Structural and Biochemical Analysis of Cullin-based Ubiquitin Ligases Reveal Regulatory Mechanisms of Ubiquitination Machinery

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Abstract

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Ubiquitin-dependent proteolysis controls protein stability and plays an important regulatory role in a broad spectrum of biological processes. At the core of the ubiquitination process are the ubiquitin ligases (UBL) that function as the central component of the ubiquitination pathway by not only catalyzing the final transfer of ubiquitin from an ubiquitin conjugating enzyme to the substrate, but also governing the specificity of the modification reaction. One class of UBLs is the multi-subunit ubiquitin ligase Skp1-Fbox-Cul1-Roc1 (SCF). The exact mechanism of assembly this complex has not been well understood until recently when Cand1 was identified. Cand1 is a 120-kDa HEAT repeat protein that forms a tight complex with the Cul1-Roc1 SCF catalytic core, inhibiting the assembly of the multi-subunit ligase. The mechanism by which this is done was unknown. Therefore, I solved the crystal structure of the Cand1-Cul1-Roc1 complex, which shows Cand1 adopting a curved superhelical structure, clamping around

the elongated SCF scaffold protein Cul1. This structure showed that Cand1 inserts a β-hairpin into an adaptor-binding site on Cul1, inhibiting its interactions with the Skp1protein. Simultaneously Cand1 buries a lysine residue on Cul1, whose modification by the ubiquitin-like protein, Nedd8, is able to block Cand1-Cul1 association. Together with biochemical evidence, these structural results elucidate the mechanisms by which Cand1 and Nedd8 regulate the assembly-disassembly cycles of SCF and other cullin-based E3 complexes. In the presence of Cand1 Cul1 cannot be neddylated. However, I determined that neddylation could occur if Skp1 and an F-box protein are present. Together these structural and biochemical details added greater detail to our understanding of SCF regulation.

Finally, I performed biochemical analysis on Cul3 based ubiquitination machinery. The Kelch-like ECH-associated protein 1 (Keap1) BTB-domain containing protein binds to Cul3 and to its substrate (Nrf2) simultaneously, serving comparative roles to Skp1-Fbox proteins concurrently. A fully functional Nrf2 ubiquitination system was achieved using all recombinant proteins. The Cul3 system was also used to identify pathways for human Dishevelled3 ubiquitination.

TABLE OF CONTENTS

List of Figures

Figure Number

Page

List of Tables

Table Number

Page

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Dedication

For Tara

Chapter 1

Introduction to the ubiquitination pathway, fate of ubiquitin-conjugates, and the role ubiquitination plays in the cell

Mechanism of Ubiquitin and UBL conjugation.

Overview

The well-conserved 76-residue polypeptide ubiquitin plays indispensable roles in eukaryotes through its covalent attachment to other intracellular proteins. Substrates marked with a polyubiquitin chain are selectively targeted to the 26 proteasome. Substrates labeled with one or a few ubiquitins are targeted for endocytosis, many of which are finally transported to the lysosome for proteolysis. Ubiquitination regulates critical cellular functions through degradation of regulatory proteins by proteasomes. Loss of control over the ubiquitin-dependent proteolysis pathway has been shown as the cause in several cancers and numerous other diseases (reviewed in Sakamoto, 2002; Yamasaki & Pagano, 2004). Ubiquitination can also affect cell processes without destroying proteins. These processes include ribosomal function (Spence *et al*, 2000), the initiation of the inflammatory response (Karin & Ben-Neriah, 2000), and the function of certain transcription factors (Conaway *et al*, 2002; Schwechheimer & Villalobos, 2004; Dhananjayan *et al*, 2005).

The ubiquitination process results in the establishment of a covalent bond between the C-terminus of ubiquitin (G76) and the ε-amino group of a substrate lysine residue. This reaction requires the sequential actions of three enzymes (reviewed in Pickart, 2001). It begins with an activating enzyme (UAE) creating a thiol ester with the carboxyl group of G76 on ubiquitin that activates the C terminus of ubiquitin for nucleophilic attack. At this point a conjugating enzyme (UBC) transitorily carries the activated ubiquitin molecule as a thiol ester to the ligase (UBL). The UBL then aids in transfer of the activated ubiquitin from the UBC to the substrate (or a previously attached ubiquitin) lysine residue through direct interaction with the ubiquitin or by serving as a scaffold bringing all components together (Figure 1.1). This process occurs not only for ubiquitin but ubiquitin like protein (UBL; Nedd8, Sumo, ISG15, etc) reactions, independent of whether the substrate-bound ubiquitin(s) or UBL will signal proteasomal proteolysis, endocytosis, or some other fate.

Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), each which can form an isopeptide bond with the C-terminal glycine of the next ubiquitin moiety in the polyubiquitin chain (Kim & Rao, 2006). The most common form of ubiquitin chain is the K48-type chain and both it and K29-linked chains signal degradation in proteasomes. However, K11- and K63-linked chains are relevant for DNA repair, endocytosis, and dimerization.

Ubiquitin-Activating Enzyme (UAE)

In most organisms a single UAE enzyme activates ubiquitin for all of the downstream reactions. The UAE enzymes are extremely efficient and allow the production of enough activated ubiquitin for the all of downstream conjugation reactions while having a comparable concentration to the other enzymes. The chemistry of the UAE reaction is well understood from rabbit reticulocyte lysate studies (Haas & Rose, 1982a; Haas *et al*, 1982b). The reaction is initiated by the binding of Mg-ATP to the UAE first and then ubiquitin, this leads to the formation of an ubiquitin-adenylate intermediate that serves as the donor of ubiquitin to a cysteine in the UAE active site.

Thus, each UAE carries two molecules of activated ubiquitin—one as a thiol ester, the other as an adenylate. The thiol-linked ubiquitin is transferred to the UBC.

This UAE-UBC interaction has been revealed in a homologous crystal structure of the Nedd8-UAE (APPB1-UAE3) and the Nedd8-UBC (Ubc12) (Huang *et al*, 2005). The structure and associated mutational analyses reveal molecular details of Ubc12 recruitment by NEDD8's UAE. The binding domain on the UAE for Ubc12 binding domain resembles ubiquitin and recruits Ubc12 in a manner similar to ubiquitin's. In a comparison with UBC-UBL the UAE and UBL binding sites on Ubc12 may overlap, indicating that some crosstalk may occur.

Ubiquitin-Conjugating Enzyme (UBC)

All UBCs share a conserved core domain consisting of ~150 amino acids. However, the specificity of function lies in unique UBC-UBL interactions. The UBC protein family is structurally well characterized (Haldeman *et al*, 1997; Huang *et al*, 1999; Zheng *et al*, 2000) with many of the most highly conserved UBC residues surrounding the active site cysteine. Free UBCs bind tightly to the loaded UAE molecule but weakly to free ubiquitin and free UAE; however, the free UBCs bind strongly to their cognate UBLs and UBCs with active site mutations can act as dominant negatives in vivo. It should be noted that UBCs active sites do not contain a general base that would be expected to deprotonate the thiol group side chains (Hein & André, 1997; Lin *et al*, 2002). This deprotonation would be required to catalyze ubiquitin transfer either from UAE to UBC, or from UBC to a substrate lysine, but there is no candidate for a general base positioned within 6 Å of the UBC active site thiol. While there is potential for the

UBL binding to reposition residues in the UBC active site this is not seen in any UBC-UBL crystal structures (Huang *et al*, 1999; Zheng *et al*, 2000).

Ubiquitin-Protein Ligase (UBL)

All known UBLs feature one of two structural elements. The first domain identified is known as the HECT domain for its *H*omology to the *E*6-associated protein *C*-*T*erminus and was revealed through studies on degradation of the p53 tumor suppressor (Scheffner *et al*, 1993; Huibregtse *et al*, 1993). It was shown that p53 is selectively degraded via the ubiquitin-proteasome pathway when cells are infected with human papillomaviruses (HPVs). This degradation required an HPV-E6 gene product and a 100kD associated protein E6-associated protein. The C-terminus was crucial for p53 ubiquitination and was highly homologous to many other proteins. This region now defines what is now known as the HECT domain (homologous to E6-AP carboxy terminus). Another interesting feature of the HECT domain is a conserved cysteine that is required for substrate ubiquitination as it forms a thiol ester formation with ubiquitin (Scheffner *et al*, 1995). While the C-terminus is required for ubiquitin transfer the N-terminus is required for substrate binding, all HECT-domain containing UBL's work by a similar mechanism.

