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Understanding Structural And Functional Mechanisms of Emi1 Inhibition of the Anaphase Promoting Complex

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Understanding Structural And Functional Mechanisms of Emi1 Inhibition of the Anaphase Promoting Complex

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Comments

Healthy, reproducing cells create and destroy proteins in an ordered manner. Both the concentration and localization of protein pools is important to regulate the many cellular processes necessary for the life of the cell. In order to produce new proteins, cells degrade existing materials such as proteins and organelles that are dispensable or troublesome in order to recycle their raw components. Degradation is accomplished largely through two major pathways: in bulk through processes such as autophagy and phagocytosis, or in a targeted manner through the ubiquitin-proteasome pathway. Autophagy utilizes an encompassing body to encapsulate targets and surrounding materials for decomposition in a regulated but relatively non-specific manner. The ubiquitin-proteasome pathway, however, is an exquisitely precise method of degradation capable of targeting specific pools of protein substrates. So-called ubiquitination generates a signal for degradation by the 26S proteasome machinery. In order to establish the degradation signal, the cell utilizes a cascade of 3 enzymes working in concert to organize substrates and ubiquitin. By using enzymes capable of substrate specificity, the cell can regulate large pools of proteins in a specific spatio-temporal manner.

In the present study, we employ biochemical and structure-based techniques to study proteins involved in an essential ubiquitination pathway involved in maintenance of the cell cycle. A mitotic regulator called the Anaphase Promoting Complex, also called Cyclosome (APC/C), modifies myriad substrates that control cell cycle activities. The APC/C is a ubiquitin ligase complex; it is the final enzyme in a tri-enzyme cascade and catalyzes the final step of Ub transfer from an E2 enzyme directly to substrates. A protein called Emi1 is responsible for directly binding and inhibiting the APC/C throughout interphase, when APC/C substrates are stabilized. We set out to study domains of Emi1 responsible for binding and inhibiting the APC/C and its association with E2 enzymes or substrates, with the aim of characterizing both the mechanism of the inhibitor and the essential functional requirements of the APC/C, which are not well understood. We aim to visualize the APC/C in complex with Emi1 through electron microscopy and to accurately interpret the location and orientation of Emi1 within this density.

The APC/C is a large, ~1,200 kDa multi-protein complex, and Emi1 is a single protein of only 50 kDa. Despite its small size, even a small subdomain of Emi1 potentially inhibits the APC/C through a combination of not well-characterized mechanisms. Emi1 has an APC/C recognition motif called a D-box that is typically found within APC/C substrates. Emi1's D-box is recruited to the substrate-binding sites of APC/C and serves as a pseudo-substrate inhibitor. Emi1 also inhibits through distinct mechanisms both of the E2 enzymes that coordinate with APC/C function. An essential, folded Zinc-binding Region (ZBR) and a helical "Linker" sequence cooperate to bind and block APC/C from recruiting one of two APC-specific E2 enzymes, UbcH10. Emi1 also has a conserved C-terminal motif, with charge and sequence similar to the other APC/C-specific E2 enzyme, Ube2s. Emi1 competes directly with Ube2s for APC/C recruitment, and it is through the combination of these three mechanisms that are afforded by many motifs within Emi1, that makes Emi1 a potent APC/C inhibitor. When associated with Emi1, the APC/C is inefficient at both recruiting substrates and binding E2 enzymes, allowing for stabilization of APC/C substrates, which is important for regulation of timing of cell cycle processes.

**Understanding Structural and Functional Mechanisms of Emi1 Inhibition of the
Anaphase Promoting Complex**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Edmond Randall Watson
May 2016

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DEDICATION

I dedicate this work to my loving parents, Ed and Cathy Watson,
who have believed in me and supported me for my entire life.

ACKNOWLEDGEMENTS

To properly acknowledge the contributions of others, one must first acknowledge their own inadequacies. When I joined the IPBS and began my dissertation research in Brenda's lab, I had absolutely no skill set for the work of a structural biologist, and almost no knowledge about our field or our science. Brenda courageously accepted me under her tutelage, and for years provided both the means and the direct advisement that developed me into the scientist I have become today. Her unparalleled dedication to both her work and my own have shown me what is truly possible through scientific research: the ability to attack the unknown, solve invisible mysteries, and establish a thorough and reliable groundwork for the progression of scientific information. She took me to multiple international conferences and meetings and taught me a great deal about the "behind the scenes" world of science. These first-hand experiences with so many world-class scientists and thinkers provide an important perspective that most students don't get to experience. I will be forever grateful for the opportunities that have been given to me by UTHSC, St Jude, and especially Brenda Schulman.

I would also like to acknowledge Dr. Eric Enemark, Dr. Lawrence Pfeffer, Dr. Charles Rock, and Dr. Stephen White who served as my faculty committee. They developed and mentored me through years of graduate school and offered key ideas and advice to move my project forward. Despite their incredibly busy schedules, they took the time to meet with me, plan my experiments, edit my documents, and I am very thankful for their selfless leadership.

On a daily basis, I have had the pleasure of working with an incredibly talented and motivated postdoc, Nick Brown. We have accomplished quite a bit together, and we have been a two-way sounding board for ideas and for making sure our experiments are designed and performed at the highest level. Nick has an innate drive to move projects forward at all costs, and I have benefitted not only from his discoveries and technical advice, but also from his mentorship and leadership role. The bar is now incredibly high for how to perform in my own postdoc.

At the onset of this project, Jeremiah Frye and I were in the trenches together, working to the point of complete exhaustion through desperation, and I share many of the results in this thesis with him. I especially cherish the many personal and professional discussions we shared in our office space, everything from literature to religion to science to life goals. I wish the best for this good friend, wherever life takes him.

I am so proud of the character and content of the Schulman lab over the years. Many incredibly hard-working and brilliant postdocs, senior scientists, and technicians have cycled through our lab over the course of my tenure: Matt Calabrese was my first direct mentor, and the combination of positivity and effectiveness he brought to every day in the lab has left me something to aspire to. Ryan made incredible improvements to generation and purification of APC/C and his selfless efforts make all of our work possible. Hari, Steve, and Asad were among "the greats" that I looked up to from my

first days. Danny and Dave, leaders of the fraternity environment within the lab, make it clear that a life in science can be interesting, light-hearted, and still top-notch and massively productive. KP, Qiu, Masaya, Manjeet, Brian, and all of the rest of the postdocs show me how hard I must work at the next step. These guys are incredible and I am humbled to work along side them. I hold Shanshan near and dear as we both developed alongside each other and had a friendly relationship on and off campus. Peter has been a great friend and we especially love to get pizza and chat about life and work. All of the lab youngsters, Julie, Alan, Vlad, Omar, Shein, Khee, Yumei, Michael, and David Y provide incredible promise for the future of our type of work, and have made the job more than just a job; they are a close group of friends participating in sports, celebrations, and helping each other through the tough times. Especially good memories were made with Marc and Vlad in Memphis, NYC, and Austria, and watching these guys become successful throughout life is going to be a great pleasure. Arkansas :)

The Schulman lab only works because of the extraordinary efforts of our fantastic lab manager, David Miller. He is a brilliant scientist, engineer, and organizer, and my favorite person to have long chats with in the workplace. The task of managing so many people with their many demands is an incredibly difficult one, and Miller made it happen while being a friend and mentor at the same time. Thanks for all of the stimulating lunch conversations and for making sure to put me on your right side so we could be as animated as we always are. Our lab also travels, schedules, and maintains supplies because of the kindest and warmest administrator in the world, Shelia Bozeman. We are so spoiled to have this wonderful, motherly figure making our complicated scientific careers more simple and achievable, and I wish her all the best with retirement, golf, photos, and family.

ABSTRACT

Healthy, reproducing cells create and destroy proteins in an ordered manner. Both the concentration and localization of protein pools is important to regulate the many cellular processes necessary for the life of the cell. In order to produce new proteins, cells degrade existing materials such as proteins and organelles that are dispensable or troublesome in order to recycle their raw components. Degradation is accomplished largely through two major pathways: in bulk through processes such as autophagy and phagocytosis, or in a targeted manner through the ubiquitin-proteasome pathway. Autophagy utilizes an encompassing body to encapsulate targets and surrounding materials for decomposition in a regulated but relatively non-specific manner. The ubiquitin-proteasome pathway, however, is an exquisitely precise method of degradation capable of targeting specific pools of protein substrates. So-called ubiquitination generates a signal for degradation by the 26S proteasome machinery. In order to establish the degradation signal, the cell utilizes a cascade of 3 enzymes working in concert to organize substrates and ubiquitin. By using enzymes capable of substrate specificity, the cell can regulate large pools of proteins in a specific spatio-temporal manner.

In the present study, we employ biochemical and structure-based techniques to study proteins involved in an essential ubiquitination pathway involved in maintenance of the cell cycle. A mitotic regulator called the Anaphase Promoting Complex, also called Cyclosome (APC/C), modifies myriad substrates that control cell cycle activities. The APC/C is a ubiquitin ligase complex; it is the final enzyme in a tri-enzyme cascade and catalyzes the final step of Ub transfer from an E2 enzyme directly to substrates. A protein called Emi1 is responsible for directly binding and inhibiting the APC/C throughout interphase, when APC/C substrates are stabilized. We set out to study domains of Emi1 responsible for binding and inhibiting the APC/C and its association with E2 enzymes or substrates, with the aim of characterizing both the mechanism of the inhibitor and the essential functional requirements of the APC/C, which are not well understood. We aim to visualize the APC/C in complex with Emi1 through electron microscopy and to accurately interpret the location and orientation of Emi1 within this density.

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LIST OF ABBREVIATIONS

Å	Angstrom
ABBA motif	APC/C recruitment motif, named for presence in Cyclin A, Bub3, Bubr1, and Acm1
APC/C	Anaphase Promoting Complex/Cyclosome
ATP	Adenosine 5'-Triphosphate
BPA	<i>para</i> -Benzoyl Phenylalanine Photocrosslinker
Cdc20	Cell division cycle 20
Cdh1	Cdc20 homolog 1
CRL	Cullin RING Ligase
C-terminal	Carboxy-terminal
CV	Column volume, a resin bed with 1mL sepharose has a 1mL CV
Da	Dalton
D-box	Destruction box (RxxLxxx(x)N)
Di-Gly	Glycine-Glycine C-terminal Residues of Ubiquitin
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligating enzyme
EM	Electron Microscopy
Emi1	Early Mitotic Inhibitor 1
EtBr	Ethidium Bromide
F-box	named for presence in CyclinF
FLAG	Peptide sequence DYKDDDDK
G1	Gap Phase 1
G2	Gap Phase 2
HECT	Homologous to E6AP C-terminus
Hi-5	High-five, <i>Trichoplusia ni</i> ovarian cells
His6	Peptide sequence HHHHHH
IEX	Ion Exchange Chromatography (anion or cation)
IPTG	Isopropyl- β - D- thiogalactopyranoside
K11-linked	Lysine11 of Ubiquitin is modified with a Ubiquitin
kDa	kiloDalton, 1000 Daltons
KEN box	Lysine-Glutamate-Asparagine degron motif
LPETGG	synthetic peptide, recognition site for Sortase transpeptidation
M-phase	Mitotic Phase
MCC	Mitotic Checkpoint Complex
NEBD	Nuclear Envelope Breakdown
Nedd8	Neural precursor cell expressed developmentally down-regulated protein 8
N-terminal	Amino terminal
Plk1	Polo-like kinase 1
RING	Really Interesting New Gene
RL Tail	Arginine-Leucine C-terminal residues (also LRRL tail)

SCF	Skp1-Cul1-F-box protein
SEC	Size-exclusion Chromatography
Sf9	<i>Spodoptera frugiperda</i> ovarian cells
S-phase	Synthesis Phase
Strep tag	Peptide sequence SAWSHQFEK that tightly binds streptavidin
TEV	Tobacco-Etch Virus protease
TPR	Tetratricopeptide Repeat
tRNA	Transfer Ribonucleic Acid
UAA	Unnatural Amino Acid
Ub	Ubiquitin
WHB	Winged-Helix B
WT	Wild type
ZBR	Zinc-binding Region

CHAPTER 1. INTRODUCTION

Introduction to the Ubiquitination Pathway for Selective Protein Degradation

The attachment of a 8.5 kDa protein called Ubiquitin to target proteins is essential to eukaryotic cellular fitness and survival. This conjugation of Ubiquitin, termed Ubiquitination, to innumerable substrates is in some way involved in every process and signaling pathway that the cell employs for function. Ubiquitination, does not occur randomly, but through the activities of a selective cascade of enzymes. This form of post-translational modification is similar in scope and importance to phosphorylation. Modification of substrates with Ubiquitin (Ub) can alter the function or fate of the target by directing the localization, conformation, interactions, or degradation of the target protein through targeting to proteasomal machinery for proteolysis and amino acid recycling.

The Ubiquitin Protein as a Signaling Moiety

The Ubiquitin protein itself is small, only 76 amino acids, with a globular structure. Many important features are contained within this 8.5 kDa protein that make it a versatile signaling moiety. Notably, a C-terminal di-Gly (GG) motif directs thioester bond formation with Cysteine residues and isopeptide bond formation with primary amines typically found in Lysine side-chains within proteins. Ubiquitin itself contains 7 Lysine residues and an intact N-terminal primary amine, so is accordingly also a target for modification in 8 ways. The conserved structural fold of Ubiquitin exposes all 7 of its own Lysines (K6, K11, K27, K29, K33, K48, K63) for modification by other Ubiquitin molecules. (**Figure 1-1**) Multiple modifications of this type, called polyubiquitination, generates a variety of morphologically and functionally distinct chains (Varadan, Walker et al. 2002, Tenno, Fujiwara et al. 2004). For instance, chains formed by modification of Ubiquitin's Lys 11, have a different signaling output from chains of Lys 63. So-called K11-linked chains are targeted for proteasomal recognition, while K63 chains direct cellular signaling (Dikic, Wakatsuki et al. 2009, Komander and Rape 2012, Berndsen and Wolberger 2014). To compound the system's complexity, target proteins may be modified on several different lysines concurrently, with either homotypic or heterotypic linkages, or with branched chains consisting of a mixture of Ub-linkages. The extent and topology of substrate-linked ubiquitin chains is thought to directly influence the rates of substrate degradation and is an important area of active research (Hershko and Heller 1985, Ikeda and Dikic 2008, Komander and Rape 2012).

Ubiquitin Is Attached to Substrates by an Enzymatic Cascade

Regulation of such a complex signaling system is achieved by directed attachment of Ubiquitin through the concerted action of three classes of enzymes. (**Figure 1-2**) Firstly, a Ubiquitin activating enzyme, also called E1, binds and activates Ubiquitin by

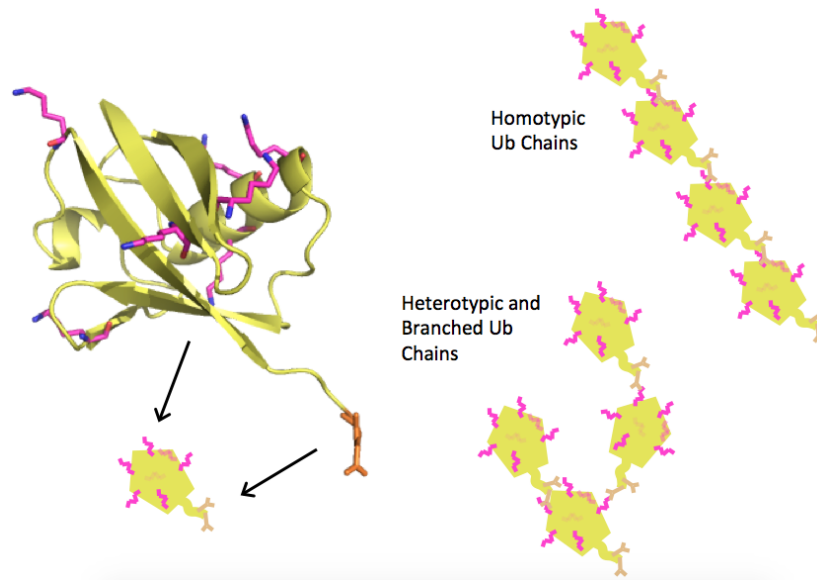


Figure 1-1. Ubiquitin as a Signaling Moiety.

Structural Representation of Ubiquitin from the Protein Data Bank (1UBQ.pdb) with 7 surface-exposed Lysine residues represented as magenta sticks, and the C-terminal Gly-Gly motif represented as orange sticks. Heterotypic chains contain multiple different Ubiquitin linkages, whereas homotypic chains arise from repeats of a single Ub linkage.

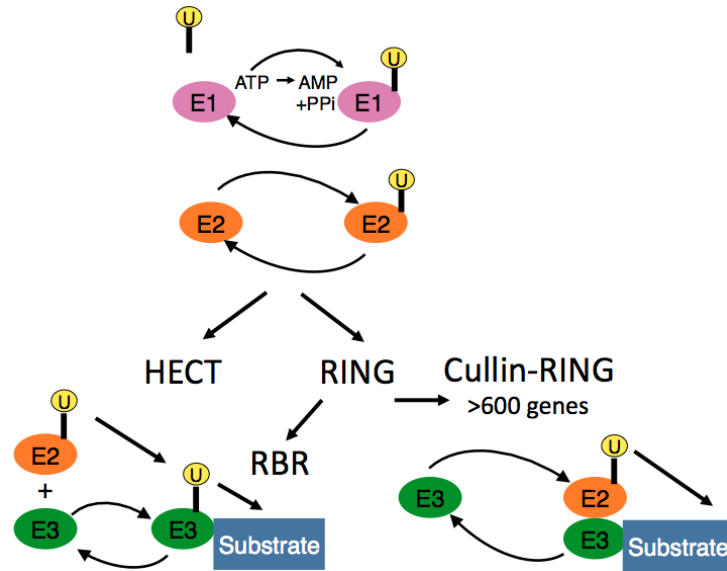


Figure 1-2. Enzymatic Cascade for Substrate Ubiquitination.

Ubiquitin, yellow sphere, is conjugated to the E1, shown in pink, in an ATP-dependent step. E1~Ub then transfers to an E2 enzyme, shown in orange, which collaborates with E3 enzymes of several types, shown in green. Cullin-RING and Simple RING E3s coordinate E2~Ub and substrates for direct transfer, whereas HECT and RBR E3s participate in the reaction.

ATP-dependent adenylation of its C-terminal Glycine residue. Following thioester linkage of Ubiquitin to a Cysteine residue within the E1, Ubiquitin's C-terminus is transferred to a Cysteine residue within a conjugating enzyme, also called E2. This E2~Ubiquitin bond is also a labile, thioester bond, and Ubiquitin is easily discharged to form an isopeptide bond with substrate Lysines when the E2~Ubiquitin conformation is appropriate and substrate is properly positioned as an acceptor (Pickart and Eddins 2004, Eletr, Huang et al. 2005, Ye and Rape 2009). In many cases this catalytic architecture is achieved through a third, ligating enzyme, also called an E3 enzyme. Over 600 E3 ligases are known to catalyze Ubiquitin transfer through a variety of mechanisms discussed later in this document. Generally speaking, E3 ligases recruit both Ubiquitin-loaded E2s and substrates to both position and mechanistically facilitate Ubiquitin transfer.

Understanding the Transfer of Ubiquitin from E1 to E2 Enzymes

Much of what we currently understand about the nature of the E1-E2 Ubiquitin transfer reaction comes from early work in our own lab (Huang, Miller et al. 2004, Huang, Paydar et al. 2005). By studying an analogous cascade of Neddylation, wherein a Ubiquitin-like protein called Nedd8 is transferred through a similar tri-enzyme cascade, the authors showed how a heterodimeric E1, APPBP1-UBA3, recruits the E2, UBC12. APPBP1-UBA3 harbors an N-terminal strand from UBC12 for optimal recruitment, and simultaneously provides a Ubiquitin-like (UBL) domain which binds the E2 mimicking a standard Ubiquitin interaction with a Ubiquitin-binding domain. Though E1 and E2 interactions were characterized, what confounded researchers was a large gap between where Ubiquitin's C-terminus was bound to the E1, and where it was going to bind on the E2 catalytic cysteine. The authors suggest that a substantial conformational reorientation would be required to place the E1 and E2 catalytic cysteine residues in close proximity. This rearrangement was later visualized for Ubiquitin through active-site disulfide crosslinking of UBA1-UBC4~Ubiquitin (E1-E2~UB) (Olsen and Lima 2013). These observations underscore much of the mechanism of ubiquitination intermediates: proteins are first recruited together through a variety of interactions, and subsequent conformational changes create a chemical environment suitable for faithful transfer of Ubiquitin through the enzymatic cascade or to the substrate.

E2 Enzymes Coordinate with Functionally Distinct Families of E3 Ligases to Transfer Ubiquitin to Substrates

E3 enzymes have been categorized into functional classes including HECT-type, RING-IBR-RING, simple RING, and Cullin-RING. HECT and RING-IBR-RING ligases not only bring Ub~E2 in proximity to substrate, but also receive Ubiquitin to participate in the reaction. Cullin-RING proteins, on the other hand, utilize substrate-binding modules called substrate adaptors to provide multifunctionality to a small group of nine Cullin proteins (**Figure 1-2**). By recognizing first a wide variety of adaptors that

can each recognize a variety of substrates, cells can achieve both regulation and substrate specificity across the many thousand targets of Cullin-RING ubiquitination.

