Insights into product release in the hydrolysis reaction catalyzed by the bacterial deubiquitinase SdeA

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Crystal structure of the Cys-Ala mutant of the deubiquitinase (DUB) domain of the Legionella pneumophila effector SdeA (SdeADUB) reveals intermolecular contacts of the prokaryotic DUB with the ubiquitin (Ub) product. Most of the interactions are preserved in the product bound structure relative to the complex of the DUB with the suicide inhibitor ubiquitin vinylmethyl ester (Ub-VMe), a complex whose structure closely mimics the structure of the substrate bound state of SdeADUB. These interactions are also preserved in the product bound state of the enzyme in solution as revealed by NMR titration studies. Isothermal titration calorimetry (ITC) and NMR titration data reveal a significant difference in affinity for Ub between the wild type and the Cys-Ala mutant enzyme, with the mutant displaying a significantly higher affinity. We attribute this difference to repulsive interactions between the thiolate ion of the catalytic Cys and the carboxylate ion of the terminal Gly76 residue of the Ub, a product feature that results upon hydrolysis of the isopeptide bond linking Ub to a protein a target. Thus, product release is likely facilitated by a repulsive interaction between the catalytic Cys (thiolate anion) and the carboxylate group produced from the hydrolysis of the isopeptide bond of the ubiquitinated substrate. Similar repulsive interactions may underlie a general mechanism of product release in hydrolysis of deubiquitinases and hydrolases of ubiquitin like protein modifiers where extensive protein-protein interactions are utilized in the enzyme-substrate engagement.

In Search of the Syk Phosphorylation Mechanism

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Spleen tyrosine kinase (Syk) has a central role in the transmission of activating signals within B-cells of the adaptive immune system. Abnormal function of Syk is implicated in many hematopoietic tumors and autoimmune diseases. Therefore, the activation mechanism of Syk is of particular biological and pharmacological interest. Our Syk fragment comprises two SH2 domains separated by a linker region. Nuclear magnetic resonance spectroscopy (NMR) and molecular dynamics simulations (MD) have revealed that there is an increased disorder in SH2-SH2 domain structure, or uncoupling of the two SH2 domains, when Syk is phosphorylated at the linger region. This behavior is the basis of a novel phosphorylation mechanism that is entropically driven. The question remains how phosphorylation in the linker region induces domain separation. MD results suggest, upon phosphorylation, the linker region has increased interaction with a few basic residues (R67 & K164) and R45 changes interaction partners. To test if these interactions are important we substituted these residues with alanine (a small uncharged amino acid). If these interactions are important; mutations should affect the uncoupling mechanism with phosphorylation mimics of Syk. However, we still observed domain uncoupling in NMR measurements. One potential way to prevent interaction after phosphorylation is to increase salt concentration, which screens the charge introduced in phosphorylated residues. The results of introducing salt to uncovering the phosphorylation mechanism of Syk will be presented and future directions discussed.

The Pasteurella multocida PfhB2 toxin represents a new class of cysteine proteases belonging to the papain enzyme family

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Pasteurella multocida is a Gram-negative bacterium that causes respiratory disease in a broad range of animals, as well as opportunistic infections in humans. P. multocida secretes a multidomain toxin, called PfhB2, that is important for virulence. In addition to its FHA (filamentous hemagglutinin) and Fic (filamention induced by cAMP) domains that are implicated in attachment and immune evasion, respectively, PfhB2 contains a YopT-like cysteine protease domain at its C-terminus - a role for which has remained elusive. PfhB2's YopT domain (herein called PfhB2-YopT) belongs to a large family of papain-like proteases that rely on a Cys-His-Asp catalytic triad for activity, features that are highly conserved in PfhB2-YopT as well. Taking a structure biology approach to characterizing the PfhB2-YopT domain, we successfully crystallized the catalytically inactive (and stable) Cysteine 3733 to Serine mutant of PfhB2 (PfhB2-YopTC3733S). Presented here is the crystal structure of PfhB2-YopTC3733S solved by experimental phasing with Seleno-methionine derived crystals at 2.39Å resolution. We find that PfhB2-YopT exists as four molecules arranged as an asymmetric unit consisting of two dimers, where the two molecules of the dimers are in a head-to-tail conformation. Further, this structure revealed an unexpected mode of catalytic triad formation where dimerization is essential for reconstituting the papain active-site fold. Such an unconventional orientation of the catalytic triad has never been observed for any class of Cysteine proteases. Interestingly, even though we detected enzymatic activity with wild type PfhB2-YopT, it failed to form a stable and crystallizable dimer, suggesting the need for a cofactor or target protein for its dimerization. Our PfhB2-YopT structure represents a novel cysteine protease fold. We predict that many proteins in the YopT superfamily likely function similarly, and aim to use PfhB2-YopT as a model to understand this new class of cysteine proteases.

High-throughput protein correlation profiling reveals evolutionary diversification of protein complexes in oligomeric states across plant species

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Multiprotein complexes are essential for coordinating cellular processes evolved through millions of years. However, the components, conservation and functions of these protein complexes remain enigmatic in planta. To analysize the preservation of protein complexes across plant species, we employed a new combination method of protein correlation profiling (PCP) and shutgon proteomics that provides large-scale datasets on protein oligomerization. This technology permited a high-throughtput comparative analysis of the elution profiles of mass spectrometry-characterized proteins among diverse plant models. To test for species-specific differences and similarities in ubiquity and dimensions of protein complexes across plant species, here four plant species were selected both for their distance in evolutionary divergence and also for their relevance as model organisms to biology research and crop production: two Malvids (leaves of Arabidopsis thaliana and fibers of Gossypium hirsutum), a Fabid (leaves of Glycine max) among dicot plants, and one monocot species of the Poales (aleurone layer of Oryza sativa). Protein complexes in the four representative species were fractionized by size exclusion chromatography (SEC) and followed by a label-free quantitative mass spectrometry analysis. For cross-species comparisons, annotated proteins in each plant species were combined into orthologous groups using Phytozome V12 plant ortholog information, and then assessed by the analysis of variance (ANOVA) F-test. Interestingly, some of orthologous protein complexes are not conserved in terms of complex size, suggesting evolutionary divergence may have shaped protein number or size distributions. To get more insight into this protein complex evolution, protein complex composition has been accomplished in Arabidopsis and rice, and then the degree of overlap of protein subunit composition will be analyzed.