The crystal structure of E6-AP was a key addition towards understanding of HECT UBL-Ligases (Huang *et al*, 1999). However, in this structure the N-lobe that binds the UBC enzyme is 40 Å away from the C-lobe that contains the active site cysteine residue. A more recent crystal structure of another HECT UBL ligase, WWP1/AIP5 was solved with the N-lobe in a completely different position than the first structure and brings the active site cysteines, assuming the UBC binds the same way with both ligases, to only 16 Å apart (Verdecia *et al*, 2003). While this is not close enough for transfer to occur it does represent the flexibility that is possible within the HECT UBL ligases and produces a model where ubiquitin transfer between UBC-UBL-substrate is possible (reviewed in Zheng, 2003). Another class of UBL ubiquitin ligases are called RING ubiquitin ligases and are discussed in detail below.

Sorting and Degradation of Ubiquitinated Proteins

Within the cytosol and nucleus of all eukaryotic cells the 2.5-MDa 26S proteasome catalyses the degradation of multi-ubiquitin conjugated proteins in an ATP-dependent manner (reviewed in Hochstrasser, 1995; Pickart, 1997; Ciechanover, 1998). This multi-subunit complex contains a proteolytic active 20S core complex and a 19S regulatory complex that is attached to one or both ends of the 20S cylinder. The base sub-complex contains six ATPase subunits that are required for the substrate-unfolding step of the degradation pathway, without this step the substrates cannot enter the 20S core for degradation (Elder *et al*, 2002; Ortolan *et al*, 2000; Layfield *et al*, 2001).

Ubiquitination can also play a role in internalization and endocytosis of receptors, including several receptor tyrosine kinases and sodium channels (Hicke, 1997; Hershko & Ciechanover, 1998). In contrast to proteasome degradation single ubiquitin moiety attachment is required for this type of substrate regulation. Ub-conjugates are sorted from the cell surface to different cell compartments by Ub-receptors that are compartmentalized along the endocytic pathway, which function as specific checkpoints for ubiquitinated cargo at different steps in the endocytic route (Levkowitz *et al*, 1998). An example of these receptors are Eps15 and epsins that act as plasma membrane Ub-receptors that bring together the mono-ubiquitin-containing RTKs with the machinery of the clathrin-coated pits (van Delft *et al*, 1997; Wendland & Emr, 1998; Klapisz *et al*, 2002). They bind directly to mono-ubiquitin that is attached to receptors via their UIMs and at the same time interact with the clathrin adaptor protein AP-2 through specific motifs. In addition to a single ubiquitin being attached, multiple mono-ubiquitination with each ubiquitin attached onto a unique substrate lysine residue, but not formation of ubiquitin chains, is possible (Miyake *et al*, 1998). While this is only one example of how ubiquitin can lead to the sorting of proteins there a many others (reviewed in Urbé, 2005 and Umebayashi, 2003).

Ubiquitin-dependent proteolysis regulates diverse biological functions.

Poly-ubiquitination, mono-ubiquitination, and ubiquitin like protein modifications regulate a diverse array of biological functions. Movement through the eukaryotic cell cycle is driven by changes in the activities of cyclin-dependent kinases (Cdks) (reviewed in Murray, 1995; Wolf & Jackson, 1998; Peters, 2005; Nakayama & Nakayama, 2006). Cdk action is controlled by synthesis and degradation of both positive regulators and cyclin kinase inhibitors (Ckis). There are different cyclins for specific phases of the cell cycle (G1, S-, or M-phases) that accumulate and activate Cdks at the appropriate times during the cell cycle and then are degraded, causing kinase inactivation. A similar effect is also seen for Ckis, which specifically inhibit certain cyclin/Cdk complexes. Specific regulation of cyclins, Ckis, and other cell-cycle regulators by the ubiquitin machinery play important roles in cell-cycle control.

The activity of several transcription factors, tumor suppressors, and oncoproteins are controlled by proteolysis via the ubiquitin pathway. NF-κB (nuclear factor κB) (reviewed in Blackwell & Christman, 1997), the tumor suppressor protein p53, (Meek, 2004; Yang *et al*, 2004; Brooks & Gu, 2006), c-JUN, β-catenin, and UBCF-1 are all regulated by ubiquitination and proteasome degradation (reviewed in Wilkinson, 1999; Yamasaki & Pagano, 2004). The ubiquitin pathway also regulates cytosolic and nuclear proteins. However, ubiquitination also affects membrane proteins at the ER, due to mis-folding, and for endocytosis once at the cell surface (Hershko & Ciechanover, 1998; Sigismund *et al*, 2004; Urbé, 2005). While most cell surface receptors appear to be targeted to the lysosome/vacuole, several cases have been reported in which ubiquitinylation targets membrane proteins to the proteasome. For example, the PDGFR is ubiquitinylated following ligand binding and is degraded by a process inhibited by proteasome inhibitors (Mori *et al*, 1992; Mori *et al*, 1993; Mori *et al*, 1995). ER degradation represents another type of ubiquitin-mediated degradation of membrane proteins, distinct from that of mature cell surface proteins. The ER is the site of folding and modification of nascent chains and of assembly of multisubunit complexes (Sommer & Wolf, 1997; Lord *et al*, 2005; Meusser *et al*, 2005). Therefore, it must be able to edit proteins that fail to fold properly or to oligomerize due to aberrant folding, mutation or excess subunits of large complexes. The Cystic fibrosis transmembrane conductance regulator (CFTR) is synthesized as an approximately 140-kDa glycosylated protein that is associated initially with the cytosolic chaperones Hsc70 and then with the ER chaperone calnexin (Ward *et al*, 1995; Xiong *et al*, 1999). Only 25–50% of the wild-type protein matures to the cell surface, whereas most of the protein molecules do not fold properly and are degraded in the ER. A single mutation at Phe508 is the underlying cause of most cases of cystic fibrosis, as it results in the protein never being released from the chaperone and therefore becomes degraded by the ubiquitin proteasome pathway (Fuller & Cuthbert, 2000).

Cullin-based ubiquitin ligases represent a super-family of multi-subunit RING UBL machinery.

Another class of UBL called the RING (Really Interesting New Gene) finger is defined by a group of histidine and cysteine residues, which allow for the coordination of two zinc ions, and is based on an alpha helix that protrudes like a finger from the complex (Deshaies, 1999; Borden, 2000). There are numerous RING finger proteins identified by sequence and while there is homology of sequence it is not this that is important for RING finger activity, but the spacing of the zincs. It is not clear if all RING finger proteins act as UBLs.

RING UBLs come in two varieties, the first of which is a single-subunit UBL, which is best characterized by the RING finger protein c-Cbl (Sawasdikosol *et al*, 2000; Duan *et al*, 2004; Thien & Langdon, 2005; Swaminathan & Tsygankov, 2006). c-Cbl is involved in the ubiquitination of numerous proteins including the epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) receptor. c-Cbl recognition of its substrates requires the N-terminal tyrosine kinase-binding (TKB) domain (Peschard *et al*, 2001). The structural details of c-Cbl were revealed in the crystal structure of the c-Cbl–UbcH7 complex that also explains many of the UBC-binding properties (Zheng *et al*, 2000). The crystal structure showed that many of the UbcH7 residues that contact the RING are the same ones that contact the E6-AP HECT domain. The RING-UBC interaction seen in this structure is probably similar for a number of RING UBL-UBC pairs, certain UBCs must have additional modes of interaction to dictate specific UBC-UBL interactions and allow formation of different types of ubiquitin chains, such as linking of ubiquitin chains via K48 or K63.

A main function for all RING finger proteins is their functioning as scaffolds, which is supported by the structure of the c-Cbl–UbcH7 complex. The structure implicates that the proximity of the TKB-bound substrate and the linker/RING-bound UBC is the key feature promoting ubiquitination. The structure provides no evidence that binding to the RING causes a conformational change that activates the UBC by putting a base near the active site, thus how the isopeptide bond forms remains unclear. However, the crystallized protein was not full-length and it is possible that additional interactions are present, but were not seen in the structure.

RING finger proteins can also be subunits of large complexes that regulate ubiquitination. Three types of multi-subunit UBLs are now that contain a RING finger protein as an essential component: SCF UBLs (Skp1-Cullin-F-box protein), the APC, and the VCB UBL(s) (Peters, 1998). The 100-residue RING finger protein known as (ROC1/Apc11) plays a key organizational role in each complex. ROC1 interacts strongly with a subunit belonging to the cullin protein family (Cul1 in the SCF UBLs, Cul2 in VCB) and concurrently bind the UBC to form a functional ubiquitin system (Kamura *et al*, 1999b). The APC also contains a RING finger protein, Apc11, among its many subunits (Page & Hieter, 1999). Disruption of either RING finger, ROC1 or Apc11, severely inhibits the respective ubiquitination reactions in vitro (Chen *et al*, 1998; Gmachl *et al*, 2000).