Cullin-RING ligases (CRLs) are the largest family of E3 ligase complexes. Within this family, architecture nucleated by a Cullin protein precisely positions the Ub-thioester-E2 adjacent to the substrate (Willems, Schwab et al. 2004, Sarikas, Hartmann et al. 2011). This example of structural scaffolding underscores one important feature of E3 ligase activity: E3s utilize several accessory proteins to recruit and organize a variety of substrates with specific Ub-loaded E2s to mediate catalysis. Serving as an assembly platform for the E2 and substrate is not the only role for E3 enzymes, though. E3s also activate E2 enzymes for transfer of otherwise stable E2~Ub conjugates. (Wu, Hanlon et al. 2003, Summers, Pan et al. 2008).

Cullin-RING E3s Utilize a Cullin Protein to Scaffold E2 and Substrate Interactions with a Flexibly Tethered RING Domain

Though only nine Cullin proteins have been discovered so far, their utilization of a variety of types of substrate adaptor complexes gives them an impressive family size of over 600 genes, more than are involved in the well-studied process of protein phosphorylation (Deshaies and Joazeiro 2009). Included in these genes are those that encode proteins containing a RING domain (Really Interesting New Gene)(Freemont, Hanson et al. 1991, Deshaies and Joazeiro 2009), collectively called RING proteins. Cullin and RING proteins coordinate with substrate adaptors and E2 enzymes, and are commonly referred to as the CRL pathway of protein ubiquitination, for Cullin-RING ligase. Essential to the catalytic function of CRLs, RING proteins are responsible for binding and activating E2 enzymes for Ubiquitin release. RING domains, which are flexibly tethered to Cullins by association into an intermolecular Beta sheet, are capable of loose rotation relative to Cullin. Through this motion, RINGs bind a loaded E2 on a conserved face of the RING's surface, and bind the substrate proximal to the acceptor lysine (Dou, Buetow et al. 2012, Plechanovova, Jaffray et al. 2012, Pruneda, Littlefield et al. 2012, Scott, Sviderskiy et al. 2014).

Introduction to the Cell Cycle

Cellular reproduction occurs first by duplication of all cellular components and then separation to equal halves of the cell before splitting into two daughter cells. Faithful duplication and separation of genomic DNA is essential to proper cell division and cellular fitness, so the events surrounding these processes have been divided into four functional phases of a highly regulated cell cycle. Catastrophic failure of either genome duplication or DNA distribution leads to cell cycle arrest and eventual cell death. Failure to arrest can lead to aneuploidy, developmental defects such as trisomy, and cancer (Penas, Ramachandran et al. 2011, Bochis, Fetica et al. 2015). Though the relative fraction of the four phases of the cell cycle is variable with cell type, a considerably large fraction of eukaryotic cell life is spent in Gap-phases where the cells recover from

stresses and prepare for growth and development with time-consuming processes such as protein expression and organelle growth (**Figure 1-3**).

The Cell Cycle Consists of Four Distinct Phases

Following a Gap-phase called G1, cells enter the synthesis phase (S-phase) wherein the entire genome is faithfully replicated to produce an identical set of twin chromatids, called sister chromatids. These sister chromatids are held together by several protein complexes throughout the next Gap-phase, G2, until entering the division phase, called M-phase (Mitotic phase). During mitosis, cells progress through a number of checkpoint stages characterized by molecular functions occurring within the cell. A crucial checkpoint in mitosis occurs between metaphase and anaphase, where the cell must decide whether DNA can be properly segregated into 2 identical halves and can therefore proceed through cytokinesis, or division of the cell body to two daughter cells. Without completion of this Spindle Assembly Checkpoint, for instance, cells will arrest in late metaphase and wait for molecular signals suggesting they are ready to progress. Completion of the Spindle Assembly Checkpoint releases the aptly named Anaphase Promoting Complex, which ubiquitinates and destroys many essential cell-cycle regulatory proteins. Degradation of these proteins drives the cell cycle through metaphase to Anaphase, allowing for eventual cell division. These duplicated cells then enter G1 phase again, growing and preparing to repeat the process again.

Cells Polarize Their Genetic Information and Organize Division in the Mitotic Phase

During mitosis, two sister chromatids of identical genetic information are organized along a central axis and divided to opposite poles through the action of a large number of protein activities. To do this consistently in a regulated and concerted way, the intracellular space undergoes severe morphological changes that can be classified to a number of distinct mitotic phases named Prophase, Pro-metaphase, Metaphase, Anaphase, and Telophase. In Prophase and Pro-metaphase, following nuclear envelope breakdown (NEBD), the DNA is compacted and folded into chromatids held together by kinetochores to make mitotic chromosomes. These chromatids are aligned to a central axis by spindle fibers in Metaphase. Following proper bi-orientation, where each sister chromatid of each chromosome is tethered to opposite spindle poles, the APC/C is released from the Spindle Assembly Checkpoint and becomes active. Immediately prior to Anaphase, APC/C-mediated degradation of a substrate called Securin allows an enzyme called Separase to cleave Cohesin. Cohesin cleavage disassembles the kinetochore and allows for each chromatid to be separated by microtubule motor proteins to opposite spindle poles within the cell. During Telophase, two nuclear membranes reform around the chromosomes, which unwind into chromatin. At the end of Telophase, the cell body is split in a process called cytokinesis and two identical cells enter G1 phase to complete and restart the cycle.

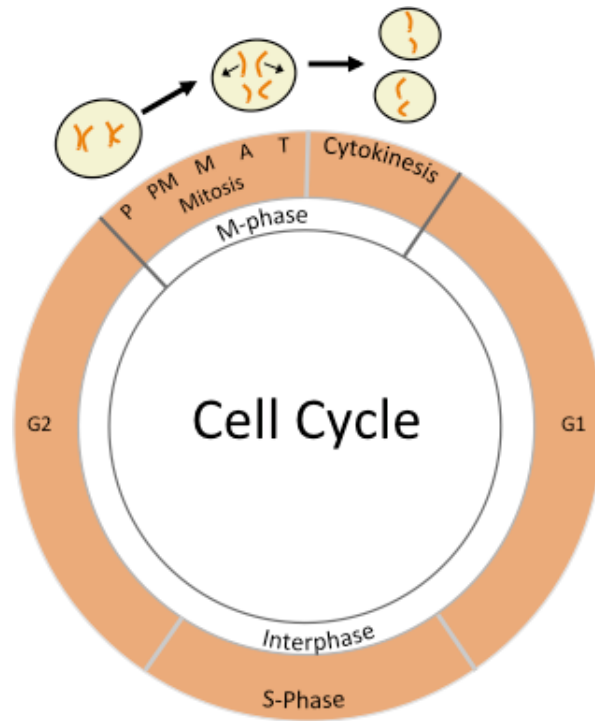


Figure 1-3. Schematic Diagram of the Cell Cycle.

The Anaphase Promoting Complex/Cyclosome

The Anaphase Promoting Complex, simultaneously discovered and named the Cyclosome, is a large assembly of at least 18 proteins expressed from 14 genes. It was discovered as an E3 Ubiquitin ligase for its role in Cyclin degradation at the end of mitosis in collaboration with an E2 enzyme called Ubch10 (King, Peters et al. 1995, Sudakin, Ganoth et al. 1995).

The APC/C remains assembled throughout the life of the cell but exists in an inhibited state following mitosis and completely through interphase. This allows for accumulation of APC/C substrates that are essential for chromosomal duplication and cell maintenance. Only when released from two major inhibitors, Emi1 and MCC, and by association with accessory coactivator proteins, Cdc20 or Cdh1, does the APC/C become active for ubiquitination of target substrates. Substrates of the APC/C include a variety of cell-cycle regulatory proteins, notably mitotic Cyclins and a number of kinases. It has been suggested that the final stages of mitosis are governed exclusively by dephosphorylation of Cdk substrates and by the APC/C-mediated degradation (Morgan 2007).

The APC/C Is Comprised of Many Subunits

The APC/C is a large complex of structurally diverse proteins (Peters 2006). The complex has been divided into rough halves, where an arc-lamp provides structural scaffolding through a number of semi-repetitive TPR-repeat containing proteins as well as substrate recruitment, and a platform provides the basis for catalysis (**Figure 1-4**). Structural conservation among the TPR repeat proteins APC7, APC8, APC3, and APC6, present as homodimers and held together by Cdc26 and APC16, provide a wall of helices surrounding an inner core (Zhang, Chang et al. 2013). These arc-lamp proteins also provide multiple docking points for the coactivators Cdh1 and Cdc20; two proteins responsible for recruitment of substrates to the complex, discussed later in this document. The structural diversity of the platform provides several mobile points of contact for catalytic machinery such as E2 enzymes and substrates. This gives rise to an overall architecture that looks similar to a cupped left hand. With fingers for the arc-lamp and the palm for platform, a central cavity is formed, inside which catalysis is presumed to occur. Importantly, along with APC1, APC4, APC5, APC15, and APC10, the APC/C platform contains a cullin and RING module comprised of APC2 and APC11, respectively (Schreiber, Stengel et al. 2011). APC2 primary sequence suggests it contains 3 cullin-repeats at its N-terminus, a C-terminal four-helix bundle and alpha-beta domain and is terminated by a WHB domain, similar to other cullin proteins (Duda, Borg et al. 2008). APC11 is a small protein of only 9.8kDa, but it is predicted to be structurally homologous to the other small RING proteins Rbx1 and Rbx2 with a number of conserved Cysteine residues for coordination of 3 Zinc ions. Therefore the APC/C is speculated to utilize a Cullin-RING type mechanism, as discussed previously.

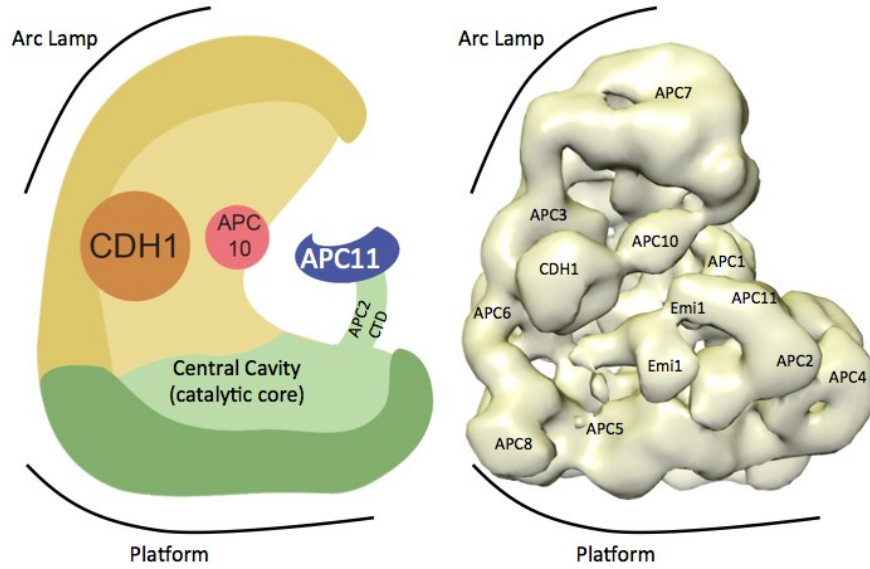


Figure 1-4. Overall Architecture of the APC/C.

Left, Schematic representation of the APC/C with Cdh1 and APC10 poised to corecruit substrate D-boxes, and APC11 positioned to perform catalysis. **Right**, EMDB-2354 shows APC/C-Cdh1-Emi1-Skp1 with labels for subunits contributing to density.

The APC/C Coordinates with Coactivator Proteins Cdc20 and Cdh1 to Recruit Substrates and Activate the Platform for Catalysis

Cdc20 (Cell Division Cycle 20) and its homolog Cdh1 (for Cdc20 Homolog 1) were discovered for their role in maintenance of cell division in *Saccharomyces cerevisiae* (Visintin, Prinz et al. 1997). Both of these proteins binds to the APC/C at the same position, and therefore compete for binding (Nagai and Ushimaru 2014). Levels of Cdc20-bound and Cdh1-bound APC/C are distinct throughout the cell cycle, as pools of these proteins are not constant and binding is competitive. Early in mitosis, Cdh1 levels are decreased and APC/C-Cdc20 exists predominantly. Following the metaphase to anaphase transition, Cdc20 becomes a target of APC/C-mediated degradation and APC/C-Cdh1 is the dominant complex throughout the rest of the cell cycle (Morgan 2007). The APC/C collaborates with Cdc20 and Cdh1 for two primary reasons; substrate recruitment and platform activation. The 7-bladed WD40 B-propeller domain common to both Cdc20 and Cdh1 provides a single, well-studied docking surface for recruitment of a “degron-motif” common to APC/C substrates, as well as other surfaces that aid in recruitment of secondary motifs. Substrates present an exposed RxxLxxx(x)N motif which is recruited by bipartite mechanism to both the coactivator and a nearby subunit called APC10 (da Fonseca, Kong et al. 2011). Secondary substrate motifs such as the ABBA (first characterized in Cyclin A, Bub3, BubR1, and Acm1) motif and KEN-box (Lys-Glu-Asn) are further coordinated by the coactivator (Tian, Li et al. 2012, He, Chao et al. 2013). APC/C recognition of substrates by multiple motifs of varying affinity is thought to be a mechanism for coordinating specificity and timing of substrate turnover. In more recent studies (Chang, Zhang et al. 2015) it has been shown that an N-terminal C-box motif of Cdc20 and Cdh1 enhances coactivator binding and APC/C activation independent of substrate recruitment.

Many Substrates Are Targeted for Destruction by the APC/C

The APC/C ubiquitinates a large number of proteins involved in cell cycle regulation. In one example, Cyclins are a group of proteins that were named for the cyclic fluctuation of their expression levels throughout the lifetime of *S. cerevisiae* cells. The characterization of these proteins, of which there are several, to be found in repeating cycles of high and low concentration was the first evidence for what is known today as the cell cycle (Evans, Rosenthal et al. 1983). Cyclins are regulatory proteins for Cyclin-dependent Kinases (CDKs), a group of kinases with critical functions throughout the cell cycle. Therefore cyclic levels of Cyclin proteins leads to fluctuations in CDK activity, and drives the cell cycle forward. A- and B-type Cyclins are bonafide substrates of the APC/C, and this underscores the role of the APC/C in regulating cell-cycle progression. The APC/C also targets many other mitotic proteins including Aurora, Polo-like Kinase 1 (PLK1), Nek2a, Securin, and parts of the APC/C machinery itself including the Cdh1 and Ubch10 (Ostapenko, Burton et al. 2012, Lu, Hsiao et al. 2014, Lu, Girard et al. 2015). Degradation of this large group of cell-cycle regulatory proteins in an ordered and specific way allows the APC/C to orchestrate and direct progression through mitosis and then regulate its own activity in trans following completion of anaphase. Detailed

information about substrate selection and temporal regulation of ubiquitination surrounding the cell cycle represents an active area of research.

Primarily Two E2 Enzymes Work in Conjunction with the APC/C to Deliver Ubiquitin

The APC/C is a Cullin-RING E3 ligase. As such, it brings together a substrate and a Ub-loaded E2 to catalyze direct ubiquitination of the substrate. The APC/C has two dedicated E2 enzymes called Ubch10 (sometimes called Ube2c), and Ube2s. Ubch10 utilizes canonical activating residues on the APC11 RING domain, and thus participates in a relatively standard Ubiquitination process. It has been shown that Ubch10 prefers to modify lysines on substrates rather than on Ubiquitin itself, and therefore builds only very short chains and many single Ubiquitins decorated on the substrate surface (Yu, King et al. 1996, Lin, Hwang et al. 2002). Ube2s, however, is a chain-elongation enzyme similar in function to Cdc34 for other CRLs. Ube2s can only attach a Ubiquitin to the Lysine (typically K11) of another Ubiquitin and has essentially no capacity to modify APC/C substrate Lysines directly (Garnett, Mansfeld et al. 2009, Wu, Merbl et al. 2010). Though the Ube2s reaction is simplified to a single modification, it occurs rapidly and repeatedly, building long K11-linked homotypic chains, which have been shown to be essential for mitotic progression (Min, Mevissen et al. 2015). Interestingly, mutations of RING residues within APC11 that are deficient for canonical E2 enzyme activities have no profound effect on Ube2s activity, suggesting a novel, yet undiscovered mechanism of ubiquitination (Brown, Watson et al. 2014).

The Mitotic Checkpoint Complex Binds to the Core of APC/C and Inhibits Function

The APC/C is engaged not only by E2 enzymes, coactivators, and substrates for activity, but also by inhibitors such as the Mitotic Checkpoint Complex (MCC) and Emi1. In the early stages of mitosis, an MCC comprised of four proteins (Mad2, Bub3, BubR1, Cdc20) binds directly to the APC/C and inactivates destruction of several substrates. Interestingly, certain APC/C substrates like Nek2a and Cyclin A are still targeted, presumably by an MCC-bound APC/C through unknown mechanisms. Following cell cycle progress cues, the APC/C is released from MCC, and causes destruction of its full suite of substrates, including Cyclin B and Securin – factors responsible for promotion to Anaphase. A low-resolution electron microscopy structure of an MCC-bound APC/C reveals a probable model for how the MCC blocks APC/C activity (Herzog, Primorac et al. 2009, Chao, Kulkarni et al. 2012). The structural data, when analyzed both as Apo-APC/C and MCC-APC/C, shows substantial electron density within the central cavity of APC/C only when bound by the large MCC. The MCC likely binds to and blocks APC/C catalytic functions as a competitive inhibitor through direct binding of the catalytic core of APC/C,.

Emi1 Is Responsible for Inhibition of the APC/C Throughout Interphase

Another protein called Emi1, for Early Mitotic Inhibitor 1, is also thought to directly bind and inhibit the APC, albeit through unknown mechanisms. In stark contrast to the multi-protein, ~300 kDa checkpoint complex and the ~1,200 kDa APC/C, Emi1 is relatively small at only 50 kDa. Unlike the MCC, Emi1 has not been structurally characterized in complex with the APC/C, and its mechanism is presumed to be unique due to the massive difference in size.

Emi1 – The Interphase Inhibitor of APC/C Polyubiquitination

Emi1 was first discovered as Rca1 in fruit fly genetic screens in 1997 (Dong, Zavitz et al. 1997), and was introduced as Fbxo5 in 1999 from a yeast two-hybrid screen for proteins that bind to Skp1, a well-known substrate-adaptor for the Cullin-RING family of E3 ligases (Cenciarelli, Chiaur et al. 1999, Latres, Chiaur et al. 1999). It was then rediscovered in *Xenopus laevis* in 2001 using similar methodology, and characterized as an inhibitor of APC/C activity early in mitosis (Reimann, Gardner et al. 2001). A closely related homolog, Emi2, shares similar domain architecture with Emi1, and plays a similar role in the *Xenopus* meiotic cycle, so for the sake of simplicity, I will highlight only the features of Emi1. In addition to cycles of expression and assembly of the APC/C, Emi1 association and inhibition also occurs cyclically. Throughout interphase the APC/C is found in its inhibited state and only at the onset of mitosis is Emi1 removed and degraded, allowing the cell to precisely control both the timing and order of substrate degradation, targeted by the APC/C, which is an important prerequisite for maintenance of the cell cycle.

Multiple Domains of Emi1 Are Suggested to Participate in APC/C Inhibition

At the onset of our investigation, putative domains of Emi1 had been discovered, but had not been thoroughly characterized for APC/C-specific function (**Figure 1-5**). Emi1 residues ~250-300 contain an F-box motif (named for its discovery first in Cyclin F) by which Emi1 was discovered. F-box domains are structural motifs that mediate protein-protein interactions, and are usually involved in Cullin-mediated ubiquitination pathways. The F-box fold interacts with other motifs such as the Leucine-Rich Repeat (LRR) and WD-40 motifs that are often involved in CRL substrate presentation (Cenciarelli, Chiaur et al. 1999). Residues ~360-420 of Emi1 contain primary sequence hallmarks that are conserved in a structural motif called a Zinc-Binding Region (ZBR). Zinc ions are coordinated by regularly spaced Cysteine and Histidine residues and are necessary for maintaining tertiary structure of this motif. Indeed, mutation of Cysteine residues thought to coordinate Zinc abrogates Emi1 function (Reimann, Freed et al. 2001, Tang, Wu et al. 2010). An extreme C-terminal Arginine-Leucine (RL) motif, called the

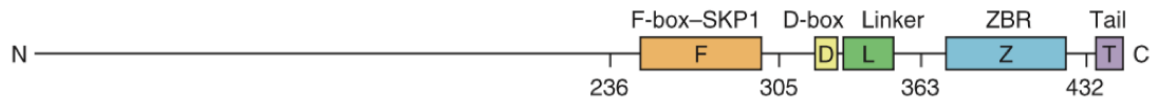


Figure 1-5. Domain Orientation of Emi1 to Scale.

Emi1 has a large N-terminal domain unimportant for APC/C association, a folded F-box domain that associates with Skp1, a small D-box motif, Linker Region, Zinc-Binding-Region, and C-terminal LRRL tail.

RL-tail, has been proposed to aid in recruitment of both Emi1 and Emi2 to APC/C through direct binding (Ohe, Kawamura et al. 2010). Deletion of these residues of Emi2 ablates binding to endogenous APC/C, as evidenced by an inability to co-IP APC/C from *Xenopus* oocytes.