Identifying critical genes and microRNAs that potentiate *KRAS*;p53-driven lung tumorigenesis

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Lung cancer is the leading cause of cancer-related deaths. KRAS and TP53 are two of the most commonly mutated genes in lung cancer, and their mutations are well recognized drivers of tumorigenesis. Directly targeting these drivers still remains a challenge for cancer therapeutics. Instead, targeting genes that potentiate KRAS/p53-driven tumorigenesis is likely an effective alternative. Therefore, we hypothesize that loss of certain genes and microRNAs can drive transformation of two non-tumorigenic KRAS;p53/Kras-mutated mammalian lung systems into cancerous states. To address this, we performed CRISPR knockout screens in these two systems using the small guide RNA (sgRNA) libraries. We identified several sgRNAs that could support anchorage-independent growth of the originally anchorage-dependent human bronchial epithelial cells that harbored KRASG12V;sh-p53 (HBEC-KP), and in concordance with this, these sgRNAs were enriched in two-dimensional culture over four-months of growth. KrasG12D;Cas9 mouse line was also generated and genome-wide knockouts will be induced directly in murine lungs for evaluating genetic contributions to tumor development.

Structure, Interactions, and Conformations of the E. coli Condensin Complex

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Structural maintenance of chromosome (SMC) proteins are major components in maintaining the essential genetic material for an organism's survival. DNA organization, condensation, cohesion, segregation, and repair functions are performed by the different members of the SMC family, cohesion, condensin, and SMC 5/6. Despite the diversity of functions performed, this family of proteins shares a similar and unique structure by where a globular ABC-ATPase domain and globular hinge domain are connected via an approximately 50 nm long anti-parallel coiled coil. SMC proteins in vivo are always complexed with additional binding partners. In bacteria, if any of the components in the SMC complex are null or nonfunctional, the cells display a temperature sensitive phenotype and when grown under fast growth conditions the population of anucleate cells increases. In humans there are a number of diseases that have been linked to mutations in SMC proteins such as Cornelia de Lange Syndrome, Robert's Syndrome, and myeloid leukemogenesis. Despite the clear basic biological importance of this family of proteins it is still far from clear how these proteins function on a mechanistic level. Over the last couple of years new and exciting results in the field have emerged, and the field is beginning to converge on a single mechanistic model known as loop extrusion for condensin function. This model describes the active compaction of DNA through the extrusion of loops. Despite these exciting advances we still do not understand the molecular basis and structural transitions that are required for loop extrusion and other SMC functions. My project will use the condensin in Escherichia coli, MukB, as a model system to address these remaining question. Our hypothesis is that large-scale conformational changes, mediated by binding factors, drive the mechanism of action of SMC proteins. More specifically my project focuses on understanding the relationship and importance of specific structural elements of MukB to overall function with crystallography. I will also monitor movements and interactions at specific regions and interfaces in MukB in the context of binding partners and substrates with cross-linking. This will allow us to draw conclusions about how proximal and distal regions of MukB are moving relative to each other, which will provide valuable insight into the conformations that are used during its mechanism of action. Furthermore, we will then be able to compare our finding with those of distantly related SMC proteins such as BsSMC. We can begin to identify underlying patterns and distinct difference with in the family of SMC proteins.

Engineering a Color Palette of FRET-BRET ATP Sensors

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ATP, a key player in energy metabolism, is involved in various cellular processes and is shown to be an indicator of cellular health. There are several methods to measure ATP levels, and genetically-encoded sensors are excellent tools for visualizing energy metabolism in live cells. Currently, however, there has been a lack of color options. By building new variants of hybrid FRET-BRET (Förster or bioluminescence resonance energy transfer) ATP sensors, our goal is to increase the flexibility and utility of these tools with different experimental formats. For example, differently colored sensors could be used to simultaneously monitor ATP levels in different individual cells or cell types as well as in different compartments within a single cell. Here, we have developed ATP sensors which employ different fluorescent protein FRET pairs in conjunction with the NanoLuc luciferase. We characterized the both the fluorescence and bioluminescence purified sensor proteins in response to ATP. Furthermore, we carried out live-cell imaging experiments to validate that our sensors can be used for ratiometric quantification of ATP levels. These new color variants of hybrid FRET-BRET ATP sensors will be an effective method for studying energy metabolism both in vitro and in vivo.

Cryo-EM Structural Analysis of Human Deubiquitinating Enzyme USP7 Illustrates Tethered-Rheostat Mechanism of Catalysis

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Ubiquitin-specific protease 7 (USP7) is a human deubiquitinating enzyme that is implicated in numerous cancers and neurodegenerative diseases. The family of USPs all contain a fingers-palm-thumb catalytic domain and a Cys-His-Asp catalytic triad. Due to their similarity in sequence and structure, structure-activity relationship studies for therapeutic design has been a challenge. USP7 is a unique member of this family of enzymes in that it has 7 domains, many of which are not shared by closely related enzymes. These domains are hypothesized to work in concert, exhibiting a large degree of conformational heterogeneity and unique intramolecular regulation. Due to these features, the field has enjoyed limited success in x-ray crystallographic studies, relying on individual domain truncations. This has left researchers to piece together the mechanism of catalysis through biochemical analysis alone. The following work represents the first comprehensive structural data to support USP7's hypothesized tethered-rheostat mechanism of catalysis. Further, the structural analysis herein describes the location of the TRAF and HUBL1-5 domains in relation to the catalytic domain, which has eluded researchers for the past decade.