Substrate recognition is delegated to a separate subunit in the multisubunit RING UBLs. In the SCF UBLs, a variable substrate-specific family, F-box proteins, are recruited to SCF complexes through the invariable adaptor protein Skp1, which recognizes the F-box motif (Skowyra *et al*, 1997; Feldman *et al*, 1997). The substrate-recognition subunit of VCB is pVHL (product of the Von Hippel–Lindau tumor suppressor gene). pVHL is recruited to the complex through interactions with the heterodimeric adaptor Elongin B/C (Stebbins *et al*, 1999).

The SCF UBLs and VCB are both regulated via modification with an ubiquitin-like protein, Nedd8/Rub1 (Liakopoulos *et al*, 1998; Read *et al*, 2000). Nedd8/Rub1 is conjugated to a specific lysine in the respective cullin subunits of each type of UBL. This modification requires active ROC1, while Nedd8 has its own UAE and UBC (Kamura *et al*, 1999a). Cullin modification by Nedd8 strongly stimulates, or is required for, substrate polyubiquitination by the respective holo-UBLs. The Cullin-based ubiquitin ligases are discussed in more detail below.

Architecture of Cullin-ligases

The cullin and cullin-like-family share a conserved Cullin Homology domain amongst the five human cullins (Cul1, Cul2, Cul3, Cul4A/Cul4B, and Cul5) and three cullin-like proteins (Apc2, Cul7, and Parc) that have been identified (reviewed in Jackson & Eldridge, 2002; Cardozo & Pagano, 2004). All of the proteins in the cullin super-family bind a RING domain protein, ROC1, and together form the core of a scaffold that creates multi-subunit RING UBL ligases. The remainder of the scaffold is made up of adaptor proteins that bind a large variety of substrates and allow the regulation of an extensive variety of cellular functions (Figure 1.2).

As outlined above Cullin-ligases adopt elongated structures with the highly conserved C-terminus binding Roc1 while the N-terminus binds substrate-adaptor proteins. All cullins are believed to share this arrangement. Cul1 binds to Skp1, which then in turn binds to many F-box proteins. Cul2 and Cul5 recruit substrates through Elongin B/C adaptor. Cul3 binds to BTB-domain containing proteins as its adaptor and Cul4A is believed to recruit substrates through its adaptor DDB1.

Despite the diversity each Cullin has the ability to target numerous substrates by forming unique ligases, but all with the same core ubiquitination mechanism. The binding of the adaptor to the cullin core can occur by multiple proteins or a single protein. In the case of Cul1 and Cul7, Skp1 binds to the N-terminus of Cul1 and the complex then binds F-box proteins, with Skp1 making most of the interactions with the F-box proteins. In the case of Cul3 only a single protein is bound with two notable domains. The first domain is the BTB domain that has high structural homology to Skp1 and second domain contains a unique substrate-binding domain. It is the vast number of variable proteins that can bind to a common cullin-core that allow for this mechanism to regulate thousands of substrates within cell. The best known Cullin-based UBL is Cullin1.

Cullin1-based UBLs

The SCF (Skp1-Cul1-ROC1-F-box-proteins) complex is the prototype of all cullin-based multi-subunit ubiquitin ligases. The SCF ubiquitin ligase complex was identified in budding yeast as a trigger of DNA replication due to its ubiquitination of the S phase cyclin-dependent kinase inhibitor SIC1 (Skowyra *et al*, 1997; Feldman *et al*, 1997). SCF is made up of four components Skp1, Cul1, and ROC1, as well variable F-box proteins. The F-box proteins dictate substrate specificity by binding a unique substrate or set of substrates as well as putting the substrate in correct position for ubiquitination. All F-box proteins contain a F-box motif and a unique substrate-binding motif on their carboxy-terminal that is most commonly a WD-40 repeat or leucine-rich repeat (LRR) (Skowyra *et al*, 1997). The F-box motif links the F-box protein to other components of the SCF complex by binding Skp1.

Structural studies provided information about the role that Cul1-ROC1 played in SCF and showed how RING UBLs promote substrate ubiquitination. The crystal structure shows the quaternary structure of Cul1, ROC1, Skp1, and the F-box motif of Skp2 (Zheng *et al*, 2002). Cul1 serves as a scaffold holding Skp1-F-box^{Skp2} at its N-terminus while simultaneously holding the UBC binding ROC1 at its highly conserved C-terminus, consequently separating these proteins by over 100 Å. The Cul1 N-terminal domain (NTD) consists of three repeats made up by five-helices. The tip of the first N-terminal repeat binds Skp1 that in turn binds F-box proteins (Figure 1.2). The sequences in the NTD of cullin paralogues are diverse, explaining how different cullins bind to distinct adaptor and substrate-binding subunits. In contrast to the NTD, the C-terminal domain of cullins are highly conserved amongst orthologues and paralogues. It is at Lys-720, which is completely conserved in all cullin orthologues and paralogues, where neddylation occurs (Osaka *et al*, 1998; Wada *et al*, 1999; Morimoto *et al*, 2000).

ROC1 is a 108-amino acid protein that can be divided into two domains: a 54-amino acid N-terminal domain and a 67-amino acid C-terminal domain that contains the RING-H2 finger. Both domains are conserved among eukaryotic ROC1 homologues and all known proteins that contain cullin homology domains can assemble with RING-H2 proteins like ROC1 (Bordallo *et al*, 1998; Xie & Varshavsky, 1999). Mutation of conserved cysteine and histidine residues in the RING-H2 domain block the ability of ROC1 to catalyze ubiquitin ligase activity. The RING-H2 mutants still bind to Cul1, suggesting that their defect arises from impaired interaction with the UBC, most likely due to changing their ability to bind zinc ions.

F-box proteins come in many different forms. All F-box proteins share the 45-amino acid F-box domain, but are otherwise very dissimilar (Skowyra *et al*, 1997). This region frequently contains leucine rich repeats (LRR) or WD-40 repeats that form β-propellers, both of which are protein-protein interaction domains that bind with phosphorylated substrates. Numerous mutagenesis studies have confirmed that an intact

F-box is required for association with Skp1, yet the exact residues vary somewhat (Kitagawa *et al*, 1999; Nakayama *et al*, 2000). The most highly conserved residue in the F-box is a proline near the N-terminal boundary that is typically preceded by a leucine. This region is required for binding of many substrates, but not all; such as Skp2 which still binds even with the proline residue being mutated. There is structural evidence that the F-box domain also forms some interactions with Cul1.

While the specificity of substrate binding to the SCF complex occurs due to the variable region of F-box proteins, another layer of control is introduced with the requirement for substrate phosphorylation (Kong & Chock, 1992; Yee *et al*, 1994; Deshaies *et al*, 1995; Chen *et al*, 1995). All known SCF substrates must first be phosphorylated upstream prior to binding their associated F-box protein, such as p27 ubiquitination by CDK2 (Montagnoli *et al*, 1999). By linking the SCF-dependent ubiquitination of substrates to the action of distinct kinases upstream of ubiquitination, it is possible to have substrate-specific degradation in response to numerous environmental or intracellular cues. This requirement for SCF substrate phosphorylation makes certain that the attention of the ubiquitination machinery is limited to a specific subset of substrate molecules.

Cullin3-based UBLs

Skp1 and ElonginC display structural similarity to the BTB-domain fold. BTB domains were originally found in the Drosophila transcription factors Bric à Brac, Tramtrack, and Broad Complex (Geyer *et al*, 2003; Xu *et al*, 2003; Stogios & Privé, 2004). All eukaryotic species express a large variety of proteins that contain a BTB domain. The structural homology between BTB proteins and Skp1 or ElonginC led to the hypothesis that BTB proteins might directly interact with cullins. This prediction has now been experimentally confirmed by several recent studies showing several BTB proteins interact with Cul3, but not with other cullins, by carrying out two-hybrid and biochemical studies. These results raise the possibility that BTB proteins may generally function as subunits of Cul3-based UBL ligases (Xu *et al*, 2003; Wilkins *et al*, 2004; Dieterle *et al*, 2005; Weber *et al*, 2005; Wang *et al*, 2006).

