wnt signaling in mammalian development, homeostasis and disease.

Studies Toward the Total Synthesis of Daphniglaucin B: Exploring the Diastereoselectivity of a Au (I)-Catalyzed Carbocyclization

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Lead candidates for effective anticancer drugs largely include two major sources of molecules: synthetic molecules made in the laboratory and natural products isolated from living organisms. In addition to the exhaustibility of the original source, isolating and purifying natural products from their original source typically results in difficult purifications with low yields. Thus, in order to more fully evaluate the properties of natural product lead candidates and their derivatives, synthetic access to these molecules must be established in the laboratory that does not rely on the natural source. With this in mind we have initiated a program towards the total synthesis of the alkaloid daphniglaucin B. Daphniglaucin B is one of two quaternary polycyclic alkaloids extracted from the leaves of *Daphniphyllum glaucescens* that exhibits cytotoxicity against murine lymphoma L1210 cells and human epidermoid carcinoma KB cells *in vitro*. Furthermore, daphniglaucin B contains an unprecedented fused polycyclic skeleton with a 1-azaniatetracyclo-[5.2.2.0.1,604,9] undecane ring system possessing eight contiguous stereocenters including two quaternary carbons and one quaternary ammonium.

In the construction of daphniglaucin B's 1-azaniatetracyclo-[5.2.2.0.1,604,9] undecane core, one of the key all-carbon quaternary stereocenter-forming reactions employs an intramolecular Au (I)-catalyzed carbocyclization of a silyl enol ether. However, the carbocyclization results in a 3:2 mixture of diastereomeric products. Studies are underway to bias the diastereoselectivity of the reaction in favor of the desired product. Toward this end, protecting groups on a nearby alcohol will be examined to probe their steric and electronic effect on the Au (I) cyclization diastereoselectivity. Improving the diastereoselectivity of this key step will increase the overall yield and viability of the synthesis of daphniglaucin B.

Cooperation of the MAPK and PI3K Pathways on Growth and Viability of Thyroid Cancer Cell Lines

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Thyroid cancers are associated with a high prevalence of non-overlapping mutations of the genes encoding the receptor tyrosine kinases *RET* and *TRK*, as well as of *NRAS*, *HRAS*, *KRAS* and *BRAF*, which all signal via the classical MAPK pathway. However, thyroid cancers are also associated with mutations of genes encoding effectors signaling along the PI3K pathway, especially in its advanced stages. The MAPK and PI3K pathways transduce growth factor-initiated signals that mediate cell proliferation and survival. Their significance in thyroid cancer pathogenesis is compelling, as almost all known thyroid oncogenes encode constitutively active mutants of effectors in these pathways. This is supported by marked effects on thyroid tumor growth in mice harboring activating mutations in *HRas* or *Braf* with *Pten* null alleles. We hypothesize that the MAPK and PI3K pathways cooperate in thyroid tumorigenesis by increasing cell proliferation and modifying the dependency of these cells lines on MAPK signaling, thus rendering them less sensitive to MEK inhibition. To test this, we knocked down PTEN expression by shRNA as a means of activating the PI3K pathway in human thyroid cancer cell lines harboring either BRAF or RAS mutations. In this isogenic cell model, we found no difference between the growth rate of parental and PTEN knockdown cells when cells were grown in high serum, as well as no differences in IC50 or growth inhibitory effects of a selective MEK inhibitor. We are now investigating the effects of PTEN loss on growth, cell cycle progression and signaling in low serum concentration. Future studies will explore effects of combinatorial inhibition of the MAPK and PI3K pathways.

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