Chemical homing to unlock the potential of neuropeptides as therapeutics to heal bone fractures

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INTRODUCTION: Bone fractures represent a significant disease burden, especially in patients over the age of 65. Non-invasive drug therapies are not available for bone fractures, which represents a problem for this population. It has been observed that nerves that express substance P, vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP) innervate the bone. Additionally, nerves branch in response to a traumatic injury to the bone such as a fracture. These peptides have been shown to have demonstrated an activating and proliferative effect on osteoblasts. Their osteogenic effects have been demonstrated in vitro. However these peptides have not been developed as therapies, because their receptors also play roles in the brain, intestines, immune system, and endocrine system. We have demonstrated that acidic oligos can be used to selectively localize both small molecules and peptides to bone fractures. We hypothesized that bone-targeting ligands could be used to limit these peptides' localization just to the fracture site, thus minimizing their toxic off-target effects and increasing their efficacy. METHODS: Targeted conjugates of substance P, VIP, and PACAP were synthesized using standard Fmoc solid-phase peptide synthesis. To ensure the conjugates' activity, MCTC3-E1 cells were treated with the targeted and untargeted compounds for three days at concentrations from 1pM to 100 nM. After three days of treatment, the cells were harvested, and the RNA was purified from the cells. Expression levels of ALP, RUNx2, OSX, OPN, Col-1A, OPG, RANKL, SOST, and OC were quantified via RT-qPCR. Once the biological activity of the conjugates was confirmed, they were tested in vivo in a fracture model. Aseptic surgical techniques were used to place a 23-gage needle as in intramedullary nail in the femur of anesthetized, 12-week-old Swiss Webster mice for internal fixation before fracture. Femur fractures were induced using a drop weight fracture device from RISystem. The mice received buprenorphine for three days post fracture. The mice were dosed subcutaneously each day for three weeks. Fracture healing was assessed using microCT (Scanco Medical Ag). Morphometric parameters were quantified in the 100 widest slices of the fracture callus. Trabecular thickness (Tb.Th.), trabecular spacing (Tb.Sp.), total volume (TV), and volume of calcified callus (BV) were calculated. Fractured femurs were tested for strength in a four-point bend to failure using an Electro Force TestBench (TA Instruments). Lower supports were 10 mm apart on the anterior face of the femur in contact with the proximal and distal diaphysis. Upper supports were 4 mm apart and spanned the entire fracture callus on the diaphysis. Force was applied from the posterior face of the femur with a displacement rate of 0.3 mm/sec. Peak load, yield load, stiffness, displacement post yield, work to fracture, and deformation data were generated. Statistical analysis was performed using a two-way analysis of variance (ANOVA) and a Tukey post-hoc analysis with significance reported at the 95% confidence level. All animal experiments were performed in accordance with protocols approved by Purdue University's Institutional Animal Care and Use Committee (IACUC). RESULTS: PACAP targeted with e10 dosed at 10 nmol/day resulted in a significant increase in BV/TV with a 39% increase of BV/TV over saline control, with a sample size of 5. PACAP targeted with e10 dosed at 1 nmol/day resulted in a significant increase in maximum load, with an 87% increase in maximum load and a 57% increase in stiffness over saline, with a sample size of 5. VIP targeted with D10 at 1 nmol/day resulted in a significant increase of 97% in BV/TV over saline control with a sample size of 5. Substance P Targeted with D10 resulted in a significant increase in BV/TV with a 58% increase at 1 nmol/day, 82% at 10 nmol/day, and 65% at 100 nmol/day over saline with a sample size of 5. Substance P targeted with D10 also resulted in a significant 87% increase in maximum load over saline control at the 1 nmol/day dose with a sample size of 5. DISCUSSION: No significant difference was seen in the osteogenic activity in vitro of the conjugated peptides when compared to the natural unconjugated fragments. Therefore, conjugation did not affect the biological activity. The attachment of a bone-targeting ligand allowed these neuropeptides to be used to improve fracture healing. They all show promise as future therapies, because they all significantly improve the healing rate of bone fracture in an in vivo model. The study was limited by the sample size, so many improvements were not powered enough to be significant. However, the toxicities that would have been associated with these neuropeptide hormones typically were absent in all but very high doses of VIP. Bone-targeting molecules can be used to improve the efficacy and lower the toxicity of potential bone anabolic agents. SIGNIFICANCE / CLINICAL RELEVANCE: Delayed fracture healing is a major health issue involved with aging, and strategies to improve the pace of repair and prevent non-union will substantially improve patient outcomes and lower healthcare costs. Here, we demonstrate that neuronal regulation of fracture repair can be utilized to accelerate fracture healing. This opens the possibility for the development of these compounds and other related bone anabolic neuropeptides as therapeutics for bone fracture repair.

Time-resolved crystallographic measurements elucidating the mechanism of bacterial HMG-CoA reductase

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HMG-CoA reductase (HMGR) is the rate limiting enzyme in the Mevalonate pathway that converts 3-hydroxy-3-methylglutarylCoA (HMG-CoA) into Mevalonate, a precursor compound used in isoprenoid biosynthesis. The enzyme is the target for cholesterol-lowering drugs and a potential target for novel antibacterials in gram-positive bacteria. Previous studies have suggested that its substrate HMG-CoA undergoes conversion into two intermediates, Mevaldyl-CoA and Mevaldehyde, before it is converted into Mevalonate. However, only Mevaldyl-CoA, has been structurally observed. Current work on HMGR focuses on identifying conditions that are suitable for running the enzymatic reaction in the crystal and also altering the rate of the reaction within the HMGR crystal to trap and observe the reaction intermediates formed during catalysis thereby testing the reaction mechanism model. By doing so, we also hope to identify the structural and chemical changes that facilitate the enzymatic reaction in HMGR.

