Friday, March 20, Session III

L10

MODULATION OF ENZYMATIC ACTIVITY AND AMYLOID FORMATION BY BIOMOLECULAR CONDENSATES

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Biomolecular condensates are dynamic, membrane-less compartments within cells, formed by proteins and nucleic acids, that phase-separate from the surrounding cytoplasm. They concentrate specific molecules and allow cells to rapidly react to environmental changes and to control reactions in space and time. The mechanisms by which biomolecular condensates regulate enzymatic reactions and how those scales with compartment size remain poorly understood. In our work, we generate synthetic membranelles compartments by fusing various enzymes with intrinsically disordered sequences known as low complexity domains (LCDs), which play a crucial role in tuning the interactions responsible for the phase separation. We demonstrated that the enhancement of enzymatic activity is not merely a consequence of increased concentration but is highly dependent on the specific microenvironment created by the LCDs. Notably, we showed that the enhancement in enzymatic rate happens across a wide range of condensate sizes, from nanoclusters to microns size condensates [1-5]. While the formation of condensates is normally highly beneficial in cells, under pathological conditions, e.g. neurodegeneration, some of these condensates mature into amyloids. The molecular mechanism governing the interplay between amyloids and dynamic phase-separated compartments is not yet fully understood. In our work, we investigated the ALS-linked RNA binding protein, hnRNPA1, and showed that amyloid formation is promoted at the condensate interface, indicating that the interplay between condensation and fibril formation is beyond a simple increase of local protein concentration [6]. Subsequently, we demonstrated that the multi-component nature of condensates adds another level of complexity. We showed that the heterotypic protein-protein or protein-RNA interaction can have either protective [7] or acceleration [8] effects depending on the context. Finally, we investigated the importance of hnRNPA1-RNA interactions, and we revealed that at intermediate RNA concentrations, RNA promotes both condensation and amyloid formation. Importantly, we showed that higher RNA concentrations suppress phase separation according to the well-known re-entrant phase behavior [8,9], but do not prevent amyloid formation of hnRNPA1A over longer time scales. Overall, these results show that RNA can modulate hnRNPA1A phase transitions in a concentration-dependent manner [8].

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L11

ENGINEERING PROTEIN STRUCTURAL DYNAMICS WITH A NOVEL GENETICALLY ENCODED RAMAN-ACTIVE AND CHEMICALLY-REACTIVE NON-CANONICAL AMINO ACID

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Recent advances in genetic code expansion enable the site-specific incorporation of over 500 diverse non-canonical amino acids (ncAAs) into proteins. For instance, in vibrational spectroscopy, which can reveal the temporal evolution of protein conformational changes upon light absorption, ncAAs can serve as "transparent window" site-specific vibrational reporters to alleviate spectral congestion (overlapping bands). On the other hand, "click" and bioorthogonal chemistries enable the site-specific attachment of small, bright and photostable fluorophores to ncAAs carrying suitable reactive groups directly in cells. However, ncAAs that combine Raman activity with chemical reactivity are scarce. We hereby converged these two different applications into our newly developed ncAA, diacetylene-phenylalanine (DAF), bearing a conjugated diyne (C C–C C) that functions both as a Raman tag and a reactive handle. We synthesized DAF and evolved a specific orthogonal translation system for genetic encoding of this ncAA. Our DAF-specific amino acyl-tRNA synthetase from *Methanomethylophilus alvus* (MaDafRS) has advantages over systems in terms of its orthogonality in eukaryotes, superior fidelity and enhanced suppression yields. We genetically encoded DAF in *E. coli* and incorporated it at multiple sites in the bacterial blue-light transcription factor EL222 to track light-induced folding and signal propagation using steady-state and time-resolved femtosecond-stimulated Raman spectroscopy. The diyne group also undergoes thiol–yne and bioorthogonal reactions (Copper-catalyzed azide–alkyne cycloadditions and tetrazine ligation), enabling stable conjugation with sulfhydryl-, azide-, and tetrazine-containing dyes. Using these chemistries, we monitored the photocycle-dependent and DNA-dependent conformational changes of EL222 via Fluorescence Resonance Energy Transfer (FRET) and also, generated an intramolecularly crosslinked EL222 mutant locked in the dark state conformation. Together, DAF unites vibrational probing and protein conjugation in a single amino acid providing a compact, minimally invasive multifunctional tool to interrogate and engineer protein structure and dynamics.