The first identified complex containing CUL-3 and a BTB protein was Cul3-MEL-26, which is required for the ubiquitin-dependent degradation of the microtubule-severing protein MEI-1 in early embryos of C. Elegans (Pintard *et al*, 2003b; Pintard *et al*, 2003a). Degradation of MEI-1 by the CUL-3/MEL-26 complex after meiosis is essential for the assembly of the mitotic spindle and can be reconstituted with the CUL-3/MEL-26 complex sustaining polyubiquitination of MEI-1 in vitro. These results identified a novel class of cullin-based UBL ligases containing BTB proteins. In contrast to the SCF complex, these BTB domain-containing proteins fulfill the roles of both Skp1 and the F-box proteins by binding to Cul3 and substrate simultaneously.

Another interesting feature of BTB proteins is the evidence to suggest that dimerization of substrate-specific adaptor proteins may be of functional importance for UBL-ligase activity. For example, hetero-dimers of the F-box proteins Pop1 and Pop2 are observed in vivo, and appear to be required for the degradation of Cdc18 in S. pombe (Kominami *et al*, 1998; Wolf *et al*, 1999). Similarly, it has been shown that the BTB protein MEL-26 forms dimers at least by yeast two-hybrid assay. Interestingly, the product of the mel-26(sb45) allele interacts with MEL-26 but not with MEI-1. This allele acts in a dominant-negative manner in vivo, suggesting that dimerization of BTB proteins may be important for the activity of the CUL-3 complex (Stogios *et al*, 2005).

The discovery that BTB proteins can assemble into a novel class of UBL ligases is of general importance, given the high number of BTB-containing proteins that are present in the genome of different organisms. Some of these BTB proteins are characterized and are known to be involved in a variety of biological processes, including regulation of microtubules and microfilament dynamics, transcription, and apoptosis (van den Heuvel, 2004; Pintard *et al*, 2004; Stogios & Privé, 2004). However, based on the published results, connections to ubiquitin-dependent degradation pathways can currently only be made for a few BTB proteins and it thus remains to be demonstrated how many BTB proteins indeed function in UBL-ligase complexes in vivo

Cullin-based ubiquitin ligases are regulated by Nedd8 modification and CAND1.

Nedd8 is covalently conjugated to a cullin protein through a process termed neddylation. Initially, Nedd8 forms, via its C-terminal glycine residue (Gly-76), a thiol-ester bond with a heterodimeric UAE-activating enzyme called APP-BP1/UAE3. Following an exchange reaction, Nedd8 is then linked to a conserved cysteine on the Ubc12 UBC conjugating enzyme (Tanaka *et al*, 1998). Finally, neddylation is completed by formation of an isopeptide bond, linking the carboxyl-end of Nedd8 Gly-76 to the -amino group of a conserved cullin lysine residue. Neddylation has been reconstituted in vitro with purified components and can add a Nedd8 moiety to all cullin proteins. In contrast to ubiquitin Nedd8 differs from Ub by forming principally mono-neddylated species.

Using site-directed mutagenesis, neddylation sites have been determined for human hCUL1 (K720) hCUL2 (K689) (sp)CUL3 (K729), and spCUL4 (K680) (Wada *et al*, 1999; Morimoto *et al*, 2000; Wu *et al*, 2005). These sites are within a conserved IVRIMKMR region, although the significance of the amino acids flanking the lysine residue in neddylation has yet to be determined. The X-ray crystallographic analysis of the CUL1-Skp1-F-box^{Skp2} complex provided structural information with respect to the neddylation site in K720 in CUL1 (Figure 1.2) (Zheng *et al*, 2002). It is positioned at the rim of a 'cleft' formed by conserved residues from the CUL1 WH-B helix, four-helix bundle, as well as the ROC1 RING domain. As revealed by in vitro studies, neddylation requires ROC1 as mutations disrupting the RING domain abolished the reaction (Gray *et al*, 2002; Morimoto *et al*, 2003). The Nedd8 conjugation pathway is fundamentally important for cell cycle control, embryogenesis, and survival. Depleting Nedd8 results in stabilization of SCF substrates, indicating it is crucial for functional SCF ubiquitination activity (Osaka *et al*, 2000; Read *et al*, 2000). The best example of this is stabilization of IB, p27 and HIF were stabilized in the ts41 hamster cells at high temperatures, under which condition the native Nedd8 UAE-activating enzyme was inactivated (Querido *et al*, 2001).

Nedd8 may also play a role in localizing Cul1 to the proteasome. With Nedd8 serving as a tether to hold Cul1 close to the proteasome. While in close proximity to the proteasome the SCF complex could then ubiquitinate substrates that would immediately be degraded by the proteasome. This was first hypothesized with the identification of Nub1, which was identified as a Nedd8-interacting protein (Kito *et al*, 2001). It possesses an N-terminal Ub-like domain that interacted with S5a of the 19S proteasome activator (Kamitani *et al*, 2001; Tanji *et al*, 2005). There is also over-expression evidence that Nub1 results in association of Nedd8 conjugates with GST-tagged S5a, suggesting that Nub1 may recruit Nedd8 and its conjugates to the proteasome for degradation (Kamitani *et al*, 2001; Hipp *et al*, 2004; Tanji *et al*, 2005; Tanji *et al*, 2006).

The final known role for Nedd8 arose with the discovery of p120/CAND1, which was found to selectively interact with Nedd8-free CUL1, and ROC1 (Oshikawa *et al*, 2003; Hwang *et al*, 2003; Min *et al*, 2003). Moreover, Cand1-bound Cul1 was not found associated with Skp1 and inhibited the SCF UBL ligase activity. Intriguingly, in vitro treatment of the p120/CAND1 IPs containing CUL1-ROC1 with the Nedd8 conjugation agents resulted in CUL1 modification and selective separation of neddylated CUL1 from p120/CAND1, suggesting a role for neddylation in dissociating the CUL1-p120/CAND1 complex. These observations further imply that neddylation may play a role in reversing the p120/CAND1-mediated inhibition.

It is possible to remove Nedd8 specifically through the isopeptidase activity of the Signalsome/CSN (Lyapina *et al*, 2001; Wei & Deng, 2003). CSN is strongly associated with SCF interacting proteins when SCF is isolated from cells. Addition of purified CSN to yeast extracts containing neddylated CUL1 results in removal of Nedd8 from its target, implicating a direct role for the CSN in promoting the cleavage reaction. The JAMM metalloenzyme domain within the CSN-5/Jab1 subunit of the CSN contains the deneddylase activity (Cope *et al*, 2002).

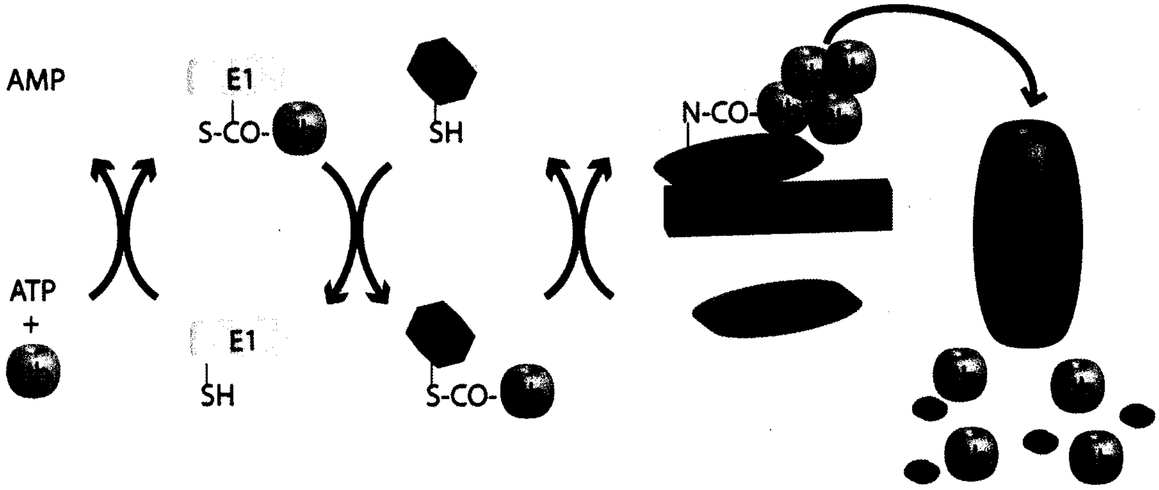

Figure 1.1. The UAE-UBC-UBL cascade in ubiquitin dependent proteolysis. The ubiquitin cascade is initiated by the E1 (UAE) that activates ubiquitin via nucleophilic attack on GLY76. It then transfer ubiquitin to the E2 (UBC) as both cysteine residue active sites on the E1 and E2 chemical identical to ubiquitin. The E2 then works together with the E3 (UBL) to attach a poly-ubiquitin chain to a substrate associated with the E3. The poly-ubiquitin chain is then identified by the proteasome and the proteasome destroys the ubiquitin-tagged substrate.

