NE-CAT Communications

A Biannual Newsletter of the Northeastern Collaborative Access Team Winter 2012

Message from the Director

Steve Ealick

NE-CAT has concluded another busy and productive year. We have accomplished many tasks, both big and small. We have implemented the PILATUS detector, made several hardware improvements, continued to upgrade our facilities, developed new software, submitted our grant application and begun to research new techniques for better data collection.

In addition, in November, NE-CAT hosted our site visit for our NIH grant review panel. We provided both talks to illustrate our future goals and guided tours of our facility to show our current status. During the visit, we were also supported by several Primary Investigators of our Driving Biological Projects who offered examples of how NE-CAT is essential to their research. All signs appear positive. We are keeping our fingers crossed and hoping that Congress does not fail to act before March 1.

Remote data collection has become very popular with the advent of our remote access suite and staff video chat. However, the vast expertise of the NE-CAT staff is still best experienced through an on-site visit to our beamlines. For further information on our beamline capabilities or to check out available beamtime, please visit our website at http://necat.chem.cornell.edu.

Beamline Developments

1. Puck Storage Rack

Some users have been unable to test and/or collect on

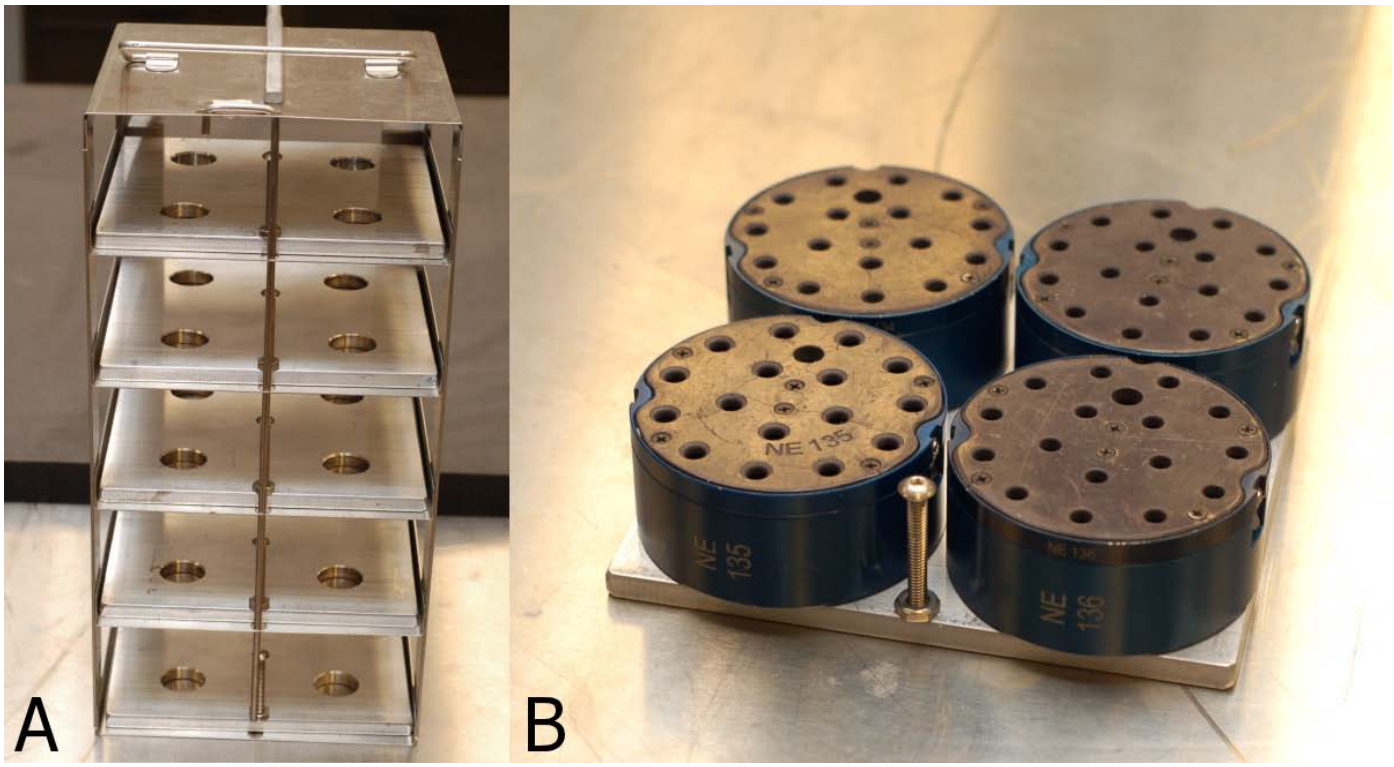


Fig. 1 A) The five-level rack for storing cryopucks in the large storage dewar. Each level has a plate which can hold up to 4 pucks. B) An individual plate with four pucks.

