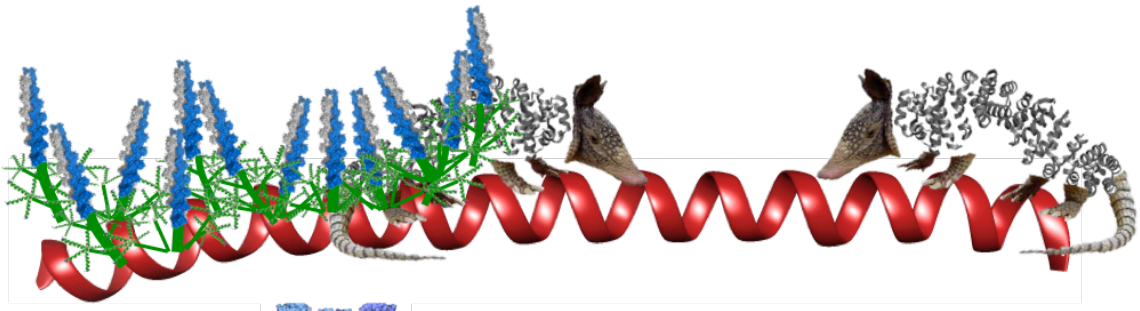
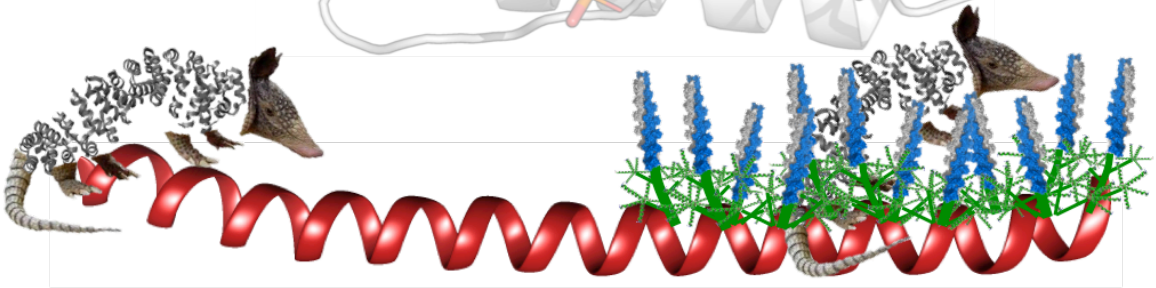
2019 TEXAS PROTEIN FOLDERS & FUNCTION MEETING



27th Texas Protein Folding and Function Meeting



April 12-14, 2019
The Retreat at Artesian Lakes
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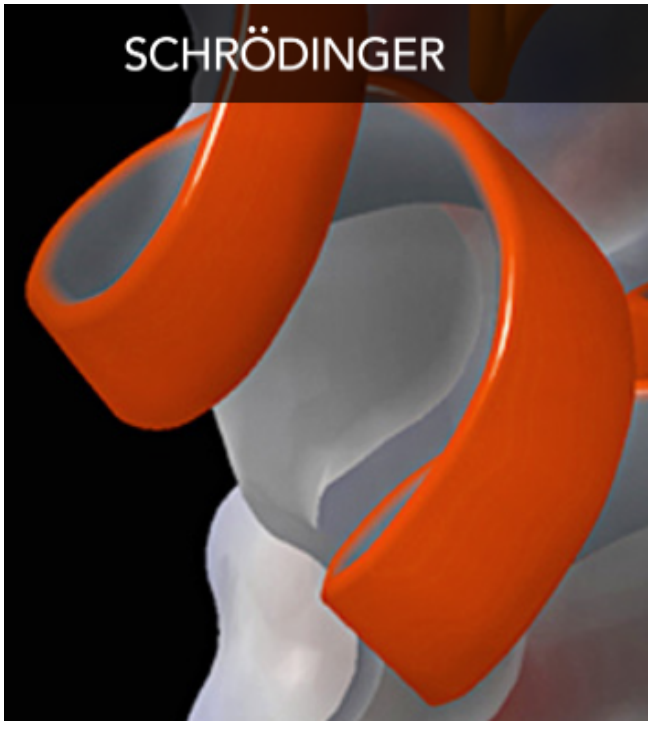

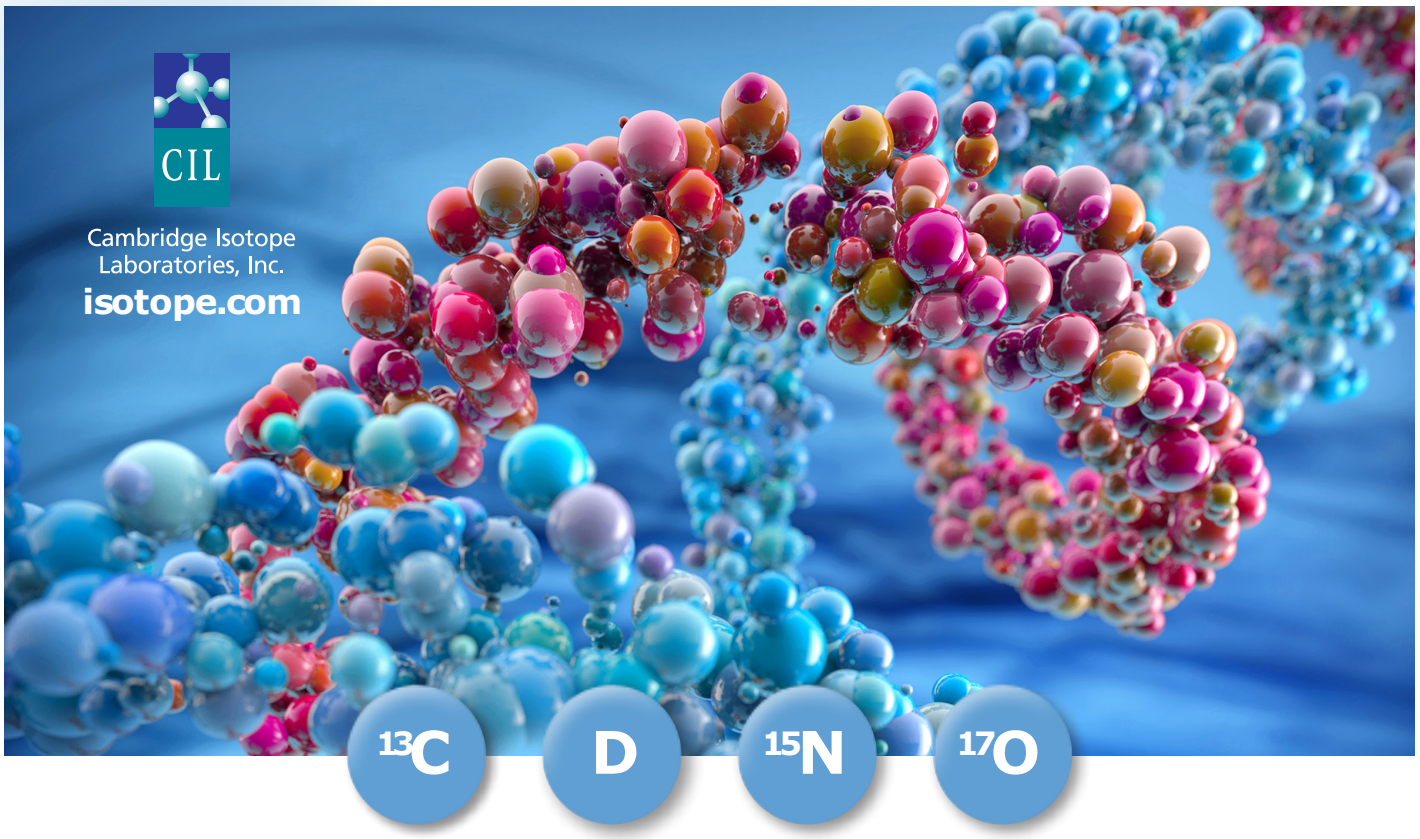

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2019 TEXAS PROTEIN FOLDERS & FUNCTION MEETING

PROGRAM

Friday April 12, 2019

4:00 PM Arrival and Registration

6:00 PM Dinner

7:30 PM **Introduction**

7:35 PM Speaker 1: **Sukyeong Lee** (Baylor College of Medicine) "**Cryo-EM Structures of the Hsp104 Protein Disaggregase Captured in the ATP Conformation"**

8:10 PM Keynote speaker 1: **James Bardwell** (University of Michigan) **"How do Chaperones Fold Proteins?"**

9:10 PM Social, Discussion, and S'mores

Saturday April 13, 2019

8:00 AM Breakfast

9:00 AM Speaker 2: **Mikaela Stewart** (Texas Christian University) **"Uncovering a Function Beyond Folding for the BRCA1 Binding Partner, BARD1**"

9:35 AM Speaker 3: **Kendra Frederick** (UTSW) **"Structural Biology in Cellular Environments Using Sensitivity Enhanced NMR"**

10:10 AM Short talk 1: **Dat Truong** (Texas A&M) **"The Important Role of a Second-Shell Amino Acid in Determining *N*-Succinylamino Acid Racemase Reaction Specificity"**