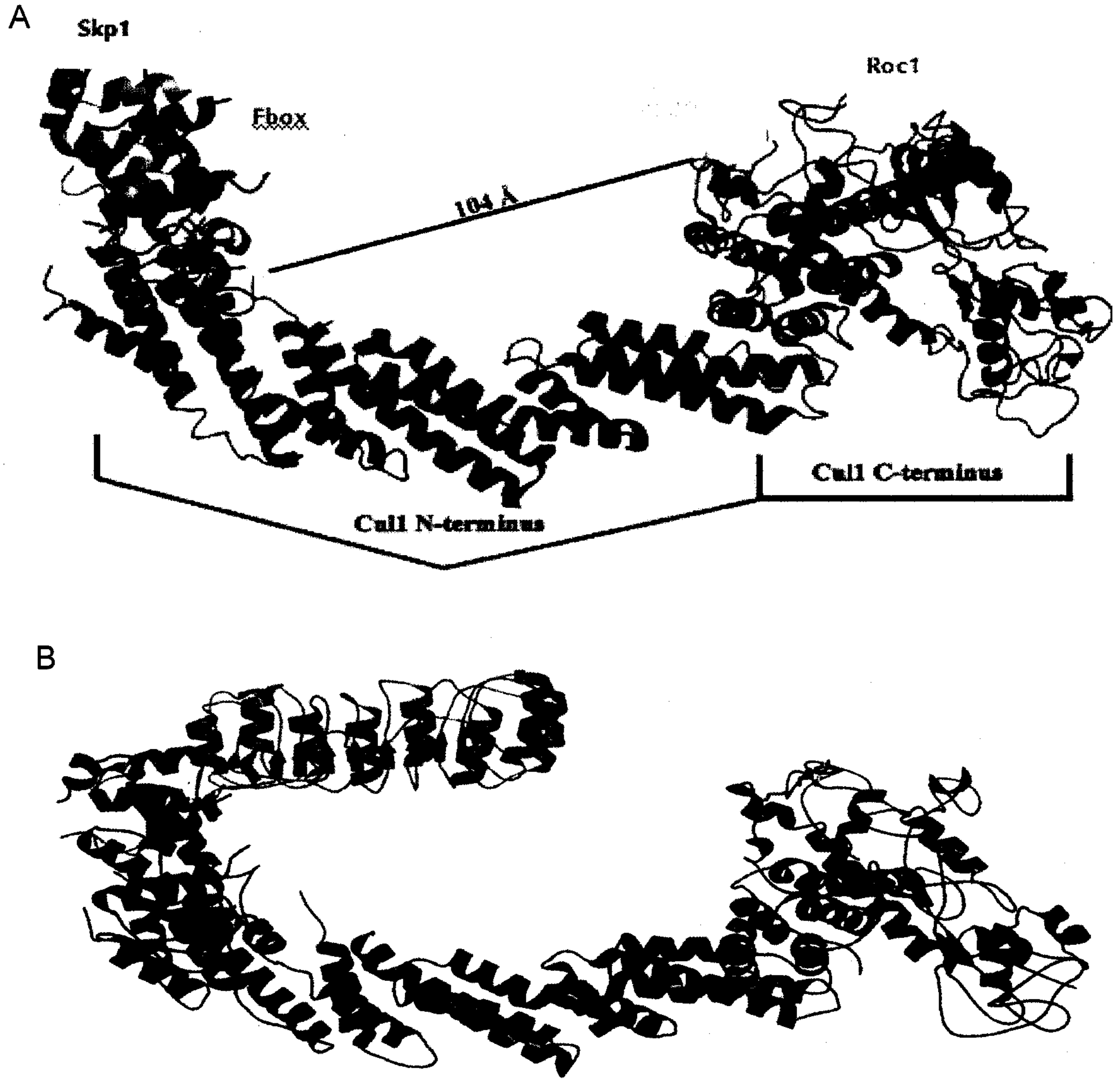

Figure 1.2. Skp1-F-box^{Skp2}-Cul1-Roc1 crystal structure (1LDK.pdb), model of multi-subunit UBL Ligases. (A) Skp1-Fbox ^{Skp2} structure provides information about Cul1-based ubiquitin ligases. Note the large distance between the Skp1 binding site and Roc1,which binds the ubiquitin-conjugating enzyme (UBC). This distance seems very far, especially since the structure lacks the full length Skp2 structure. (B) Using the Skp1-F-box^{Skp2}-Cul1-Roc1 structure and the known Skp1-Skp2 structure (1FS1.pdb) to create a model of SCF by aligning the Skp1-Fbox^{Skp2} proteins bound to Cul1-Roc1 with the full length Skp1-Skp2 structure is possible to get more exact model of SCF. The model shows how Skp2 helps traverse Cul1 and points the substrate binding domain towards the UBC binding site on Roc1. Model helps explain how substrate could be localized close to UBC binding site that was not clear in the Skp1-F-box^{Skp2} structure alone.

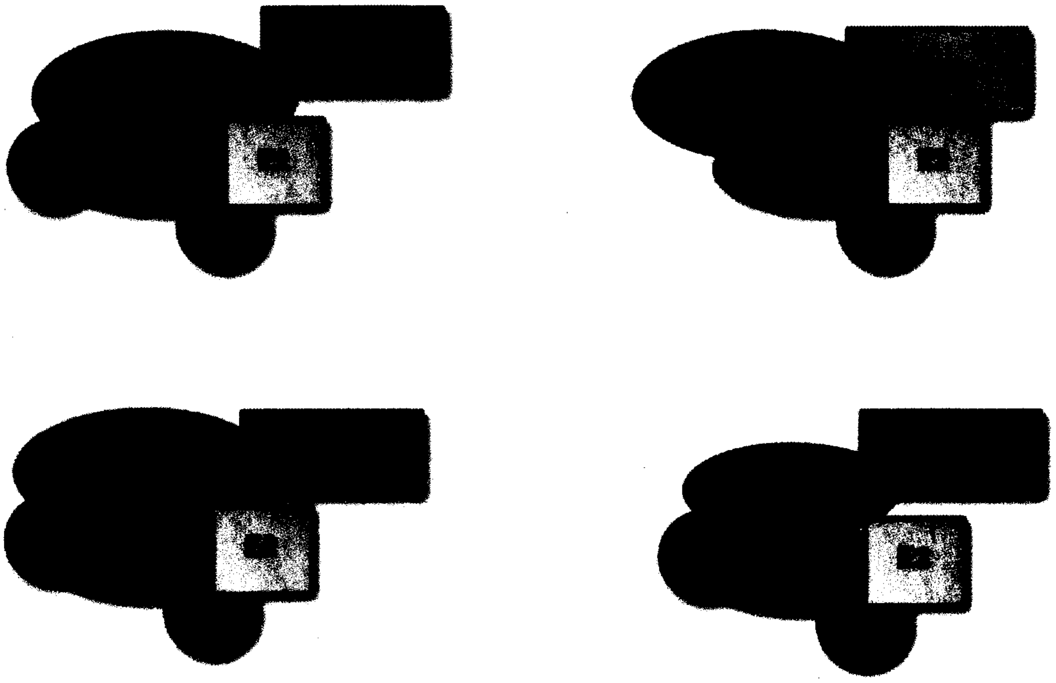

Figure 1.3. All Cullin based UBL's share a similar assembly with adaptor proteins. While the SCF complex is the prototype of the cullin family all cullin proteins share a similar architecture. Each cullin protein serves as the core of a multi-subunit ubiquitin ligase. Roc1 associates with each cullin protein and is responsible for binding the E2 (UBC). The substrates are bound by unique substrate-adaptor proteins that vary between cullins. The f-box proteins bind to Cul1-based ubiquitin ligases while Broad Complex, Tramtrack and Bric à Brac (BTB) proteins bind to Cul3, DDB1 to Cul4, and longin C to Cul2.