all their samples during a trip. With increased use of the sample automounter, NE-CAT needed a sample storage facility for cryopucks. During the September shutdown, Ed Lynch made a rack for storing cryopucks (Fig. 1A). This rack goes into the large NE-CAT long term storage dewar and users can leave a puck full of samples in it for their next trip. Some users have already taken advantage of our on-site storage and kept pucks in this holder between trips. The holder can carry up to 4 pucks per level (Fig. 1B), for a total of 20 pucks. The design is available to any group that would like to adopt for their use. Note that with 20 pucks, the rack gets rather heavy.

2. PILATUS Processing Unit

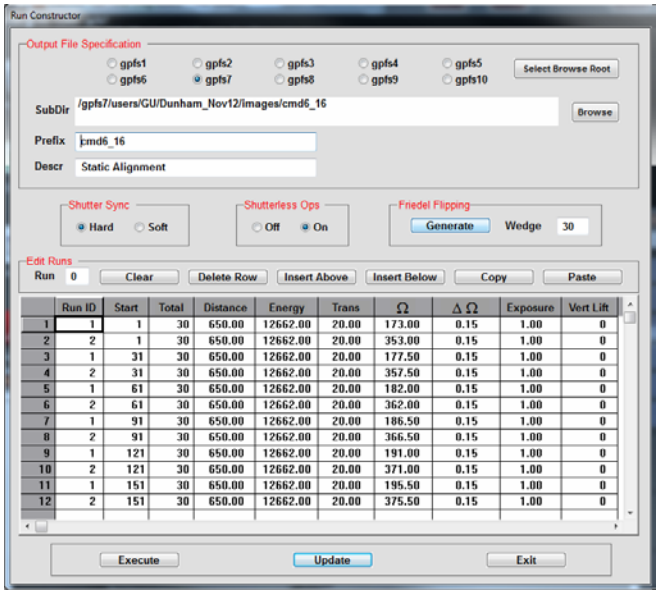
As both a hardware and software upgrade, we acquired a PILATUS Processing Unit (PPU) during the September shutdown. The PPU is a dedicated data transfer compute server made by Dectris, the manufacturer of the PILATUS. The PPU is a high-end server which provides massive processing power and dedicated software packages. The PPU is designed to interface beamline IT infrastructures with modern Redhat Linux-based operating systems such as the CentOS used at NE-CAT. It comes with the latest compilers and a pre-installed 10Gbit/s high-speed network card (SFP+).

To transfer data from the PILATUS computer to our data storage filesystem, we use software called FURKA/GRIMSEL. This software is developed by Dectris and designed to transfer files from the PILATUS. This software was originally installed directly on a non-dedicated local computer handling the GPFS file system. We were aware that this caused occasional loss of data frames.

The FURKA/GRIMSEL software package comes pre-installed on the PPU which will now interface between the PILATUS and our GPFS file system. The PPU also has a large RAMdisk which will buffer the incoming data before sending it out to the GPFS file system. This will both provide an increased rate of file transfer as well as improve reliability of the data transfer. During the commissioning, we were able to collect thousands of data frames at 25Hz without any loss.

3. Beamline Computers OS Upgrade

Also during the September shutdown, the operating system (OS) on the computers running the Master and Auxiliary control scripts were upgraded to 64-Bit Windows 7 on both beamlines. The reason for the OS change is the ending of extended support for Windows XP in 2014. In anticipation of the end-of-support, Malcolm Capel spent the past year porting the beamline control program, CONSOLE, to Windows 7. Extensive tests were carried out to assure stable operation prior to changing the OS.