10:25 AM Short talk 2: **Suman Shrestha** (University of Texas at Arlington) **"Evolution of Specificity and Stability in the Folding Trajectory of Caspase"**

10:40 AM Group photo then Coffee break

11:05 AM Speaker 4: **Jae-Hyun Cho** (Texas A&M) "**Molecular Recognition of Human Phosphoinositide 3-Kinase by Nonstructural Protein 1 of Pandemic Influenza Virus"**

11:40 AM Short talk 3: **Wei Peng** (UTSW) "**Using Cryo-EM to Unveil the Structural Basis for Virulence of a Bacterial Toxin"**

11:55 AM Short talk 4: **Shantanu Guha** (Tulane) **"Ebola Virus Delta-Peptide is an Enterotoxic Viroporin *in vivo*"**

12:15 PM Lunch

4:30 PM Posters and Social

6:00 PM Dinner

7:10 PM Speaker 5: **Mahesh Narayan** (UTEP) **Exploring Outcomes Associated with Amyloid Particle Infiltration of Heterotypic Neurons**

7:45 PM Speaker 6: **Lukasz Joachimiak** (UTSW) "**Native Tau Structure is Disrupted by Disease-Associated Mutations that Promote Aggregation"**

8:20 PM Keynote speaker 2: **Ceclia Clementi** (Rice University) **"Learning Protein Models from Microscopic Simulation and Experimental Data"**

9:20 PM Social, Discussion, and S'mores

Sunday April 14, 2019

8:00 AM Breakfast

9:10 AM Speaker 7: **Sheena D'Arcy** (University of Texas at Dallas) "**Conformational Analysis of Mtr4 using Hydrogen-Deuterium Exchange**"

9:45 AM Short talk 5: **Elahe Masoumzadeh** (Texas Tech) **"Characterizing the Structure and Dynamics of CstF-64 RRM-RNA Complexes Using NMR Spectroscopy"**

10:00 AM Short talk 6: **Drew Roth** (Texas A&M) "**Investigating the Impact of Small Heat Shock Proteins on Aggregate Inhibition and Disassembly"**

10:15 AM Short talk 7: **Daniel Moss** (Tulane) "**Deimmunizing Mutations Alter Conformational Stability and Reshape T-Cell Priming for Recombinant Pseudomonas Exotoxin"**

10:30 AM Short talk 8: **Donna Iadarola** (Texas A&M) **"Molecular Mechanisms Underlying Mitochondrial Phospholipid Import"**

10:45 AM Coffee break

11:00 AM Short talk 9: **Zachary Boswell** (Texas Tech) **"Cancer Associated Mutations Perturb Rad50 D-loop to Circumvent Allosteric Regulation"**

11:15 AM Short talk 10: **Cecilia Bores** (UTMB) **"dsDNA Packed inside Phage Capsids: Structure and Defects Emergence"**

11:30 AM Speaker 8: **Matthieu Gagnon** (UTMB) "**How RRF and tRNA Disassemble the Ribosome During Recycling**"

12:05 PM Lunch and Departure

2019 TEXAS PROTEIN FOLDERS & FUNCTION MEETING

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Future Meetings:

Anticipated dates are March 27-29, 2020

The latest information about past, present, and future meetings can always be found at: http://txprotein.org

KEYNOTE SPEAKER FRIDAY

How do Chaperones Fold Proteins?

James Bardwell

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI

Protein folding in the cell relies heavily upon chaperones, yet the process of how chaperones help proteins to fold remains unclear. This is in large part due to a lack of structural information on protein folding while bound to chaperones. To this end, the chaperone field has been attempting for some time to determine the structure of a chaperone-bound protein in the act of folding. By utilizing a novel hybrid crystallographic method we have succeeded in determining the structural ensemble of the chaperone Spy in complex with an in vivo substrate, Im7. We demonstrate how this chaperone assists protein folding by allowing Im7 to bind in multiple unfolded and partially folded conformations. We have also made a detailed examination of the forces involved in chaperone-client interaction. Our work reveals how chaperones rapidly bind to proteins, how the chaperone-client complex is stabilized, how a chaperone can facilitate substrate folding, and that substrate folding can trigger its own release, without the need of allosteric regulation of the chaperone. Contrary to the commonly held belief that chaperones recognize unfolding intermediates by their hydrophobic nature, we discovered that the model chaperone Spy uses long-range electrostatic interactions for its rapid binding to its unfolded client protein Im7. Short-range hydrophobic interactions follow, which serve to stabilize the complex. Hydrophobic collapse of the client protein then drives its folding. By burying hydrophobic residues in the core structure of the client, the affinity to Spy decreases, which causes the release of the client, making Spy the first self-regulating chaperone. By allowing the client to fold itself, the chaperone circumvents the need for substrate-specific folding instructions and beautifully explains how chaperones can facilitate the folding of various unrelated proteins. Finally I will discuss how our chaperone discovery efforts have gone into yeast with the aim of addressing the role that host factors play in amyloid formation.

KEYNOTE SPEAKER SATURDAY

Learning protein models from microscopic simulation and experimental data

Cecilia Clementi

Department of Chemistry, Rice University

The last years have seen an immense increase in high-throughput and high-resolution technologies for experimental observation as well as high-performance techniques to simulate molecular systems at a microscopic level, resulting in vast and ever-increasing amounts of high-dimensional data. However, experiments provide only a partial view of macromolecular processes and are limited in their temporal and spatial resolution. On the other hand, atomistic simulations are still not able to sample the conformation space of large complexes, thus leaving significant gaps in our ability to study molecular processes at a biologically relevant scale. We present our efforts to bridge these gaps, by exploiting the available data and using state-of-the-art machine learning methods to design optimal coarse models for complex protein systems. We show that it is possible to define simplified molecular models to reproduce the essential information contained both in microscopic simulation and experimental measurements.

Cryo-EM Structures of the Hsp104 Protein Disaggregase Captured in the ATP Conformation

Sukyeong Lee[1], Soung Hun Roh[1], Jun Liu[2], Francis T.F. Tsai[1]

[1]Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, USA; [2]Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut 06520, USA.

Hsp104 is a ring-forming, ATP-driven molecular machine that recovers functional protein from both stress-denatured and amyloid-forming aggregates. Examining the 3D structure of Hsp104 poses considerable challenges because Hsp104 readily hydrolyzes ATP, whereas ATP analogs can be slowly turned-over and are often contaminated with other nucleotide species, and different and seemingly conflicting 3D structures have been reported. Here we present the single-particle cryo-EM structures of a catalytically inactive Hsp104 double Walker B variant ($Hsp104_{DWB}$)in the ATP-bound state. Surprisingly, we observe that the $Hsp104_{DWB}$ hexamer adopts distinct ring conformations despite being in the same nucleotide state. The latter underscores the structural plasticity of Hsp104 in solution, with different conformations stabilized by nucleotide binding. At this meeting, I will discuss the mechanistic implications of our structural observations.

Uncovering a function beyond folding for the BRCA1 binding partner, BARD1

Mikaela Stewart*, Tom Walsh**, Abhinav Dhall**, Champak Chatterjee**, Mary-Claire King**, Rachel Klevit**

*Texas Christian University, Fort Worth, TX; **University of Washington, Seattle, WA

Despite decades of research on the tumor-suppressor known as breast cancer 1 early onset protein (BRCA1), fundamental understanding of its function and how it prevents breast and ovarian cancer risk is lacking. For example, although it has been known for over 15 years that the BRCA1-associated RING domain protein (BARD1) is required for proper folding and function of BRCA1, the role BARD1 plays in catalytic activity remains enigmatic. We have applied biochemistry and nuclear magnetic resonance techniques to characterize BRCA1/BARD1 heterodimers that harbor cancer-associated missense mutations in the RING domain of BARD1. Paradoxically, although heterodimerization with BARD1 is required for BRCA1 ligase activity, we found that the BARD1 RING *per se* is dispensable *in vitro* for the ubiquitylation of several substrates. However, the BARD1 RING domain mutants discovered in families with a history of cancer all show specific loss of ubiquitylation activity towards histone H2A in nucleosomes. Investigation of the interaction between nucleosomes and the BRCA1/BARD1 heterodimer reveals a substrate binding site on the BARD1 RING domain. I will present our biochemical and structural findings, a working model for the role of BARD1 in BRCA1 ligase function, and its implications for breast cancer risk.

Structural biology in cellular environments using sensitivity enhanced NMR

Kendra Frederick

Department of Biophysics, University of Texas Southwestern Medical Center

The misfolded proteins associated with neurodegenerative disease can adopt a variety of different conformations, some of which are toxic. Because these proteins have identical amino acid sequences, the cellular environment clearly influences the final state, yet most structural studies do not include the cellular context and, perhaps because we are not studying the correct conformation, not a single therapeutic strategy for these diseases addresses the underlying protein misfolding pathology. Using new sensitivity-enhancement technology for solid state NMR spectroscopy, we study protein structure in native environments - inside living cells - to reveal how both healthy and disease-relevant cellular environments influence protein structure.