L12

STRUCTURAL MECHANISMS OF Nedd4-2 AUTOINHIBITION AND REGULATION BY MEMBRANES, CALCIUM AND 14-3-3

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The HECT-family E3 ubiquitin ligase Nedd4-2 (NEDD4L) controls ion transport by ubiquitinating key membrane proteins, most notably the epithelial sodium channel (ENaC). Nedd4-2 dysfunction is linked to Liddle syndrome and hypertension, and has also been connected to epilepsy and other cardiopulmonary disorders. Multiple regulatory inputs have been suggested, including intramolecular autoinhibition [1,2], Ca^{2+}-dependent control via the C2 domain [1], and binding of 14-3-3 proteins [2,3], but the structural basis of these mechanisms remains puzzling. To investigate how Nedd4-2 is kept inactive and how it is regulated, we used an integrative structural and biochemical approach. We combined cryo-electron microscopy (cryo-EM), small-angle X-ray scattering (SAXS), hydrogen–deuterium exchange mass spectrometry (HDX-MS) and analytical ultracentrifugation with

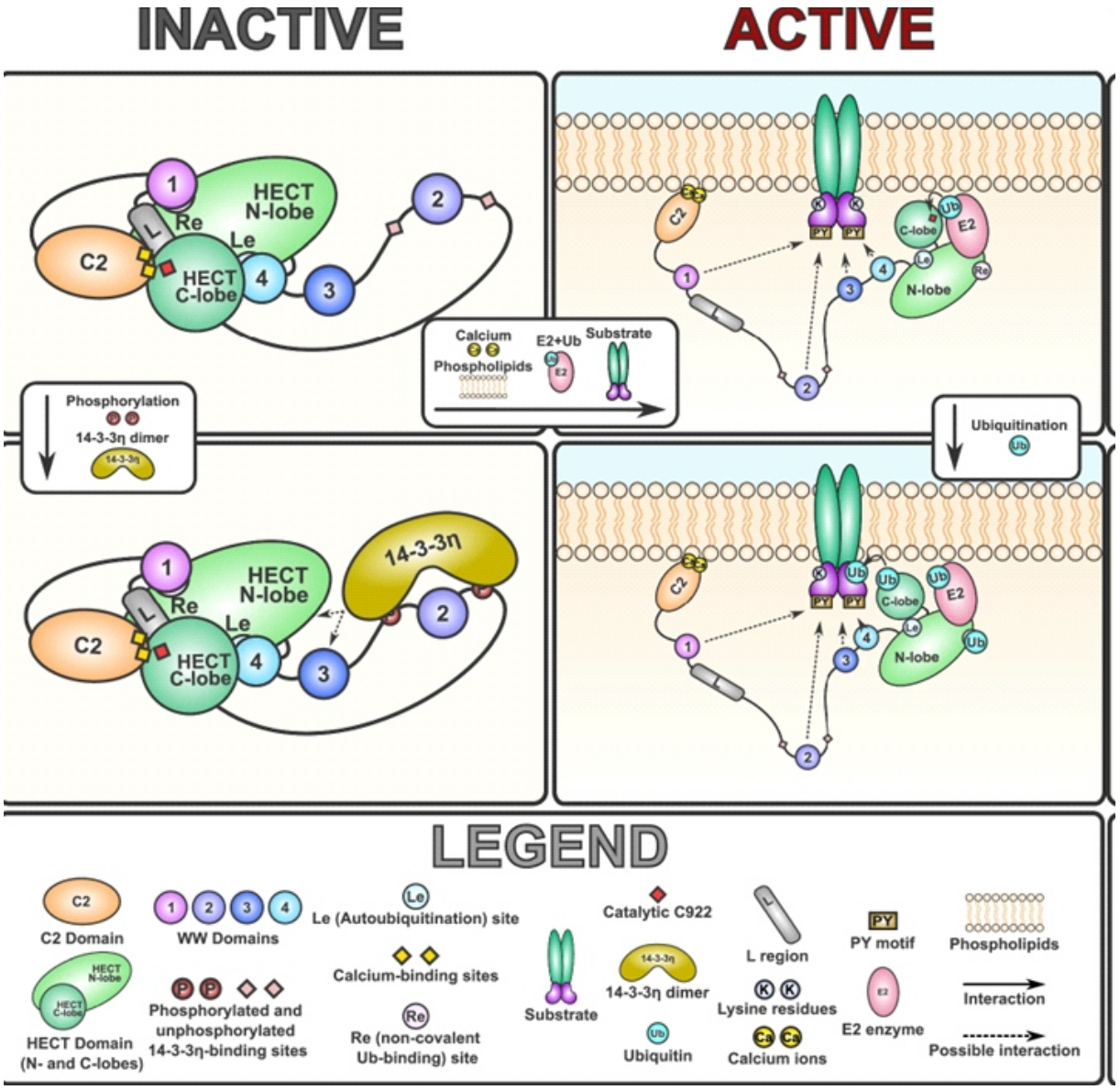

Figure 1. Graphical scheme of Nedd4-2 regulation via calcium, autoinhibition and 14-3-3. [1]

liposome-binding assays and in vitro ubiquitination assays. Using these methods, we analyzed full-length Nedd4-2 and a complex of Nedd4-2 with a 14-3-3 homodimer. Our cryo-EM data show that full-length Nedd4-2 adopts a compact autoinhibited conformation. Ca^{2+} is required for strong liposome association and we identify residues that contribute to Ca^{2+} coordination, but Ca^{2+} binding alone does not cause large structural changes and does not activate ubiquitination in our assays. In contrast, binding to phospholipid membranes is required to disrupt the C2–HECT interaction and release the autoinhibited state, indicating that membrane binding is the