	Run ID	Start	Total	Distance	Energy	Trans	Ω	Δ Ω	Exposure	Vert Lift
1	1	1	30	650.00	12662.00	20.00	173.00	0.15	1.00	0
2	2	1	30	650.00	12662.00	20.00	353.00	0.15	1.00	0
3	1	31	30	650.00	12662.00	20.00	177.50	0.15	1.00	0
4	2	31	30	650.00	12662.00	20.00	357.50	0.15	1.00	0
5	1	61	30	650.00	12662.00	20.00	182.00	0.15	1.00	0
6	2	61	30	650.00	12662.00	20.00	362.00	0.15	1.00	0
7	1	91	30	650.00	12662.00	20.00	186.50	0.15	1.00	0
8	2	91	30	650.00	12662.00	20.00	366.50	0.15	1.00	0
9	1	121	30	650.00	12662.00	20.00	191.00	0.15	1.00	0
10	2	121	30	650.00	12662.00	20.00	371.00	0.15	1.00	0
11	1	151	30	650.00	12662.00	20.00	195.50	0.15	1.00	0
12	2	151	30	650.00	12662.00	20.00	375.50	0.15	1.00	0

Fig. 2 Example of the runs dialog after friedel flipping wedges have been generated by the Auxiliary script.

4. Friedel Flipping

After moving away from the ADSC data collection GUI to the CONSOLE script on Auxilliary, users who wished to perform Friedel flipping during SAD or MAD data collection needed to manually enter the wedge angles and frame numbers for each run individually. During the 2012-3 run cycle, the CONSOLE software was updated to automatically generate all the necessary wedges for a chosen rotation range in the Static Alignment data collection mode (Fig. 2).

5. Remote Beamline Access

Remote data collection on 24-ID-E has been operational during the entire 2012-3 run cycle and 65% of our groups used the 24ID-E beamline via the remote access suite. It has been a great success and the unique, simple web-based design is very popular among our users. It works in all operating systems that support HTML5-capable web browsers and from any location that supports high-speed internet access.

There will be many new features added to remote data collection in the upcoming months. Among the many additions users can look forward to seeing are vector-based scanning techniques, integration with RAPD,

integrated text chat between users and support staff and improved data backup to the home institution. For users who wish to have the additional capabilities of a tunable beamline with a mini-Kappa, we anticipate that remote data collection will be possible on 24-ID-C in late 2013.

Research Highlights

How Bacteria Store Their Ribosomes During Stress

Thomas A. Steitz, Sterling Professor of Molecular Biophysics and Biochemistry, Department of Chemistry, Yale University, New Haven, CT

Yury Polikanov, Postdoctoral Associate

Bacteria are subjected to various stresses in their natural environments that lead to specific and regulated adaptive responses. Entry into a stationary phase is one of them, which occurs when the supply of nutrients is exhausted and results in nearly complete arrest of protein biosynthesis. In this phase bacterial cells are highly resistant to external stresses, including inhibition by antibiotics.

When bacteria actively grow and divide, they continually synthesize proteins according to the genetic information encoded in their DNA. Large macromolecular complexes called ribosomes translate, with the help of adaptor molecules (transfer RNA or tRNAs), the genetic information encoded in the messenger RNA (mRNA) into the protein sequences. Rapidly growing cells require an adequate supply of amino acids to cope with the demands of protein synthesis. Consequently, in times of nutrient starvation, when there are not enough amino acids, bacteria slow down protein synthesis and convert ribosomes into stable inactive forms. One such form, referred to as 100S ribosome dimer is important for cell survival during stationary phase. In this form individual 70S ribosomes are arranged in pairs.

The process of 100S dimer formation in bacteria *Escherichia coli* involves two small protein factors, Ribosome Modulation Factor (RMF) and Hibernation Promoting Factor (HPF). RMF binding results in dimerization of individual 70S ribosomes into 90S particles, which then become stabilized as 100S dimers following HPF binding. Although the *rmf* gene, which encodes for RMF protein, is dispensable during normal growth conditions, it becomes essential for cell viability during starvation, which highlights the importance of 100S dimer formation during stress. In contrast to *rmf*, a deletion of the *hpf* gene neither affects the cell viability, nor impacts dimer formation.

Fig. 3 Yury Polikanov (left) and Thomas A. Steitz (right) with models of the ribosome.

Alternatively, another protein factor YfiA (also called protein Y) can promote the formation of an inactive 70S ribosome monomer. Although YfiA and HPF have homologous amino acid sequences, their effect on 100S dimer formation is different. HPF converts 90S dimers into 100S particles, while YfiA inhibits RMF-dependent ribosome dimerization. Previous studies suggested that HPF and YfiA can interfere with protein synthesis simply by competing with the binding of tRNAs to the ribosome.