Emi1 Itself Is Regulated by Phosphorylation and Ubiquitination by SCF

Emi1 itself is a substrate of CRL-mediated ubiquitination and subsequent proteasomal degradation. The N-terminal domain of Emi1 contains a variety of motifs which are recognized by both kinases and CRL machinery to provide controlled mechanisms of Emi1 destruction. First, a pair of Serines at residues 145 and 149 within Emi1 are phosphorylated by Polo-like Kinase 1 (PLK1) in an Evi5-dependent manner. When Evi5 is not associated with Emi1, PLK1-mediated phosphorylation creates a well-characterized phospho-degron DSGxxS (Residues 144-149) motif for SCF recruitment. β -TrCp, an SCF substrate adaptor protein, binds Emi1's phospho-degron and promotes SCF-dependent Emi1 turnover (Margottin-Goguet, Hsu et al. 2003, Hansen, Loktev et al. 2004). Despite knowledge of these mechanisms of Emi1 destruction, the context of its regulation is not known. Two possible scenarios could describe the relationship between Emi1's own regulation and its inhibition of APC/C. In one scenario, Emi1's N-terminus could be protected from recognition by interaction with the APC/C. Alternatively, Emi1 N-terminus may be only flexibly tethered to the APC/C through C-terminal contacts, allowing constant phosphorylation, ubiquitination, and degradation. In the second scenario, it is also conceivable that phosphorylation or ubiquitination of Emi1 could either enhance APC/C binding, or provide a mechanism for liberation of APC/C. However, there is no direct evidence for regulation of Emi1 while associated with APC/C yet, and the mechanism of dissociation is not well understood. Emi1 is also regulated at the transcriptional level by a transcription factor called E2F. Its transcription is induced at the G1/S-phase transition, enhancing APC/C inhibition and substrate stabilization (Hsu, Reimann et al. 2002, Verschuren, Ban et al. 2007).

Specific Timing and Localization of APC/C Inhibition Within the Cell Cycle

Emi1's inhibition of the APC/C is critical to prevent premature degradation of Cyclins and other cell cycle kinases. Emi1 levels increase following mitosis and APC/C remains in an inhibited state throughout interphase. Even during the early stages of mitosis, while Cyclin B activates Cdk1 to drive spindle assembly, APC/C must be inhibited. To inhibit APC/C specifically at the location of spindle assembly, Emi1 is recruited through an interaction with a NuMa/Dynein-dynactin complex. This localizes Emi1 to the spindle poles and therefore any APC/C at the poles is efficiently inhibited, and Cyclin B is not degraded (Ban, Torres et al. 2007). Following phosphorylation and degradation of Emi1, the APC/C is bound by MCC until completion of the Spindle Assembly Checkpoint (SAC). By unknown mechanisms, MCC disassembles from the APC/C, which is finally able to rapidly degrade a number of cell cycle regulatory proteins and drives the cell cycle forward.

Aims of This Study

In the present study, I aim to characterize how Emi1, a protein dwarfed in size by its target, is capable of reducing the activity of APC/C to negligible levels throughout most of the cell cycle; this allows a functional APC/C to be assembled and ready to efficiently degrade cell cycle regulatory components only when the cellular environment is ready. Protein-protein interactions often mediate the activity or accessibility of enzymes and thus change the outcome. As mentioned above, the Anaphase Promoting Complex utilizes these interactions to attract its substrates and cofactors and catalyze the degradation of these substrates. As a form of regulation, the cell has also developed another protein that potently inhibits the APC/C by unknown mechanisms, and it is speculated that this inhibition is also mediated through protein-protein interactions..

To characterize Emi1 function, I will establish protocols for expression and purification of many constructs of the Emi1 gene. With sufficient quantity of pure Emi1 proteins, and the capacity to make mutations, we can begin to probe the functionality of Emi1 in APC/C-dependent ubiquitination assays. Using established *in vitro* enzyme assays designed to monitor APC/C ubiquitination efficiency, we can saturate with Emi1 preemptively to counteract the activity of APC/C. I will utilize this system to establish baselines for how effectively mutants of Emi1 inhibit these reactions.

In parallel, Emi1 will be used both as the subject and critical tool for electron-microscopy studies with APC/C-Cdh1. EM density calculations systematically exclude mobile elements within protein complexes. Further micro-fluctuations of the meta-structure of large complexes limits the resolution obtainable by this method. Emi1 will therefore be used in EM studies not only in attempt to characterize its location and function, but also in attempt to stabilize the APC/C and therefore improve the structure determination processes.

Following a comprehensive analysis of Emi1 interactions with APC/C, the interaction will be further exploited to develop methods by which other APC/C interactions can be studied. With Emi1 as a positive control for APC/C association, protocols will be developed for incorporation of a photoactivatable crosslinking reagent designed to inform on site-specific localization of protein-protein interactions. In brief, an unnatural amino acid called *p*-BPA will be incorporated to a single site within Emi1. Following association with APC/C, crosslinking will be activated and information about the interaction is revealed. This technique is both complementary to other biochemical and structural studies of Emi1-APC/C, and also provides a framework to study less tractable APC/C interacting partners. For instance, Ube2s interacts with the APC/C only transiently, and cannot be visualized through electron microscopy. Following optimization and confirmation of Emi1 crosslinking, analagous experiments can be performed with Ube2s to stabilize and characterize otherwise unobservable interactions.

CHAPTER 2. EXPRESSION AND PURIFICATION OF EMI1 AND UBE2S FOR BIOCHEMICAL AND STRUCTURAL STUDIES

Emi1 Expression and Purification

Initial characterization of Emi1 includes describing which of the known or yet undiscovered motifs are contributing to the functionality of Emi1. To this end, we designed expression constructs systematically isolating or deleting a number of domains of Emi1 for functional testing against APC/C-mediated ubiquitination *in vitro*. Utilizing recombinantly expressed APC/C and its bona-fide cell cycle substrates, we can recapitulate the activities of cellular APC/C and analyze loss or gain of function variants of Emi1 in *in vitro* ubiquitination assays.

Expression of Recombinant Emi1-Skp1 Protein Complex in *E. coli* Cells

With rough approximations of a regulatory N-terminus, an F-box, a D-box, a linker sequence spanning between the D-box and a folded ZBR, and the extreme C-terminal tail, we made whole domain truncations as described in **Table 2-1**. For the sake of simplicity, I establish and hereafter utilize motif-specific nomenclature for constructs of Emi1, namely N, F, D, L, Z, and T for N-terminus, F-box, D-box, Linker region, ZBR, and Tail, respectively. Therefore Emi1 DLZT contains the latter four domains and does not contain the N-terminus or F-box, and so on.

Bacterial expression in *Escherichia coli* has long been a standard technique for promoter-driven protein over-expression due in part to the ease of culture maintenance, relative affordability, and robustness of expression. Therefore all gene constructs were initially cloned into a robust expression vector with an N-terminal GST affinity tag. A modified version of pGEX-4T1 (GE Healthcare), with a TEV protease recognition site replacement of the Thrombin protease recognition site, was an ideal choice for initial protein expression for several reasons. First, the genetic linkage of Emi1 to a GST tag allows for significant improvement in purity from affinity chromatography over the more widely used hexa-Histidine tag, and subsequent TEV-mediated liberation of the GST tag yields high-quality protein with nearly native amino acid sequence. This also subverts the need for immobilized metal affinity chromatography, which may interfere with Emi1's Zinc-binding region. Second, the pGEX vector is under control of the Lac operon and therefore protein expression is directly induced with a relatively inexpensive chemical called isopropyl-thiogalactopyranoside, IPTG. As such, this vector has been widely used to produce high quantities of well-purified protein samples.

For Emi1 constructs that contain the folded F-box motif, dual-plasmid co-expression with a Skp1 clone was found to be necessary, as uncomplexed Emi1 F-box yields very little protein. Following standard expression and induction protocols, the initial quantity and quality of GST-Emi1 produced for these constructs was widely

Table 2-1. Emi1 Constructs Are Named According to Their Inclusive Domains.

Construct	Residues	Skp1 Coexpression	Expression System
Emi1-NFDLZT (full-length)	1-447	Yes	High-five
Emi1-NFDLZ	1-432	Yes	High-five
Emi1-NFDL	1-362	Yes	High-five
Emi1-FDLZT	236-447	Yes	BL21 Gold
Emi1-DLZT	305-447	No	BL21 Gold
Emi1-ZT	363-447	No	BL21 Gold
Emi1-T		No	Synthesized

Emi1-NFDLZT contains the N-terminus, F-box with Skp1 bound, D-box, Linker Domain, Zinc-Binding-Region, and essential Tail residues. Smaller constructs lack one or more of these domains and are named accordingly.

variable. Whereas milligram quantities of pure Emi1-DLZT or ZT are obtained per liter of expression, only microgram quantities of relatively impure Emi1-NFDLZT are expressed in *E. coli*, even when co-expressed with Skp1.

Expression of Emi1-Skp1 in High-Five Insect Cells

Notably, constructs containing sequence more N-terminal than the F-box were unable to be expressed in bacteria in sufficient quantity for purification. These clones were transferred to a baculovirus-containing plasmid for transfer to an insect cell expression system (Philipps, Forstner et al. 2005). The Bac-to-Bac vector system (Invitrogen) allows for viral incorporation of Emi1 genes, and subsequent transfection into insect cells in a number of steps. A plasmid called a Fastbac is first transformed to chemically competent cells harboring baculovirus DNA and incorporates the viral DNA in a process termed transposition. Bacmid DNA is then purified from these cells, which harbors the Emi1 gene, a reporter GFP, and necessary components to produce live virus. Transfection of this bacmid DNA to adherent SF9 insect cells initiates the viral life cycle and virus can be harvested in the extracellular medium 3 days post-infection (d.p.i.). This process can be repeated to increase viral titers until mature, concentrated viral media can be used to infect large-scale suspension cultures of another insect cell line, High-five (Hi5). Using this expression system, N-terminal Emi1 constructs could be generated in sufficient quantity and quality for purification and use in assays (**Figure 2-1**). With the combination of these two expression systems, high-level overexpression of all Emi1 constructs is possible, and procedures for purification of these proteins can be developed.

Individualized Purification of Emi1 Constructs

A single, standard purification protocol was applied en bloc to all of these constructs, with various outcomes. While some constructs behaved quite well and were readily purified through affinity, ion-exchange (IEX), and size-exclusion (SEC) chromatographies, others were unable to be purified in this way for varied reasons. This section will highlight some of the most important observations and the protocols that were developed to produce large quantities of optimally pure protein in the most time- and cost-effective way for the variety of Emi1 constructs.

N-terminal Constructs of Emi1 Are Unstable and Require High Ionic Strength

Full-length Emi1-NFDLZT as well as constructs containing the N-terminus through residue 432 (NFDLZ), or residue 362 (NFDL), were expressed in high-five cells as previously described. These protein complexes (each are co-expressed with Skp1) are observed to be relatively unstable and require high ionic strength for solubility. Following GST immobilization chromatography, or GST pulldown, overnight dialysis to 50mM Tris pH 7.6, 50mM NaCl, 5mM DTT resulted in significant precipitation present

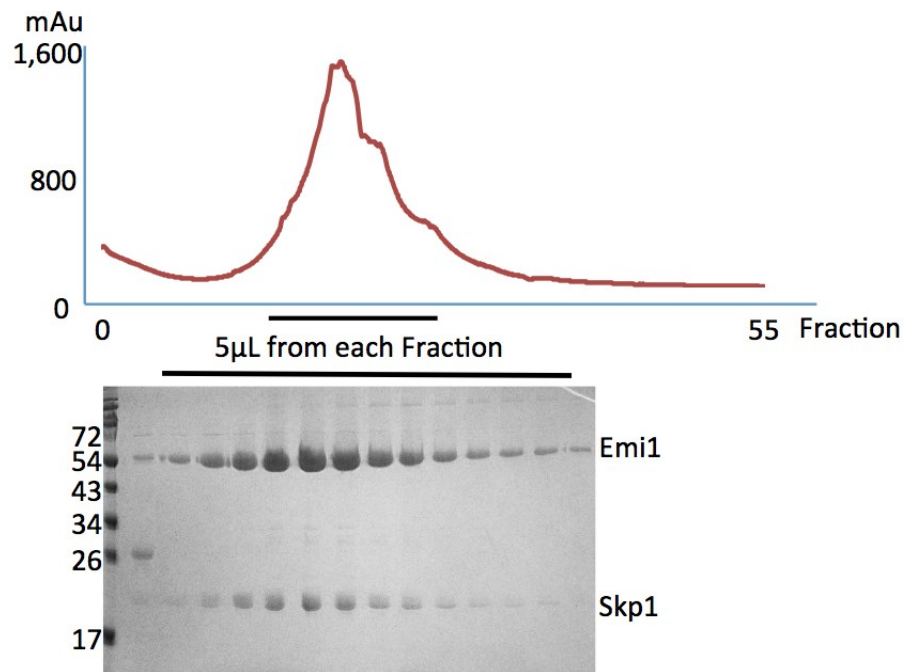


Figure 2-1. Emi1-NFDLZT Purification on Cation Exchange.

Above, representative gradient elution from Cation Exchange collected in several 5mL fractions. Below, 5μL each fraction under the peak is analyzed for purity and concentration on SDS-PAGE.

inside the protein-containing dialysis tubing, and insignificant Emi1 protein remaining in the soluble fraction. To overcome this issue, following affinity chromatography these constructs are dialyzed and cleaved in a buffer containing 50mM MES pH 6.0, 200mM NaCl, and 5mM DTT. By maintaining physiological NaCl concentration, no precipitation is observed, and the slightly basic pH of the MES buffer affords tighter binding to cation exchange resin in the next step. To further enhance the affinity with cation exchange resin, gradual dilution to 100mM NaCl while mixing is required and immediate cation exchange is used to remove all liberated GST and several other impurities. Protein precipitation is observed at this dilution step on a time-course and NaCl dilution titration, necessitating immediate loading to the IEX resin. Elution with a shallow NaCl gradient yields a strong protein peak that correlates with fractions containing Emi1 (**Figure 2-1**). Following IEX, the protein is further purified and buffer exchanged to 20mM Tris pH 7.6, 150mM NaCl through SEC on a 10/300 superdex column.

Emi1 FDLZT Is Easily Purified in Complex with Skp1

Despite containing the F-box and Skp1 co-expression like other N-terminal constructs, FDLZT (residues 236-447) gives moderately good yield from bacterial cultures, and is purified without precipitation or loss of activity using a standardized protocol. Following GST pulldown and glutathione-mediated elution, FDLZT constructs are treated with ~1% w/w TEV protease in elution buffer at 16°C. To enhance binding to IEX resin, the protein is diluted four-fold in buffer absent of salt, bringing the concentration of NaCl to 50mM. No precipitation is observed at low NaCl, perhaps implicating the N-terminal 235 residues of Emi1 in NaCl-dependent solubility. Following elution from cation exchange by a NaCl concentration gradient, the protein is further purified through SEC, concentrated to ~100µM and flash-frozen in liquid nitrogen for storage at -80°C.

The C-terminal Inhibitory Region of Emi1 Requires Optimization of Purification Protocols

Emi1-DLZT and ZT protein purification are performed similarly to FDLZT through GST-affinity chromatography. However, glutathione-mediated elution is inefficient and requires up to 20 CV before Emi1 is fully removed from the column. The massive volume of eluent is problematic for downstream purification procedures, and is unable to be concentrated despite several hours of centrifugal pressure in a centrifugal concentrator. To first identify the underlying biophysical events causing these problems, glutathione eluent protein samples were loaded to an Ethidium Bromide (EtBr) agarose gel typically used for purification of nucleotide products. Indeed, Emi1 DLZT purified from BL21 cells and purified through GST affinity is visible through EtBr staining suggesting the protein sample forms intermolecular crosslinks with nucleotides. Due to these non-specific crosslinks, elution from all types of sepharose columns is impractical and leads to damage and clogging of columns and concentrating membranes. Following

subsequent cation exchange, fractions from the IEX unbound and wash pools contain nucleotides, whereas protein eluate does not stain with EtBr (**Figure 2-2**).

To overcome this, multiple approaches were attempted simultaneously. Inclusion of DNase had negligible effect when added either to the lysis or elution buffers, and purifications performed in 1M NaCl yield half the protein but do not positively affect the glutathione elution profile (**Figure 2-3**). Though on-column cleavage is typically avoided to prevent enzymatic contamination of resins and columns used in future purifications, it was tested as an alternative to glutathione elution for these constructs. An improvement in elution was not expected, as the GST-tag itself is not implicated as the problem and Emi1 would still be capable of association with nucleotides as before. Much to our surprise, on-column cleavage with TEV allowed for liberated Emi1 to elute from the column in only 2 column volumes, making four-fold dilution of NaCl in downstream steps non-prohibitive. Furthermore, by leaving the liberated GST tag on the column, the eluent of on-column cleavage is nearly full-purity Emi1 (**Figure 2-4**). To remove the small amount of remaining impurities, we further purify by cation exchange using free resin in batch and transferring to gravity columns.

A combination of factors makes this purification protocol scalable to both high and low number of constructs, at high or low culture volume; a single construct of 24 Liters of cells yields protein of similar quality to multiple constructs at 1 Liter scale with similar demands on time and technical performance. By performing purification steps manually in plastic columns instead of through automated FPLC runs, many constructs are simultaneously purified, and eluted samples do not require time-consuming centrifugal concentration steps prior to IEX as they are robustly expressed and eluted at small volumes. This protocol makes it possible to purify upwards of 60 different constructs of Emi1-DLZT, Emi1-FDLZT or full-length Ube2s at 1 Liter scale within 48 hours by a single person (**Figure 2-5**). This provides the framework for development of mutagenesis experiments that require multiple variants of a single Emi1 construct at approximately equal purity. Following functional assay screens of the many mutants purified this way, protein constructs with desired behaviors can be followed up by larger scale purification ending in SEC for final polishing and full purification.

Utilizing Assays That Segregate the Functions of the APC/C-specific E2s Ubch10 and Ube2s

In order to be able to test Emi1 function, we utilize established *in vitro* ubiquitination assays in a variety of ways. By modifying pure proteins with fluorescent markers, we can monitor the ubiquitination of APC/C substrates in fluorescent scans of SDS-PAGE gels as slower-migrating (and therefore more modified) species. For the majority of assays presented in this work, we have chosen an N-terminal fragment containing the N-terminal 95 residues of Cyclin B. This construct contains several lysines for modification with Ubiquitin, and behaves well as an *in vitro* substrate in published studies (Dimova, Hathaway et al. 2012). Ubiquitination assays are performed

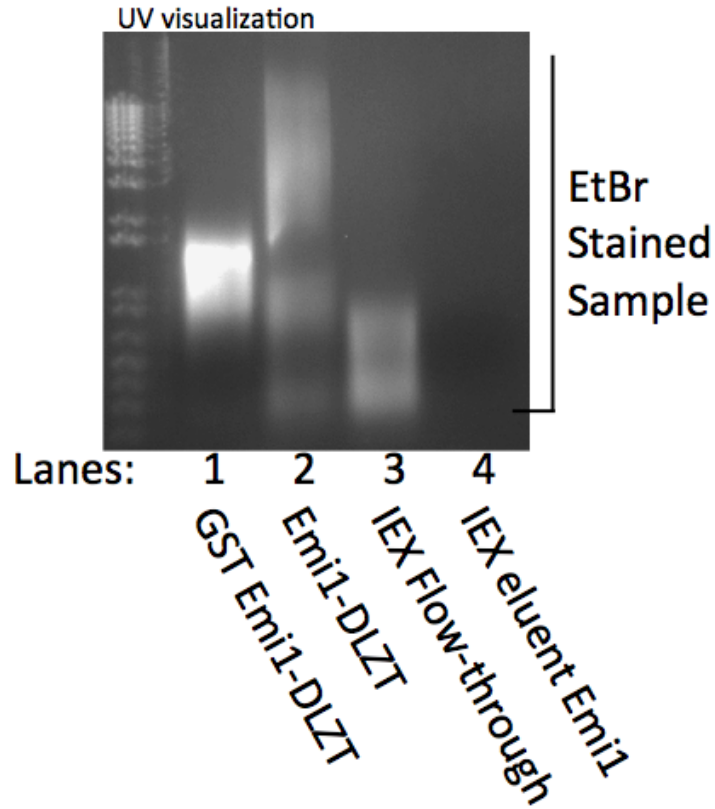


Figure 2-2. Emi1-DLZT Dissociates from Nucleotides during IEX.

Protein samples are loaded to 0.8% agarose gel and stained with EtBr. **Lane 1** contains GST-Emi1-DLZT elution from GST-pulldown, **Lane 2** contains product of TEV cleavage, **Lane 3** shows the unbound fraction from ion exchange, and **Lane 4** shows pure Emi1 protein eluent from ion exchange.

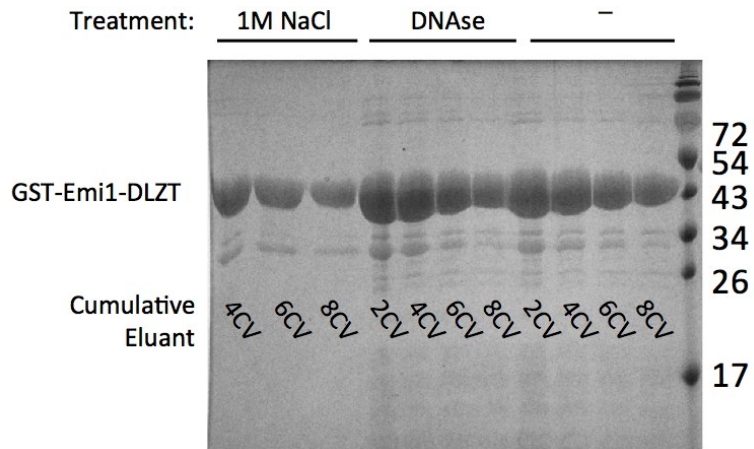


Figure 2-3. Increased Ionic Strength and DNase Treatment Do Not Prevent Nucleotide Binding.