Dimerization of the yeast Isoprenylcysteine Carboxyl Methyltransferase, Ste14

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Isoprenylcysteine carboxyl methyltransferase (Icmt) is an membrane protein localized to the ER that is responsible for the carboxyl methylesterification of CaaX proteins. Currently little is known about the structure and mechanism of Icmt. Our model, the yeast Icmt Ste14, is comprised of six transmembrane domains in which TM1 contains a dimerization motif, G31XXXG35XXXG39. Previous studies proposed Ste14 forms functional higher order oligomers. Thus, to determine the sequence and structural determinants for dimerization, we used cysteine-scanning mutagenesis to generate single cysteine mutants for every residue in TM1. Each mutant was characterized via immunoblot analyses to assess expression, activity, and the ability to form dimers. Additionally, several of the residues proposed to lie on the same face of TM1 formed dimers upon the addition of sulfhydryl specific cross-linkers. We further validated the homodimerization of wildtype Ste14 with SEC-MALS and SAXS. Together, we suggest that functional form of the Ste14 Icmt is, in fact, a homodimer and will ultimately be useful as we further explore the mechanism of action of Ste14.

A Phosphatidic Acid Sensitive Domain in P-Rex1 Involved in Cell Migration and Metastasis

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P-Rex1 is a guanine nucleotide exchange factor for Rac and thereby controls cell migration and proliferation. It contains a tandem DH/PH domain catalytic module, two dishevelled, EGL-10 and pleckstrin (DEP) and two PDZ domains, and a C-terminal region that contains weak homology to inositol polyphosphate 4-phosphatase. P-Rex1 is regulated by both intra-domain interactions and interactions with other proteins such as Gβγ, PKA, mTOR, PAK, the phospholipid PIP3 which help dictate its intracellular localization as well as exert allosteric effects. P-Rex1 is over-expressed in multiple cancers, breast, prostrate, ovary and melanoma. The understanding of regulatory mechanisms influencing P-Rex1 would therefore aid in development of anti-cancer drug therapies. Currently, only the structures of the DH/PH tandem and PDZ1 domains of P-Rex1 have been determined. This study aims to study the role of the DEP domains towards P-Rex1 function using structural and biochemical techniques. DEP domains are approximately 90-100 amino acids in length and function in signalling events via protein-protein interaction and recruiting proteins to cell membrane. For our study, P-Rex1 DEP1 and DEP2 domains were expressed and purified individually as well as in tandem from Escherichia coli cells as MBP-His6-tagged proteins. For crystallization trails, the MBP-His6 tag was cleaved. The tandem DEP1-DEP2 and DEP2 proteins were not stable. The DEP1 domain however crystallized and diffracted to 3 Å. It has a characteristic / fold, comprising of the conserved helical core and a protruding -ribbon. Previous studies from DEP domains in other proteins implicate the -strand residues in facilitating membrane recruitment, and similarly DEP1 has a stretch of polybasic residues which we hypothesized would be involved in membrane recruitment. The binding of DEP1 to phospholipids was tested using protein-lipid overlay assays. The P-Rex1-DEP1 showed binding to phosphatidic acid (PA), whereas a mutant with alanine substituting for basic residues in the -ribbon did not. From the results of this study, we conclude that P-Rex1-DEP1 structure has a characteristic / fold and the polybasic stretch in its -ribbon enables DEP domain to bind to PA. Future experiments will focus on testing the role of DEP1 in membrane localization of full length P-Rex1 and if PA regulates its activity.

Structural Insights into Phospholipase Cε Function

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Phospholipase Cε (PLCε) is a member of the PLC family of enzymes that hydrolyze phosphatidylinositol lipids downstream of G protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). PLCε is unique among the PLC superfamily as it contains an N-terminal CDC25 domain, which has guanine nucleotide exchange factor (GEF) activity towards small G proteins Rap1A, and two C-terminal Ras association (RA) domains. The best characterized pathway leading to PLCε activation is mediated through β-adrenergic receptor (βARs) signaling. Stimulation of these receptors culminates in the activation of the small GTPase, Rap1A, which binds directly to PLCε and translocates the complex to the Golgi. There, PLCε hydrolyzes phosphatidylinol-4-phosphate (PI4P). Prolonged activation of this pathway results in increased expression of hypertrophy-related genes. However, how the structural basis of PLCε activity under basal conditions and upon activation by Rap1A are unknown. Towards this end, we have obtained the first high-resolution insights into a catalytically active PLCε variant. These studies, together with biochemical analysis of the intramolecular contacts observed in the structure, provide the first molecular insights into this enzyme.

Molecular Mechanism of Rap1A-Dependent Activation of PLCepsilon

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Phospholipase C (PLC) enzymes hydrolyze phosphatidylinositol lipids to produce diacylglycerol (DAG) and inositol phosphates, leading to the activation of protein kinase C (PKC) and downstream signaling pathways, including cell growth and survival. The PLCepsilon subfamily is a key player in cardiovascular function, where it contributes to maximum contractility. However, prolonged activation of PLCepsilon results in cardiac hypertrophy and heart failure through its ability to regulate the expression of hypertrophic genes. This process is regulated by the small GTPase Rap1A, which is activated downstream of Beta-adrenergic receptors. Rap1A binds to the C-terminal Ras association (RA) domain of PLCepsilon, simultaneously translocating the complex to the perinuclear region and activating PLCepsilon. PLCepsilon also contains an N-terminal CDC25 domain, which has guanine nucleotide exchange factor (GEF) activity for Rap1A resulting in a feed forward activation loop and sustained lipid hydrolysis. However, the molecular mechanism of this process is not known. In this work, we seek to characterize the interactions between Rap1A and PLCepsilon using structural and functional studies to map the Rap1A binding site on PLCepsilon and determine whether activation results in conformational changes that release autoinhibition and/or increase membrane association. These studies provide the first molecular details of the Rap1A-dependent activation of PLCepsilon and open the door to the development of new therapeutic strategies for treating cardiac hypertrophy.