Chapter 2

Structure of the Cand1-Cul1-Roc1 complex

Introduction

Ubiquitin-dependent proteolysis controls protein stability and thus plays a crucial role in numerous biological processes. Targeting a protein for degradation by the ubiquitin pathway requires the successive action of the ubiquitin-activating enzyme (UAE), ubiquitin-conjugating enzyme (UBC), and ubiquitin-ligase (UBL) (Hershko & Ciechanover, 1998). Together these enzymes work together to covalently link a poly-ubiquitin chain to a protein, which then allows for that protein to be recognized by the proteasome for destruction. The last step in the cascade, the UBL ligases, are the most significant constituent of the cascade as they bring all the components together to allow for ubiquitination to occur (Pickart, 2001).

The best-characterized multi-subunit UBL-ligase is the SCF (Skp1-Cul1-F-box protein) complex. It is involved in the ubiquitination of several key cellular regulators (Deshaies, 1999). The SCF complex is made up of the invariant Skp1, Cul1, and Roc1 proteins that form the core of the scaffold. Roc1 binds the UBC conjugating enzyme via its conserved zinc finger domain. Skp1 binds to variable F-box proteins that each binds a substrate or set of substrates. Both Roc1 and Skp1 are held at opposite ends of Cul1 with the F-box proteins traversing the 100Å distance along Cul1, bringing the substrate close to the UBC (Zheng *et al*, 2002). There are over 60 F-box proteins in humans, which allows for many protein substrates to be targeted by this complex. In addition to Cul1, there are many other members of the cullin family (Cul2, -3, -4A. -4B. and -5) that all work in a similar manner, namely, that bind Roc1 and substrate-adaptor proteins to form ubiquitin ligases.

The majority of previous research into cullin-ligases has been focused on substrate recruitment by F-box proteins. However, the exact manner in which these multi-subunit complexes are assembled and dissembled is less clear. Understanding the regulation of these complexes is critical due to their regulating function of key cellular proteins. If they were constitutively active, then their target substrates would constantly be degraded and would not be able to carry out their prescribed functions. Equally troubling, a constitutively inactive cullin complex would result in constitutively active key cellular proteins. Therefore, it is extremely important that we understand how these UBL ligases are regulated. One manner of regulation is by auto-ubiquitination of the F-box proteins (Zhow and Howley, 1998). Another observation is that all cullin proteins can be modified by with the ubiquitin-like protein Nedd8.

The biochemical evidence for this model was unclear until Cand1 was identified. It had been shown that unneddylated Cul1 and Cand1could bind and form a complex. However, upon Cul1neddylation, the Cand1-Cul1 complex could no longer be detected. Also, in the presence of Cand1, a functional SCF complex could not be formed (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Yogosawa et al., 1996; Zheng et al., 2002). These lines of evidence provided the key information for Nedd8's role in Cul1 regulation, namely, to dissociate an inhibitor of the SCF complex. While this information did not address directly the deneddylation role of CSN, nor how deneddylation was required, it did allow for a circular SCF^{Nedd8} model of: SCF^{Nedd8}-dependent substrate-ubiquitination, SCF deneddylation, disruption of SCF by Cand1, followed by Cullin neddylation and re-assembly of the SCF complex. To confirm this model and the mechanisms involved we solved the 3.1Å crystal structure of the Cand1-Cul1-Roc1 complex (Table 2.1) and examined the structure to give us indications of how the mechanisms of SCF regulation might be occurring.

Materials and Methods

Protein Overexpression and Purification

Full length human Cand1 was overexpressed as a Glutathione-S-Transferase (GST)-fusion protein in E. coli. Full length Cul1 and full length Roc1 were overexpressed in both E. coli and insect cells as previously reported (Zheng et al., 2002b). For bacterial expression of the Cul1-Roc1 complex, a "Split-and-Coexpress" scheme was used. The resulting protein complex retained its full function and intact structure (Zheng et al., 2002b). All proteins were isolated from the soluble cell lysate by glutathione affinity chromatography. After cleavage by thrombin, the Cand1 protein was further purified by anion exchange and gel filtration chromatography and concentrated by ultrafiltration. The Cul1-Roc1 complex, Skp1-F-box^{Skp2} complex, Skp1-Skp2, and the Nedd8 UAE were purified following previously reported procedures (Schulman et al., 2000; Walden et al., 2003; Zheng et al., 2002b). The Nedd8 UBC (Ubc12) and Nedd8 were both produced in E. coli as GST-fusion proteins and further purified by anion exchange and gel filtration chromatography.

Crystallization and Data Collection

The purified Cand1 protein (13mg/ml) and the bacteria-expressed Cul1-Roc1 complex (8.8mg/ml) were mixed at 1:1 molar ratio in a solution of 20mM Tris-HCl pH=8.0, 200mM NaCl, 5mM dithiothretiol (DTT). Crystals of the ternary complex composed of native proteins were grown at 4°C by the hanging drop vapor diffusion method by mixing the complex with an equal volume of reservoir solution containing 100mM Tris-HCl pH=8.0, 7-10% PEG8000, 5mM DTT. The Cand1-Cul1-Roc1 crystals form in space group $P2_12_12_1$, with a = 108.4 Å, b = 151.3 Å, c = 215.9 Å, and contain one complex in the asymmetric unit. The ternary complex of Cul1-Roc1 and seleno-methionine (Se-Met) labeled Cand1 produced crystals of significantly smaller sizes, which did not diffract beyond 8 Å. A mixture of wild type and Se-Met labeled Cand1 at 1:1 ration together with Cul1-Roc1 was then prepared and crystals of normal sizes were obtained. Only a small population of Se-Met labeled Cand1, however, exists in the crystals as judged from the low selenium fluorescence signals. All the data sets were collected at BL5.0.2 beamline at the Advance Light Source using crystals flash-frozen in the crystallization buffer supplemented with 25% ethylene glycol at –170°C. Reflection data were indexed, integrated, and scaled (Table 2.1) using the HKL2000 package (Otwinowski and Minor, 1997).

Structure Determination and Refinement

The structure of the complex was determined by molecular replacement using the native data set. Molecular replacement calculations were carried out with the program AMORE using the Cul1-Roc1 structure as the search model (CCP4, 1994). After rigid-body refinement of the top Cul1-Roc1 solution, which clearly stood out from the rest, model phases were calculated and underwent density modification using CNS (Brunger et al., 1998). 3.2 Å Fourier map calculated with the resulting phases showed interpretable electron densities for a large number of Cand1 helices throughout the molecule.

Moderate discrepancies between the model and the calculated density map around Cul1's first and second cullin repeats were also clear. After correcting the Cul1 model, about two thirds of Cand1 helices with poly-alanine sequences were built into the continuous electron densities in O (Jones et al., 1991). Care was taken not to over-interpret the map in ambiguous regions. The resulting model was then refined by rigid body refinement, followed by density modification of the model phases. Five additional cycles of model building and refinement were performed before all Cand1 helices and most connecting loops were found and side-chains were built into the model. Although the electron densities of the Cand1 β-hairpin could be recognized at the very early stage, its polypeptide backbone and side-chains were built at the last step. Selenium anomalous experiments using crystals containing Se-Met labeled Cand1 were used to confirm the sequence registry. The entire structure has been checked using simulated annealing composite omit maps. The final refined model does not contain four Cand1 loops (120-123, 308-343, 409-423, 787-790) due to the absence of their electron densities. It is assumed that these loops are disordered in the crystals. The final Cand1-Cul1-Roc1 model has 97.2% of the residues in the favored and allowed regions and none in the disallowed region of the Ramachandran plot. All figures were prepared with PyMol (DeLano, 2002).

Results and Discussion

Protein Purification and Crystallization

For crystallization purified protein was extracted from bacteria. Cul1-Rbx1 was purified as previously described (Zheng et al, 2002) while Cand1 was purified to homogeneity by similar methods. Cand1 was purified as a GST-fusion protein from E. Coli with the GST-tag removed by thrombin digestion (Figure 2.10A). The resulting Cand1 and GST were separated by anion exchange chromatography and gel filtration chromatography (Figure 2.10B and 2.10C). Cand1 was concentrated to 13mg/ml and Cul1-Roc1 was concentrated to 8.8mg/ml. All crystallization was done via hanging-drop vapor diffusion. Cand1 crystals were grown in 14.5-16.5% PME5K, 1M Na Citrate pH=5.3, 2M NaCl, and 5mM DTT. Cand-Cul1-Roc1 crystals were grown in 100mM Tris-HCl pH=8.0, 7-10% PEG8000, and 5mM DTT. The Cand1 crystals did not diffract any better than 9Å while the Cand1-Cul1-Roc1 crystals were able to diffract to 3.0Å, which was sufficient for data collection and solving of the Cand1-Cul1-Roc1 structure via molecular replacement, as described above.