Molecular recognition of human phosphoinositide 3-kinase by nonstructural protein 1 of pandemic influenza virus

Jae-Hyun Cho

Department of Biochemistry and Biophysics, Texas A&M University,

1918 influenza A virus (IAV) caused the worst flu pandemic in human history. Nonstructural protein 1 (NS1) is an important virulence factor of the 1918 IAVs. NS1 antagonizes host defense mechanisms through interactions with multiple host factors. However, the molecular recognition mechanisms underlying the hijacking host proteins by the pandemic 1918 NS1 remain unknown. One important pathway by which NS1 increases IAV virulence is to activate phosphoinositide 3 kinase (PI3K) through binding to the p85β subunit of PI3K. Here, using NMR spectroscopy, small-angle X-ray scattering, and X-ray crystallography, we investigate the molecular basis by which the 1918 NS1 effector domain ($NS1^{ED}$) binds human p85β subunit. Structure determination of 1918 $NS1^{ED}$ in the free and p85β-bound forms, and NMR relaxation dispersion experiments revealed that the free 1918 $NS1^{ED}$ undergoes a dynamic conformational switch in the sub-ms timescale between p85β-binding-incompetent and -competent states. Combined with kinetic analysis, we present that binding of 1918 $NS1^{ED}$ and p85β is well-represented by conformational selection binding mechanism, in which the dynamic motion of free 1918 $NS1^{ED}$ controls the overall binding process. Moreover, comparison of $NS1^{ED}$ structures of diverse IAV strains indicated that the structural adaption occurring through dynamic conformational exchange might be a prevalent mechanism among NS1 proteins.

Exploring outcomes associated with amyloid particle infiltration of heterotypic neurons

Mahesh Narayan

Department of Chemistry and Biochemistry, The University of Texas at El Paso

Until recently, the ability to induce neurodegenerative pathogenicity through a self-seeding homotoxic mechanism was thought to be exclusive to the prion-protein. However, emerging data reveal that other proteins including Amyloid β, tau, and α-synuclein are capable of self-propagating; make them “prion-like” or “amyloidogenic”. In a study with outcomes that would potentially tie Alzheimer’s pathology to Parkinson’s onset, we investigated whether the presence of an amyloid particle in a non-native neuronal locus elicits heterotoxicity. The results from this study are discussed.

Native tau structure is disrupted by disease-associated mutations that promote aggregation

Lukasz A. Joachimiak

Center for Alzheimer's and Neurodegenerative Diseases
Department of Biochemistry
University of Texas Southwestern Medical Center

Tauopathies are neurodegenerative diseases characterized by intracellular amyloid deposits of tau protein. Missense mutations in the tau gene (*MAPT*) correlate with aggregation propensity and cause dominantly inherited tauopathies, but their biophysical mechanism driving amyloid formation is poorly understood. Many disease-associated mutations localize within tau's repeat domain at inter-repeat interfaces proximal to amyloidogenic sequences, such as ^{306}VQIVYK^{311}. Using cross-linking mass spectrometry, intramolecular FRET, recombinant protein and synthetic peptide systems, *in silico* modeling, and cell models, we conclude that the aggregation prone ^{306}VQIVYK^{311} motif forms metastable compact structures with the upstream sequence that modulates aggregation propensity. Disease-associated mutations, isomerization of a critical proline, or alternative splicing are all sufficient to destabilize this local structure and trigger spontaneous aggregation. These findings provide a biophysical framework to explain the basis of early conformational changes that may underlie genetic and sporadic tau pathogenesis.

Conformational Analysis of Mtr4 using Hydrogen-Deuterium Exchange

Sheena D'Arcy, Ph.D.

Department of Chemistry and Biochemistry, The University of Texas at Dallas

Mtr4 is a 3' to 5' DExH-box RNA helicase that is highly conserved among eukaryotes. It plays an essential role in regulating the degradation of various RNA transcripts by stimulating their unwinding and delivering them to the exosome. The exosome is a multi-subunit complex with 3' to 5' ribonucleotlytic activity. Mtr4 can activate the exosome either on its own, or as part of a complex. In the nucleus, one such complex is the Trf4/Air2/Mtr4 polyadenylation complex or TRAMP. The Trf4 subunit of TRAMP is a noncanonical poyl(A)polymerase that adds an oligo-adenylated tail to the 3' end of the RNA substrate, while the Air2 subunit of TRAMP contributes RNA-binding motifs.

Despite the presence of various high-resolution structures of Mtr4, questions remain regarding its RNA helicase activity, RNA binding activity, and how it is altered in the TRAMP complex. To address these questions, we have completed a series of hydrogen deuterium exchange experiments in the presence and absence of RNA substrate. We have analyzed Mtr4 both alone and in the TRAMP complex. The addition of RNA substrate causes large changes in Mtr4 deuterium uptake due to both direct binding and associated conformational change. Intriguingly, we can distinguish these events by different kinetics of deuterium uptake. The exchange data are complemented by biochemical experiments. Future work will expand our analysis to include the exosome.

How RRF and tRNA disassemble the ribosome during recycling

Matthieu G. Gagnon

Department of Microbiology and Immunology, and Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch

Termination of translation occurs when a stop codon on the messenger RNA (mRNA) arrives in the aminoacyl (A) site of the ribosome and is recognized by a release factor, promoting release of the nascent protein. The ribosome is left with the mRNA and deacylated transfer RNAs (tRNAs) in the peptidyl (P) and exit (E) sites. In bacteria, the ribosome post-termination complex (PoTC) is recycled by the concerted action of the ribosome recycling factor (RRF) and the elongation factor G (EF-G). Although many models for ribosome recycling have been proposed, the mechanism by which the 70S ribosome is dissembled into the individual small 30S and large 50S subunits remains elusive. Structure determination of the ribosome complexed with EF-G and RRF has been challenging, which hampered our understanding of ribosome recycling. We have determined a 3.5 Å-resolution crystal structure of the *Thermus thermophilus* 70S ribosome PoTC containing RRF, EF-G, and two deacylated tRNAs in both the P and E sites. The deacylated P-site tRNA has moved into a previously unsuspected state of binding (peptidyl/recycling; p/R) that is analogous to that seen during the initiation phase of protein synthesis. The p/R-tRNA forms novel interactions with the 50S subunit, including non-favorable phosphate-phosphate contacts with the 23S ribosomal RNA (rRNA). The rearrangements in the tRNA propagate to the anticodon loop, weakening the base pairing interactions with the mRNA. The RRF-mediated displacement of the deacylated P-site tRNA on the ribosome appears to follow the reverse path of tRNA binding during initiation, providing a missing link in understanding the role of RRF and tRNA in ribosome splitting.

The Important Role of a Second-Shell Amino Acid in Determining *N*-Succinylamino Acid Racemase Reaction Specificity"

Dat P Truong[1], Simon Rousseau[1], Jamison Huddleston[2], Frank M. Raushel[1,2], James Sacchettini[1,2], & Margaret E. Glasner[1]

[1]Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU,College Station, Texas 77843 [2]Department of Chemistry, Texas A&M University, 3255 TAMU, College Station, Texas 77843

Several lines of evidence show that catalytic promiscuity, which refers to the ability of an enzyme to catalyze more than one reaction in the same active site, plays a role in the evolution of new enzyme functions. Studying catalytic promiscuity can help identify structural features that predispose an enzyme to evolve new functions. Our research will address this problem using the catalytically promiscuous *N*-succinylamino acid racemase/*o*-succinylbenzoate synthase (NSAR/OSBS) subfamily, which is a branch of the OSBS family. We found that the residue R266 is conserved in the NSAR/OSBS subfamily, in which most members catalyze both NSAR and OSBS reactions. However, the homologous position is usually hydrophobic in other nonpromiscuous OSBS subfamilies, which lack NSAR activity. R266 is a second-shell amino acid that is close to the catalytic K263, but it does not contact the substrates, suggesting that R266 affects the catalytic mechanism, rather than substrate binding. Mutating R266 to glutamine in *Amycolatopsis* NSAR/OSBS reduces both NSAR and OSBS activities, but it is more deleterious for NSAR activity. Specifically, the R266Q mutant decreases the rate of proton exchange between the alpha proton of the NSAR substrate and the general acid/base K263 without affecting that of the other general acid/base K163. Analysis of the crystal structure of *Amycolatopsis* NSAR/OSBS R266Q shows that K263 forms a salt bridge with the conserved metal ligand D239, which normally forms a salt bridge with R266 in the wild type protein. This interaction reduces the acid/base reactivity of K263 in the NSAR reaction. However, this interaction is less deleterious for the OSBS reaction because K263 forms a cation-π interaction with the OSBS substrate and/or the intermediate, rather than acting as a general acid/base. Together, the data explain the switch in the reaction specificity by the R266Q mutant and show that R266 is important for determining NSAR reaction specificity.

