small-but-measurable net unwinding (~7 deg/protein). A torsional free energy model couples this unwinding to observed limits in cooperative cluster size. These results predict that AGT will partition in favor of torsionally-relaxed, relatively protein-free DNA structures like those near replication forks. AGT binds O^6-methylG-C and O^6-methylG-T lesions with a specificity ratio (K_S/K_N) far too low for efficient lesion search. This suggests that other factors are needed to direct AGT to lesion sites. Recently we have found that AGT binds the human MutSbeta homologue and PCNA proteins. We propose that these proteins target AGT to sites of mismatch repair (including those containing O^6-methylG-T pairs) and to sites near the replication fork, where O^6-methylG lesions may be repaired before their potential for mutagenesis can take effect. This work was supported by NIH grant GM070662 to MGF.

944-Symp
DNA Supercoiling Enhances Cooperativity and Efficiency of an Epigenetic Switch
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Upon infection by bacteriophage λ the λ repressor protein, CI, interacts with the λ operator DNA, thereby regulating protein expression and deciding between the lysogenic and lytic state. This λ switch is a model on the basis of which epigenetic switch regulation is understood. In order to study the interaction between naturally supercoiled DNA and the DNA associating protein CI, we invented a novel assay where supercoiled circular DNA plasmids were individually tethered by peptide nucleic acid (PNA) handles [1]. We used this tethered plasmid assay for a single molecule investigation of the dynamics of supercoiled DNA and studied both the dynamics of the molecule itself and its interactions with the regulatory CI protein. The dynamics of the construct was analyzed by tracking the tethered bead. This revealed that compared with relaxed DNA, the presence of supercoils greatly enhances juxtaposition probability [2]. When CI was added to the supercoiled assay, the protein would attach to the operator sites thereby looping DNA. Our studies reveal that the efficiency and cooperativity of the epigenetic λ switch are significantly increased in the supercoiled system compared with a linear assay, thus increasing the Hill coefficient [2,3]. In contrast to other single molecule assays, the current methodology allows for studying DNA dynamics and DNA-protein interactions of DNA in its naturally supercoiled state.
[1] K. Norregaard, M. Andersson, P.E. Nielsen, S. Brown, L.B. Oddershede, Nature Protocols vol. 9 p.2206 (2014).
[2] K. Norregaard, M. Andersson, K. Sneppen, P.E. Nielsen, S. Brown, L.B. Oddershede, PNAS vol. 110 p. 17386 (2013).
[3] K. Norregaard, M. Andersson, K. Sneppen, P.E. Nielsen, S. Brown, L.B. Oddershede, Bacteriophage vol. 4 p. e27517-(1-5) (2014).

Symposium: Mechanisms of Actin Filament Nucleation and Mechanotransduction

945-Symp
Mechanosensitivity of Formin-Actin Interactions
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Cells are able to sense and adapt to their physical environment, translating mechanical inputs into chemical outcomes. The molecular basis for this fascinating property, referred to as ‘‘mechanotransduction’’, remains largely unexplored. The actin cytoskeleton, which generates and transmits forces throughout the cell, is certainly a key player in this process. Here, I will focus on the situation where tension is applied to actin filaments that are interacting with formins at their barbed ends. In cells, formins are typically anchored to a substrate and thus undergo a mechanical force as the filament is put under tension. We have studied this situation in vitro, by manipulating individual filaments with a microfluidic flow, as they are nucleated and elongated by formin mDia1 anchored to the bottom of the microchamber. I will show how filament tension in the picoNewton range modulates the elongation rate of the actin filament, the processivity of the formin and its competition with other end-binding proteins. Quantitatively probing these mechanical properties also provides an interesting angle to test molecular models of formin-assisted filament elongation.

946-Symp
WISH/DIP/SPIN90 Proteins Activate Arp2/3 Complex to Create Linear Actin Filaments that Seed Assembly of Branched Actin Networks
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Arp2/3 complex nucleates branched actin filaments responsible for powering diverse cellular processes, including cellular motility and endocytosis. WASP-mediated activation of Arp2/3 complex requires preformed filaments, ensuring that nucleation and branching are coupled, but leading to the biochemical consequence that branched network assembly cannot be initiated without a preformed seed filament. We recently discovered a class of Arp2/3 complex activators, the WISH/DIP/SPIN90 (WDS) proteins, that activate Arp2/3 complex without preformed filaments. WDS proteins have several sequence features distinct from WASP proteins, making their mechanism of activation unclear. In addition it is unknown if linear filaments produced by WDS-activated Arp2/3 complex can seed assembly of branched actin networks. Here we use single molecule TIRF microscopy to determine the mechanism of Dip1, a WDS protein from fission yeast. We directly demonstrate that linear filaments nucleated by Dip1 and Arp2/3 complex are substrates for branching by WASP-bound Arp2/3 complex. Dip1 remains bound to Arp2/3 complex at the pointed ends of the linear filaments it nucleates for hundreds of seconds, unlike WASP proteins, which are released before nucleation. Using engineered cysteine crosslinking, we show that a structural change in the complex that brings Arp2 and Arp3 into a filament-like (short pitch) conformation bypasses the need for Wsp1 but not actin filaments in activation. Therefore, we propose a model in which a step-wise set of conformational changes activate Arp2/3 complex. This model explains both how Dip1 bypasses the need for preformed actin filaments and why Wsp1 requires them, thereby providing important new insights into the molecular regulation of the assembly of actin filament networks.