Overall Structure of the Cand1-Cul1-Roc1 Complex

Cand1 forms a compact structure with Cul1-Roc1 (Figure 2.1) by wrapping tightly around the elongated Cullin core. The Cul1-Roc1 protein retains its previously reported elongated N-terminal structure and globular C-terminal structure as previously reported

(Zheng et al, 2002). Cand1 is a nearly all-helical protein made up of 27 Tandem HEAT repeats. Each HEAT repeat is ~40 residues long with two anti-parallel helix motifs making up each HEAT repeat. The HEAT repeats are found in tandem with each HEAT repeat packing with the next repeat and making numerous intramolecular contacts. The continuous interaction of the HEAT repeats of Cand1 results in the coiling of Cand1 around the full length of Cul1 in a head-to-tail fashion with N-terminus of Cand1 interacting with the C-terminus of Cul1, while the C-terminus of Cand1 interacts with the N-terminus of Cul1. Cand1's repeats adopt a right-handed turn through the first half of the protein, switching in the middle and adopting a left-handed turn through the C-terminal half (Figure 2.1 and 2.2). The result of this is Cand1 forming a clamp around Cul1; Roc1's non-Cul1 interaction regions remain exposed to solvent. Cand1 does not completely cover the Skp1 binding site on Cul1's N-terminus. However, a β-hairpin on Cand1 does interact with a few residues of Cul1 that were previously shown to interact with Skp1 (Figure 2.1 and 2.4). On the other end of Cul1 (C-terminus) Cand1 interacts very tightly with the known Cul1 neddylation site, Lys720, and completely buries the lysine residue through a tridentate interaction with two of Cand1's aspartic residues and a tyrosine residue on Cul1.

Structure of Cand1 Structure

All of Cand1's helical segments resemble HEAT repeats that have been previously described (Chook and Blobel, 1999; Cingolani et al., 1999; Collins et al., 2002; Groves et al., 1999). A pair of anti-parallel helices with a connecting loop characterizes the HEAT repeat motif (Figure 2.2A). Typically the first helix of the heat repeat is kinked due to the presence of a proline residue, but is not a completely separate helix as in an ARM repeats. Cand1 has this HEAT repeat structure with the first helix of each repeat (helix A) covering the outer surface with the second of each repeat (helix B) making up the inner surface of Cand1 and making most of the interactions with Cul1. The 27 consecutive Cand1 HEAT repeats yield a curved structure with two halves bearing opposite handedness, making Cand1 distinct from all known HEAT-repeat structures. Specifically, the N-terminal half of Cand1 forms a right-handed superhelix, whereas the C-terminal half adopts a left-handed superhelical structure (Figure 2.2A). The change of the handedness predominantly owes to a drastic shift in the packing orientations between repeats 19 and 20. The Cand1 structure has three distinct regions. The first of these is the base region that connects the two anti-parallel arms. The N-terminal arm is slightly longer than the C-terminal arm, with each arm being made up of ten repeats and six repeats, respectively. The elongated N-terminal arm allows Cand1 to wrap over Cul1 and cover the lysine that is neddylated. The conserved surface of Cand1 is concentrated with those regions that overlap at the Cul1 neddylation site, the Skp1 binding site, and the core of the base responsible for the change in Cand1 handedness (repeats 19 and 20). These conserved sites may give clues to Cand1's role in Cul1 regulation as well as regulation of other cullins.

Interaction between Cand1 and Cul1

The Cand1-Cul1 interactions are extensive and make multiple interactions while burying a total of ~8900 Å^2 accessible surface area from the two proteins. The elongated Cul1 N-terminus is made of three copies of the five-helix cullin repeat motif and extends over 100Å. In complex with Cand1, the Cul1 NTD becomes slightly less curved; due to the interactions between the first cullin repeat and the Cand1 C-terminal arm (Figure 2.9). The most pre-dominant feature of the globular Cul1 CTD is the four-helix bundle (4HB), which also packs against the third cullin repeat in the NTD (Figure 2.3C). Its main role is to bind Roc1. The Cand1 N-terminal arm wraps around the Cul1 C-terminus making interactions with multiple residues on Cul1 as well as limited interactions with Roc1.

The Cand1 C-terminal arm interacts with the Cul1 N-terminus by interacting with the first two cullin repeats (Figure 2.3B). Cand1's 25th HEAT repeat is unique from all of the others as it has a β-hairpin structure as its intra-repeat structure, as opposed to the standard loop. The tip of this Cand1 β-hairpin protrusion reaches the N-terminal half of Cul1's first cullin repeat, interacting simultaneously with three Cul1 helices, two of which have been previously shown to be involved in Skp1-binding (see below). At the numerous interfaces between the Cand1 C-terminal arm and the Cul1 N-terminus, several buried intermolecular salt bridges are formed in addition to a large number of van der Waals contacts and hydrophobic interactions. More than half of the Cand1 amino acids at the interface are completely conserved (Figure 2.3B), although the interacting Cul1 residues are not conserved in Cul1 orthologues or in Cul1 paralogues (Figures 2.6-2.8). The Cand1 central base supports Cul1-binding by providing three major anchoring points that each come in contact with one of the three Cul1 helical repeats (Figure 2.3A). Throughout the Cand1-Cul1 at Cul1's N-terminus most of the interacting residues on the Cand1 side, but not the Cul1 side, are again highly conserved (Figure 2.3A and 2.8-2.10).

Distinct from the C-terminal arm, the Cand1 N-terminal arch uses only a strip of its concave surface that is close to the apical ridge to interact with Cul1 (Figure 2.3C). The first two Cand1 HEAT repeats at the N-terminal tip of the arch make extensive interactions with the Cul1 CTD and represent the most conserved Cand1-Cul1 interface in the whole complex. The apical ridges of these two Cand1 HEAT repeats make additional direct contacts with the RING domain of Roc1.

Collision of Skp1 and Cand1 at Cul1's-Skp1 binding Site

A superimposition model of the Cand1-Cul1 structure and the Cul1-Skp1 structure shows that Cand1's β-hairpin would collide with Skp1 (Figure 2.4). The highly conserved β-hairpin (Figure 2.3 and 2.8) on Cand1 is ~25Å long and is made up of two anti-parallel β-sheets with a very tight turn characterized by four amino acids, MGFP, with the GFP repeat being conserved throughout the Cand1 protein in all species. The β-hairpin interacts with a hydrophobic groove on Cul1's first cullin repeat and interacts with Cul1's Tyr42 residues, which was shown to be involved in Skp1 binding previously (Zheng et al, 2002). The interactions at this region buries ~1000Å^2 of surface area between Cand1 and Cul1. As shown in Figure 2.4B Skp1 and Cand1 collide when the structures are superimposed. While this result is intriguing it does not show conclusively that this is the mechanism by which Cand1 blocks Skp1 binding to Cul1; therefore, regulating SCF formation. Further biochemical evidence is discussed in chapter 3. However, the lack of conservation amongst cullin proteins in this region (Figure 2.6 and 2.7), due to their unique substrate-adaptors, brings into question Cand1's role as a universal regulator of cullin ligase function. Yet, the interacting residues on Cand1 are highly conserved and do suggest that they might disrupt similar, yet unique interactions on other cullins.

Interaction between Cand1 and the Cul1 neddylation site.

In contrast to the lack of mutual conservation at the Skp1 binding site of Cul1 and Cand1, the neddylation site of Cul1 and Cand1 interact with the most highly conserved regions in both proteins. The first two Cand1 HEAT repeats fit tightly against the surface of Cul1, characterized by the WH-B and 4HB domains of Cul1 and the RING domain of Roc1. These interactions are very strong and are made up by hydrophobic interactions, salt bridges, and hydrogen bonds between the two proteins. Most of these interactions occur at highly conserved residues (Figure 2.5). Of particular interest is the known Cul1 neddylation site, Lys720. In previous structures (Zheng et al, 2002) this lysine residue is unbound and completely exposed to solvent. However, in the Cand1-Cul1-Roc1 complex the lysine residue is bound by a tridentate interaction formed by Cand1's Asp19 and Asp21, separated by Lys20 (DKD repeat is completely conserved amongst all known Cand1 homologues), and Tyr776 on Cul1 (Figure 2.5B). This interaction completely buries the otherwise solvent exposed Lys720 so that it cannot be covalently linked with the C-terminal glycine of nedd8. The ε-amino group of the lysine must be available for the Nedd8 specific UBC to create the Nedd8-Cul1 isopeptide bond; with the lysine bound by Cand1 this seems unlikely. This striking structural evidence creates a clear picture of how Cand1 binding to Cul1 and Cul1 modification by the Nedd8 moiety are mutually exclusive, not only due to the mutual binding of Cul1, but also due Cand1's first two HEAT repeats resting directly on the surface groove where Nedd8 most likely sits after being covalently attached to Cul1 (Figure 2.6A).