Evolution of Specificity and Stability in the Folding Trajectory of Caspase

Suman Shrestha and A. Clay Clark

Department of Biology, University of Texas at Arlington

Proteins must fold properly and efficiently to accomplish cellular functions. Over the course of evolution, energy landscape must be maintained such that the protein folds in the right conformation however, how protein's energy landscape is maintained or altered is unclear. To study how a protein's energy landscape changed over time, we characterized the folding trajectories of caspase homologs and their common ancestor. Caspases are an ancient class of cysteinyl proteases that control apoptosis, responsible for cell differentiation, and maintain cellular homeostasis in multicellular organisms. The caspase family is an excellent model to study protein evolution because substrate specificity and conformational selection are retained through hundreds of millions of years of evolution. Some regulatory features are ancient, and therefore common, while other features are modern and cluster specific. In this study, we performed enzyme kinetic assay using synthetic tetrapeptides and substrate phage display using peptide library on ancestral reconstructed and extant proteins to study evolution of substrate specificity. In addition, we examined urea-induced equilibrium unfolding properties to evaluate thermodynamic stability and folding pathways. We found that common ancestor of effector caspases showed promiscuity over substrates (D/L/I/VXXD) which is specified later on in modern proteins in different lineages; caspase-3 and -7 prefers DXXD whereas, caspase-6 prefers VXXD. On the other hand, equilibrium unfolding result suggests, in their optimal pH all the human effector caspases unfolds via four-state process; the native dimer (N2) undergoes an isomerization to a dimeric intermediate (I2) and to a monomeric intermediate (I) before completely unfolded (U). In lower and higher extremities of pH, caspases unfolds via three-state process with either dimeric or monomeric intermediates, or two-state process. Overall, the data show that the folding landscape was established in the early ancestor (>650 MYA) and enzyme specificity evolved from the common landscape. However, there is discrepancy in the overall ΔG^0_{conf}; caspase-6 being most stable with ΔG^0_{Total} 39 kcal/mol and caspase-7 is a least stable with ΔG^0_{Total} 19 kcal/mol. This discrepancy of stability in the folding process between extant caspases may be due to subsequent mutations in the subfamily.

Using Cryo-EM to Unveil the Structural Basis for Virulence of a Bacterial Toxin

Wei Peng[1,4], Marcela de Souza Santos[1], Yang Li[2], Diana R. Tomchick[2,3], Kim Orth[1,3,4]*

[1]Department of Molecular Biology, [2]Department of Biophysics, [3]Department of Biochemistry, [4]Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390, United States

Advances in the field of **cryo-EM** single particle reconstruction have made it a powerful method and tool to reveal molecular mechanisms for biological questions. Using cryo-EM, we have gained insights into structural bases of virulence for bacterial toxins and effectors.

Pore-forming toxins (PFTs) are important bacterial virulence factors. Among the PFT family, hemolysin **ClyA** belongs to the α-helical subfamily, which can be found in *E. coli, Salmonella* Typhi, and *Shigella flexneri*. ClyA is shown to be a dodecamer in the crystal structure. Taking advantage of cryo-EM, we were able to observe that the ClyA pore complexes can exist as dodecamer, tridecamer, and tetradecamer. The reported resolutions for the three oligomeric complexes are 2.8 Å, 3.2 Å and 4.3 Å, respectively, allowing us to reveal the assembly mechanisms. We also show a stabilized intermediate state of ClyA during the transition process from soluble monomers to pore complexes. Unexpectedly, even without the formation of mature pore complexes, ClyA can permeabilize membranes and allow leakage of particles less than ~400 Daltons. In addition, we are the first to show that ClyA forms pore complexes in the presence of cholesterol within artificial liposomes. These findings provide new mechanistic insights into the dynamic process of pore assembly for the prototypical α-PFT ClyA.

Ebola virus delta-peptide is an enterotoxic viroporin *in vivo*

Shantanu Guha, Lilia Melnik, Jenisha Ghimire, Jing He, Robert F. Garry, William C. Wimley

Department of Biochemistry and Molecular Biology, Tulane University School of Medicine, New Orleans, LA

Ebola virus (EBOV), part of the filovirus family, has a genome that encodes for a partly conserved, 40-residue polypeptide, called the delta peptide, which is produced during Ebola virus disease pathogenesis. Sequence-structure-function analysis and *in vitro* data show that the delta peptide is a viroporin, a term used to describe a diverse family of virally encoded pore-forming peptides and proteins involved in the replication and pathogenesis of numerous viruses. Full-length and conserved C-terminal EBOV delta peptide fragments permeabilize synthetic lipid bilayers and multiple cell types *in vitro*. Activity requires that the two conserved cysteines are connected by a disulfide linkage. Here, we test delta peptides in an established mouse model of diarrheal pathology focusing on the small intestine. We hypothesized that the cell permeabilization observed against numerous cell types *in vitro* would correspond to intestinal pathology, *in vivo*. We found that the delta peptide exerts potent enterotoxic activity against mouse intestine upon introduction to a closed intestinal ileal loop, resulting in a diarrheal syndrome in the mice. The peak activity of the peptide occurs 9-12 hours after introduction of peptide into the intestinal loop. Histological examination of the intestinal architecture showed severe damage and loss of goblet cells. Our *in vivo* results demonstrate that the EBOV delta peptide is a potential novel therapeutic target, and neutralizing it could ameliorate some of the severe enterotoxic burden characteristic of EBOV disease pathogenesis. Accordingly, our recent focus has been to engineer and subsequently characterize a high-affinity neutralizing antibody to block the activity of the delta peptide *in vitro* and *in vivo* as a novel and efficacious therapeutic agent against active EBOV infections.

Characterizing the structure and dynamics of CstF-64 RRM-RNA complexes using NMR spectroscopy

Elahe Masoumzadeh[1], Petar N. Grozdanov[2], Clinton C. MacDonald[2], and Michael P. Latham[1*]

[1] Department of Chemistry & Biochemistry, Texas Tech University [2] Texas Tech University Health Sciences Center

CstF-64 is a member of the Cleavage stimulation Factor (CstF) protein complex which plays an important role in mRNA 3'-end processing and in transcription termination of poly(A) sites. CstF-64 contains an RNA recognition motif (RRM) that binds to the G/U rich RNA sequences located downstream of the cleavage and polyadenylation site and hence is an important part in the regulation of the cleavage and polyadenylation. There are several studies examining RNA binding for different families of RRMs, but the exact RNA binding mechanism and how the CstF-64 RRM protein correctly recognizes the G/U sequence is not still well-understood. Here with the use of NMR techniques, we were able to calculate the RRM structure with and without bound RNA. We also studied the fast (pico-to-nanosecond) and slow (micro-to-millisecond) timescale dynamics of the RRM in the RNA free and bound forms. Interestingly, our millisecond time scale NMR dynamics data reveal another process after RNA binding. We hypothesized that there are two populations of RRM-RNA complex: the major population corresponds to the RRM-RNA complexes (1:1) whereas the minority of RRM-RNA complexes undergo a transition to another conformation, possibly a dimeric RRM state, after binding.

Investigating the Impact of Small Heat Shock Proteins on Aggregate Inhibition and Disassembly

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Cells have evolved a set of highly conserved proteins known as chaperones to assist in cellular function and homeostasis by correcting and preventing protein misfolding and aggregation. However, under extended stress, some proteins still misfold and aggregate, resulting in diseases such as cardiomyopathies and numerous neuropathies. The small heat shock proteins (sHsps) are a class of chaperones capable of inhibiting aggregation and assisting in protein disaggregation. However, the mechanisms by which the sHsps carry out these functions are poorly understood. In order to address this problem, we utilize the *E. coli* sHsps*,* IbpA and IbpB (IbpAB) with an established aggregation-prone substrate RuBisCO (from *R. Rubrum)*. We have found that RuBisCO forms either amorphous or fibril-like aggregates under slightly different conditions. Because standard bulk assays cannot be used to characterize the state distribution of protein aggregates in detail, we have developed a single particle fluorescence technique known as Burst Analysis Spectroscopy (BAS), which permits the minimally perturbative, free-solution observation of aggregate nano-particle size distributions. Using BAS, we observe that IbpAB can limit aggregate particle growth to a strikingly similar but limited size range, which is independent of the aggregation pathway. Using a two-color variant of BAS (MC-BAS), we show that IbpAB displays distinctive binding patterns when interacting with structurally different RuBisCO aggregate particles, suggesting IbpAB can sense and react to specific aggregate characteristics. Using ensemble inter- and intra-molecular FRET assays, we show that the binding of IbpAB to RuBisCO aggregates alters both the average relative proximity of different RuBisCO monomers within an aggregate, as well as the average conformation of the RuBisCO monomer itself. Time-resolved BAS measurements of aggregate disassembly in the presence of the *E. coli* bi-chaperone disaggregase, consisting of DnaK, DnaJ, GrpE, and ClpB (KJE/B), demonstrate that (1) IbpAB dramatically enhances RuBisCO aggregate disassembly and (2) that IbpAB release from aggregate particles is co-incident with disassembly of the aggregate itself. Additionally, IbpAB can inhibit aggregate growth of an aggregating RuBisCO sample depleted in the non-native monomer pool however, stimulated disaggregation by the KJE/B disaggregase is lost. Overall, these observations are most consistent with a model in which IbpAB bind to early aggregating states of RuBisCO, incorporate into the nascent aggregate particle and directly alter the conformational properties of the aggregate so that both ongoing particle growth is blocked and subsequent aggregate disassembly is enhanced.