947-Symp
Two Types of Actin Nucleators, Three Ways to Make Actin Filaments?
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Spire (Spir) and Cappuccino (Capu) are both actin nucleators, which are essential for Drosophila oogenesis. Despite similar biochemical activities, Spir and Capu are not redundant: mutations in either gene cause the same phenotypes, including loss of an actin mesh throughout the oocyte, mislocalization of polarity determinants and female sterility. Why are two actin nucleators required? To address this question we study how Spir and Capu nucleate independently and together.
Capu is a formin. Like other formins, it uses conserved FH1 and FH2 domains for nucleation and processive elongation. Capu does not have a DAD domain. It nevertheless uses its similarly-positioned tail in nucleation and autoinhibition, like other formins. Further, the Capu-tail enhances processivity. Our work indicates that this is a conserved function of formin tails.
Spir is a WH2-nucleator. The last two WH2 domains (of four) and their linker are sufficient to nucleate (C3D). Interestingly, when these WH2 domains are reversed (D3C) nucleation activity is lost. C3D binds two actin monomers with positive cooperativity. In contrast, D3C binds actin with negative cooperativity - it binds only one actin monomer. A similar construct with Linker 3 mutated (C(gs)5D) also binds only one actin monomer. Thus domain order and Linker 3 are necessary for cooperative binding.
Direct interaction between Spire and Capu is essential for actin mesh formation, subsequent oogenesis and fertility. New data indicate that actin binding but not necessarily nucleation by Spir is necessary during oogenesis. Combined, these point to a role for Spire as a Capu activator. Indeed, in bulk in vitro assays, Spir stimulates Capu’s actin assembly activity in the presence of capping protein and profilin, conditions under which Spir alone does not promote filament formation. We continue to study this interaction in vivo and in vitro.

948-Symp
Molecular Mechanism of Actin Filament Nucleation by Leiomodin (Lmod)
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Leiomodin (Lmod) is related to tropomodulin (Tmod), and the two proteins have similar localization in striated muscle, i.e. near the M-line, in association with the pointed end of the actin filaments. However, in vitro the two proteins have fundamentally different activities: Lmod is a powerful actin filament nucleator, whereas Tmod caps the pointed end. Originally, it was assumed

that the different domain organizations of the two proteins explained their different activities. Thus, Tmod has two tropomyosin (TM)- and two actin-binding sites, organized as: TMBS1-ABS1-TMBS2-ABS2. Lmod is longer, featuring a C-terminal extension that contains a Pro-rich region and a WH2 domain, which constitutes a third actin-binding site. The presence of the WH2 was considered to be the major feature distinguishing Lmod from Tmod. Here we show that this is not the case. Among the main findings are: 1) Lmod not only lacks TMBS2, but also ABS1, such that the entire region N-terminal to ABS2 has very little effect on nucleation, 2) The C-terminal extension of Lmod has also a limited effect on nucleation, and adding it to Tmod produces a very modest increase in nucleation, 3) Despite being relatively well conserved, the major feature distinguishing Lmod from Tmod is ABS2, consistent mostly of a Leu-rich repeat domain. Structural analysis shows that ABS2 can bind up to 3 actin subunits, and subtle differences between Lmod and Tmod dictate the affinities of their interactions with actin, and thus their roles in nucleation vs. capping. Understanding these differences allowed us to engineer an ABS2 Tmod-Lmod hybrid with nucleation activity equal to that of full-length Lmod.

Symposium: Molecular Basis for Mitochondrial Signaling

949-Symp
Systems Approaches to Mitochondrial Calcium Signaling
Jennifer Wettmarshausen[1], Yiming Cheng[2], **Fabiana Perocchi**[1,2].
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Mitochondria are essential hubs of calcium-mediated signaling networks. The organelle can take up, buffer, and release calcium ions to effectively shape intracellular calcium transients, stimulate ATP production and regulate cell death. Although, the basic mechanisms of mitochondrial calcium homeostasis have been firmly established for decades, the molecular identity of the mitochondrial calcium signaling toolkit has evaded classical bottom-up approaches. Our previous studies (1,2) have provided a compelling example of the power of systems approaches applied to mitochondrial calcium signaling to discover hitherto unknown molecular components of the calcium uniporter. Currently, we are developing computational and experimental frameworks for a systematic reconstruction of calcium-dependent signal transduction cascades in mitochondria. By combining evolutionary genomics and loss-of-function genetic and chemical screens, our systems approach holds the potential to shed light on yet unanswered questions in the field of mitochondrial calcium signaling.
1. Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Koteliansky V, Mootha VK (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature. 476(7360):341-5.
2. Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK (2010). MICU1 encodes a mitochondrial EF hand protein required for calcium uptake. Nature. 467(7313):291-6.