DNase and 1M NaCl concentration each fail to clear nucleotides from Emi1-DLZT protein preps. With protein elution requiring many column-volumes (CV), downstream purification steps are impossible.

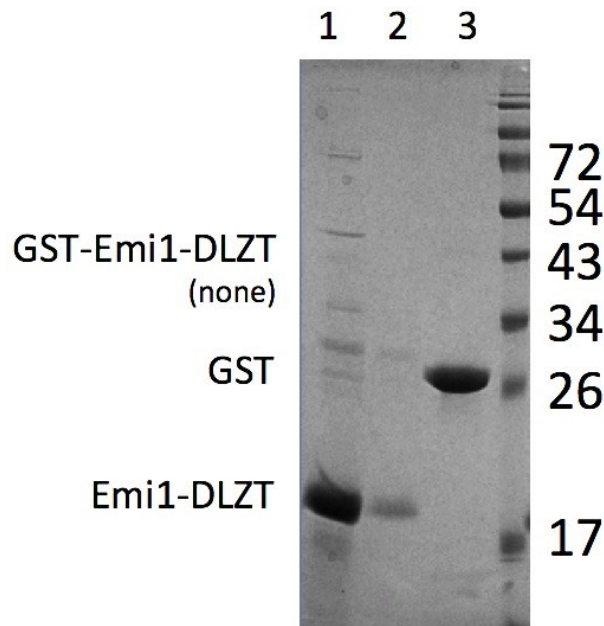


Figure 2-4. On-column Cleavage Effectively Purifies Emi1-DLZT from GST in Small Volumes.

On-column cleavage is performed for Emi1-DLZT and protein products are visualized by SDS-PAGE. **Lane 1** contains the first column-volume elution using wash-buffer, **Lane 2** contains the fifth column-volume elution using wash-buffer, and **Lane 3** contains the first column-volume elution with Glutathione. This suggests Emi1 is completely liberated from GST, is eluted in low volume, and is well-purified from GST or contaminants following wash-buffer elution. Wash-buffer contains 50mM Tris pH 7.6, 200mM NaCl, 5mM DTT, and no Glutathione.

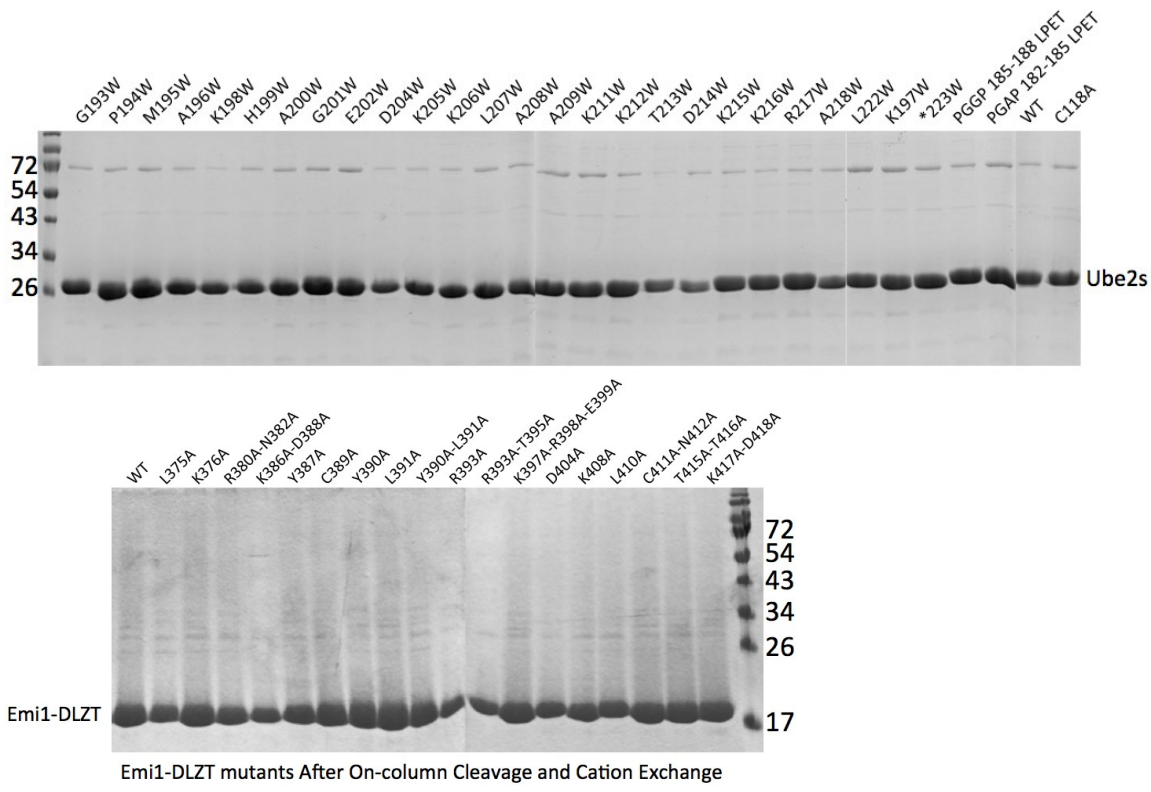


Figure 2-5. Optimized Purification Strategy Yields Pure Ube2s and Emi1.

Optimized purification of Ube2s (**Top**), Emi1-DLZT, (**Bottom**), or Emi1-FDLZT-Skp1 enables preparation of 60 constructs purified to high quality at the same time. This purification strategy makes Tryptophan-, Alanine-, and Amber-Scanning Mutagenesis experiments possible at large scale.

by combining two independent mixtures: one contains APC/C, 5 mM MgCl₂, 5 mM ATP, 0.25 mg/mL BSA, 1 μM Cdh1, and 100 nM UBA1, and the second mixture contains E2, 125 μM bovine Ubiquitin (Sigma) and fluorescent substrate. APC/C in the first mixture is incubated with Cdh1 on ice for at least 30 min prior to mixing. This combination prevents autoubiquitination of the E2 enzyme, which occurs when the E2 is capable of loading but does not have a substrate to target (Jarvis, Brown et al. 2016).

The APC/C works with two distinct E2s in a sequential manner. The requirement for the substrate to be ubiquitinated before Ube2s can function makes it difficult to segregate any E2-specific functions of Emi1 inhibition. To circumvent this problem, a Ubiquitin protein is genetically fused to the N-terminus of Cyclin B 1-95 (UbCyclin B) that is labeled and purified as before. This mimic of ubiquitinated Cyclin B bypasses the requirement for the initiating E2, Ubch10. Therefore assays separating the functions of each E2 can be tested in Emi1 inhibition assays.

After establishing baselines for APC/C activity along a timecourse, Emi1 can be tested as an inhibitor of APC/C function. Emi1 is added to the mixture containing APC/C-Cdh1 and allowed to incubate 1 hour before starting the reaction with E2 and substrate. This allows complexes of APC/C-Cdh1-Emi1 to form at equilibrium regardless of the affinity of mutant versions of Emi1. Emi1 is a potent inhibitor of APC/C-Cdh1 mediated ubiquitination of the fluorescent Cyclin B 1-95 either with Ubch10 or Ube2s alone (**Figure 2-6**). This provides the first evidence that Emi1 may be acting as a general APC/C inhibitor and not targeting the individual functions of the E2s.

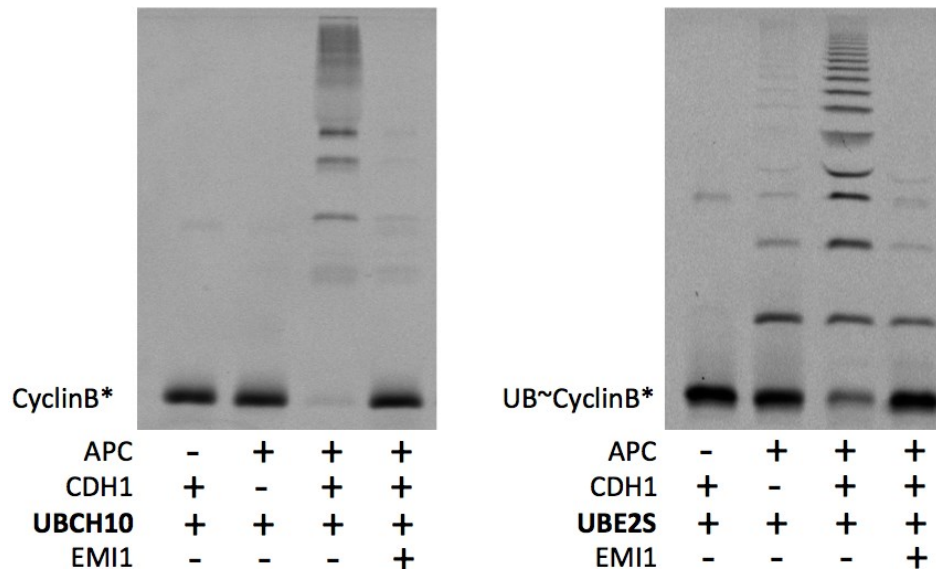


Figure 2-6. Emi1 Is a Potent Inhibitor of APC/C Mechanism in Assays That Segregate Both E2 Enzymes.

Fluorescent scan of SDS-PAGE results of ubiquitination assays monitoring fluorescent substrate modification by APC/C in presence or absence of Emi1-FDLZT. Ubiquitination of Cyclin B 1-95* by Ubch10, **Left**, and Ub~Cyclin B 1-95* by Ube2s, **Right**, are both inhibited by 50nM Emi1-FDLZT in a 12-minute reaction containing 12.5nM APC/C-Cdh1.

CHAPTER 3. MAPPING THE DOMAINS WITHIN EMI1 THAT CONTRIBUTE TO APC/C INHIBITION

Introduction to Emi1 as a Multi-Modal Inhibitor

Three essential motifs and domains of the Emi1 protein have been discovered prior to this study, and provide a basis for understanding Emi1 activity and for our thorough biochemical characterization. Critical to Emi1's proposed mechanism is a canonical D-box degron motif. This motif is commonly found in APC/C substrates and represents the major mode of substrate recruitment and targeting. The relative simplicity and disordered nature of the D-box motif presents a conundrum for APC/C substrate recruitment: conceivably many proteins contain Arginine-x-x-Leucine (RxxL) sequences, but are not intended APC/C targets. Indeed, Emi1 itself has two such sequence motifs and is predicted to be largely intrinsically disordered, fulfilling the two requirements for D-box binding. In preliminary studies, mutation of the conserved D-box of recombinant human Emi1 (Ala₃₂₂-x-x-Ala₃₂₅) reduces immunoprecipitation of APC/C from HeLa extracts (Reimann, Gardner et al. 2001) and does not sustain M-phase arrest in the *Xenopus laevis* meiotic homolog Erp1 (Ohe, Inoue et al. 2007, Tischer, Hormanseder et al. 2012).

The same studies introduce a Zinc-dependent folded domain which is similarly conserved in Emi1 and Emi2. This domain, called the Zinc-Binding Region (ZBR) coordinates two zinc ions using 8 conserved Cysteine and Histidine residues. Metal ion coordination is often a requirement for structural integrity within these types of domains, and this is evidenced for Emi1 by loss of function in Emi1 proteins mutated for one of the conserved zinc-coordinating Cysteine residues (Miller, Summers et al. 2006). Similarly, removal of Zinc itself with a specific chelator, TPEN, abrogates Emi1 function, which can be rescued by addition of excess Zinc (Reimann, Gardner et al. 2001). These results suggest that Emi1's ZBR domain is either capable of a folding-unfolding transition that is dependent on Zinc, or participates in metal-dependent catalytic mechanism. Either may provide a mechanism for sensing cellular cues or localization-dependent regulation of Emi1 function.

Emi1 D-box and ZBR domains were its first functional elements to be discovered, and are both evolutionarily conserved and retained within functional homologs of Emi1. Closer inspection of the data characterizing these regions does not account for the full capacity of Emi1, however. A third conserved, functional element was later speculated to account for these discrepancies. At the extreme C-terminus of Emi1 and homologs is a so-called RL tail consisting of a penultimate Arginine and C-terminal Leucine. In studies of both Emi1 and Emi2, deletion or Alanine substitution of these residues prohibits association with APC/C as detected in immunoprecipitation experiments in human embryonic kidney cells (Ohe, Kawamura et al. 2010).

The preliminary data generated in these studies suggest that Emi1 requires a variety of motifs for function, but the mechanism of inhibition is still unknown.

Wholesale deletion or mutation of critical domains in Emi1 makes it a defunct inhibitor, but neither the loss-of-function nor the wild-type mechanism is convincingly explained. Having overcome the technical hurdles of expressing both active APC/C-Cdh1 and a variety of Emi1 constructs recombinantly, and with ubiquitination assays that segregate the functions of the APC/C's E2 enzymes, studies characterizing and ranking the diverse modalities of Emi1's domains can be performed. Our in vitro ubiquitination system does not have the limitations associated with experiments performed in cells or lysates. The purified system both increases the capacity for experimentation and reduces the complexity of result analysis. With this toolbox of techniques and knowledge, we are well equipped to study mechanism and structure of Emi1 inhibition of the APC/C.

The N-terminus of Emi1 has been implicated in Emi1's own regulation through phosphorylation, association with CRLs, and subsequent destruction, though it is not clear whether it is required for APC/C-specific activity. To isolate regions of Emi1 that are critical for APC/C inhibition, constructs are designed with wholesale deletion of a variety of Emi1 domains. We first establish that constructs that delete either 235 residues or 304 residues (FDLZT, DLZT) from the N-terminus are equally potent inhibitors to full-length Emi1-Skp1 in qualitative assays. In agreement with previous studies, constructs which impinge on either the Dbox (residues 322-325) or the extreme C-terminus (residues 444-447) are deficient suggesting that approximately residues 300-447 represent the minimal Emi1 construct that exhibits wild-type activity (Frye, Brown et al. 2013).

Alanine Scanning Mutagenesis of the Conserved Zinc-binding Region of Emi1

As the name suggests, Emi1's ZBR domain has been shown to bind Zinc and this metal coordination is in some way important for Emi1's function. To further characterize the structural requirement for this domain, I developed an alanine scanning mutagenesis experiment. Many clones harboring single, double, or triple amino acid substitutions were prepared by site-directed mutagenesis. The structure of the folded ZBR was recently determined in our lab through NMR experiments, allowing selective mutation of only surface-exposed residues which would be candidates for intermolecular interactions. By mutating surface residues that are presumed not to be necessary for the overall fold of the ZBR, we can determine if any one residue or set of residues are important for binding and inhibiting the APC/C.

Selective Mutant Emi1 Proteins Exhibit Deficiency for APC/C Inhibition in an E2-independent Manner

To be thorough our mutational analysis includes 27 mutations covering the entire surface of the ZBR, a 45-residue domain, and these mutations were cloned in duplicate to Emi1-FDLZT and Emi1-DLZT to eliminate spurious effects and false-positives. We first utilize assays with Ubch10 and APC/C catalyzing ubiquitination of Cyclin B with titrations of WT Emi1 inhibitor to determine a sensitive range for inhibition. Within this

range, Emi1 concentrations allow near-complete inhibition of APC/C activity, but are not in excess such that subtle defects are compensated for. Many features of ubiquitination assays are malleable, especially timecourses and relative enzyme/substrate concentrations. These parameters are systematically altered to find the optimal conditions to visualize real defects in Emi1 inhibition.

Following assay optimization, purified Emi1-DLZT mutant proteins are screened for potency at two concentrations, 300nM and 60nM. Although the majority of mutant Emi1 proteins are efficient inhibitors, defects are observable for 9 clones and are colored according to rank-order (**Figure 3-1**). Prompted by these results, we designed experiments to test whether the defects observed for Emi1 inhibition of this Ubch10 assay would persist for Ube2s. It is conceivable that the Emi1 ZBR somehow blocks Ubch10 activity or association with the APC/C, but does not affect Ube2s. This hypothesis is supported by unpublished data suggesting that Ube2s utilizes distinct surfaces of the Cullin-RING machinery within APC/C for its function. In this scenario, it is also possible that the mutations causing defects for Ubch10 would be benign in a Ube2s assay, read out as wild-type inhibition capacity. To test this directly, we utilize UbCyclin B which bypasses Ubch10 as previously described, and monitor Ube2s-APC/C mediated ubiquitination. As before, the assay is optimized with wild-type Emi1 to establish the same sensitive range for inhibition.

Surprisingly, Emi1 surface mutations that render it inefficient at inhibiting Ubch10 have the same effect for Ube2s. There are two logical explanations for this result: either the mutations disrupt the folding of the ZBR domain, or they suggest that Emi1's ZBR contributes globally to inhibition of the catalytic mechanism of the APC/C, not necessarily interrupting either E2 interaction specifically. To gain insights about the ZBR fold respective to these deleterious mutations, HSQC NMR experiments were performed on purified Emi1-ZT wild-type and mutant proteins. We were able to disavow mutation R393/T395 in the middle of a hydrophobic Beta-sheet through this screen, but other defective mutants exhibit the hallmarks of native ZBR folding, namely L375A and K376A (data not shown). This affirms that surface residues of Emi1 ZBR that are not required for native tertiary structure formation are important for APC/C inhibition.

Discovering a Single Surface of the Emi1 ZBR That Mediates Activity

The residues defective in our ZBR Alanine scan are distant in primary sequence space, ranging from L375 to L410. However, when studying the NMR structure, these residues localize to a single surface within the folded domain. This provides evidence that one surface of Emi1's ZBR domain mediates contact with the APC/C either through inter- or intra-molecular interactions (**Figure 3-2**). Performing the entire screen again using Emi1-FDLZT-Skp1 further validated these results. The same ZBR mutant residues perturb Emi1 activity in proteins from a different construct and clonal source, confirming there is no experimental or systematic error with the proteins or the assays.

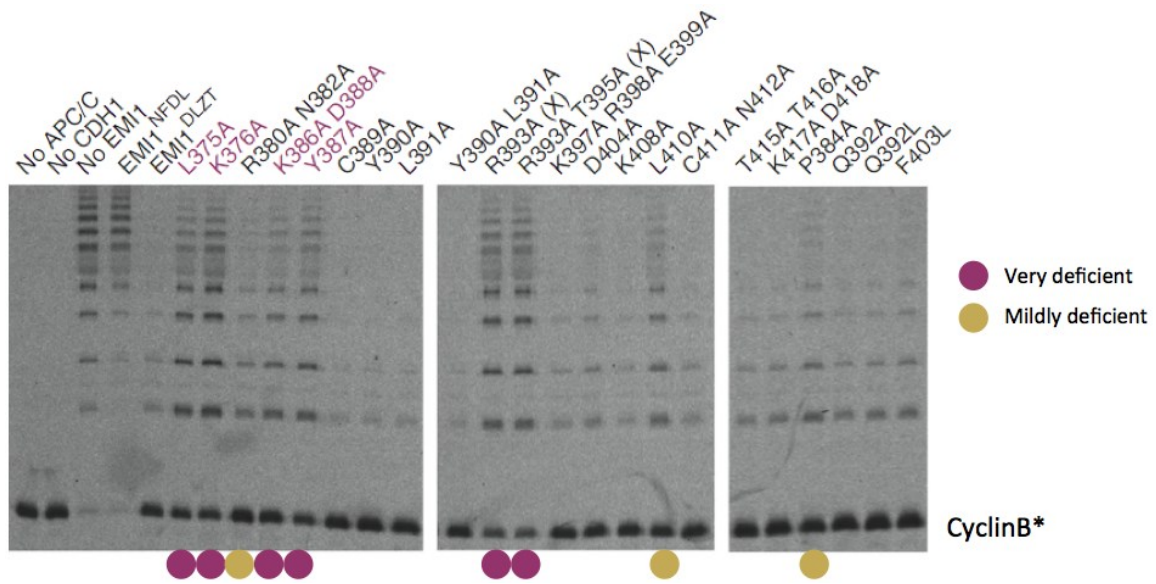


Figure 3-1. Alanine Scan of Emil's ZBR Reveals Amino-acids Required for Function.

Ubch10-mediated ubiquitination of Cyclin B 1-95* is inhibited by 60nM Emi1-DLZT. Some mutations of Emil at the indicated positions hinder this function. Magenta circles indicate strongly deficient mutations, and orange circles indicate mildly-deficient mutations.