Deimmunizing mutations alter conformational stability and reshape T-cell priming for recombinant pseudomonas exotoxin

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Effective adaptive immune responses depend on activation of $CD4^+$ T-cells via the presentation of antigen peptides in the context of MHC class II. The structure of an antigen strongly influences its processing within the endolysosome and potentially controls the identity of peptides that are presented to T-cells. A recombinant immunotoxin, comprised of pseudomonas exotoxin A domain III (PE-III) and a cancer-specific antibody fragment, has been developed as a treatment for cancer, but its effectiveness is limited by the induction of neutralizing antibodies. Immunogenicity was substantially reduced by mutating six residues within PE-III. Although the mutations targeted T-cell epitopes, we demonstrate that reduced conformational stability and protease-resistance were responsible for reduced antibody titer and altered T-cell priming. Analysis of mouse T-cell responses coupled with biophysical studies on single-mutant versions of PE-III suggest that modest but comprehensible changes in T-cell priming can dramatically perturb antibody production.

Molecular Mechanisms Underlying Mitochondrial Phospholipid Import

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Mitochondrial membrane biogenesis requires import of phospholipids; however, the molecular mechanisms underlying this process remains elusive. Recent work has implicated membrane contact sites between the mitochondria, endoplasmic reticulum (ER), and vacuole in phospholipid transport. Utilizing a genetic approach focused on these contact site proteins, we identified Vps39 as an essential protein required for phosphatidylethanolamine (PE) transport from the ER to the mitochondria. Vps39 abundance and recruitment to the mitochondria and the ER is dependent on PE levels in each of these organelles, directly implicating Vps39 in the transport process. Contrary to the established roles of Vps39 in vesicular fusion and contact site formation, we find that the function of Vps39 in mitochondrial PE import is independent of both of these processes; a finding that uncovers a completely novel role of Vps39 in intracellular PE trafficking. Our work provides molecular underpinnings of PE transport from the ER to the mitochondria.

Cancer associated mutations perturb Rad50 D-loop to circumvent allosteric regulation

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Detection and repair of DNA double strand breaks are critical for cellular survival and cancer prevention. The Mre11-Rad50-Nbs1 (MRN) protein complex is a primary responder to DNA double strand breaks, tethering damaged DNA strands and preparing the broken ends for downstream homologous recombination or non-homologous end joining. Rad50, a member of the ATP-Binding Cassette (ABC) ATPase super-family, regulates the conformational and functional states of MRN via ATP-induced association of the two nucleotide binding domains (NBDs) followed by ATP hydrolysis. Mutations of two conserved residues in Rad50 D-loop (leucine and aspartic acid) are implicated in cancer with the leucine to phenylalanine mutation resulting in a curative outlier response in a human patient with metastasized cancer. Here, we show that these two D-loop mutations have gain-of-function ATPase activity, which profoundly impairs ATP-dependent regulatory functions of the protein complex, while disparately affecting Rad50 structure and dynamics. The conserved leucine is critical for maintaining an “inactive” Rad50 ground state, and mutation to phenylalanine serves to populate an “active” state that preemptively orients Rad50 for ATP hydrolysis and circumvents allosteric regulation. Conversely, mutation of the conserved aspartate minimally impacts structure and dynamics, suggesting an alternate mechanism to reach an activated ground state. From detailed characterization of this hypersensitive motif, we propose a molecular mechanism for allosteric regulation of Rad50 ATP hydrolysis and NBD dissociation required for proper DNA double strand break repair initiation. These studies describe the relationship between function, structure, and molecular motions in improperly regulated Rad50, which could reveal the underlying mechanism for how these cancer mutations affect the cell.

dsDNA Packed inside Phage Capsids: Structure and Defects Emergence

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DNA packaging and ejection are two critical moments in dsDNA bacteriophages lifecycle and their understanding is decisive for the effective application of phages as an alternative to antibiotics. The forces needed to pack the DNA molecule to near crystalline density (~0.5g/ml) combined with the geometrical constrains of the phage capsid determine the conformation of the confined DNA. Most theoretical studies that have been performed to better understand how is the conformation of the DNA inside bacteriophage capsids considered DNA as a perfect elastic rod and predict highly ordered structures. However, the emergence of more disordered conformations exhibiting defects such as knots, kinks, loops, … that hinder both the insertion and extraction of the DNA molecule in the phage capsid is also plausible. This raises the central question of the present work - how much order and disorder is reasonable (or required) when DNA is confined inside the phage capsid? We have performed Molecular Dynamics simulations using oxDNA model for dsDNA and a purely repulsive harmonic wall representing the proteic capsid of the bacteriophage to mimic the packing process in phage ϕ29. We have thoroughly analyzed the DNA conformation by means of density profiles and correlation functions during packing finding different results depending on how fast the DNA is being inserted. DNA structure predicted by these simulations show patterns that agree with experiments, cryoEM and X-ray diffraction, but many features in a more realistic capsid model – presence of multivalent ions, torsional forces, and local attractive/repulsive sites in the capsid or an elongated shape – might contribute to the emergence of these or other characteristics.

Synthesis of Multi-part Pf Rad50 for NMR Spectroscopy Analysis of Coiled-coil Domain in Full-length Pf Rad50

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The Mre11-Rad50 complex is important for DNA double strand break repair in all organisms. Research has suggested that conformational changes in the globular, enzymatic region of the Rad50 protein propagates long-range allosteric changes across the coiled-coil region of the protein, a distance of several hundred Ångstroms. Methyl-labeling in combination with nuclear magnetic resonance (NMR) spectroscopy provides a means for understanding the structural changes within the protein complex the size of Mre11-Rad50 (~240 kiloDaltons); however, there currently is no method for isolating the methyl-labeling to specific regions on the coiled-coil for analysis of full-length Rad50. Methyl-labeling a small segment within the full-length protein would greatly simplify analysis of NMR data, providing an opportunity to study this long-range allostery in more detail. We purified three segments of Rad50 consisting of the N Terminal coiled-coil region, zinc hook domain, and C Terminal coiled-coil region, and attempted to join the strands using a combination of sortase and intein chemistry in various chemical conditions. We anticipate that the strands will be fused yielding a protein that is structurally and functionally indistinguishable from its full-length counterpart allowing us to study changes in Rad50 structure that occur over large distances.

Investigating the mechanism of Mre11 nuclease activities

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Our genomic DNA is exposed to a number of stress conditions such as reactive oxygen species, ionizing radiation, chemical agents, and ultraviolet light all of which can result in DNA double strand breaks (DSBs). Inefficiency of the proteins involved in the DNA damage response to repair the damage might have tumorigenic potential. The initial complex involved in DNA DSB repair is Mre11-Rad50-Nbs1 (MRN). This complex is thought to be involved in binding to and and unwinding the damaged DNA double helix, processing the broken DNA ends and recruiting other downstream proteins to repair the damage. Mre11 has Mn^{2+}-dependent 3'-to-5' dsDNA exonuclease and ssDNA endonuclease activity. We do not understand the mechanistic details of these activities, but several conserved histidine residues have been implicated in the catalytic mechanism. Here, we are using pH dependence of the activities to understand the role of these histidine residues. Exonuclease and endonuclease data show that Mre11 is exonuclease inactive but still endonuclease active in higher pH. Advances in solution state NMR spectroscopy have enabled us to selectively label and study large protein complexes like MR (~200 kDa). Our lab uses side chain methyl group labeling of hyperthermophilic P. furiosus (Pf) Mre11 and Rad50 to study the structure and dynamics of the individual proteins and their complex. Two dimensional $^{1}H,^{13}C$ correlation spectra of labeled isoleucine, leucine, valine and methionine show changes in chemical shifts of several methyl groups upon changing pH. The existing X-ray crystal structure of the Pf Mre11 nuclease and capping domains was used to see the effect of changing the pH for each residue in structure. Our data suggests that the separation of endonuclease and exonuclease activity may come from different active sites or a different catalytic mechanism for these two activities.

Building a Better Biosensor: Materials enhance and stabilize incorporated proteins

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Green fluorescent protein (GFP) has been developed as a sensor for various targets, including protein-protein interactions, ion concentrations, and intracellular pH. One proof-of-concept sensor system that has been developed is a set of truncated circular permutants of GFP, collectively known as leave-one-out GFP (LOO*n*-GFP), in which the n^{th} β-strand has been 'left out' of the protein. Without this β-strand the GFP is unable to fold properly and is not fluorescent. Fluorescence can be reconstituted with the addition of the left-out peptide, making this construct a self-reporting biosensor for the missing piece of its sequence. LOO-GFPs have a tendency to oligomerize, which (i) hampers ligand binding and (ii) leads to fluorescence in the unbound state. Monomerization of LOO-GFP on beads reduces the background but also decreases binding affinity. To overcome these issues, we genetically fused LOO-GFP to *Drosophila* Ultrabithorax (Ubx), which self-assembles into a variety of materials *in vitro*. Ubx stabilizes incorporated proteins, so that EGFP-Ubx materials can be autoclaved or stored dry for 9 years and retain fluorescence. Ubx offers a transparent support system for LOO-GFPs and has the potential to maintain LOO-GFP stability and binding affinity while preventing LOO-GFP aggregation. Indeed, LOO-GFPs bound to Ubx materials are capable of left-out peptide removal and rebinding with low amounts of background fluorescence. To assess the utility of this composite material, we are examining assembly of LOO-GFP-Ubx fibers, measuring binding affinities, and quantifying on- and off-rates for the left-out peptides. In the long term, the LOO-GFP-Ubx system can be engineered to bind and detect other peptide sequences.