Developing Chikungunya virus-like nano-assemblies for diagnostics and nanovaccines

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Every year during the monsoon season, clusters of cases for Chikungunya are reported sporadically from several countries and in various states of India., at present no vaccine or antiviral drug is available against CHIKV in the market. VLNPs contain crucial immunologic molecular structures of the viral pathogen. These do not contain the replicative viral genomes and are easy to produce in large scale using heterologous protein expression and are also expected to be safe and effective. This study focuses on developing chikungunya VLNPs which have an engineered fluorescent protein on their surface. CHIKV vaccine strain 181/25 cDNA has been used to produce VLNPs and tagged with the foreign fluorescent protein. To produce CHIKV VLNPs, a portion of capsid has been removed which is responsible for viral RNA encapsidation. Alphaviral promoters were used for making VLNPs of CHIKV in mammalian cell lines. The fluorescence tagged VLNPs of CHIKV have been partially purified using viral precipitation method and centrifugation for application in fluorescence-based molecular diagnostics assay.

2.6 Å Structure of Tulane Virus With Minor Mutations Leading to Receptor Change

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It has been a common practice to eliminate mutations during virus propagation in vitro. However, for low yield viruses, like Tulane virus (TV) and Hepatitis B virus (HBV), numerous efforts have been made to increase the virus titer at every steps of virus propagation and purification. In this study, we were able to obtain a high yield TV mutant and whole genome sequencing has revealed several mutation sites. A 2.6 Å resolution structure of the TV mutant was solved using cryo-EM with EM density consistent with the sequence variations. Most importantly, via the enzyme-linked immunosorbent assay (ELISA) experiment, the TV mutant has been found to lost the binding ability to its original cellular receptor, the histo-blood group antigens (HBGAs). Therefore, it is appealing to propose that it may have switched to a new receptor to better adapt the cell culture system.

Molecular determinants of the differential modulation of Cav1.2 and Cav1.3 by nifedipine and FPL 64176

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Nifedipine and FPL 64176 (FPL), which block and potentiate L-type voltage-gated Ca2+ channels respectively, more potently modulate Cav1.2 than Cav1.3. To identify potential strategies for developing subtype-selective inhibitors, we investigated the role of divergent amino acid residues in transmembrane domains IIIS5 and the extracellular IIIS5-3P loop region in modulation of these channels by nifedipine and FPL. Insertion of the extracellular IIIS5-3P loop from Cav1.2 into Cav1.3 (Cav1.3+) reduced the IC50 of nifedipine from 289 nM to 101 nM, and substitution of S1100 with an A residue, as in Cav1.2, accounted for this difference. Substituting M1030 in IIIS5 to V in Cav1.3+ (Cav1.3+V) further reduced the IC50 of nifedipine to 42 nM. FPL increased current amplitude with an EC50 of 854 nM in Cav1.3, 103 nM in Cav1.2, and 99 nM in Cav1.3+V. In contrast to nifedipine block, substitution of M1030 to V in Cav1.3 had no effect on potency of FPL potentiation of current amplitude, but significantly slowed deactivation in the presence and absence of 10 μM FPL. FPL had no effect on deactivation of Cav1.3/DHPi, a channel with very low sensitivity to nifedipine block (IC50 ~ 93 μM), but did shift the voltage-dependence of activation by ~-10 mV. We conclude that the M/V variation in IIIS5 and the S/A variation in the IIIS5-3P loop of Cav1.2 and Cav1.3 largely determine the difference in nifedipine potency between these two channels, but the difference in FPL potency is determined solely by divergent amino acids in the IIIS5-3P loop.

Competitive protein-based fluorescent assay for glucose detection in exhaled breath condensate

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Despite recent advances in glucose biosensors and glucose detection, reliable measurement of glucose at the very low concentrations in non-invasive fluids is highly desired. The current gold standard for glucose measurement are the electrochemical sensors based on glucose oxidase (GOx); however, high dissociation constant of the enzyme prevents it from being able to resolve sub-micromolar changes of glucose at low concentrations. Based on the need for a highly sensitive biosensor to detect glucose at the sub-micromolar level, we have based our assay on the E. coli glucose binding protein (GBP) that has a stronger and more specific affinity to glucose (KD = 0.35 μM) compared to that of GOx (KD = 20 mM). We take advantage of the competitive binding of glucose (KD = 0.35 μM) and galactose (KD = 1.4 μM) to GBP to develop a fluorescent biosensor that is quenched when bound to a galactose-quencher and fluorescent when bound to glucose. We have engineered GBP to contain a biorthogonal azide (12-ADA) tag that allows selective conjugation to fluorophores, quantum dots, or another matrix of interest directly from cell lysate. We have found that our chemoenzymatic tagging method enables us to selectively and efficiently bind the 12-ADA-GBP from cell lysates containing overexpressed proteins to fluorophores of interest without any purification steps. This presentation will discuss our work in selectively conjugating the protein to highly-photostable quantum dots using copper-free click chemistry and subsequent characterization of competitive fluorescent quenching when interfaced with a galactosamine-quencher. This will enable the competitive detection of glucose via galactose-analog displacement upon interaction with a glucose-containing fluid.