The study conducted by the researchers from the Steitz group at Yale University investigated the crystal structures of the bacterial 70S ribosome in complex with either RMF (Fig. 4A), HPF (Fig. 4B), or YfiA (Fig. 4C), to better understand how these proteins act. Using data collected at the APS beamline 24ID-C, the researchers solved crystal structures of each the three hibernation factors in complex with the bacterial ribosome to show how bacteria sequester the number of actively synthesizing ribosomes during stationary phase. Their results revealed that RMF and HPF can bind simultaneously and act together to interfere with initiation of protein synthesis. In their structure the binding of RMF interferes with initiation of protein synthesis by preventing the crucial interactions between the ribosome and the mRNA – a carrier molecule of the genetic information. Because of the sequence similarity the globular domains of HPF amd YfiA in their binding sites are the same and overlap with those of the mRNA, and tRNAs, as well as with initiation factors. However, only the binding of RMF and HPF, but not YfiA, enables the structural re-arrangements of the ribosome required for the formation of 100S dimers.

The results of this study clarify how bacterial cells preserve ribosomes in a translationally inactive yet stable form during starvation and possiblly during other stresses such as exposure to antimicrobial agents. Consequently, preventing this important mechanism to play in bacterial cells might be worthy further considerations during the development of new strategies to combat the rise of antimicrobial drug resistance in pathogens.

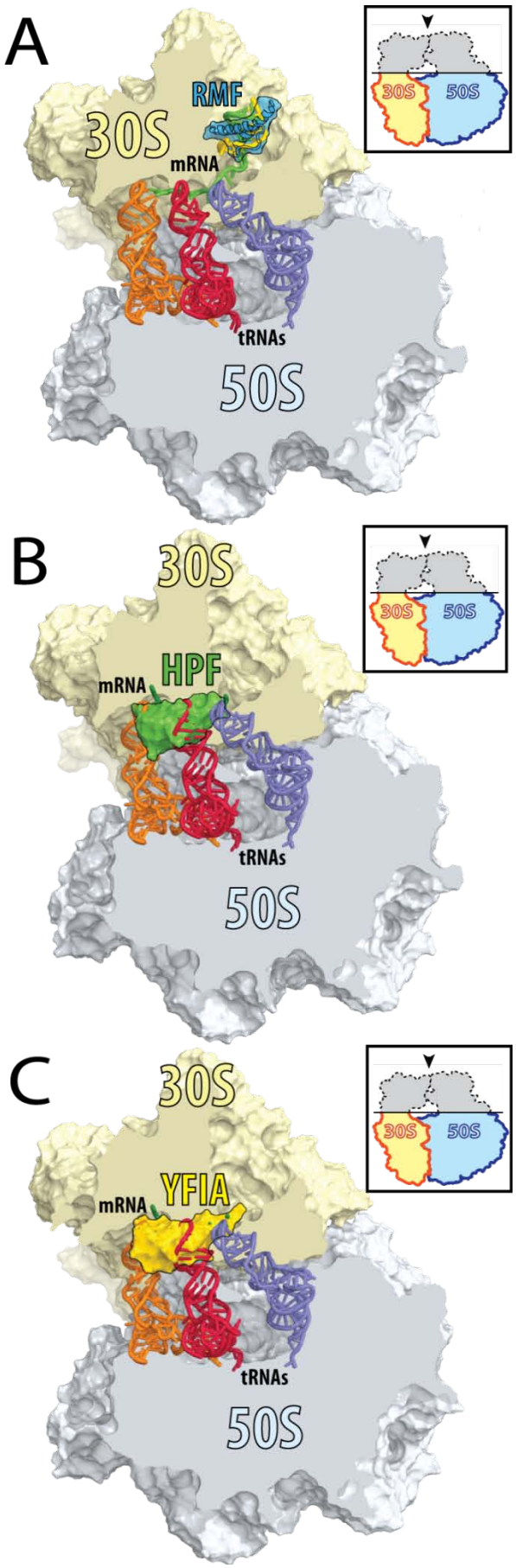
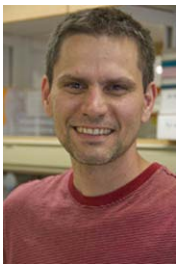


Fig. 4 The structures of RMF (A), HPF (B) and YfiA (C) hibernation factors bound to the ribosome. Small ribosomal subunit (30S) is shown is light yellow, large ribosomal subunit (50S) is in light blue. Key players of the protein synthesis cycle - mRNA and three tRNA molecules, not actually present in the studied complexes, are shown in green, orange, red and blue, respectively, to illustrate how hibernation factors inhibit protein biosynthesis. The views are indicated by the insets in each panel. (A) RMF (blue) prevents pivotal ribosome-mRNA interaction essential for initiation of protein synthesis by clashing with mRNA (green) and part of the ribosome (yellow). (B, C) HPF (green) and YfiA (yellow) bound to the ribosome prevent mRNA and tRNA molecules from interacting with the same ribosome.