main trigger for activation under our conditions. Finally, we show that 14-3-3 binds to phosphorylated motifs near the WW2/WW3 region and inhibits Nedd4-2 function. The Nedd4-2:14-3-3 complex is not affected by Ca^{2+}, but it reduces both membrane binding and catalytic ubiquitination. A low-resolution cryo-EM model of the complex supports a mechanism where 14-3-3 stabilizes the autoinhibited conformation. Overall, these results define domain-level interactions that control Nedd4-2 activity and provide structural targets for modulating this E3 ligase in disease-relevant pathways.

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L13

THE ACTIVITY OF CDK16 IS REGULATED BY 14-3-3

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CDK16, a member of the PCTAIRE family of cyclin-dependent kinases (CDKs), plays a crucial role in various physiological processes, including neurite outgrowth, vesicle trafficking, spermatogenesis, glucose homeostasis, and muscle differentiation. [1] However, its activity is also associated with the progression of various cancers, including breast, lung, endometrial cancer, melanoma, and others. [2] Given the essential role of CDK16 in promoting cancer cell proliferation, understanding its regulatory mechanism at the molecular level is crucial. Unlike other CDKs, CDK16 activation is mediated through interaction with cyclin Y, phosphorylated at residues Ser100 and Ser326, in complex with 14-3-3, as depicted in Figure 1. [3] Two phosphorylation sites in the N-terminal extension of CDK16 kinase domain (NTE), Ser119 and Ser153, have been identified as binding motifs for the 14-3-3 proteins, which affect the function of a wide spectrum of targets, including the modulation of enzymatic activity. Specifically, phosphorylation at S153 has been suggested to hinder