Enhancement for MAINMAST, De Novo Main-Chain Tracing Method: Symmetric Multi-Chain Modeling, Local Refinement, and Graphical User Interface

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The significant progress of the cryo-EM poses a pressing need for software for structural interpretation of EM maps. Particularly, protein structure modeling tools are needed for EM maps determined around 4 Å resolution, where finding main-chain structure and assigning the amino acid sequence into EM map are still challenging problems. We have developed a de novo modeling tool named MAINMAST (MAINchain Model trAcing from Spanning Tree) for EM maps for this resolution range. MAINMAST builds main-chain traces of a protein in an EM map from a tree structure constructed by connecting points with a high density in the map without referring to known protein structures or fragments. The method has substantial advantages over the existing methods: i) MAINMAST directly constructs protein structure models from an EM density map without requiring reference structures; ii) The procedure is fully automated and no manual setting is required; iii) a pool of models are produced, from which a confidence score is computed that indicates accuracy of structure regions. Here, we report substantial improvements of MAINMAST in three aspects. The largest improvement is that the method now can perform automatic map segmentation and structure modeling for symmetrical multi-chain complexes. The tree-graph structure that connects dense points are traced for multiple chains simultaneously in a symmetric fashion. Figure 1 and 2 are showing the examples of the multi-chain segmentation by MAINMAST and Segger v1.9.5 (plugin in UCSF Chimera molecular visualization software) on EMD-6551 (Magnesium channel CorA at 3.8Å resolution) and EMD-8118 (TRPV1 at 3.28Å resolution). In these two maps, MAINMAST successfully find the individual protein regions from the EM maps. Moreover, the accuracy of a model is significantly improved by a new implementation of local sequence matching and structure refinement. The local matching protocol is also useful for identifying missing regions in a structure model, i.e. regions with a low density, in an EM map. Finally, we developed a software plugin of MAINMAST for the UCSF Chimera, so that users can monitor structures at each step of a modeling procedure. The major functionalities include to generate and to display tree structures from local dense points in the map, main-chain traces, and reconstructed all-atom models. Through the interface, users can easily control parameters of MAINMAST and save and restore sessions.

Conformational Dynamics Contribute to Phospholipase Cbeta Activity

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Phospholipase C (PLC) enzymes hydrolyze the lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to produce the second messengers inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Production of these second messengers leads to many diverse physiological responses, including vascular smooth muscle contraction and inflammation. Previous structural and functional studies of PLCbeta have revealed a highly conserved catalytic core that adopts a compact, globular structure and forms the minimal fragment that retains lipase activity. However, a growing body of evidence suggests that the PLCbeta PH domain, which interacts with EF hands and TIM barrel of the catalytic core in crystal structures, may be flexible in solution. Using a combination of small angle x-ray scattering (SAXS), cross-linking studies, and biochemical assays, we provide the first structural data demonstrating that the PH is in fact conformationally flexible in solution. The PH domain adopts two major states: an open state wherein the PH domain is extended away from the core, and a closed state consistent with the crystal structures. We also provide evidence that these conformational states are functionally significant. These findings provide new evidence of conformational heterogeneity of PLC enzymes in solution and reveal new insights into their roles in cell signaling and cardiovascular disease.

Protein intakes greater than versus at the RDA differentially affect lean body mass responses to purposeful catabolic and anabolic stressors: A systematic review and meta-analysis of literature

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Under stressful conditions such as energy restriction (ER) and resistance training (RT), recommended dietary allowance (RDA) for protein of 0.8 g/kg/d may no longer be appropriate. We aim to assess the effects of protein intake greater than versus at the RDA on changes in whole-body lean mass. Articles were screened using PubMed, Scopus, CINAHL, and Cochrane databases. Twenty-three comparisons from 19 randomized-controlled, parallel studies were included. Among all comparisons, protein intakes greater than the RDA attenuated lean mass loss after ER [0.41 kg (0.15, 0.67); WMD (95% CI), n=15 comparisons], but not without ER [0.23 kg (-0.44, 0.89), n=8]. Protein intakes greater than the RDA increased lean mass after RT [0.77 kg (0.23, 1.31), n=3], but not without RT [0.29 kg (-0.04, 0.62, n=20]. Protein intakes greater than the RDA positively influence changes in lean mass when adults are purposefully stressed by dietary energy restriction and/or resistance training.