DOMAIN STABILIY AND FUNCTIONAL ANALYSIS AT THE AD3 LOCUS OF SYNAPTOTAGMIN 1 C2 DOMAINS

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Synaptotagmin 1 (Syt1) is a member of a family of proteins that play a crucial role in facilitating Ca^{2+}-mediated vesicle fusion in neurotransmitter release. They are comprised of two tandem C2 domains, C2A and C2B, which are tethered to the membrane by a single transmembrane helix. Generally, the C2 domain motif is found in a broad range of proteins that bridge the interaction between protein and membrane. A single point mutation (Y311N) in a highly conserved region of the C2B domain of Drosophila Syt1 has the ability to cripple Ca^{2+}-mediated vesicle fusion, despite the protein's ability to fold correctly and target the membrane. In addition to this mutation, we introduce another point mutation (Y311F) to combat the hindered thermodynamic stability of the domain from the original mutation. We show that the AD3 locus appears to regulate a delicate balance between the structural stability of the general C2 fold and its Ca^{2+} sensitivity by acting as a "brace" through the combination of steric bulk and hydrogen bonding potential. We aim to elucidate the importance of the AD3 locus of Synaptotagmin 1 through the use of X-ray crystallography, isothermal titration calorimetry (ITC), and guanidine denaturation coupled to circular dichroism (CD).

Extension protein engineering improves protein stability and binding

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It is desirable to modify proteins to have higher protein stability and stronger interactions with their binding partners and protein termini still remain an unexplored region for optimization. We hypothesized that certain termini extensions will form desirable intra-molecular interactions, while maintaining the protein's original structure/function. As such, we have developed a method called extension protein engineering (EPE) that screens libraries of variants containing artificial extensions to their natural sequence for improved stability and binding. The method uses recently developed high throughput protein folding equilibrium and kinetic assays and a custom data analysis program. EPE was applied to a hybrid protein that connects the yeast SH3 domain found in Abp1 (AbpSH3) to its target peptide from Ark1 (ArkA15) using a flexible linker. In this hybrid protein, we first mutated the last peptide residue, K(-8), at the C-terminus of the hybrid, to 15 other amino acids (and a stop codon) and found little improvement in stability. The ArkA15 peptide was then extended beyond this position for an additional 4 residues using a limited library. Each residue in the extension contains one of 4 residues (Y, S, P, H) at each position generating a library size of 256 variants. A number of extended peptides (enriched in tyrosine) were identified from the library that increased hybrid stability through slower unfolding rates compared to wild-type. NMR spectroscopy shows that the peptides appears to bind to a pocket centred on L38, extending it's known binding site. EPE with a similar 256 variant library was also used at the N-terminus of AbpSH3 alone to yield 4-residue extended variants also enriched in tyrosine with increased domain stability, however these were predominantly stabilized through faster folding rates compared to wild type. Interestingly, the domain extension appears to bind to the same L38 region that was previously found for the hybrid, suggesting this is a good additional binding region for AbpSH3. Taken together, simple artificial extensions can indeed be optimized to increase protein stability and peptide binding without affecting core structures/functions. EPE promises to be a simple method to improve the properties of many other proteins or peptides.

Discovering novel antimicrobial peptides using high-throughput screening and rational variation.

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Antimicrobial peptides (AMPs) have long been attractive drug candidates for the next generation of clinical antibiotics due to their potent antimicrobial activity and low propensity for inducing resistance in pathogens. However, due in part to toxicity concerns and activity loss *in vivo*, AMPs have yet to have any impact clinically. Our lab hypothesized that the presence of host cells could cause depletion of free peptide available to target bacterial cells and showed this to be true for some AMPs using concentrated human red blood cells (RBCs) as a model eukaryotic cell. To solve this problem, we synthesized a combinatorial peptide library based on the potent AMP, ARVA, and screened the library for activity in the presence of concentrated RBCs. We isolated nine unique, but similar sequences from the screen. We designed a consensus sequence based on the nine peptides and synthesized it using only D-isomer amino acids to form D-NOGCON. D-NOGCON displays excellent antimicrobial activity against multiple human pathogens in the presence and absence of concentrated RBCs, causes very little hemolysis, and is not susceptible to cleavage by cellular or plasma proteases. D-NOGCON also has high activity against bacterial biofilms and does not readily induce leakage. In this work, we created rational variants of D-NOGCON with truncations, insertions and mutations to test various hypotheses about the basis for highly potent antimicrobial activity with low hemolysis and low toxicity against nucleated cells. In the near future, we will use this information to design a next generation combinatorial peptide library based on D-NOGCON that will be screened for i) clinically relevant antimicrobial activity in the presence of serum and concentrated red blood cells, ii) negligible hemolysis, and iii) negligible toxicity against nucleated cells.

Mechanism of Yeast N-BAR Protein Membrane Fission Activity

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Extracellular and membrane substrates are internalized through endocytosis and delivered to early endosomes in membranous vesicles. Formation of these carriers requires membrane fission proteins and regulatory factors. One well-characterized fission protein is Dynamin, which is considered as a universal fission agent in Eukaryotes[1]. Dynamin is a multidomain GTPase, thought to remodel membranes into tubules and accomplishing fission through conformational changes of helical polymer, caused by GTP hydrolysis[2]. While the dynamin homolog is absent on the plasma membrane of *S. cerevisiae,* an N-BAR homolog, Rvs161/167p is required for endocytosis[3]. Using a single particle fluorescence technique, called Burst Analysis Spectroscopy (BAS)[4], we observe Rvs161/167p induce membrane fission in a physiological rate (~10 s/fission reaction)[5]. Meanwhile, we observe the termination of fission activity by truncating N-terminus, indicating Rvs161/167p may follow amphipathic helix insertion model to cause membrane fission, instead dynamin paradigm. The similar result has been achieved with N-BAR of *C.elegans*, AMPH-1, indicating N-BAR may function as another universal fission proteins.

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The Molecular Mechanism and Structural Analysis of Membrane Interaction via FerA Domains in Ferlins

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Ferlin proteins participate in such diverse biological events as vesicle fusion in *C. elegans*, fusion of myoblast membranes to form myotubes, Ca^{2+}-sensing during exocytosis in the hair cells of the inner ear, and Ca^{2+}-dependent membrane repair in skeletal muscle cells. Mutations in dysferlin can cause muscular dystrophies and abnormalities in myoferlin expression are linked to several types of cancer. Ferlins are Ca^{2+}-dependent, phospholipid-binding, multi-C2 domain-containing proteins with a single transmembrane helix that spans a vesicle membrane. The overall domain composition of the ferlins resembles the proteins involved in exocytosis; therefore, it is thought that they participate in membrane fusion at some level. But if ferlins do fuse membranes, then they are distinct from other known fusion proteins. Here we show that the central FerA domain from dysferlin, myoferlin, and otoferlin is a novel four-helix bundle fold with its own Ca^{2+}-dependent phospholipid-binding activity. Small-angle X-ray scattering (SAXS), spectroscopic, and thermodynamic analysis of the dysferlin, myoferlin, and otoferlin FerA domains, in addition to clinically-defined dysferlin FerA mutations, suggests that the FerA domain interacts with the membrane and that this interaction is enhanced by the presence of Ca^{2+}.

In all disorder a secret order: the interactions of the intrinsically disordered region of BRCA1

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The intrinsically disordered region of breast cancer 1 early onset protein (BRCA1) binds to the partner and localizer of BRCA2 (PALB2), which leads to the recruitment of both PALB2 and BRCA2 to areas of DNA damage. Genetic mutations in *PALB2* that disrupt this interaction are associated with defects in DNA damage repair and higher risks of breast cancer. In order to understand the molecular details of this vital interaction, we have developed a system to study the interaction of BRCA1 and PALB2 *in vitro*. We find that minimized binding regions of BRCA1 and PALB2 proteins participate in a high-affinity interaction *in vitro.* Our preliminary circular dichroism and nuclear magnetic resonance data conflict with the previously predicted model of BRCA1 forming a coiled coil upon interaction with PALB2. We will also be using this *in vitro* system to explore the change in binding affinity in constructs with mutations that have been associated with development of breast and ovarian cancer. We will present our structural findings and new insights regarding this important DNA damage response recruiting event.

Structural investigations into the serum endonuclease Dnase1L3, as it relates to Systemic Lupus Erythematosus

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Dnase1L3 is a serum endonuclease in the Dnase1 family. As contrasted with the better known Dnase1, Dnase1L3 is more active in apoptotic microparticle degradation, chromatin DNA laddering, and is uninhibited by actin. Presence of undigested apoptotic DNA in serum results in anti-dsDNA antibodies and anti-neutrophil cytoplasmic antibodies, which characterize Systemic Lupus Erythematosus (SLE). SLE is an autoimmunological disease known for multisystem inflammation. The differences between activities of Dnase1 and Dnase1L3, have previously been shown to play a key role in the progression of SLE. Lack of functional Dnase1L3 can cause pediatric onset lupus which corresponds to a high incidence of lupus nephritis. Based on homology models, the 23 amino acids on the C-terminus of Dnase1L3 represent a substantial structural difference from Dnase1 and modulate key physiological roles of the enzyme with respect to Dnase1. We hypothesize that the Dnase1L3 C-terminus changes secondary structure in the presence of DNA and/or interacts with and disrupts lipid membranes. To test this hypothesis, we have undertaken structural analysis of the C-terminus with Circular Dichroism. This has allowed us to measure the secondary structure changes of the C-terminus during a variety of physiologically relevant intermolecular interactions. The phospholipid interaction has been assayed by co-sedimentation assay. In addition, we have obtained valuable structural information through protein crystallography. Any work in further developing this enzyme as a potential enzymatic therapy, or a myriad of other biotechnical applications, will rest on the structural information gained from our investigations into the structural and biochemical nature of Dnase1L3.