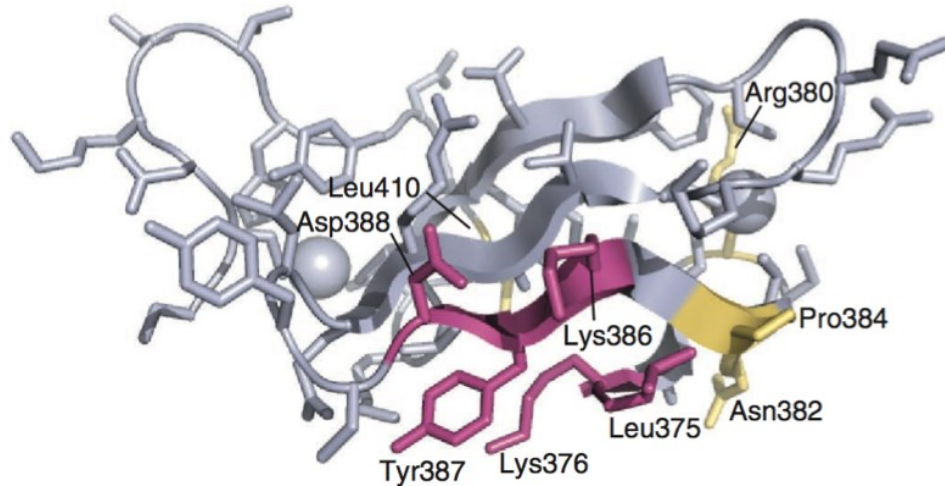


Figure 3-2. Alanine Scanning Mutagenesis Reveals a Single Surface Required for Inhibition.

Cartoon representation of the ZBR domain of Emi1 with results from Alanine scanning mutagenesis experiments highlighted in magenta (very deficient), or orange (mildly deficient). Mutants containing R393A have not been colored magenta, as they do not report on APC/C inhibition. Side-chains are shown in gray for all mutations in the screen that were not deficient in APC/C assays.

Discovery of an Essential Linker Domain Between Emi1's D-box and ZBR Domains

Both the ZBR and the D-box were implicated in previous studies as functionally relevant motifs of Emi1, described above. Again utilizing our in vitro expression and assay system, mutations of Emi1's D-box can be tested in the context of Emi1's inhibitory C-terminus. The Arg₃₂₂-X-X-Leu₃₂₅ mutation to Ala-X-X-Ala characterized as D-box dead is in fact required for Emi1 function, but not to the extent suggested from immune-precipitation experiments found in literature (Reimann, Gardner et al. 2001, Miller, Summers et al. 2006). This mutant, hereafter D^{mut} is less harmful than mutation of the ZBR in a single assay (**Figure 3-3, lanes 2, 3, 4, 13**). A wholesale deletion of the D-box in a construct retaining both wild-type ZBR and the RL tail (Emi1-ZT) does not, however, mimic the simple D-box mutation. This completely unexpected result leads us to consider other differences between Emi1-D^{mut}LZT and Emi1 ZT. We hypothesize that there may be a functional motif in the linker domain which connects Emi1's D-box to the ZBR. Functionality of this previously undiscovered region would rectify the disparity in Emi1 activity between our mutants, as it is left intact in Emi1-D^{mut}LZT, and deleted in Emi1-ZT.

Wholesale Deletion of a Linker Region of Emi1 Reveals a Functional Requirement for This Region

To test this hypothesis, systematic deletion mutants targeting neither the D-box nor ZBR, but specifically the previously benign Linker domain between them were designed and prepared. We begin with a relatively mild deletion of 5 residues in the approximate center of this Linker domain, and further extend deletions in both directions to a total deletion of 10, 20, or 30 residues of this 50-residue sequence (**Figure 3-3, top**). Both Ubch10 and Ube2s-specific assays present identical functional consequence of Emi1 Linker deletion: 10-residue deletions are tolerated well in these assays, while 20- and 30-residue deletions render Emi1 functionally incompetent. Importantly, both the D-box and ZBR domains are intact in these constructs, pointing to a novel functional requirement for Emi1 inhibition.

Residues Within Emi1's Linker Region Are Required for Inhibition

Two possible scenarios account for this unexpected but striking result: either there are specific amino acids in this Linker region which are mediating contact with APC/C, or the distance spanned by these ~40-50 amino-acids is important to allow simultaneous D-box recruitment and ZBR engagement. Much of Emi1 is predicted to be intrinsically disordered, potentially to maximize the distance achievable with limiting sequence space. To test first whether only distance is required, I supplant the defective 20-residue deletion with 20 inherently flexible and extended Glycine, Serine, and Threonine residues. This full-length but mutant sequence of Emi1 does not robustly compensate for the 20-residue deletion, suggesting a functional role of specific amino-

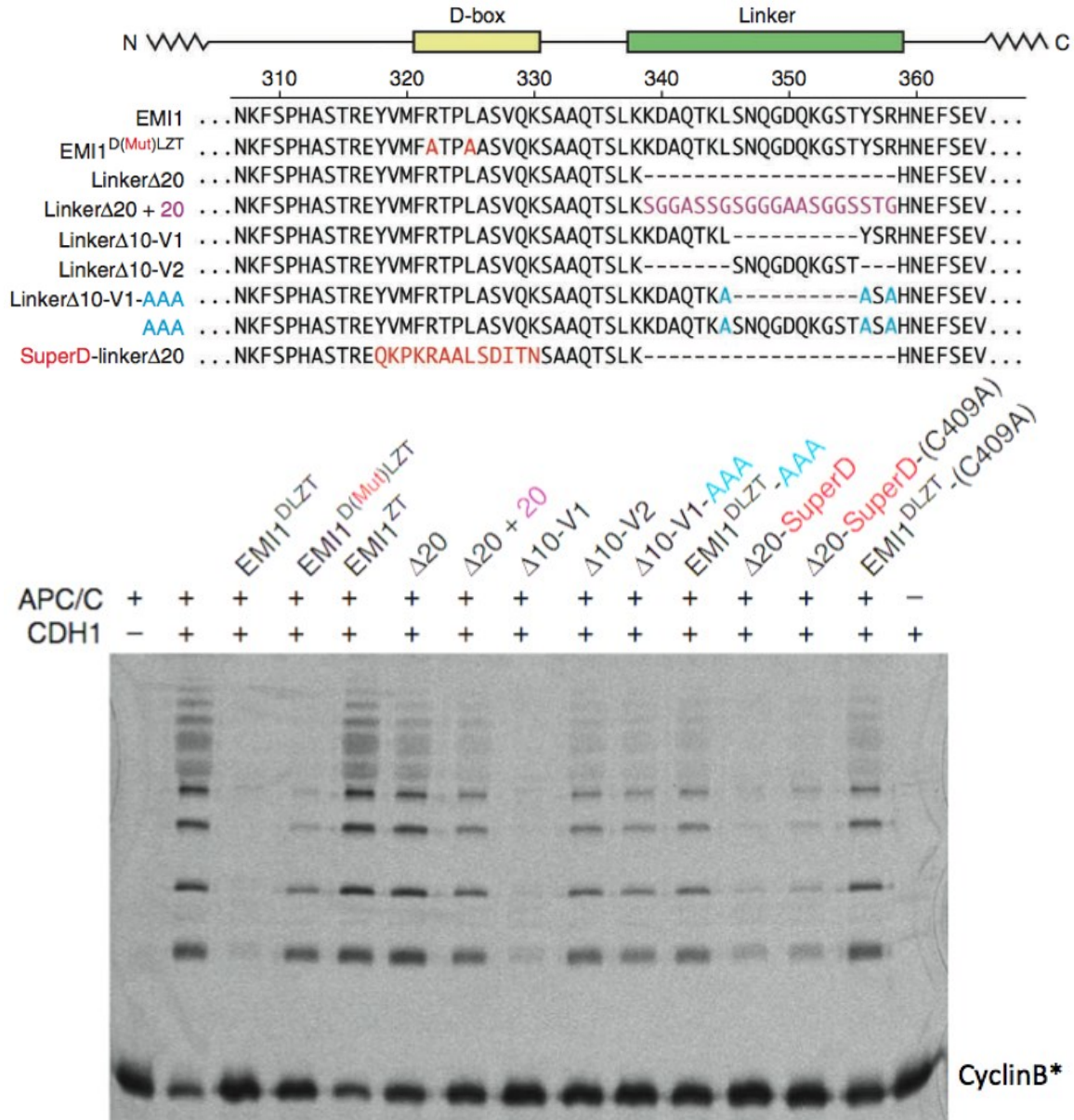


Figure 3-3. Relative Analysis for Mutations of Emi1 D-box, ZBR, and Functional Linker Domain Between Them.

Top, protein sequence and domain representation for Emi1 D-box and Functional Linker domain. Mutations in this region are annotated on the left, and color-coded for clarity. **Bottom**, fluorescent scan of SDS-PAGE results from inhibition of APC/C-Ubch10-mediated ubiquitination. Emi1-DLZT mutant proteins, as ascribed, are tested for their ability to inhibit Cyclin B 1-95* ubiquitination by APC/C.

acid side-chains in this region. In other words, since the distance can be spanned again, but the defect still exists, it is likely that the amino-acid side-chains in this region are important. Multiple lines of evidence further support this hypothesis. Firstly, knowing that a 10-residue deletion behaves normally where a 20-residue deletion is severely deficient, I hypothesize that any sequence-specific functionality would derive from the opposite 10 residues (termed V2 for version 2) which are further deleted to form the 20-residue deletion construct. In the same assay, the first 10-residue deletion behaves normally, where an orthogonal 10-residue deletion is severely deficient. Secondly, we locate specific residues within the linker that contribute to Emi1 activity. In the context of both the full-length-native, and benign 10-residue mutations, I make substitutions for patches of residues in the offending 10-residue Linker sequence. Triply mutating conserved Leu345, Tyr356 and Arg358 to Alanine residues was sufficient to substantially impair inhibition. Upon closer inspection of the data, mutation of these three residues alone, in otherwise wild-type Emi1-DLZT, prevents Emi1 inhibition to a similar degree as Emi1-D^{mut}LZT and the Emi1-DLZT ZBR mutant (C409A, **Figure 3-3, bottom**). Therefore in these experiments, the contribution towards Emi1's mechanism is approximately equal for each of these 3 domains.

Emi1 Contains a Relatively Weak D-box and an Essential RL-Tail

Strengthening Emi1's Pseudosubstrate Activity Through Fusion with the D-box Region of Hsl1

At least four domains, then, contribute to Emi1 activity: a D-box, a folded ZBR, a functional Linker between them, and a conserved RL Tail. To further characterize the relationship between these domains, and describe if they work competitively or cooperatively, we design constructs to enhance one domain and look for compensation of mutations in other domains. Emi1's D-box contributes the least to inhibition, evidenced by the relatively good inhibition of Emi1-D^{mut}LZT in **Figure 3-3, lane 4**. We tested this directly by generating peptides corresponding to the isolated D-box of both Emi1 and another D-box containing protein, the yeast APC/C substrate Hsl1. In isolation, Emi1's D-box is inefficient at blocking substrate ubiquitination, whereas an isolated peptide of Hsl1 potently blocks Cyclin B recruitment to the APC/C, presumably as a competitive inhibitor of the D-box binding site between Cdh1 and APC10 (**Figure 3-4**). Based on this information, a chimeric Emi1~Hsl1 fusion protein is generated that replaces the relatively poor sequence flanking and including the Emi1 D-box with the analogous region from Hsl1. This enhanced D-box version of Emi1, hereafter Super D-box or SuperD, is a much more potent inhibitor and nearly overcomes Emi1's requirement for either a Linker or a ZBR (**Figure 3-3, lanes 12, 13**). Though we can only explain the mechanism but not the purpose for an avidity requirement, our analysis reveals that Emi1 has evolved to utilize multiple weak motifs throughout the span of its C-terminus, which are each essential but not sufficient for inhibition.

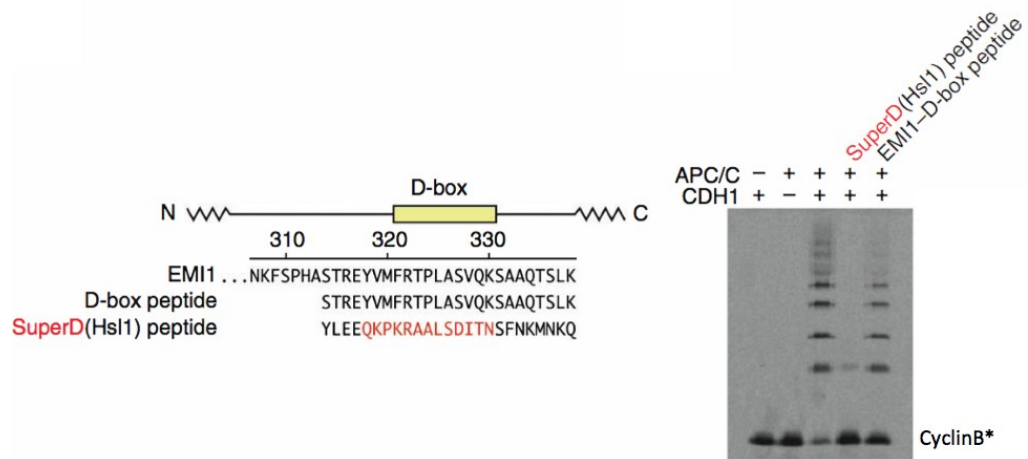


Figure 3-4. Emi1 D-box in Isolation Is an Inefficient Inhibitor of APC/C.

Left, Schematic Diagram and Sequence Information for D-box peptides generated for Emi1 and Hsl1. Hsl1 sequence in red directly replaces analogous Emi1 sequence above it in SuperD constructs. **Right**, Emi1 D-box peptide is tested for inhibition of Cyclin B 1-95* ubiquitination by APC/C-Ubch10 alongside Hsl1 D-box peptide.

Emi1's C-terminal RL-Tail Inhibits Ube2s Specifically

Emi1 terminates with a Leu-Arg-Arg-Leu Tail (LRRL, or RL-tail) that is conserved both throughout evolution and among homologs (**Figure 3-5**). Our analysis has so far characterized the contributions of Emi1's D-box, Linker, and ZBR domains as weak but cooperative for binding and inhibiting APC/C activities. Constructs of Emi1 deleted for the C-terminal Tail domain were inefficient inhibitors in data not shown, which is in accordance with published data suggesting a role for the Tail in APC/C recruitment (Ohe, Kawamura et al. 2010). To study this domain's contributions, then, we generate the Tail domain in isolation through peptide-synthetic chemistry. Despite the isolated Emi1-T being a significantly weaker inhibitor of the APC/C, at high concentrations this small peptide is capable of inhibiting specific APC/C functions, described below.

Close inspection of the C-terminal extension of one of the E2 enzymes specific for APC/C polyubiquitination, Ube2s, reveals similar distinguishing features. For instance, an extended, unique basic tail follows the structurally conserved globular core domain shared among E2s. This Lys-rich E2-tail also terminates with an identical LRRL motif (**Figure 3-5**) that is not found in Ubch10. We hypothesize that Emi1 could antagonize the activity of Ube2s, but not Ubch10, through direct competition for APC/C binding sites. We test this in a battery of Ubch10 and Ube2s specific assays, as well as ubiquitination assays which utilize both E2s and more directly recapitulate the *in vivo* function of the APC/C. What is clear from our results is that Emi1-T is a specific inhibitor of Ube2s-directed polyubiquitination. In a representative assay incorporating the activity of Ubch10 and Ube2s together, a titration of Emi1-ZT selectively inhibits Ube2s's Ubiquitin-extension mode of action (**Figure 3-6**). Qualitatively, no decrease in Ubch10-derived ubiquitination is observed (visualized as lower molecular-weight conjugates of 1 to 4 Ubiquitin proteins). Higher molecular-weight species, which are produced by Ube2s modification of Ubch10's products, are specifically inhibited upon titration of Emi1's isolated Tail. Conceptually, if Emi1-ZT were a Ubch10-specific inhibitor, we would expect that any low-MW conjugates would be efficiently polyubiquitinated and the migration pattern observed would be significantly different. This discrete function of Emi1 is corroborated in other *in vitro* assays (Wang and Kirschner 2013) monitoring radiolabeled Securin modification in the presence of Emi1 containing or lacking the C-terminal RL tail.

Visualizing an APC/C-Cdh1-Emi1 Complex Through Electron Microscopy

Our biochemical studies provide a comprehensive understanding of how Emi1 interacts with the APC/C through several domain contacts, utilizing a multimodal framework for inhibition. Evidence suggests that the Emi1 D-box engages the D-box co-receptor proteins Cdh1 and APC10 in the top of the catalytic core, in the same way that substrates have been visualized in previous studies (Passmore, McCormack et al. 2003, Carroll, Enquist-Newman et al. 2005, Buschhorn, Petzold et al. 2011). Our

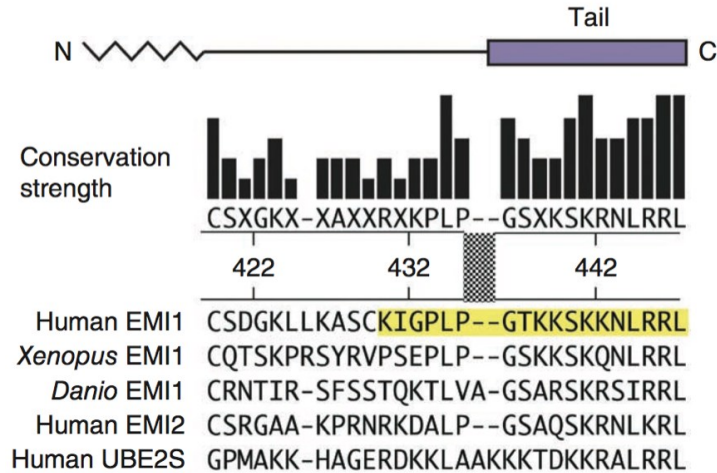


Figure 3-5. Emi1 C-terminal Tail Is Conserved Throughout Evolution, Among Homologs, and with Ube2s.

Schematic diagram and sequence information is shown for C-terminal tail of Emi1. Alignment of Emi1 from Humans, Frogs, and Zebrafish, Human Emi2, and Human Ube2s reveal a conserved LRRL tail.

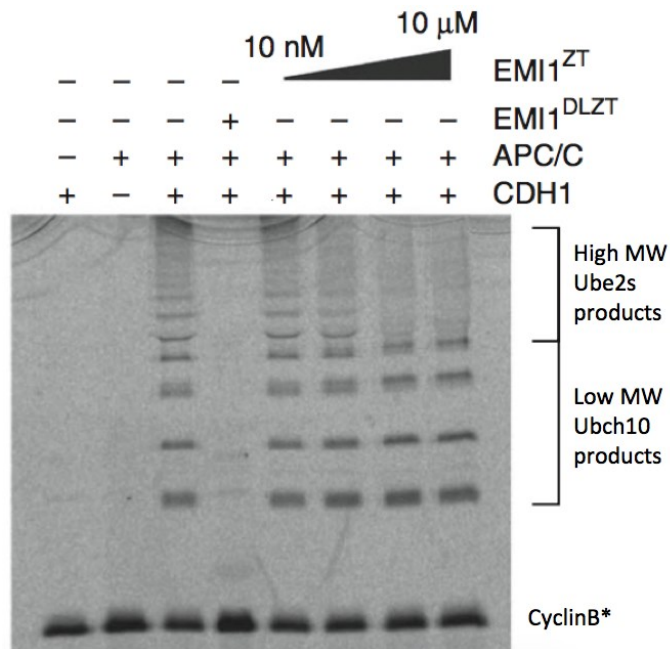


Figure 3-6. EMI1-ZT Inhibits Ube2s-generated High Molecular Weight Products.

Cyclin B 1-95* modification by APC/C with both Ubch10 and Ube2s is monitored in the presence of selected EMI1 variants. A titration of EMI1-ZT reveals selective inhibition of high molecular weight products.

characterization of a Linker and ZBR domain suggest that Emi1 is somewhat flexibly tethered to the APC/C through several important contacts, but is not spanning the maximal distance afforded by the sequence of Emi1. What is still entirely unknown, with the exception of the assumed D-box recruitment mechanism, is how and where these domains are binding to the APC/C. Though Emi1-T competes for Ube2s recruitment, and Emi1 ZBR affects catalytic efficiency, global placement of these domains is impossible with current structural information. Prior to this study, years of APC/C structural characterization revealed the approximate locations of a majority of APC/C proteins within the complex. Notably, the APC2-APC11 Cullin-RING proposed catalytic machineries have not been visualized as they are likely not rigidly attached to the body of the complex. If APC2 and APC11 behave similarly to other CRL family members, there are likely mobile elements that make their contributions to the averaged electron density map insignificant. It is believed that APC2 and APC11 function within the so-called catalytic core in the middle of the APC/C where the D-box co-receptors tether substrates. Studies of the MCC support this hypothesis, as the much larger inhibitory complex is shown to compete for the APC/C core and block APC/C activity through steric hindrance (Herzog, Primorac et al. 2009). We aim to visualize the structure of Emi1 bound to APC/C through electron microscopy to localize the sites of intermolecular contacts and understand more about the mechanism of inhibition. In collaboration with the Stark lab at the Max Planck Institute for Biochemistry in Göttingen, Germany, we perform EM studies to visualize our recombinant APC/C as an apoenzyme or in complex with Cdh1 and Emi1.

Insertions of Globular Domains Reveal Emi1 Position and Orientation on the APC/C

EM analysis reveals that despite its small size, Emi1-DLZT spans much of the central core of the APC. In order to map the location of C-terminal elements of Emi1, we purify Emi1-FDLZT-Skp1 proteins that have been engineered with large propeller insertions near specific domains within Emi1's C-terminal inhibitory region. These proteins were monitored closely throughout purification for behaviors either consistent with Emi1-NFDLZT, Emi1-FDLZT, or Emi1-DLZT so the purification strategy could be tailored to account for the insertions. Despite the large insertion nearly doubling the size of our GST-Emi1-FDLZT construct, the purification protocol was indifferent and proteins were purified to high quality using the standard Emi1-FDLZT protocol (**Figure 3-7**).