Small molecule modulation of the ubiquitin proteolysis system (UPS)

Frank Sicheri, Professor, Department of Biochemistry at the University of Toronto, Senior Investigator, Samuel Lunenfeld Research Institute at Mount Sinai Hospital, Toronto, Ontario, Canada



Derek Ceccarelli, Senior Research Associate

Stephen Orlicky, Research Associate

The ubiquitin proteolysis system controls the stability and fate of a multitude of proteins thereby impacting virtually every cellular process. Transfer of ubiquitin to lysine residues on targeted substrates is precisely controlled through the action of a conserved E1-E2-E3 enzyme cascade (Fig. 5A). The human genome encodes two E1 enzymes, at least 38 E2 enzymes, and more than 600 distinct E3 enzymes that function in various combinations to target multiple substrates (Nalepa et al, 2006). An E1 enzyme activates ubiquitin as a thioester intermediate in an ATP-dependent manner and transfers the activated ubiquitin to the catalytic cysteine residue of an E2, which then interacts with an E3 enzyme to conjugate ubiquitin as an isopeptide bond onto the substrate. E3 ligases confer substrate specificity via specific protein interaction domains and promote transfer of ubiquitin from the E2 to a substrate directly using a RING domain as in the case of the Skp1-Cullin-Fbox (SCF) multi-subunit E3s, or indirectly via an E3 ubiquitin thioester intermediate using a HECT domain. The resultant ubiquitin linkage marks the substrate for degradation by the 26S proteasome and/or alters substrate interaction with other proteins.

Structure-guided analyses have provided great insight into the inner workings of the UPS. Structures of E1s, E2s, E3s and substrate complexes have contributed to our molecular understanding of the protein interactions and catalytic mechanisms that underlie ubiquitin transfer and downstream effects of substrate ubiquitination. Gaining insight into the biological roles of specific UPS components is complicated by the myriad of potential combinations of E1-E2-E3-substrate interactions that are functionally relevant in cells.

Small molecules that modulate interactions and activities of specific UPS components can serve as useful tools to probe biological function. Towards this end, our lab has embarked on small molecule screening campaigns to identify probes that disrupt E3-substrate interactions or inhibit ubiquitin transfer function. Structural characterization of these probes with their UPS targets has provided insight into both inhibitor mechanism of action and the normal mechanism of action of the target proteins within the UPS. Two recent structural examples from our lab solved with data collected at NECAT are briefly described below.

Disrupting an E3-substrate interaction

The E3 SCF^{Cdc4} regulates stability of proteins involved in cell cycle control including Cyclin E and Sic1. Having previously characterized the complex between Cdc4, the substrate recognition subunit of SCF^{Cdc4} and the targeting phospho-peptide element within cyclin E (Orlicky et al., 2003), we adapted a fluorescence polarization based assay to screen for small molecules that disrupt this essential interaction (Orlicky et al., 2010). These efforts yielded the first known inhibitor of substrate recognition by an SCF E3, called SCF-I2. This small molecule binds to the WD40 propeller domain of Cdc4 at a site remote from the substrate binding site and through a series of allosteric changes causes disruption of the substrate binding pocket (Fig. 5B). The co-crystal structure determined at NE-CAT revealed that SCF-I2 inserts between propeller blades 5 and 6 at a site 25Å away from the substrate-binding site. This study raised the possibility that other WD40 domain interactions both within and outside the UPS may also be amenable to allosteric modulation by small molecules.

Inhibiting an E2 enzyme

The E2 enzyme Cdc34 together with the E3 SCF^{Skp2} regulates the cell cycle by targeting the cyclin dependent kinase inhibitor proteins p21, p27 and p57 for degradation. In collaboration with Celgene Corporation, our lab characterized the first reported E2 enzyme inhibitor, called CC0651 (Ceccarelli et al., 2011). This small molecule was identified through an activity-based screen that monitored the level of substrate ubiquitination in a reconstituted E1-E2-E3-substrate system. The E2 Cdc34 was identified as the small molecule target since substitution of the reaction mixture with yeast Cdc34 or Ube2R2, the closest related E2, rendered p27 ubiquitination resistant to

inhibition by CC0651. Structure determination of a Cdc34-CC0651 complex revealed the inhibitor bound within a pocket located 19Å from the catalytic site (Fig. 5C). Comparison with the Cdc34-apo structure revealed that CC0651 induced structural changes suggestive of an allosteric mode of inhibition.