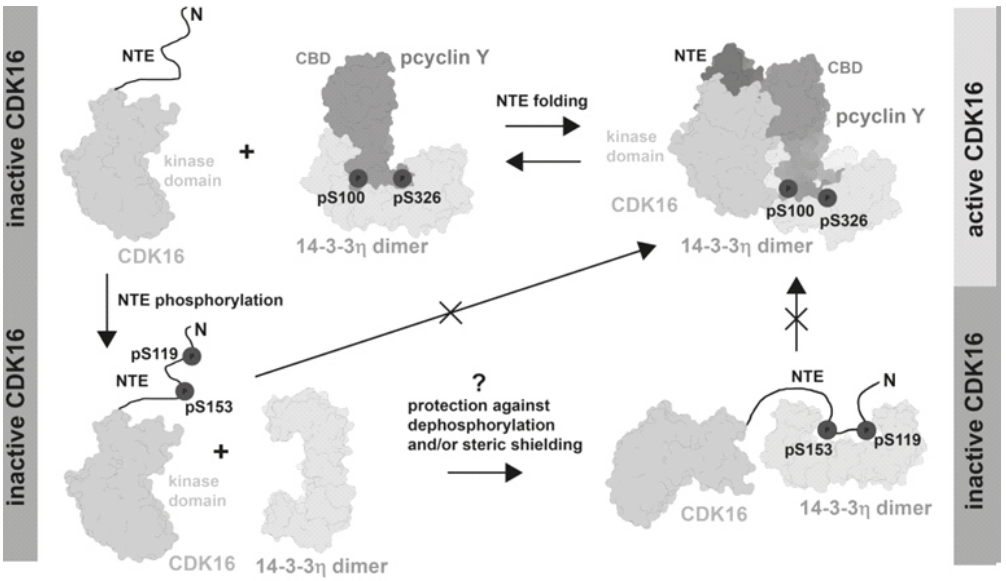

Figure 1. Graphical scheme of CDK16 regulation via cyclin Y and 14-3-3.

CDK16's activity. [4] Here, we focus on how 14-3-3 proteins control CDK16's activity.

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L14

FROM MOLECULE TO MECHANISM: INTEGRATED INSTRUMENTATION FOR STRUCTURAL BIOLOGY

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Modern structural biology depends on combining complementary technologies to understand the structure and dynamics of proteins, DNA, and RNA. This presentation will showcase biophysical analysis using Applied Photophysics (circular dichroism and stopped-flow kinetics) for studying protein folding, conformational stability, and biomolecular interactions. We will also introduce Refeyn's mass photometry for rapid, label-free measurement of molecular mass, oligomerization, and sample heterogeneity in solution. Finally, advanced imaging solutions from Leica Microsystems enable high-resolution visualization of molecular organization and protein localization within cells, linking molecular structure to biological function.

Friday, March 20, Session IV

L15

EXPLORING THE SEQUENCE SPACE OF A FLUORESCENT DEOXYRIBOZYME USING STRUCTURED LIBRARIES, SELECTION AND MACHINE LEARNING

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Finding ways to more comprehensively explore the sequence space of complex functional motifs is an important and unresolved question in nucleic acid engineering. Standard approaches use libraries in which a single variant of a motif is randomly mutagenized at a low level. This provides comprehensive coverage of sequence space over short mutational distances, but only limited information about more distant variants. Here we describe a new approach that uses libraries made up of sequences consistent with the multiple constraints of a desired target motif. Functional variants are rapidly identified in a single round of selection followed by high-throughput sequencing, and rules relating sequence to function elucidated using machine learning. This method was tested using a fluorescent deoxyribozyme recently discovered in our group called Aurora. Single-step selections showed that a secondary structure library based on Aurora contained approximately 40-fold more unique catalytic sequences than one generated by random mutagenesis. Furthermore, models developed by machine learning could quantitatively predict read numbers and identify the most active variants using small subsets of sequences as training sets. By combining secondary structure libraries, selection and machine learning in this way, sequence space can be explored far more quickly and efficiently than in standard approaches.