APC/C samples with Emi1 bound were prepared by Georg Petzold in Jan-Michael Peters lab, and Holger Stark calculated single-particle EM reconstructions for all samples, including mutants in Emi1-FDLZT-Skp1 with insertions between residues S354 and T355 in the Linker and following the C-terminal LRRL Tail. Since the C-terminal tail is required for maximal complex formation, the SuperD version of Emi1-FDLZT-Skp1 was necessary. The insertions contained the Beta propeller from *S. cerevisiae* Doa1, (Pashkova, Gakhar et al. 2010) which is an ideal choice for two reasons. Doa1's Beta propeller has its N and C termini in close proximity and therefore only minimally

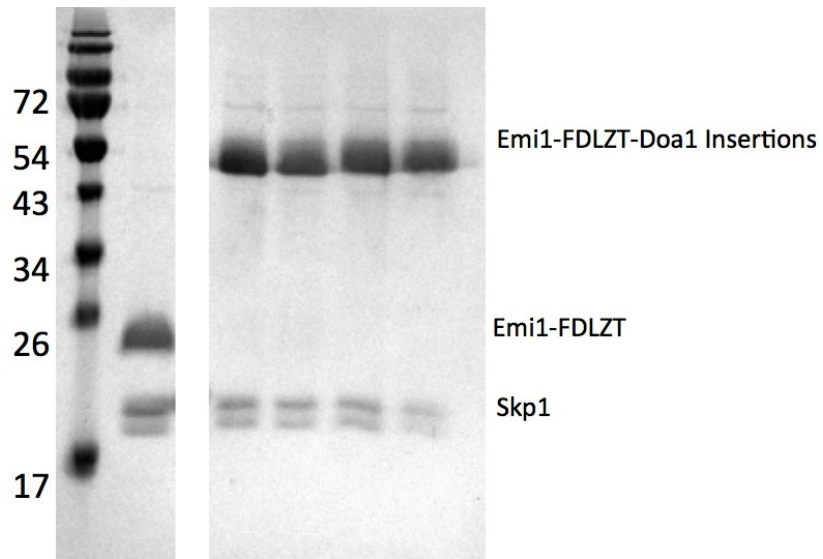


Figure 3-7. Emi1-FDLZT-Skp1 with Doa1 Insertions Are Purified Well Despite Their Relatively Large Size

disrupts structural features of Emi1, and relatively large features such as Beta-propeller folds are easily visualized as prominent density in EM. Although we cannot be certain that the insertions do not alter native interactions, our resulting structures suggest an orientation of Emi1 that agrees with biochemical data. The data are consistent with the linker projecting away from the D-box bound to Cdh1 and APC10 and toward the APC/C platform and catalytic core and with the ZBR and tail extending across the platform to the base of the APC/C (**Figure 3-8**).

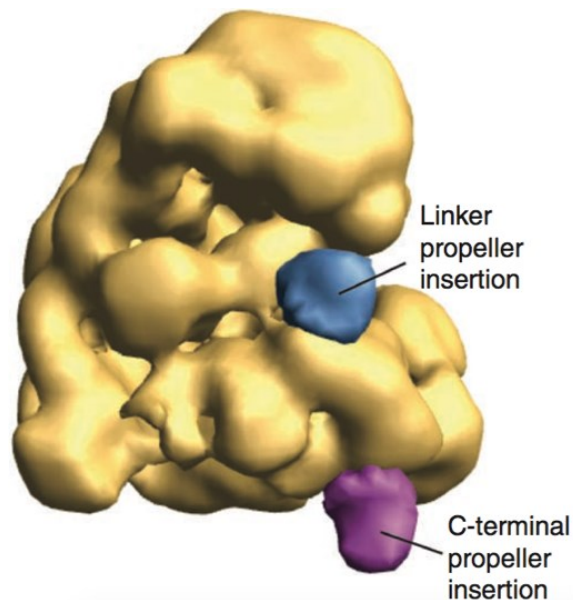


Figure 3-8. Difference Maps for Emi1-FDLZT-Skp1 Containing Beta-Propeller Insertions in the Linker Domain and C-terminus.

Density corresponding to the Doa1 Beta-propeller insertions is clear and are colored for reference. Difference density visible from insertion between residues S354 and T355 is colored blue, and C-terminal insertion density is colored magenta.

CHAPTER 4. MAPPING EMI1-APC/C AND UBE2S-APC/C INTERACTIONS THROUGH BPA PHOTOCROSSLINKING

Introduction to Photocrosslinking as a Strategy for Mapping Transient Protein-Protein Interactions

In the previous sections we both revealed functionally important motifs and domains of Emi1, and visualize Emi1 directly binding in the catalytic core of the APC/C through electron microscopy. Emi1 is tractable for EM studies with APC/C due to its high-affinity, avid binding. Studies of this nature are not possible for proteins that interact with APC/C more transiently. Alternate methods must be developed to study the nature of this type of interacting protein, and for this we turned to photoactivatable crosslinking.

Prompted by new structural and biochemical information from our studies of Emi1-bound APC/C, we asked a second related question: Where does the E2 enzyme Ube2s bind to the APC/C? Ube2s is speculated to behave in a unique way with the APC/C. It does not require the canonical E2-interacting surface of the APC11 RING, and does not require interaction with a RING at all to stimulate Ubiquitin transfer (Brown, Watson et al. 2014). How it coordinates with the APC/C to polyubiquitinate substrates, a process that is essential for proper mitotic progression (Garnett, Mansfeld et al. 2009, Bremm and Komander 2011), is unknown. We developed an orthogonal crosslinking approach to study the interaction of Ube2s with the APC/C. By allowing recombinant Ube2s to interact with APC/C and snap-crosslinking, we can trap the native interactions as they occur, even if only transiently.

***para*-Benzoyl-phenylalanine (BPA) Photo-crosslinker**

With the end-goal of locating Ube2s points of contact with the APC/C, thus giving the first glimpse at its location during ubiquitin chain-elongation, we selected a short-range, photoactivatable, non-specific crosslinker called *para*-Benzoyl-phenylalanine (hereafter BPA)(Chin, Martin et al. 2002). This benign crosslinker can be introduced to proteins in place of any standard amino acid using a variety of published methodologies (Chin, Santoro et al. 2002, Chin, Cropp et al. 2003, Chin, Cropp et al. 2003, Deiters, Cropp et al. 2003), and is called an Unnatural Amino Acid (UAA). Because the crosslinker is inactive until triggered with light, protein-protein interactions occur natively, allowing Emi1 or Ube2s to transiently associate with APC/C through normal contacts. Upon excitation with intense UV light at a specific wavelength (365nm), the crosslinker's chemistry allows for short-range nucleophilic attack of incompletely identified chemical targets on contacting protein (Yeh, Tung et al. 2016) or DNA (Pendergrast, Chen et al. 1992) molecules. Its usefulness in capturing protein-protein interactions has been demonstrated a number of times (Liu, Mack et al. 2008, Lee, Dimla et al. 2009, Chatterjee, Guo et al. 2013, Brown, Watson et al. 2014, Furman, Kang et al. 2014, Javahishvili, Manibusan et al. 2014), and we set out to develop

strategies for incorporation of BPA to Emi1 and Ube2s and subsequent crosslinking to APC/C-Cdh1.

Considerations for Substitution of a Photocrosslinker in Place of Native Amino Acids

Some important considerations were made before deciding that BPA crosslinking is an appropriate strategy for our system. Firstly, by replacing a standard amino acid with a non-native residue, we might disrupt the overall fold of the target protein and therefore lose function. Secondly, by adding an exceptionally large amino acid analog to the proposed interface of a protein-protein interaction, it is possible that the interaction would be lost entirely. Therefore before expending a large amount of effort and time on incorporation protocols, we developed a simple screen to mimic the large size of the molecule and the effects it might have on our proteins and their intermolecular interactions.

Development of Methods for Incorporation of BPA to Proteins

Tryptophan Scanning Mutagenesis

Using the standard amino acid Tryptophan, which we consider is the best natural residue mimic of BPA's bulky size, we first test the effects of having a BPA molecule within proteins. Using site-directed mutagenesis, a number of desired positions of BPA (known or speculated interacting residues on Emi1 and Ube2s) were replaced by tryptophan, protein was prepared, and assays for fold or function were employed to remove any poorly designed positions.

As a representative assay, Emi1 C-terminal residues 440-447 were mutated to Tryptophan and purified as previously described. Some of these mutants are deficient in APC/C inhibition (**Figure 4-1**), due to disruption of necessary APC/C contacts. Residues that are not contributing to inhibition (**Figure 4-1**, green indicators) tolerate Tryptophan mutations, and are suitable targets for BPA incorporation. In our scan, residues K442, N443, and R445 and an additional residue past the C-terminus, *448, are good candidates for replacement with a bulky BPA molecule. These mutants are in direct agreement with an Alanine scan performed in 2010 on Emi2 (Ohe, Kawamura et al. 2010). The analogous Emi1 residue for each of their mutants which are considered defective for APC/C association by co-immunoprecipitation experiments is also found to be defective in our screen.

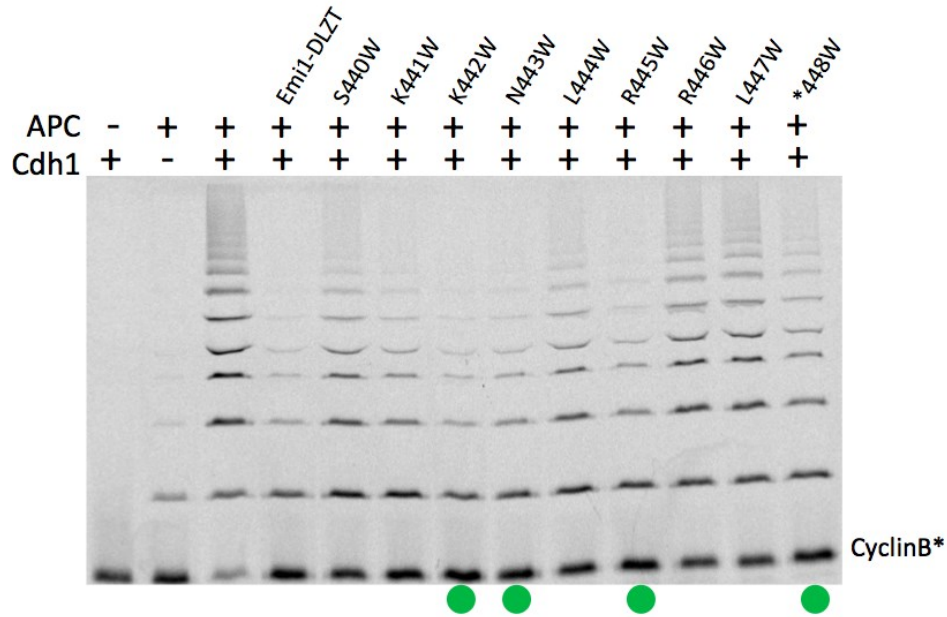


Figure 4-1. Emi1 C-terminal Tail Residues Are Mutated to Tryptophan to Mimic BPA Incorporation and Tested as Inhibitors in APC/C assays.

Cyclin B 1-95* ubiquitination is inhibited by Emi1 containing Tryptophan mutations in the C-terminal Tail. Mutants that are not defective for inhibition (shown by green spheres) are suitable positions for BPA incorporation.

***in vivo* Incorporation of BPA to Central Residues of Emi1 and Ube2s**

The most significant technical hurdle for UAA crosslinking is developing a protocol for incorporation of the chemical itself into the protein of interest. The first of two strategies we employ is amber suppression technology, which has been developed for a number of years by multiple groups (Wang, Brock et al. 2001, Wang and Schultz 2001, Wu, Deiters et al. 2004, Xie, Wang et al. 2004, Young, Ahmad et al. 2010). This *in vivo* incorporation methodology harnesses the native function of the host ribosomal translation machinery by supplying a new tRNA, an amino-acyl RNA synthetase, and a BPA amino acid precursor molecule. BPA can be translated with the protein at specific sites by including an amber stop codon at the desired UAA site. Relying on an evolved incorporation vector, amber codons do not exclusively recruit Release Factor 1 (RF1) to posit a translational “stop”, but also provide a T-stem loop structure that recruits both the BPA molecule and the synthetase. At some ratio, BPA incorporation outcompetes stoppage and generates full-length protein to the next non-amber stop. For our future studies, we utilize the most recently optimized, high-efficiency system available publicly; a vector called pEVOL-pBpF developed by Dr. Peter Schultz (Young, Ahmad et al. 2010).

Despite years of progress and development, the efficiency of BPA incorporation is highly unpredictable for this system, and invariably protein expression yields are significantly reduced, even for proteins without an amber-stop incorporation burden. This is due in part to the reliance of *E. coli* host proteins utilization of amber stop codons. Throughout the bacterial genome, hundreds of genes require the TAG codon for termination of translation, and through amber suppression technology these are efficiently replaced with BPA. Presumably, this causes a decrease in the productivity of these cells during the cumbersome task of protein over-expression following chemical induction. Nutrient-rich media partially overcomes these prohibitively low protein yields, in part through the allowance of high cell density at induction. Following the published suggestions of the system’s developers, we systematically optimize many steps of the expression protocol to increase production of purified BPA-containing protein. For Emi1 and Ube2s we find only a few factors that improve upon the previously optimized system. Auto-induction media (Studier 2005) produces approximately four-fold more protein than LB with identical incorporation efficiency, when accompanied by co-induction with previously optimized concentrations of arabinose and IPTG at an optical density of 1.6 instead of 0.8, measured at 600nm. Changing expression temperatures between 16°C and 30°C for 16 hours did not have a drastic effect, whereas 37° induction for 4 hours was insufficient. Concentration of BPA itself in the cultures was varied and found to be most effective at 0.2mM, in agreement with published findings (Young, Ahmad et al. 2010). From our pGEX vector, this improved system is capable of yielding as much as 10mg per Liter, or amounts unsuitably low for purification.

Interestingly, expression and incorporation efficiencies are changed drastically within a single construct, solely dependent upon the relative location of the TAG codon for BPA incorporation. In **Figure 4-2**, Ube2s is expressed from a single construct using

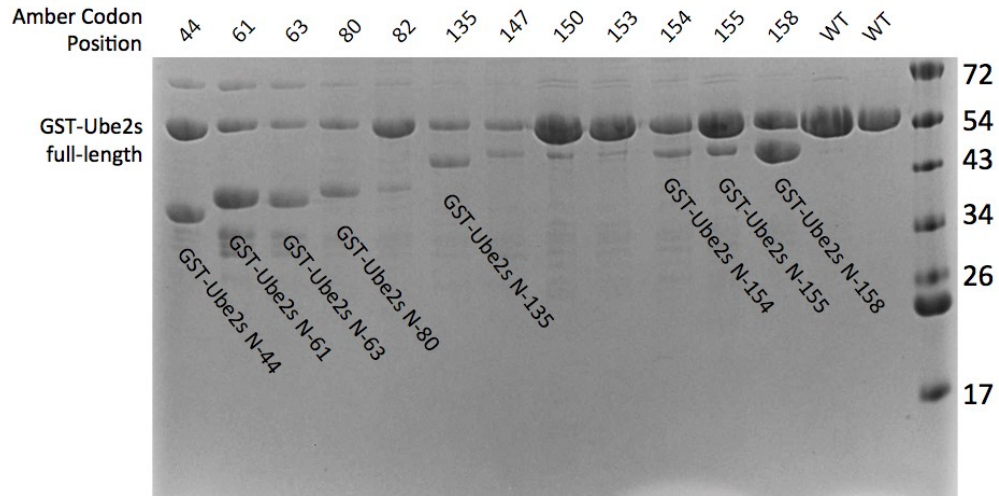


Figure 4-2. Incorporation Efficiencies Are Evident from GST-Ube2s Pulldowns Following *in vivo* BPA Incorporation.

BPA is introduced through amber suppression to several positions throughout Ube2s. At some ratio, incorporation failures are visualized as quicker migrating bands of truncated molecular weight.

identical parameters but changing the amino-acid replaced with BPA for several mutants. BPA incorporation and overall expression are changed many-fold through a seemingly random mechanism that likely points to a poorly-understood mechanism of ribosomal incorporation reliant on either DNA or peptidyl sequences flanking the amber stop. This effect is not an artifact of a single experiment, as completely new clones purified with a C-terminal LRRL deletion exhibit the same trend of expression and incorporation variation (**Figure 4-3**). Through our optimized protocols and ability to perform expression at a large 24 liter scale for any low-level expression construct, we are able to produce BPA-containing proteins at essentially any position of Emi1 or Ube2s. Using this methodology, BPA has been incorporated to over 50 constructs of Emi1 and Ube2s.

Purification of BPA-containing Emi1 or Ube2s Following *in vivo* Incorporation

Incorporation of BPA site-specifically using amber suppression technology is only partially efficient, as described above, yielding both full-length protein with BPA and protein C-terminally truncated at the site of the amber stop-codon. A number of purification strategies are used to separate full-length proteins from truncations, including C-terminal affinity tags and chromatographic separation based on size. However, C-terminal affinity tags are not optimal for Emi1 or Ube2s as both require a native C-terminal LRRL sequence for APC/C-dependent activity. Furthermore, though SEC is possible when the BPA replacement site is near the N-terminus, any C-terminal positions of BPA would create truncated proteins at nearly full-length, making separation by size impractical. Luckily, for Emi1 and Ube2s, a highly basic C-terminal ~40 amino acid stretch allows for separation from the neutral, uncharged N-terminal region of these proteins more efficiently than SEC using cation exchange. This does not, however, provide a means for purification of near full-length incorporation failures. For this we develop an entirely new method for incorporation of BPA to C-terminal fragments of disordered proteins.

Introduction of Fmoc BPA to C-terminal Residues of Emi1 and Ube2s

To produce Emi1 or Ube2s with BPA near the C-terminus, we utilize a bacterial transpeptidase called sortase to covalently attach a preformed peptide with a purified protein. This method has been used in the past for a variety of reasons (Bolscher, Oudhoff et al. 2011, Popp, Dougan et al. 2011, Guimaraes, Witte et al. 2013, Swee, Guimaraes et al. 2013, Theile, Witte et al. 2013, Strijbis and Ploegh 2014), and has been a popular method within our lab for applying N- or C-terminal fluorescent tags to proteins. The sortase enzyme itself recognizes any exposed LPET^G sequence (^G denotes site of cleavage) within proteins or small polypeptides and catalyzes simultaneous cleavage and transpeptidation of substrates with N-terminal Glycine residues (**Figure 4-4**)(Popp, Antos et al. 2009).

Before attempting BPA incorporation, we first identify a site within the C-terminus of Emi1 capable of tolerating an LPET^G motif as a final product. Assays

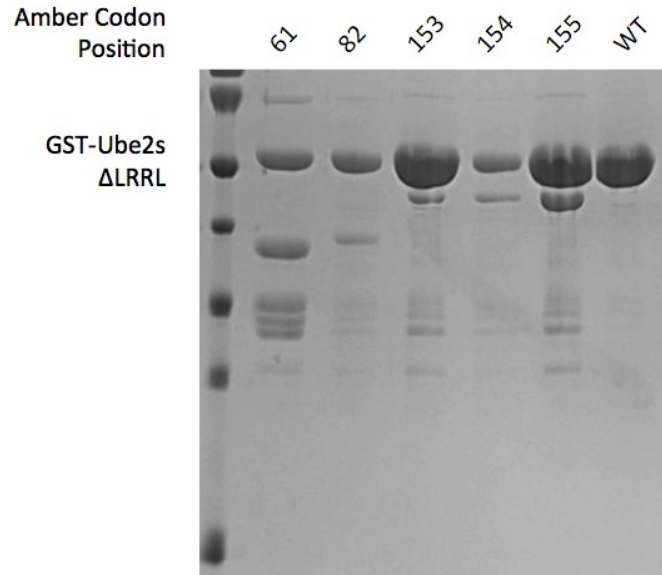


Figure 4-3. Ube2s BPA Using New Clones with C-terminal Deletion.

SDS-PAGE of samples from GST-affinity pulldown reveals identical expression and incorporation ratios for new clones with C-terminal LRRL tail deletion.

Protein-LPET/G + G-peptide

=

Protein-LPETG-peptide

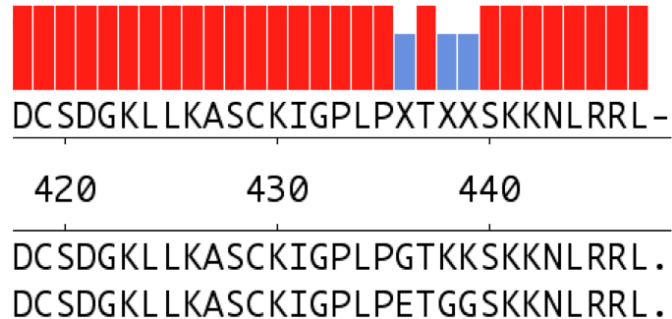


Figure 4-4. Sortase-mediated Transpeptidation Attaches an Emi1 Tail Peptide to Emi1 Protein.

Schematic Diagram and Sequence Information showing how Sortase fuses a C-terminal peptide of Emi1 sequence with purified Emi1 protein corresponding to N-terminal sequence. This technique generates 3 mutations at the site of attachment, but yields otherwise wild-type full-length protein.

designed to test these final product mimics validate a region 23 residues from the C-terminus of Emi1 as benign. As proof of principle and in order to optimize sortase parameters for this system, peptides corresponding to these residues of Emi1 were synthesized, and Emi1 terminating in LPETGG is purified. Following enzymatic reaction, full-length Emi1 can again be separated from truncated products through cation exchange and SEC.