Bone-Fracture-Targeted Dasatinib Accelerates Fracture Repair In Vivo

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INTRODUCTION: There are 6.3 million fractures each year in the USA, costing the healthcare system ~$28 billion/year (data source: National Ambulatory Medical Care Survey and the American Academy of Orthopaedic Surgeons). More importantly, greater than 300,000 hip fractures occur every year in the US, and one in four of these patients will die of associated complication1,2. Clearly, a major need exists for a systemically administered drug that can accelerate fracture repair without imposing unwanted side effects. From our perspective, a bone anabolic agent that would concentrate at the fracture site and clear rapidly from unaffected sites should satisfy these requirements. To address this need, we have designed a fracture-targeted conjugate that contains a bone-homing aspartic acid decapeptide moiety linked to dasatinib, a potent Src inhibitor, via a self-immolative linker. Upon localization to the fracture surface, free dasatinib is released from the conjugate promoting enhanced fracture repair. METHODS: • Synthesis: The Asp10 moiety of dasatinib-Asp10 conjugate (DAC) was synthesized via standard solid phase peptide chemistry using a 2-chlorotrityl chloride resin. Dasatinib was attached to the Asp10 by esterifying it to maleimidylpropionic acid which in turn was conjugated to the cysteine-containing Asp10 via Michael addition. • Animal studies: All animal protocols were reviewed and approved by Purdue's Animal Care and Use Committee (PACUC). A stabilized fracture was introduced into the femoral midshaft of anesthetized ND4 Swiss Webster mice. Fracture-bearing mice were dosed subcutaneously with vehicle, dasatinib (10 mol/kg) or DAC (10 mol/kg) at the indicated frequency. At three weeks post-fracture, healed femurs were collected and subjected to microCT (Scanco) analyses. All statistical analyses were performed with GraphPad Prism using unpaired two-tailed t-tests with a 95% confidence interval to assess statistical significance. Data are displayed as mean standard error of mean (SEM). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. RESULTS SECTION: DAC contains a dasatinib warhead, releasable ester linker, and fracture-homing aspartic acid decapeptide. Treatment with DAC essentially doubles the bone fraction (BV/TV) and bone volume relative to vehicle-treated controls by 3 weeks post fracture induction (n = 8). While non-targeted dasatinib also enhances mineralization in the fractured region, its impact was less than half that of its targeted counterpart. The microCT images show significantly denser trabecular bone microstructures in the targeted group compared to vehicle- and free dasatinib-treated controls. Trabecular thickness is also remarkably increased while trabecular spacing is drastically decreased relative to mice receiving non-targeted drug or vehicle alone. Treatment with DAC every other day for 3 weeks was equally effective as daily injections of DAC, yielding a 114% increase in bone density over PBS-treated controls (n = 7). Reducing this dosing interval to every four days, however, resulted in a measurable decline in potency. Importantly, even the weekly dosing of DAC resulted in a trabecular density that was comparable to that seen in mice treated every day with the same concentration of free dasatinib. DISCUSSION: We have shown that DAC facilitates femur fracture repair in vivo by increasing bone density at the fracture site. Although nontargeted dasatinib caused a modest improvement in healing rate, targeted dasatinib was dramatically better than free drug, enabling faster bone. We conclude that alternated day dosing of DAC constitutes a promising new therapy for acceleration of bone fracture repair. SIGNIFICANCE/CLINICAL RELEVANCE: The findings of this study will provide a new, systemically-administered therapy to the treatment of fracture repair. Such therapy is still nonexistent currently, and the findings of this study could significantly lower the morbidity and mortality rate arising from bone fractures. REFERENCES: 1. Bentler, S. E. et al. The aftermath of hip fracture: discharge placement, functional status change, and mortality. Am. J. Epidemiol. 170, 1290–1299 (2009). 2. Braithwaite, R. S., Col, N. F. & Wong, J. B. Estimating hip fracture morbidity, mortality and costs. J. Am. Geriatr. Soc. 51, 364–370 (2003).

A Systematic Evaluation Model for Educational Biochemistry Animations

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Animations have been created and used in undergraduate biochemistry curriculum with the intention to help students to perceive dynamic and complex biochemical processes. However, the effectiveness of these animations in conveying messages have not been fully explored due to the lack of a well-established evaluation metrics. This poster will present a systematic model for analysis of biochemistry animatics/animations that are intended to be used in a large biochemistry classroom. The model uses variation theory and multimodal analysis as guiding frameworks. Using semi-structured interviews, we identified the primary and secondary learning objectives set by the course instructor and the animation design team respectively, as well as the student perceptions of the animatic features. The content and presentation modes of the animatic were also analyzed using the model. Alignment between what was intended to be learned (intended objects of learning), what was depicted in the animatic (enacted objects of learning), and what was perceived by the students (lived objects of learning) governed the resulting learning objectives for the animation.

Developing Targeted Cytotoxic Therapeutics to Treat Cancer

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Folates serve as important one-carbon donors in de novo biosynthesis of purines and thymidylate, amino acid biosynthesis and metabolism, and biological methylation reactions from S-adenosylmethionine. These biosynthetic pathways are vital for cell proliferation events including tumor growth and T-cell lymphocyte activation. Folate-utilizing enzymes are therefore key targets for treatment of cancer and inflammatory diseases. FDA approved antifolate drugs to treat certain cancers and inflammatory diseases include methotrexate and pemetrexed (Alimta®). However these drugs lack specific targeting to disease tissue and cause serious cytotoxic side effects. To improve targeting to only affect diseased tissue and alleviate side effects, we have developed a series of targeted cytotoxic therapeutics (TCTs), termed AGFs. Each AGF molecule is characterized by a series of cell proliferation and cell protection assays to determine the mode of transport into the cell and its intracellular targets. A subset of our AGFs is transported via folate receptors, transporters that are overexpressed in specific cancers and activated macrophages. We further characterize these AGFs with in vitro inhibition assays and crystallization of the target-inhibitor complexes. We found individual and dual inhibition of β-glycinamide ribonucleotide formyltransferase (GARFTase) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (ATIC), enzymes required in de novo purine synthesis. Additionally, we observed that our AGFs are more potent in cell-based assays than in in vitro inhibition assays, suggesting that our molecules may undergo additional modifications in cells. Our results show that AGFs target disease tissue and inhibit de novo purine synthesis. We will continue to characterize these molecules and develop additional, more potent, molecules.

Characterization of the β-barrel assembly machinery in nanodiscs using cryo-EM

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β-barrel assembly machinery (BAM) is a 200 KDa five component complex in the outer membrane of Gram-negative pathogens. BAM is responsible for the biogenesis of outer membrane proteins (OMPs), which are essential for nutrient import, signaling, and adhesion. Previously, the structure of BAM is first solved by crystallography, which demonstrated the opening of the exit pore and rearrangement at the lateral gate in the periplasmic domain of BamA (PDB: 5EKQ), the central component for BAM. Later on, the lateral gate opening was also observed from cryo-EM (PDB: 5LJO), but in DDM, which is a detergent. In order to figure out native state of BAM in lipids, this study applied nanodisc technology and study the structure of BAM using cryo-EM. It was thus demonstrated that BAM maintains a similar structure in its native lipid environment as the one from DDM, which is consistent with previous reports, and indicates that OMPs are likely inserted into the membrane at the lateral gate during biogenesis.