As the mechanism by which E2s function is still poorly understood, CC0651 also provided a useful tool to probe the inner working of a mysterious enzyme and the biological consequences of Cdc34 inhibition. This study also raised the possibility that other E2 enzymes within the UPS may be selectively targeted for therapeutic end. Indeed, considerable interest in the therapeutic potential of UPS inhibitors has followed on the approval of proteasome inhibitors (Bortezomib and carfilzomib) for the treatment of multiple myeloma and mantle cell lymphoma. Small molecule inhibitors that selectively target specific E2s or E3-subtrate complexes are of interest as in theory they may reduce the toxicity associated with the global perturbation of the UPS by proteasome inhibitors. Our studies characterizing the mechanism of action of Cdc34 and Cdc4 specific inhibitors provides a proof of concept that other components of the UPS are in fact druggable.

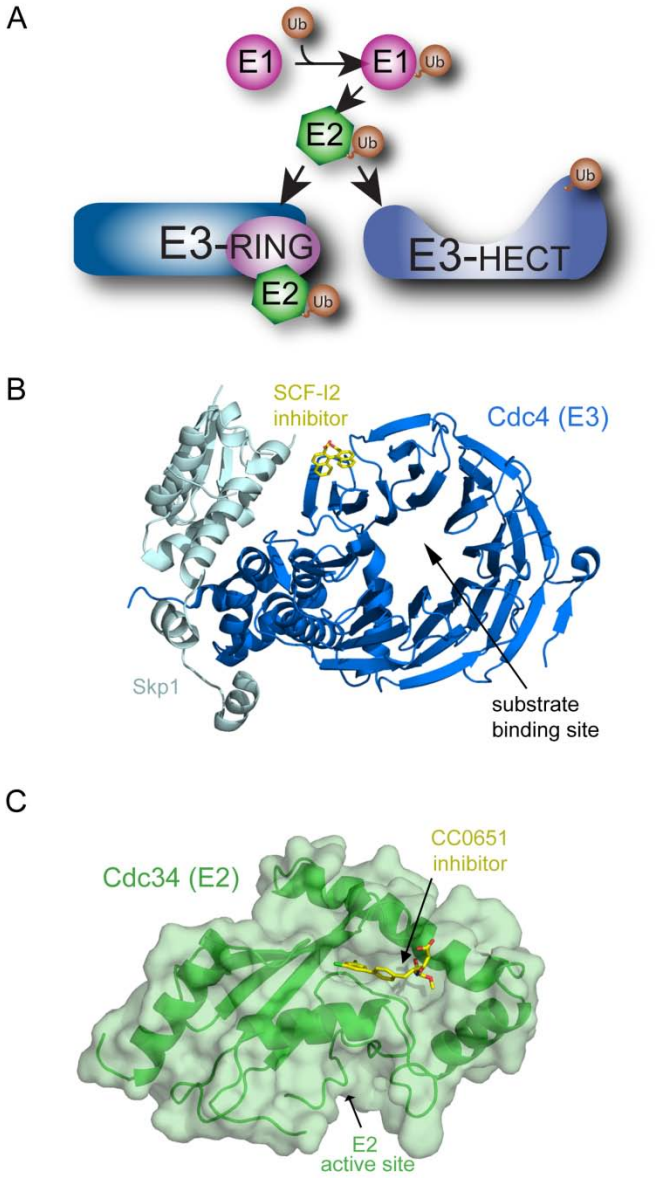


Fig. 5 (A) The E1-E2-E3 cascade of ubiquitination. (B) Structure of Skp1-Cdc4 E3 subunits in complex with SCF-I2 (PDB: 3MKS). (C) Structure of Cdc34 E2 in complex with CC0651 (PDB: 3RZ3).

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Staff Activities

Presentations

Narayanasami Sukumar. Plenary lecture "Crystallographic studies on cobalamin binding proteins in eukaryotes and prokaryotes" at the Vitamin B12 Symposium, Faculte de Medecine, University of Lorraine, Nancy, France, September 20-22, 2012.

Narayanasami Sukumar. Co-Chair of section on "Structure-based molecular characteristics and biochemistry of vitamin B12 enzymes" at the Vitamin B12 Symposium, Faculte de Medecine, University of Lorraine, Nancy, France, September 20-22, 2012.

Publications

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Acknowledgements

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