Establishing Positive Controls for Capturing Protein-Protein Interactions with BPA

To validate this relatively complicated strategy for mapping protein-protein interactions in our multi-protein system, we first develop the technique using a robust two-protein complex. In a previous study (Sato, Mimasu et al. 2011), Sato et.al were able to recapitulate a crystallizable (Nakamura, Nakano et al. 2007) interaction between mouse Gankyrin and a C-terminal domain of an AAA+ ATPase called S6 (S6C). The authors replaced residues at the crystallized interface between Gankyrin and S6C with BPA in a variety of combinations. Following extensive crosslinking and purification of the crosslinked product, the same crystals are formed and the structure is solved with a covalent BPA crosslink. The electron density map reveals the same molecular structure but with extra density corresponding to the crosslinked residues. These constructs and protocols serve as a strong positive control for many aspects of BPA crosslinking including functionality of the synthesized UAA chemical itself, correct UV activation protocols and machinery, proper incorporation of BPA to proteins and our ability to monitor native protein-protein interactions through SDS-PAGE.

As seen in **Figure 4-5**, we are able to reproduce the protein-protein interactions described in literature, and we can validate that our crosslinker is correctly incorporated, specifically replacing residue Arg85 of gankyrin, which is capable of co-purifying with S6C as predicted from the study. On a time-course of UV photoactivation, the covalently crosslinked product of Gankyrin-S6C is observed in denaturing SDS-PAGE experiments only in reactions containing the residues responsible for the interaction. Interestingly, replacement of the receiving Glutamate 356 of S6C to Aspartate, a theoretically mild mutation, is incapable of BPA crosslinking. These results are confirmatory that BPA incorporation and crosslinking behaves similarly to published results in a model system.

Using Emi1 as a Positive Control for APC/C-specific Crosslinking and Detection

With confidence that our BPA crosslinking methods report on direct protein-protein interactions, and with the ability to generate and purify Emi1 and Ube2s proteins with BPA at any position, we aim to test site-specific crosslinking in the context of the APC/C. Due to the vanishingly small quantities of recombinant APC/C we are able to generate, and to the transient nature of Ube2s-APC/C interactions, we first establish APC/C crosslinking with Emi1 as a positive control. The tight-binding nature of Emi1, and our prior domain characterization and structure determination make this an attractive system for validation of this approach.

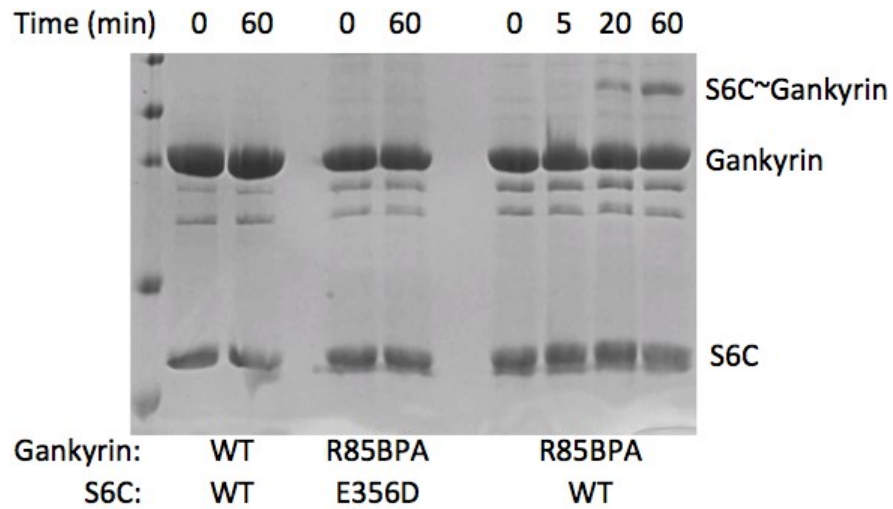


Figure 4-5. Gankyrin and S6C Are a Positive Control for BPA Crosslinking.

This coomassie-stained SDS-PAGE gel of BPA crosslinking recapitulates published crosslinking for the Gankyrin-S6 complex. Crosslinking is visualized as a higher molecular weight band appearing on a timecourse of UV photoactivation, and only in conditions where BPA within Gankyrin is expected to crosslink S6C.

We incorporate BPA to 8 positions among Emi1's D-box, Linker domain, and C-terminal tail as previously described. 1 μ M Emi1-BPA is mixed with 1 μ M APC/C-Cdh1 and incubated for 30 minutes prior to photoactivation. Photo-crosslinking is performed for 60-minutes using our optimized protocol, and reaction products are loaded to SDS-PAGE gels. Western blots with anti-His tag primary antibodies (Sigma) specific for a His-tagged Emi1 reveal high molecular-weight species indicative of APC/C crosslinking. Importantly, these crosslinks do not form when Emi1-BPA is mixed only with Cdh1 absent of APC/C (**Figure 4-6**). Interestingly, residues near the D-box of Emi1 form crosslinked products of two sizes, whereas crosslinked Tail residues migrate to a single position, indicative of binding to only one protein.

Ube2s Interactions with APC/C at Multiple Locations Are Captured Through BPA Crosslinking

In the context of the multi-subunit APC/C, specific crosslinks are visualized for Emi1-BPA. This result validates this crosslinking approach as a means to capture and monitor binding events between specific residues of APC/C associating proteins. We aim to highlight the usefulness of this tool not by recapitulating interactions that are well characterized, rather to report on previously unidentified contacts. Although from our electron microscopy studies we have information to trace Emi1's path along the APC/C architecture, very little is known about the location of Ube2s in the context of APC/C. We therefore employ photocrosslinking as an orthogonal approach to electron microscopy to glean information about the transient structure of Ube2s-bound APC/C.

Residues surrounding E153 and I154 of Ube2s were chosen for crosslinking and are of general interest because they are kinetically implicated in an activation mechanism in experiments performed by Nick Brown at the onset of this part of the project. A double mutant E153A-I154A version of Ube2s is not activated by the APC/C, and is therefore considered severely deficient in APC/C-dependent assays. However, in APC/C independent reactions, where the inherent reactivity of Ube2s is tested in Ubiquitin transfer to an acceptor Ubiquitin, no defect is observed. These results suggest that residues E153 and I154 contact the APC/C to direct Ubiquitination of APC/C-bound substrate Ubiquitins. To visualize and characterize this interaction, I target residues along the pair of helices containing E153 and I154 for BPA incorporation. Crosslinking is performed similarly to the optimized protocol established for Emi1, and products of the reaction are loaded to SDS-PAGE and monitored by western-blot analysis of a FLAG-tag on Ube2s.

C-terminal Helices C and D of Ube2s Crosslink Specifically to APC2 and APC4

BPA substitutions of several residues within this targeted region of Ube2s are crosslinked to APC/C-Cdh1 by co-mixing 1 μ M Ube2s with 1 μ M APC/C-Cdh1 for 30 minutes prior to photoactivation. Reactions are performed at sufficiently large volumes to allow the same reactions to be loaded many times to different gels, making it possible

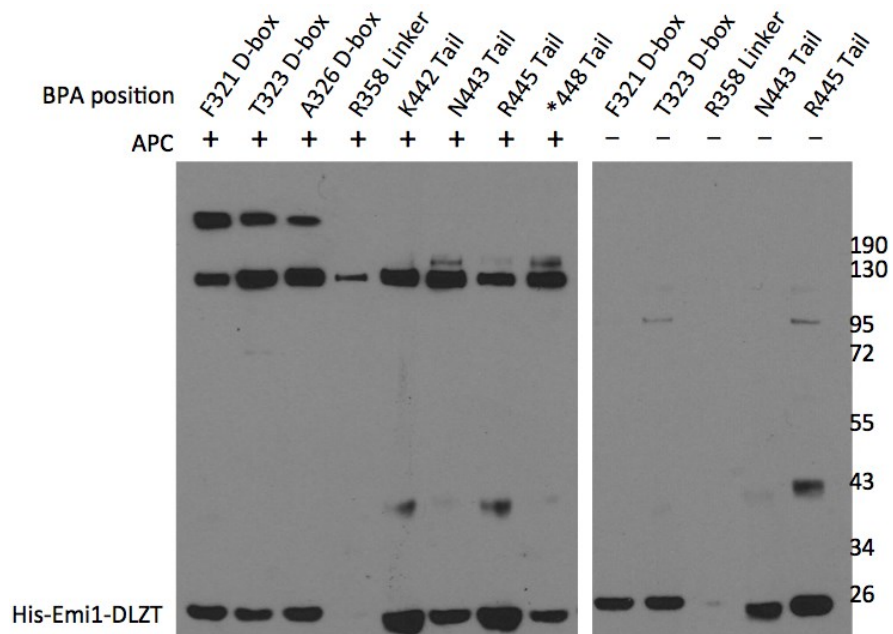


Figure 4-6. His-Emi1 with BPA Reporting on D-box, Linker, and Tail Interactions with APC/C.

This western blot of SDS-PAGE gel using anti-His antibodies specific for His-Emi1-DLZT reveals APC/C dependent crosslinking. Crosslinks observed for BPA positions surrounding the D-box are different from those observed for Emi1's Tail.

to western blot for several of the proteins involved in the interaction without introducing the variability associated with performing several experiments.

Crosslinking is performed with a number of controls to validate the specificity of the reaction: UV exposure of Ube2s-BPA in the absence of APC/C reveals any non-specific intramolecular crosslinking. Furthermore, Ube2s-BPA proteins lacking the C-terminal LRRL tail are tested to verify the crosslinking is dependent on specific recruitment of Ube2s to the APC/C, and does not report on non-specific, random interactions. Negative crosslinking results in these control experiments strengthen our confidence in the effectiveness of our system to recapitulate native interactions between Ube2s and the APC/C.

Only upon activation with UV light, significant crosslinking is observed between residues E153, I154, and A158 of Ube2s and specific subunits of the APC/C (**Figure 4-7, Figure 4-8, Figure 4-9**). Western blots specific for the FLAG-tag on Ube2s show migration patterns specific for two locations of Ube2s significantly larger than Ube2s protein itself. These two alternate migrations of Ube2s persist throughout crosslinking of the three different BPA positions in this helix. Further analysis of the same reactions reveals that only certain subunits of the APC/C are modified with Ube2s. Antibodies specific for APC4 protein and APC2 protein reveal that both of these subunits are modified with Ube2s in specific reactions. Furthermore, a second antibody for the Strep-tag on APC4 confirms there is not a problem with antibody specificity; rather the APC4 subunit is specifically crosslinked by Ube2s in these reactions. These results begin to explain the mechanism of Ube2s activation by the APC/C. The C-terminal helices of Ube2s that include residues E153, I154, and A158 make contact with the APC2/APC4 region of the platform, and this is likely responsible for either direct activation of the E2, or as a means for E2 positioning near its substrate, or both. Since the methodologies for residue-specific identification of the crosslinked protein side-chains are not fully developed, future studies will be required to elucidate what specific APC2 and APC4 contacts are being made, and how they facilitate Ube2s activity.

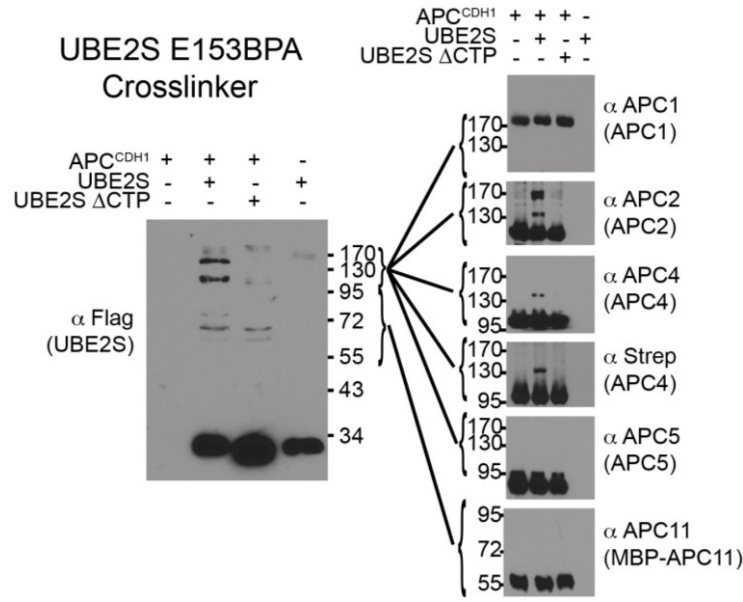


Figure 4-7. Crosslinking Between Residue E153 of Ube2s and APC/C-Cdh1.

Western blots of Ube2s-APC/C photocrosslinking reactions are performed with the indicated antibodies. Ube2s migration in SDS-PAGE gels is specifically slowed to two positions. These positions correlate with slower migration visualized for APC2 and APC4, suggesting specific crosslinking to these subunits. These results validate an RL-tail recruitment dependency for these APC/C-Ube2s interactions, and an APC/C dependency for Ube2s crosslinking.

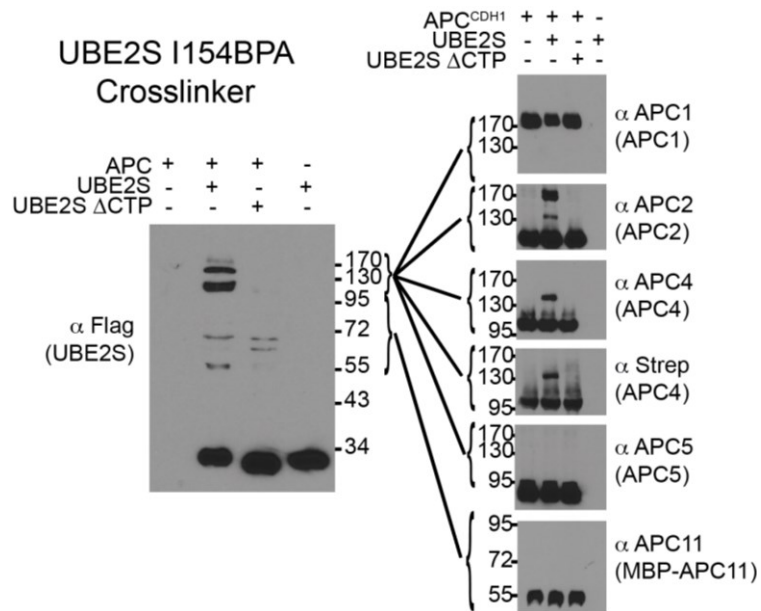


Figure 4-8. Crosslinking Between Residue I154 of Ube2s and APC/C-Cdh1.

Western blots of Ube2s-APC/C photocrosslinking reactions are performed with the indicated antibodies. Ube2s migration in SDS-PAGE gels is specifically slowed to two positions. These positions correlate with slower migration visualized for APC2 and APC4, suggesting specific crosslinking to these subunits. These results validate an RL-tail recruitment dependency for these APC/C-Ube2s interactions, and an APC/C dependency for Ube2s crosslinking.

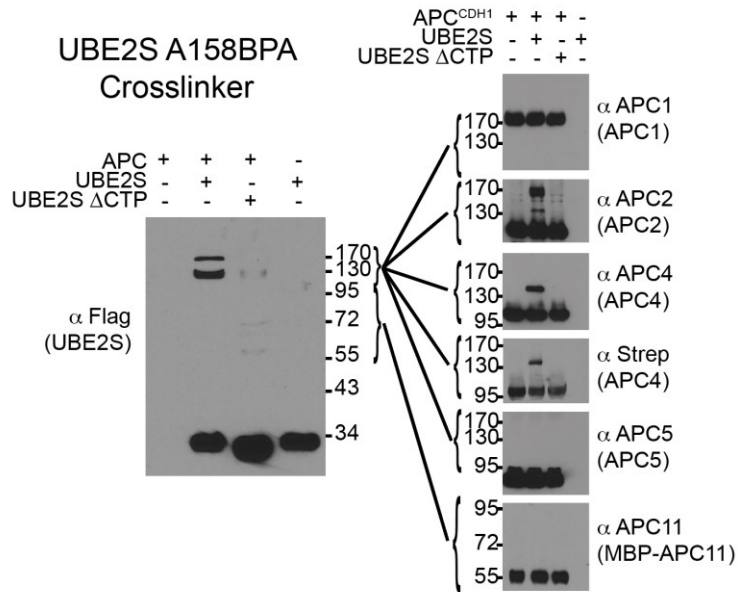


Figure 4-9. Crosslinking Between Residue A158 of Ube2s and APC/C-Cdh1.

Western blots of Ube2s-APC/C photocrosslinking reactions are performed with the indicated antibodies. Ube2s migration in SDS-PAGE gels is specifically slowed to two positions. These positions correlate with slower migration visualized for APC2 and APC4, suggesting specific crosslinking to these subunits. These results validate an RL-tail recruitment dependency for these APC/C-Ube2s interactions, and an APC/C dependency for Ube2s crosslinking.

CHAPTER 5. DISCUSSION

APC/C-Cdh1-Emi1 Structure at High Resolution

Two years after completion of the work described in this dissertation, a unique opportunity to analyze the high-resolution structure of APC/C with Emi1 bound was made possible both by data generated by our lab and our collaborators, as well as by a journal article published by the Barford lab in Cambridge, UK (Chang, Zhang et al. 2015). Both high-resolution structures combined with the structural groundwork provided by our lab (Frye, Brown et al. 2013) yield similar information about the orientation of Emi1 on APC/C and the interactions between them, and accordingly both are studied to provide insights into the results obtained prior to their discovery.

Emi1 D-box Engages the Cdh1 and APC10 Co-receptors

The most obvious region of the structure to validate is the D-box binding site between the β -propeller surface of Cdh1 and the jelly-roll fold of APC10, as this is a well-established substrate recruitment mechanism of the APC/C (Passmore, McCormack et al. 2003, Carroll, Enquist-Newman et al. 2005, Buschhorn, Petzold et al. 2011). By examining both the density map (EMD: 2924) and the model associated with it (PDB ID 4UI9)(Chang, Zhang et al. 2015), it is evident that Emi1 is recruited to these co-receptors as expected (**Figure 5-1**). Though the published model contains a modified D-box motif within Emi1, (T323I), it is reasonable to expect that the native Emi1 D-box sequence, although relatively weak, is capable of acting as a pseudo-substrate motif and inhibits substrate recruitment in this way.

Emi1 ZBR Alanine Scan Informs on Residues in the Interface

We next analyze the electron density ascribed to the Emi1 ZBR. This region of the electron density map is very convincingly correct for both placement and orientation of the ZBR structure that was solved in our lab. The structured ZBR fits quite well within map density, even at low contour and provides insight to the direction of travel from the N-terminus to C-terminus, which is useful when considering the Linker domain between the D-box motif and ZBR domain. When comparing the modeled structure with our Alanine scanning mutagenesis results (**Figure 5-2**), no major discrepancies are observed. In fact, the surface that was suggested as an APC/C interacting motif points directly to the RING subunit of APC11. When taking the biochemistry and structures together, the function of these residues is likely a mix between inter-molecular contacts with APC11 and intramolecular contacts supporting and stabilizing the structure of the ZBR itself as well as the disordered regions following the structured Linker helix. Furthermore, the APC11 RING is implicated as the catalytic module for the APC/C, and direct binding to this mobile element restrains its mobility and directly inhibits the CRL catalytic mechanism of APC/C.

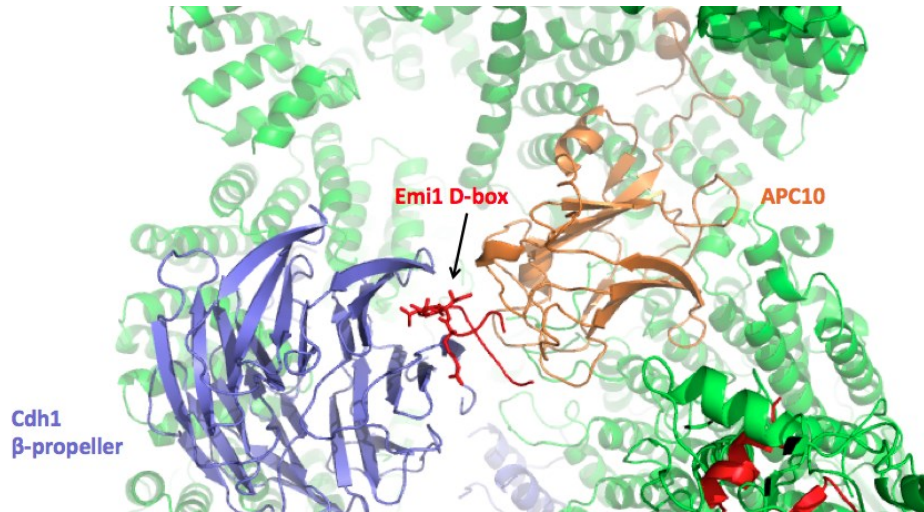


Figure 5-1. Emi1 D-box Is Co-recruited to Cdh1 and APC10.

Analysis of APC/C-Cdh1-Emi1 structural model PDB: 4UI9 from the Barford lab. Cdh1 is colored purple, Emi1 is red, and APC10 is orange, all other components of the model are green. Emi1 is co-recruited to Cdh1 and APC10 in the same way substrates have been shown to bind.