Structural and enthalpic comparisons of RNA-edited C2A and C2B domain variants of Octopus Synaptotagmin 1.

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Deamination of adenosines and cytosines in mRNA transcripts is one method by which organisms can change codons prior to translation in order to alter the sequence of individual amino acids, without permanently altering the genome. Cephalopods utilize this technique much more than mammals do. The focus of our research is to examine two such edits which octopus use to modify the sequence of Synaptotagmin 1 in the C2A and C2B domains. These domains are calcium binding domains which also bind the membrane. Synaptotagmin 1 acts as a calcium sensor in neurons, allowing vesicles to fuse with the pre-synaptic membrane when the neuron experiences a calcium pulse. We are comparing the 'wild-type' sequence (the sequence that matches the DNA) of each domain with an I to V substitution at position 248 in the C2A domain (I248V) and 395 in the C2B domain (I395B). These two variants are differentially expressed in different parts of the octopus. We are solving the structure of each domain with x-ray crystallography to make structural comparisons between the variants. We also have successfully crystallized both variants of the C2A domain both in the presence and the absence of calcium, so we are also making a comparison between calcium bound and not bound. We are also making enthalpic comparisons with isothermal titration calorimetry (ITC) data to compare the change in heat when calcium binds each domain. We are also obtaining data on circular dichroism to give additional insight into the structures. Preliminary data suggests that the I to V edit alters the calcium-binding affinity of these domains.

Stabilizing and Delivering Growth Factors to Promote Angiogenesis

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Engineering vascular tissues requires construction of biocompatible 3D scaffolds that provide protein signals to stimulate new blood vessel growth. However, such proteins are often small and unstable, and easily lost or inactivated during scaffold construction. We have generated biocompatible and mechanically robust materials derived from the *Drosophila melanogaster* Hox protein Ultrabithorax (Ubx). Because Ubx materials self-assemble in mild buffers, we can incorporate active functional proteins, such as vascular endothelial growth factor (VEGF), through gene fusion. VEGF-Ubx materials promote primary human endothelial cell attachment, activation, and survival, and can instigate and guide neovascularization *ex vivo* and *in vivo*. To enhance the reliability of this response, we have incorporated additional growth factors and created chimeric Ubx materials fused to basic fibroblast growth factor (bFGF), stromal cell-derived factor 1 alpha (SDF-1a), and VEGF alone and in combination. *In vitro* attachment and survival assays revealed that combining all three growth factors elicited the greatest endothelial attachment, activation, and survival responses. Testing is underway to determine if VEGF-, bFGF-, and SDF-1a-Ubx materials also enhance development of angiogenic vessels in the chick chorioallantoic membrane and following subcutaneous implantation in mice. Our data suggest that Ubx-based materials will be useful for promoting vascularization, demonstrating that precise delivery of growth factors can be accomplished by these unique biomaterials.

Directed Evolution of NSAR Activity

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The fitness landscape of proteins that evolve new activities and the propensity to evolve along multiple or different mutational paths is not well understood. Promiscuous proteins, which have a second chemical activity that is biologically irrelevant, provide a suitable system to study protein evolution. Promiscuous and in cases biologically relevant NSAR (*N*-succinyl amino acid racemase) activity evolved in an ancestral OSBS (*o*-succinyl benzoate synthase) in the NSAR/OSBS enzyme subfamily and later evolved into a biologically relevant function. However, the amino acid substitutions and structural changes that were required to gain NSAR activity are not known. Given the relatively unknown nature of the fitness landscape and the large sequence diversity among NSAR/OSBS proteins, are there multiple mutational trajectories to gain NSAR activity? Furthermore, do these trajectories differ based on which protein sequence is used as the template? To answer these questions, a directed evolution experiment has been devised in which survival of an L-methionine auxotrophic strain requires the conversion of D-methionine to L-methionine using an NSAR-dependent metabolic pathway from *Geobacillus kaustophilus*. The mutations in the survivors will identify which residues are required for the gain of NSAR activity. Starting from different non-promiscuous protein templates, the mutational trajectory leading to increases in NSAR activity will be tracked by determining effects of mutations that affect NSAR activity in successive rounds of evolution. Collectively, these results will show how evolvable NSAR activity is in the NSAR/OSBS family and if it is possible have different mutational trajectories to evolve the same activity.

Conserved Residues Have Different Roles in Homologous OSBS Enzymes

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Conserved amino acids are commonly presumed to have the same function in homologous proteins. However, the role of any amino acid is also tied to its structural context. To investigate how the roles of conserved amino acids depend on their structural context, we examined *o*-succinylbenzoate synthase enzymes from *Thermobifida fusca* (TfOSBS) and *Escherichia coli* (EcOSBS) that share only 22% sequence identity. Rapid divergence of these enzymes due to sizable insertions and deletions led to differences in active site size and electrostatic interactions. Analysis of the structural interactions between the two proteins, however, suggests that two conserved active site residues (R128/G254 in TfOSBS and R159/G288 in EcOSBS) play similar roles in substrate binding. Surprisingly, mutating these amino acids severely decreased the catalytic efficiency of EcOSBS, but was only mildly deleterious in TfOSBS. Instead, the G254A mutation in TfOSBS compromises thermostability, while G288A in EcOSBS does not affect thermostability. To determine whether this difference arises from the mesophilic versus thermophilic natures of the two species, we determined the effects of the same mutations in *Amycolatopsis mediterranei* OSBS (AmOSBS), a mesophilic Actinobacteria in the same subfamily as TfOSBS. The R124M and G252A mutations in AmOSBS had the same effects as the mutations in TfOSBS, indicating that the divergent roles of these amino acids stem from structural divergence of the two subfamilies, rather than adaptation to thermophilicity. Extending this analysis to other active site positions demonstrates that many other positions play different roles in thermostability and activity in the two enzymes. These differences are due not only to structural divergence between the enzymes, but also changes in conformational dynamics.

Structure and Dynamics of DnaJB8 Co-chaperones in their Oligomeric State

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The DnaJ (Hsp40) family of proteins are a diverse set of co-chaperones that encode a functionally conserved J domain, which cooperates with Hsp70 proteins to maintain cellular homeostasis. In mammalian cells, the DnaJ family encodes over 40 diverse members but a mechanistic understanding of protein folding for many of these members remains limited. A member of the B subfamily of the DnaJ family, DnaJB8, has been implicated in suppression of a poly-glutamine expanded huntingtin protein aggregation but little is known about the mechanics of this process. Structural information on this chaperone has been limited due its propensity to oligomerize. Here we used a multidisciplinary approach that includes crosslinking mass spectrometry (XLMS), solid state NMR (ssNMR) and biochemistry to study the dynamics and oligomerization of DnaJB8. The protein encodes a functionally conserved N-terminal J-domain (JD) two low complexity domains (F/G and S/T rich) and a C-terminal domain (CTD), implicated in HDAC activity, but only a structural model is available for the JD. Using full length DnaJB8 protein, we show that this co-chaperone forms oligomeric assemblies *in vitro* and *in vivo*. Using cross-linking mass spectrometry (XLMS) we show that the distal JD and CTD domains form electrostatically driven contacts. Assembly of the DnaJB8 oligomer is perturbed by increases in ionic strength and a concomitant loss of contacts between the JD and CTD. Next, using ssNMR we show that the DnaJB8 oligomer is rigid and that the JD is stably immobilized, but when ionic strength increases this interaction is lost and the dynamism of oligomers increases. Recombinant JD and CTD domains are well folded, yield monodisperse peaks by SEC and XLMS pairs for the JD are consistent with the NMR model. Finally, we reconstitute the JD:CTD interaction *in vitro* and using XLMS and chemical shift mapping validate that the interaction is electrostatically driven. Our data supports an evolutionarily conserved mode of interaction in DnaJB8 between two distal domains, JD and CTD, that drive oligomeric assembly, modulate the conformation of the low complexity domains, and likely play a role in substrate recruitment and regulation of Hsp70 binding.

The Characterization and Crystallization of the Human Oxidoreductase, Pyrox-D1: A New Described Cause of Early-Onset Myopathy in Humans

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Mutations in the oxidoreductase, Pyrox-D1, have been linked to a newly described, early-onset recessive myopathy from five families with four different recessive variants. This new myopathy presents with a histopathology with a distinct combination of multiple pathological hallmarks characteristic of different myopathies; central and multicore disease, centronuclear, myofibrillar and nemaline myopathy. Patients present in infancy with slowly progressive proximal and distal weakness, facial weakness, nasal speech, swallowing difficulties, and mild to moderately elevated serum Creatine Kinase (CK) levels. The link between the mutations in the Pyrox-D1 gene and the disease phenotype are not yet understood.