950-Symp
The Mitochondrial Calcium Uniporter: Molecular Composition and Physiological Role
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Mitochondria rapidly accumulate Ca^{2+} through a low-affinity uptake system (the mitochondrial Ca^{2+} uniporter, MCU) because they are exposed to high $[Ca^{2+}]$ microdomains generated by the opening of ER Ca^{2+} channels. These rapid $[Ca^{2+}]$ changes stimulate Ca^{2+}-sensitive dehydrogenases of the mitochondrial matrix, and hence rapidly upregulate ATP production in stimulated cells. Ca^{2+} also sensitizes to cell death mediators, e.g. ceramide. In my presentation, I will present the most recent molecular information on MCU, identified by our group in 2011, and the newly identified regulators (MCUb, MICU1, MICU2). I will also show how the availability of molecular tools for MCU now allows to carry out experiments in intact cells and whole organisms that highlight and clarify the importance of mitochondrial calcium homeostasis in physiology and pathophysiology.

951-Symp
Molecular Mechanisms of Mitochondrial Ca^{2+} Uptake: Role of MICU1 and its Paralogs
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Mitochondrial Ca^{2+} uptake is central to cell metabolism, signaling and survival. Recent studies identified MCU as the pore of the mitochondrial Ca^{2+} uniporter and MICU1 as its critical regulator. MICU1 and its paralogs, MICU2 and MICU3 are EF-hand proteins and are the primary candidates to confer Ca^{2+} sensitivity to the Ca^{2+} uniporter. We will present studies of the molecular mechanisms of the MICU-dependent closure of the uniporter at low $[Ca^{2+}]$ levels and its cooperative activation when $[Ca^{2+}]$ increases. Furthermore, we will present clues to the MICU-dependence of the tissue specific mitochondrial Ca^{2+} uptake profiles.

952-Symp
High-Affinity Interaction with VDAC Links Cytosolic Proteins to Mitochondrial Regulation in Health, Cancer, and Neurodegeneration
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We address the question of how mitochondria control cell survival and cell death by studying the voltage-dependent anion channel (VDAC). VDAC, the major channel of the mitochondrial outer membrane (MOM), is a well-recognized key conduit for ATP and other bioenergetics metabolites fluxes across MOM. We have found that dimeric tubulin, the subunit of microtubule, induces highly efficient reversible blockage of VDAC reconstituted into planar lipid membranes. Although the tubulin-blocked state still conducts small ions, it is virtually impermeable to ATP. We propose that by modulating VDAC permeability for ATP and other respiratory substrates, tubulin controls mitochondrial respiration. These findings are supported by experiments with isolated mitochondria and human hepatoma cells, thus uncovering a mechanism of regulation of mitochondrial energetics by free tubulin and also suggesting how cancer cells preferentially use inefficient glycolysis rather than oxidative phosphorylation (the Warburg effect).
We also found a functional interaction between VDAC and α-synuclein (α-syn), an intrinsically disordered neuronal protein intimately associated with Parkinson disease (PD) pathogenesis. Importantly, in addition to regulation of VDAC permeability by α-syn, our data indicate that VDAC facilitates translocation of α-syn across MOM where it could target complexes of the mitochondrial respiratory chain in the inner membrane. Supporting our *in vitro* experiments, a yeast model of PD shows that α-syn toxicity in yeast depends on VDAC. Considering that VDAC is a major conduit for respiratory substrates across the mitochondrial outer membrane, we conclude that the α-syn/VDAC functional interaction reveals the elusive physiological and pathophysiological roles for monomeric α-syn in PD and also in general neurodegeneration.

Platform: Electron Microscopy and Solution Scattering

953-Plat
GFP for EM: Site-Specific Labeling of Proteins for Electron Microscopy
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Structural analysis of macromolecules by electron microscopy (EM) has been facilitated by a recent technological revolution in instrumentation and data processing, which has led to the achievement of their visualization at atomic resolution. However, moderate resolution electron density maps of protein complexes can be misleading, resulting in ambiguity when ascribing subunits to particular locations within the architecture of complexes. To this end, investigators have traditionally performed subunit mapping by N- or C-terminal fusions with tags, such as maltose binding protein (MBP), with mixed success. Toward the accurate determination of the location and orientation of protein subunits, as well as large scale movements of protein complexes, the establishment of a highly specific labeling technique would be a major breakthrough. Here we present a site-specific labeling strategy that exploits a unique chemical handle introduced by the incorporation of the unnatural amino acid (UAA). The UAA permits a site-specific copper-free click reaction for labeling with a modified label, such as MBP or Nanogold. We use this method to label a subunit