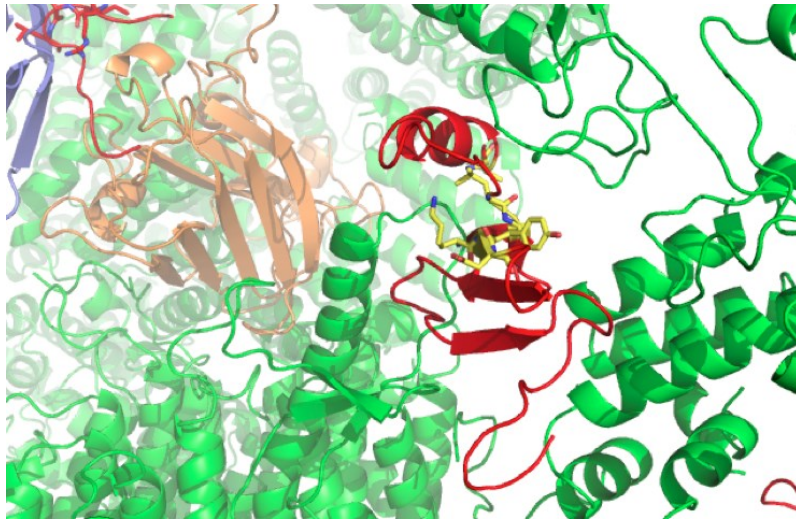


Figure 5-2. Emil ZBR Associates with APC11 and Utilizes the Surface Discovered in the ZBR Alanine Scan, and the Functional Linker Forms a Helix Between APC11 and APC1

Emil, red, is represented as a cartoon with “very deficient” mutants colored in yellow. These mutations map to a surface that helps to organize the helical, essential Linker domain, and may bind directly to APC/C.

Functional Linker Domain Between the D-box and ZBR Is Visualized and Localizes to APC1 and APC11

In the electron density maps, there is evidence for a structured region of Emi1 that protrudes from the N-terminus of the folded ZBR domain. The Linker domain of Emi1 is perfectly suited to fill this density, as it is directly N-terminal of the ZBR and has been shown through mutational analysis to be essential for function. Though it is difficult to be certain of the register of the helix shown in the maps due to lack of side-chain density at the achieved resolution, the published model for Emi1 in this region places residues Y356 and R358 in positions for contact with APC1 and APC11. Since mutation of these residues, and not deletion of 10 residues directly N-terminal of them, causes a defect in Emi1's APC/C inhibition, the model likely confirms our mutational analysis of this region.

Emi1 Tail Binds APC/C at the Base of the Platform and Inhibits Ube2s Tail

Finally, a small helical portion of Emi1's Tail is visualized binding to the base of the APC/C platform region, and makes contacts specifically with APC2 and APC4. Importantly, the published manuscript also visualizes the C-terminal tail of Ube2s binding to this same region in a moderate-resolution structure of a Ubch10-Ube2s fusion protein. Though the model suggested by the authors does not place the perfectly conserved and critical LRRL tail of these proteins in the density, it does account for Emi1's ability to specifically inhibit the activity of Ube2s in our assays. Furthermore, the overall orientation of the entirety of Emi1 is exactly as we report it to be, with the D-box bound to the coactivator Cdh1 at the top of the catalytic cavity, the ZBR interfering with catalytic machinery in the peripheral center of the cavity, and the Emi1 C-terminal tail associating near the base of the platform (**Figure 5-3**).

Retrospective Analysis of BPA Incorporation Methodologies

A combination of two methodologies for incorporation of BPA to target proteins was presented in this dissertation. Amber suppression technology has been developed for more than 10 years and the newest systems are easy to use and are capable of generating milligrams of pure BPA-containing protein per liter of culture. Though this technique has limitations and drawbacks, I would suggest that amber suppression for *in vivo* incorporation of a wide variety of unnatural amino acids is the more robust system for a number of reasons. By scaling up the volume of bacterial culture, it is quite easy to overcome the problem of inefficient incorporation and reduced expression. Furthermore, these problems can likely be overcome by shifting the position of the amber stop codon to residues surrounding the residue of interest as this seems to have a random but strong effect on both protein expression and UAA incorporation. Typically genes of interest are expressed with C-terminal affinity tags, allowing for immediate separation of prematurely truncated proteins, and this makes amber suppression a powerful and simple technique for scanning through several positions of a protein to isolate conditions that

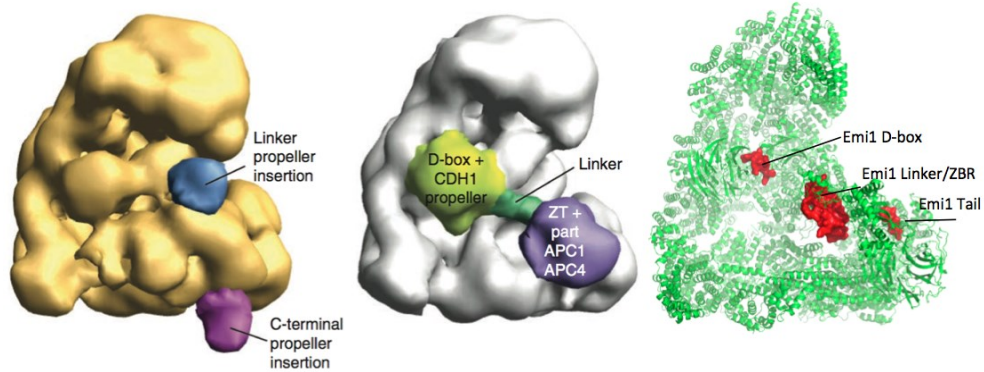


Figure 5-3. Emi1 Traverses the APC/C Architecture as Visualized by Negative Stain EM Experiments.

Left, Negative stain reconstructions of APC/C-Emi1 with Doa1 propeller insertions, compared with Emi1-DLZT lacking insertions, **Middle**, allowed us to visualize the overall topology of APC/C-Cdh1-Emi1. This is consistent with Emi1 spanning the catalytic core as in PDB ID 4UI9, **Right**.

enable site-specific crosslinking and inform on the biology of protein-protein interactions.

Both Emi1 and Ube2s have essential C-terminal tail residues that must be preserved and therefore these proteins cannot tolerate affinity tags at the C-terminus. As explained previously, this makes *in vivo* incorporation of BPA to residues near the C-terminus impossible as the incorporation defects are unable to be isolated from full-length proteins. To circumvent this issue, we established a method that allows for BPA incorporation to a C-terminal peptide, which is subsequently ligated to the protein through sortase-mediated transpeptidation. The unmodified protein is then smaller than the modified protein by an entire ~20-40 residue peptide, even in cases where the UAA is at positions near the extreme C-terminus, and this allows for purification by size, or in our case by ion exchange chromatography mediated by the highly basic C-terminal tail.

The second method, although essential for overcoming our specific problem, has a number of drawbacks that make it an impractical method for large-scale use. Firstly, the final yield of protein is limited by the initial yield of peptide. By pre-incorporating BPA via Fmoc chemistry, the final quantity of peptide represents the upper limit for peptide-incorporated protein. In fact, for C-terminal labeling, the best sortase reaction efficiency is achieved by having the peptide in vast excess over protein, and this makes the yields of full-length protein quite limiting, on the order of 50µg to 500µg, depending on the scale of the enzymatic reaction and the success of the purification strategy. Purification from the enzyme following the reaction is also critical, as any contaminating sortase enzyme can readily cleave the product and remove the BPA-peptide. For this, we purify our 1mL reaction mixtures over gradient NaCl IEX, followed by SEC, a strategy which further decreases the overall yield. A variety of other methodologies, including self-splicing C-terminal inteins (Batjargal, Walters et al. 2015) and cell strains that lack the ability to truncate (Lajoie, Rovner et al. 2013) have been developed recently, and are likely a more practical and useful method for overcoming this problem.

Multisite Binding as a General Mechanism of Inhibition

Multivalency Is Common in Biology

We discover here a multisite, avid mechanism of APC/C inhibition by Emi1. Emi1's exhibition of multivalent inhibition is not unique; rather it is representative of an entire class of inhibitors, both natural and designed. Even beyond inhibition, multivalency mediates many biological and synthetic interactions, and investigation into the properties of other multivalent interactions of both the protein and drug-design frameworks helps to better place Emi1's functionalities and critical role among a well-established mechanism of biological function.

Multivalent interactions can be loosely defined as cooperative interactions of multiple connected binding groups during a single binding process. An oversimplified

example is Velcro, in which many very weak hook-fiber interactions are connected on a single surface and work together to create a stronger interaction. This definition necessitates both a multisite receptor (APC/C in our case) and a multisite ligand (Emi1 in our case), as a large number of ligands with only one site of interaction for the receptor do not exhibit cooperativity as a connected binding group. Early studies of these types of phenomenon derived from characterization of interactions in inorganic chemistry including chelation and other metallic properties, and only later were similar cooperative binding effects discovered occurring in organic molecules and subsequently throughout biology. It is now known that multivalent interactions occur throughout the protein interactome, with extensive characterization of lectin interactions with carbohydrates and sugars (Lepenies, Lee et al. 2013, Wittmann and Pieters 2013, Visini, Jin et al. 2015), and toxin interactions with cell-surface receptors (Wolfhagen, Torensma et al. 1994, Nishikawa 2003). Other, more simple 2-site interactions such as a single p27 protein binding both to Skp2 and Cks1 (Hao, Zheng et al. 2005), Cand1 inhibition of Cull1-Roc1 (Goldenberg, Cascio et al. 2004), Cbl proteins as multivalent adapters for tyrosine kinase functions (Tsygankov, Teckchandani et al. 2001, Swaminathan and Tsygankov 2006) exhibit the same principle of multi-site interactions, and recently, useful tools such as structural databases (Meyer and Knapp 2014) have been developed to aid in the molecular understanding of these natural interactions.

One such biological interaction is observed and found to be essential at the telomeres of DNA by multivalent association of the proteins Rif1 and Rif2 with Rap1. These proteins bind to the telomeric DNA repeats and form a sterically occlusive cap called a telosome that inhibits telomerase cleavage and transcriptional silencing. A recent study aims to characterize how single or multivalent interactions between Rif1 and Rap1 as well as Rif2 and Rap1 lead to higher-order associations with DNA, considering only Rap1 is believed to bind DNA (Leuthauser, Thomas et al. 1992, Shi, Bunker et al. 2013). Two Rif1 sites are capable of binding two different, distant Rap1 sites or molecules, which can simultaneously be bound by another Rif1 or Rif2 molecule. In this case, multivalency seems to be less important for avidity affects leading to high affinity interactions, and more important for establishing a network of linked interactions which, through multiple binding sites for both Rif1 and Rif2 at distinct locations within Rap1, enables the dissociation of each of these single protein-protein interactions to be overcome by having multiple association events. From this perspective, Emi1 multivalency can also be characterized as a series of connected binding events. Not only do the multiple binding sites of Emi1 each contribute to the combined kinetics of association with APC/C, but they also lessen the overall dissociation constant, increasing the stability of the APC/C-Emi1 complex. For instance, temporary dissociation of the relatively weak D-box is overcome by association kinetics of the Linker, ZBR, and Tail regions of Emi1, such that Emi1 does not wholly dissociate, but self-stabilizes on the APC/C. This may account for the ability to maintain stoichiometric complex throughout the relatively long purification of APC/C-Emi1 complexes for EM, including an overnight gradient centrifugation step *in vitro*, and throughout interphase of the cell-cycle *in vivo*.

Perhaps more importantly, though, are the applications of this natural phenomenon to the rational design of synthetic inhibitors and multivalent drug-like molecules for treatment of a wide range of human pathologies. Indeed, both targeted development of specific systems, such as trimeric PCNA binding peptides (Bozza, Yang et al. 2012), selectivity of inhibitory compounds of the BCL-2 family (Bruncko, Wang et al. 2015), and hundreds of other structure guided drug design efforts, as well as broadly developed biotechnologies like multivalent architectural scaffolds and nanostructures (Li, Choi et al. 2014) have enabled significant improvements in therapeutic discoveries in recent years. Rational drug design efforts typically utilize known structural information of both the target and the inhibitor to intelligently enhance drug potency or specificity. This enables biochemists to build larger or more kinetically productive inhibitors, or to specifically target only certain proteins or complexes within structurally or functionally similar families.

Analogous to rational drug design, sequence-directed population studies of libraries of modified inhibitory peptides are also a powerful technique for improving known inhibitors. For example, a group of Italian researchers modify a known CamKII inhibitory peptide and established a library of similar but non-identical peptides to probe the functional consequences of their mutations (Gomez-Monterrey, Sala et al. 2013). The authors identify a 17-mer peptide with improved specificity for CamKII that is capable of penetrating cells and inhibiting the RAS/RAF/MEK/ERK cellular signaling pathway. To a certain extent, our work mimics this mutational analysis approach for peptide enhancement, such as our generation of a Super-D-box chimera, and it is possible that even milder mutations of this APC/C recruitment motif would be superior to Emi1's native D-box, like the T323I mutation observed in the Barford structure. In consideration of developing peptides or chemicals that mimic Emi1 form or function, studies like these provide an exciting glance at the possible outcomes of these works. Similarly, the structural and biochemical information we have discovered about Emi1 presented here could be developed further into more potent or selective APC/C inhibitory compounds. Through visualization of the surfaces of APC/C that are important for catalysis, and by studying the interactions that dictate Emi1 association with several functionally diverse regions of the complex, it is easy to imagine development of small molecules or peptides which would mimic these functions.

Targeting APC/C with Pharmacological Inhibitors

A variety of activating mutations have been shown to dysregulate the APC/C and lead to cancers (Penas, Ramachandran et al. 2011), and blocking mitotic exit is an effective approach for stimulating tumor cell death. For these reasons, the APC/C has very recently become an attractive target for development of pharmacological inhibitors. Only recently has sufficient biochemical and structural data been generated to enable APC/C targeted drug development, and as such, only 2 non-biological inhibitors have been established for the APC/C to date; APCin (APC inhibitor) and TAME (tosyl-arginine-methyl-ester) (Zeng and King 2012, Sackton, Dimova et al. 2014, Lub, Maes et al. 2015). APCin shares structural similarity to the RxxL D-box motif of substrates, and

a Cdc20-APC α in cocystal structure reveals that the inhibitor binds competitively to the coactivator's D-box binding site. This substrate-recruitment inhibitor causes stabilization of APC/C substrates in mitotic *Xenopus laevis* extracts, making for a promising target in APC/C regulation. TAME mimics an essential C-terminal IR tail of Cdc20, Cdh1, and APC10. Each of these proteins is coordinated by an IR-tail binding groove within APC3 (Yamaguchi, Yu et al. 2015). Both APC α in and TAME inhibit the APC/C at distinct sites, and their combination treatment yields a synergistic effect in *Xenopus* extracts (Sackton, Dimova et al. 2014).

These pharmacological inhibitors have been developed to mimic and therefore block the biological activation of APC/C and have established therapeutic potential by stimulating cell death of multiple myeloma cells. Our biochemical and structural characterization of the biological inhibitor, Emi1, reveals novel functionalities within APC/C that are ideal drug targets. Much like the C-terminal IR-tail interaction of the coactivator proteins with APC3, our conserved C-terminal RL tail of Emi1 is absolutely essential both for its function as an inhibitor, and for Ube2s's function as an E2. Evolutionary pressure has already developed a regulated inhibitor of Ube2s recruitment, and this site is likely an ideal target for small molecule mimic of an RL tail. Our studies with Emi1 also reveal an unfortunate truth: much remains unknown about the true mechanisms of action of this critically important cell cycle regulator. In our process of isolating the function of a very small inhibitory subdomain, we discovered 4 functional elements blocking 3 critical functionalities on a single face of our 1,200 kDa complex. It is quite possible that further regulation of this complex is yet to be discovered, and that a number of critical protein-protein interaction sites have not been visualized and will be viable targets for future drug discovery as well. Indeed, all of the Emi1 data generated here has highlighted the necessity of future studies of this complex and its workings, and provides a framework for piecing together the complex networks of interactions that regulate and activate the APC/C's function.

MCC Inhibition of APC/C

Utilizing what we have learned about APC/C activity from our studies with Emi1 and from recent high-resolution information, we can further analyze the mechanism of the other inhibitors, including the other major biological APC/C inhibitor, the Mitotic Checkpoint Complex. Although limited by the low resolution of current human APC/C-MCC structures, it seems likely that the MCC blocks the substrate recruitment mechanism of APC/C based on structures observed in crystals and EM (Herzog, Primorac et al. 2009, Chao, Kulkarni et al. 2012). What is unclear is whether the MCC is capable of inhibiting one or both of the APC/C specific E2s, Ubch10 and Ube2s. By directly contacting and covering much of the catalytic core, it is plausible that Ubch10 is precluded from its canonical APC/C binding sites (Brown, VanderLinden et al. 2015), but it is also conceivable that Emi1 potentially inhibits Ube2s recruitment where MCC inhibits only Ubch10 recruitment. This could provide a means for regulation of E2-specific ubiquitination activities, as it is known that the extent and topology of

ubiquitination regulates the fate of the target, from degradative targeting to signaling and other outcomes.

Cand1 Is Both a Competitor and Activator of CRLs.

Canonical CRL complexes are engaged by another protein, CAND1, which, like Emi1, was long considered to be a general inhibitor. CAND1 associates with Cullins blocking their activation by Nedd8ylation, and obstructing Skp1 association with F-box proteins. Despite structural evidence supporting competitive inhibition of SCF, and *in vitro* assays characterizing the effect, many *in vivo* experiments suggest CAND1-dependent activation of SCF activity in cells. This paradox persisted for several years until recent experiments discover a cryptic mechanism that supports both *in vitro* inhibition and *in vivo* activation of SCF by CAND1. In this case, the answer comes from binding kinetics: CAND1 drastically increases the dissociation of F-box proteins from Cullin, but has no effect on F-box association constants. By effectively removing a stably bound accessory protein, CAND1 serves as an exchange factor to enable Cullin protein assembly with the hundreds of known adaptor complexes.

Analysis of the multisite, multifunctional binding of CAND1 allows speculation about a general model of SCF assembly and activation in a broader, cellular context: Perhaps, since CAND1 is unable to bind to Nedd8-modified Cullin proteins, a novel mechanism is employed by SCF to degrade large pools of a subset of substrates, and then Cullin returns to promiscuous association with F-box proteins to sample a variety of new targets. In this model, a CRL encounters a substrate, neddylation activates the CRL, and CAND1 association is not possible. The absence of the CAND1 exchange factor allows specific, processive destruction of substrates recruited only by the currently associated F-box protein. In time, deneddylases remove Nedd8, and CAND1 association allows Cullin to collaborate with new F-box proteins, targeting new substrate pools. This model couples CRL activity with substrate availability, F-box protein expression and localization, and the competitive interplay between neddylation and CAND1 association, providing ample means of regulatory control by cellular cues.

Could Emi1's Multisite Binding Provide More Than an Inhibitory Role?

Due to the cryptic, multisite CAND1 activation mechanism, this exchange factor was mistaken as a competitive inhibitor of CRLs. Emi1 also employs a similar multisite association with the many functional domains within APC/C, which begs the question: is Emi1 doing more than APC/C association and inhibition? Kinetic studies of Emi1 as performed by a postdoc in the lab, Nick Brown, in conjunction with this work suggest that the multisite binding of Emi1 creates an overall affinity so tight that it cannot be measured in our assays, likely in the picomolar range. This distinguishes Emi1's constants for dissociation and association from those of Cand1, suggesting that it is not an exchange factor by the same mechanism. It is also possible that by associating with a variety of distant parts of the APC/C, that Emi1 could provide a means of stabilization of

the multi-protein APC/C. Indeed, Emi1 lowers the inherent flexibility of the APC/C in our EM studies and therefore allows for higher resolution structural information to be obtained.

What Is the Rest of Emi1 for?

Our characterization of Emi1 function paired with recent structural information only accounts for the C-terminal 1/3 of Emi1's sequence. Though a few residues within the N-terminal sequence of Emi1 have been shown to be responsible for Emi1's own regulation, it is not clear that this is the only function of this part of the protein. Interestingly, Emi1 contains an F-box that is benign for APC/C inhibition, but may serve other roles in the cell. F-box proteins are well-established functional partners the CRL family of ligases, and Emi1's F-box binds with high affinity to Skp1, another major Cullin-associating protein. As of yet a functional relationship between Emi1's F-box and CRL activity have not been established, but beyond Emi1's original discovery based on Skp1 association, the F-box has not been studied extensively. There may be cell-type specific, or stress response related functions of this domain that are yet to be understood.

Furthermore, residues besides the well-characterized phosphodegrons have been shown to be post-translationally modified with phosphorylation by the action of Cyclin-dependent-kinases (Moshe, Bar-On et al. 2011). This modification of Emi1's C-terminus renders it insufficient at inhibiting the APC/C, providing a Plk1-mediated degradation independent manner of Emi1 regulation. A further six phosphorylation sites in the N-terminus of Emi1 have been discovered, but no kinase or functional outcome of phosphorylation has been tested. It is plausible that environment-specific modification of Emi1 by a variety of factors represents a tunable control of Emi1 regulation, and therefore regulation of APC/C E3 ligase activity and regulation of cellular signaling pathways in which APC/C substrates are involved.

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VITA

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Publications

*Brown, N.G., *R. VanderLinden, ***E. R. Watson**, et.al, in review.

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* denotes equal author contribution