This project involves expression, purification, and crystallization of Pyrox-D1 for characterization by X-ray diffraction. Following crystallization, preliminary characterization will involve spectroscopic and enzymatic analysis to determine the co-factors used by this enzyme, and eventual structure determination by X-ray crystallography. Understanding the co-factors and biophysical characteristic of this protein could help in developing new treatments in the future for this form of myopathy.

To date, we have successfully purified and crystallized Pyrox-D1. Going forward, the goal is to determine the molecular structure of Pyrox-D1, which will help elucidate the protein's function. The structure and function can be utilized to develop pharmaceutical treatments for patients presenting with this idiopathic dystrophy.

We have shown that Pyrox-D1 functions at least in part by facilitating the transition between oxidation states of NADP and that is function utilizes FAD as an electron carrier. A potential homologous structure has been created for Pyrox-D1. Two mutations have been identified in idiopathic muscular dystrophy patients, which we believe will be found in Pyrox-D1 once we have determined the molecular structure. The protein has been successfully crystalized and is currently being refined to undergo X-Ray diffraction.

Aggregation and Fusion of Phosphatidylcholine Liposomes Induced by the Macrolittins, a Synthetically Evolved Family of Potent Pore-forming Peptides

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Peptides that form pores in lipid bilayer membranes can be potentially used in a variety of biotechnological and clinical applications including drug delivery and targeted cancer therapy. Previously we performed a synthetic molecular evolution-based high-throughput screen of a rational combinatorial peptide library that used the sequence of the modified membrane lytic bee venom peptide Melp5 as a template. As a result, a novel family of peptides called “macrolittins” were discovered that release macromolecules from phosphatidylcholine vesicles at low concentration by forming large pores. In addition to liposomal leakage caused by Macrolittins, we found that the Macrolittins cause dramatic increases in light scattering, even at relatively low P:L of 1:200, which suggests that the vesicle-macrolittin mixture undergoes a dramatic change in the physical state of the lipid vesicles. Herein, we hypothesized that macrolittins can induce liposome aggregation and fusion at a specific range of P/L ratio. The fluorescent resonance energy transfer (FRET) analysis of liposomes labeled with NBD-PE and lissamine rhodamine B dyes detected lipid exchange into unlabeled liposomes, suggesting that vesicle fusion is taking place. Furthermore, confocal microscopy and electron microscopy revealed the aggregation and fusion of liposomes in the presence of macrolittins to yield gigantic structures. These processes were not found in liposomes treated with the parent peptide Melp5, suggesting distinctive mechanisms underlying peptide-liposome interactions between Macrolittin and Melp5. More importantly, it is a good starting point to investigate potential sequence-structure-function relationship considering that Macrolittin is one of the derivatives of Melp5.

Caspases from Scleractinian Coral Show Unique Regulatory Features

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Coral bleaching is an immune response that rejects compromised symbionts. *Porites astreoides* and *Orbicella faveolata* are coral species that exhibit different response to stress. Disease resistant P. astreoides employs adaptive autophagic mechanisms when immunocompromised, while disease-sensitive *O. faveolata* activates caspase-mediated apoptosis. We characterized caspases from *O. faveolata* (O.fav-3a and O.fav-3b) and P. astreoides (P.ast-3 and P.ast-7). O.fav-3a and P.ast-7 contain a CARD-like domain found in initiator caspases despite high sequence similarity to effector caspases. Experimental results from substrate-phage display show that O.fav-3a and P.ast-7 prefer Asp in the P4 position, which is corroborated by activity against canonical substrate, DEVD-AFC. In contrast, O.fav-3b and P.ast-3 prefer Val at the P4 position, but the enzymes have very low activity in vitro. We determined the structure of P.ast-7 bound with DEVD-FMK inhibitor to 1.57Å resolution by X-ray crystallography. The data reveal a hydrogen-bonding network with a unique insertion (RYP) in one active site loop and suggest that the sequence may be autoinhibitory when substrate is not bound. In addition, the structure reveals an N-terminal peptide bound near the active site that may play a role in stabilizing the dimer interface or serve as a possible exosite. Molecular dynamics simulations show reduced substrate movement in P4 site of P.ast-7 bound with DEVD and P.ast-3 bound with VFPD compared to other substrates. Overall, the data suggest that the CARD-containing coral caspases, P.ast-7 and O.fav-3a, function as initiator caspases, but with DEVDase activity, while P.ast-3 and O.fav-3b are more similar to human caspase-6 in substrate selection.

Using pH sensitive peptides for the endosomal release of antibodies

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Most therapeutic antibodies target cell surface epitopes. However, there are many appealing cytosolic targets that have not been fully explored, largely due to the difficulty in delivering antibodies into the cell. While endosomal uptake can be achieved, the fate of endocytosed proteins is mostly degradation by the endosome/lysosome pathway. Development of a method that enables antibodies to escape this hydrolytic fate and be released into the cytosol would open up an entirely new set of cancer therapeutic targets for exploration. We are developing pH-sensitive pore-forming peptides to enable endosomal escape of endocytosed protein cargo. The pH sensitive, pore forming peptide pHD108 is a 26-residue peptide that is a gain-of-function variant of melittin, a membrane permeabilizing peptide from bee venom. Multiple acidic glutamate residues in pHD108 confer the pH-triggered membrane permeabilization activity. In synthetic unilamellar vesicles, pHD108 has no poration activity at pH 7 but at pH ≤ 5.5 it releases macromolecules from lipid vesicles at very low peptide concentration. In this work, we are i) engineering pHD108 to bind strongly to cell membranes at pH 7 to promote endosomal uptake, and ii) determining the effect of increased membrane binding on the activity profile of pHD. Specifically, we are modifying pHD108 by conjugation to fatty acids and cholesterol, and testing the effects of lipidation on small molecule and macromolecule release from lipid vesicles. Ultimately, we will study the effect of lipidated pHD108 on cells, measure their endosomal uptake, and determine their ability to enable release of proteins from endosomes. Success in this research would result in identifying a potential cancer therapeutic adjuvant as well as providing a novel means to study the blocking/neutralizing effects of antibodies against intracellular targets.

The Evolution and Mechanism of Enzyme Specificity and Stability of Caspase-3

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The caspase family of proteases is an excellent model when analyzing protein evolution. Caspases are in a large family, and proteins evolved from a common ancestor. Caspases in the apoptotic initiator subfamily form a stable monomer, and their activities result from dimerization. Apoptotic effector caspases, however, can only form stable dimers. Urea folding/unfolding studies showed that human caspase-3, an effector caspase, folds by a four-state equilibrium process in which the native dimer (N2), unfolds through a non-active dimeric intermediate (I2), a non-native monomeric state (I) and an unfolded monomeric state (U). We compared the folding process of caspases from other species in order to examine whether the folding mechanism is conserved in other species. We show that zebrafish caspase-3b also folds by the four-state model. In addition, the human caspase-3 dimer is stabilized by several salt bridges across the dimer interface, and dimerization is sensitive to pH due to the presence of two histidine residues in the salt bridges. In contrast, zebrafish caspase-3a and -3b do not use histidines in the salt bridges, so the zebrafish dimers appear more sensitive to pH change.

Following evolution of the dimer, mutations in effector caspases resulted in changes in substrate specificity. The caspase-6 subfamily shows preference to valine at the P4 position, while caspase-3 shows preference to aspartate at the P4 position. However, recent research shows that zebrafish caspase-3a has relaxed specificity and allows either valine and aspartate at the p4 position. In contrast, the closely related zebrafish caspase-3b is similar to human caspase-3 with ~100-fold preference for D over V at P4. Our data shows that there are two ways to change enzyme specificity. The first one is through improving H-bonds to the P4 aspartate by decreasing the H-bond distance between carboxyl group in P4 and asparagine from active site loop 3. A second mechanism that results in selection of D over V is by including a polar cap near the S4 site. This mechanism is observed in caspase-3b from zebrafish and appears to be a general strategy from the scaffold of the common ancestor. We show that mutations that break the salt bridge in human caspase-3 results in relaxed specificity compared to wild type protein.

In conclusion, protein conformational ensemble and enzyme specificity are two important ways to regulate caspase activity. Gaining the knowledge with regards to the mechanisms with which these proteins and their related families evolved may assist in uncovering how and why the various functions evolved in these proteins.

Computer simulations show key role stochasticity of replication fork speed plays in the dynamics of DNA replication

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Eukaryotic DNA replication is elaborately regulated to ensure that the genome is faithfully replicated in a timely manner. Replication initiates at multiple origins, from which replication forks emanate and travel bi-directionally. Activation of replication origins and fork speed are stochastic in individual cells, but reproducible population-wide. To study the complex spatio-temporal regulation of replication, models of DNA replication in S. cerevisiae have been developed, but none have considered stochastic replication fork speed. Here, we present Repli-Sim, the first model of DNA replication, which includes stochastic speed of the replication fork. Utilizing data from both wild-type and hydroxyurea-treated yeast cells, we show that Repli-Sim achieves more accurate results than models assuming constant fork speed. Due to the stochastic nature of replication, its completion in a timely fashion is a challenge. Previously proposed solutions promoted finishing replication by modifying replication initiation and origin activation, while we propose empirically-derived modification in replication speed based on distance to the approaching fork, which promotes completion of replication.