NMR investigation of Src and Abl tyrosine kinase substrate recognition

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Protein tyrosine kinases(PTKs) are important mediators in multiple cellular processes such as cell proliferation, adhesion, and motility. They are enzymes that selectively phosphorylate tyrosine in specific substrate proteins. The dysregulation of PTKs is seen in a variety of cancers, for example the production of the Bcr-Abl kinase fusion protein in chronic myelogenous leukemia (CML); the presence of Src and EGFR kinases in lung cancer tumor cells.To date, kinase-targeting drug discovery based on ATP-kinase interaction has been intensively studied. Less is known in the field of PTKs-substrate recognition. In particular, structural information on the substrate-interaction site is limited by comparison to ligands overlapping the ATP site. Obstacles include weak binding affinities and transient interactions between kinases and substrates. Understanding how kinases interact with their substrates is essential to explain signaling networks and to exploit substrate recognition as a new avenue in designing effective therapeutics. We study the binding of peptide substrate and bisubstrate to the catalytic domain of Src and Abl tyrosine kinase by solution NMR. In the study of Src kinase domain, we suggest multiple conformations of polypeptide in the bound state. Crystal structures have demonstrated a C-lobe substrate binding site of Src and other PTKs, which differs from cleft binding adopted by Serine/Threonine kinases. However, our paramagnetic relaxation enhancement(PRE) investigations indicate a substrate binding site for Src that is in the cleft between N- and C-lobe, similar to Ser/Thr kinases.

Structural basis of lactoferrin recognition by *Neisserial* lactoferrin binding protein B

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Neisseria are exclusive human pathogens causing meningitis, septicemia, bacteremia and gonorrhea diseases. During pathogenesis, Neisseria bacterium employs TonB-dependent surface receptors to obtain iron from iron-containing host glycoproteins mainly transferrin, lactoferrin, and hemoglobin. Lactoferrin binding protein (Lbp) system selectively hijacks lactoferrin and potentially extracts iron from it to evade the host nutritional immunity. Lbp system is composed of two components: lactoferrin binding protein A (LbpA), and lactoferrin binding protein B (LbpB). Additionally, LbpB provides protection against host and synthetic cationic antimicrobial peptides. However, the molecular mechanisms of Lbp system's functions are unknown due to the absence of structural and biochemical studies. Therefore, this knowledge gap has hindered the development of Lbp-based protein-conjugate vaccines. In the current study, we have determined the crystal structure of Neisseria meningitidis LbpB in complex with holo-lactoferrin at 2.85 Å. The structure details molecular interactions at the NmLbpB-hLf protein-protein interface explaining their nanomolar affinity. The interface is stabilized by extensive hydrogen bonding and salt-bridge formation between C-lobe of lactoferrin and N-lobe of NmLbpB. Structural analysis also provided insight into NmLbpB's selectivity towards holo-lactoferrin. The atomic structure can be used for deciphering molecular mechanism of iron import by Lbp system.

Understanding complex single molecule emission patterns with information based deep learning

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Analyzing single molecule emission patterns plays a critical role in retrieving the structural and physiological information of their tagged targets and, further, understanding their interactions and cellular context1. These emission patterns of tiny light sources (i.e. point spread functions, PSFs) simultaneously encode information such as molecule's location, orientation, environment within the specimen and the paths the emitted photons took before captured by the camera. However, to date, retrieving multiple classes of information beyond 3D position from complex or high-dimensional single molecule data remains challenging, due to the difficulties in perceiving and summarizing a comprehensive yet succinct model. We developed smNet, a deep neural network that can extract multiplexed information near the theoretical limit from both complex and high-dimensional point spread functions. Through simulated and experimental data, we demonstrated that smNet can be trained to efficiently extract both molecular and specimen information, such as molecule location, dipole orientation and wavefront distortions from complex and subtle features of the PSFs, which otherwise are considered too complex for established algorithms. The capability of smNet in extracting sample induced aberration through the raw single-molecule blinking data itself allows wavefront measurement deep into the specimen without guide star and will further allow continuous feedback to a wavefront-control element during single molecule imaging of a living specimen2. We expect that smNet will pave the way for multiplexed physical and physiological measurements through the emission pattern of a single molecule. References 1.W. E. Moerner, D. P. Fromm, Methods of single-molecule fluorescence spectroscopy and microscopy. Review of Scientific Instruments 74, 3597–3619 (2003). 2.P. Zhang, S. Liu, A. Chaurasia, D. Ma, M.J. Mlodzianoski, E. Culurciello and F. Huang, Analyzing complex single molecule emission patterns with deep learning. Nature Methods 15, 583–586 (2018).

Role of an insertion domain in substrate binding in the ubiquitinating transglutaminase MavC

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Bacterial effectors are often adaptations from already preexisting proteins found in host systems for the invasive pathogen to successfully infect the host. One such example is LPG2147 (MavC), a bacterial effector found in Legionella pneumophila, that can ligate a ubiquitin moiety onto the ubiquitin conjugating enzyme UbE2N and can also deamidate ubiquitin. Upon structural comparison of MavC and another known ubiquitin deamidase, CIP, we found that both structures are relatively similar, differing in a nearly 100-residue insertion in the catalytic module of MavC, named the insertion domain herein. This difference between both structures implies that the insertion domain is likely responsible for UbE2N recognition, so to test this, the insertion domain was cloned, expressed, and purified. Pulldown assays were performed to compare the ability of the insertion domain to bind to UbE2N when compared to the full-length protein, which revealed that the insertion domain plays a significant role in binding to UbE2N. A crystal structure of this protein domain was also obtained to confirm that the domain maintains its fold even outside the context of the MavC enzyme, indicating that this domain is an independently folded motif. These studies showed that the insertion domain of MavC is an important contributor of UbE2N binding. Understanding function of domains such as this one can lead to a better understanding of how bacterial effectors have evolved to infect hosts more effectively.