Conclusions

The crystal structure of Cand1-Cul1-Roc1 reveals detailed mechanisms for how Cand1 ands in assembling and dissembling the function SCF UBL ubiquitin ligase. The most highly conserved regions of both proteins (Cand1's first two HEAT repeats and Cul1's neddylation site) bind tightly and serve as the anchor for the rest of the Cand1 protein to interact with Cul1. Due to Cand1's highly sinuous superhelical structure it can wrap around Cul1, and most likely other cullins, with mostly hydrophobic interactions bringing Cand'1s highly conserved β-hairpin close to the adaptor protein-binding site. The β-hairpin then interacts with same region on Cul1 as the invariable adaptor protein Skp1, thus blocking it's binding as well as the variable F-box proteins. The β-hairpin may interact with other cullin proteins in a similar manner, despite the cullin protein family's variability at the N-terminus. All of the structural evidence provides a clear picture of how Cand1 blocks formation of a functional Cul1-based UBL ubiquitin ligase. The mechanism for Cand1 disruption of other cullin-based UBLs may be similar to that of Cul1 as the interactions between Cand1 and Cul1 at the Cul1 neddylation site occurs at highly conserved region amongst all cullins. Thus allowing all Cullins to interact with Cand1 and bringing Cand1's β-hairpin in close proximity to disrupt the assembly of the multi-subunit ligase.

Table 2.1. Statistics from the crystallographic analysis. Rsym = $\Sigma_h\Sigma_i |I_{h,i}-I_h|/\Sigma_h\Sigma_i I_{h,i}$ for the intensity (I) of i observations of reflection h. R-factor = $\Sigma|F_{obs}-F_{calc}|/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. R-Free = R-factor calculated using 5% of the reflection data chosen randomly and omitted from the start of refinement. RMSD: root mean square deviations from ideal geometry and root mean square variation in the B-factor of bonded atoms.

Resolution (Å)	50 - 3.1
Observations	331559
Unique reflections	66264
Redundancy	5.0
Data coverage (%)	99.5 (99.9)
Overall I/s	18.9 (2.5)
R_{sym} (%)	8.1 (54.4)
Refinement statistics	
Total atoms	15530
R-factor (%)	24.8
R-free (%)	31.4
RMSD bonds (Å)	0.008
RMSD angles (°)	1.54

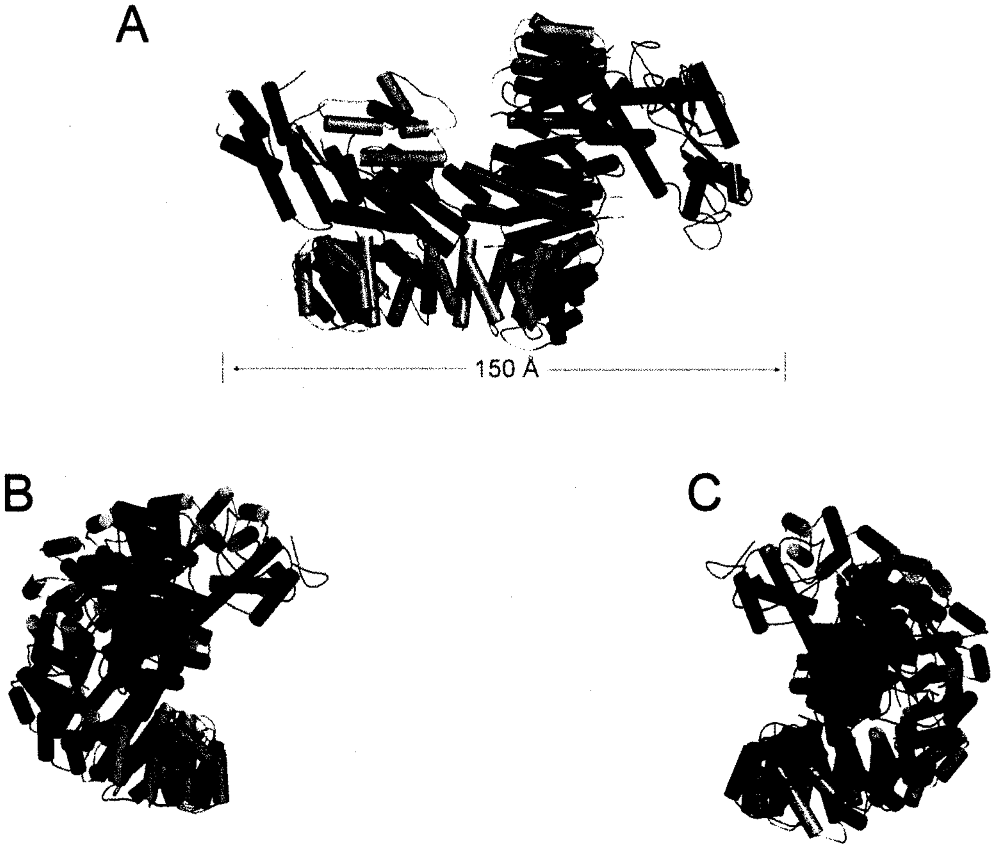

Figure 2.1. Cand1 Forms a Ternary Complex with Cul1 and Roc1. (A) Overall structure of Cand1(orange)-Cul1(green)-Roc1(red). (B) Side-view showing N-terminus of Cul1 and C-terminus of Cand1. (C) Side-view showing the globular C-terminus of Cul1, Roc1, and the N-terminus of Cand1. Cand1 is made up of 27 Tandem HEAT repeats, each HEAT repeat is made up of two anti-parallel helices. This can be seen in all views. Cand1 has a right-handed motif on it's N-terminal half and left-handed motif on its C-terminal half. This change in handedness allows Cand1 to form a clamp that binds tightly to Cul1.

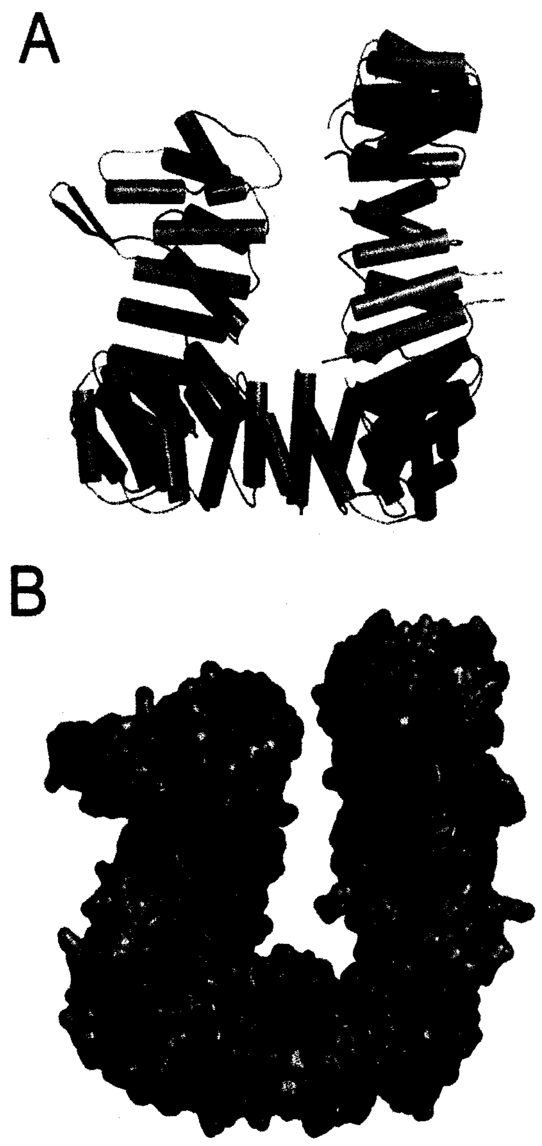

Figure 2.2. Overall Structure of Cand1 is defined by its 27 HEAT repeats. (A) The general shape of the Cand1 protein when it is in complex with Cul1-Roc1. The 27 HEAT repeats are each made up of two anti-parallel α-helices, as shown in this rendering with all alpha helices as cylinders. Each HEAT repeat then packs tightly against the next to form the overall curved structure. (B) A surface representation of Cand1 is shown with the conserved residues colored in blue. The conserved residues cluster on Cand1's termini.

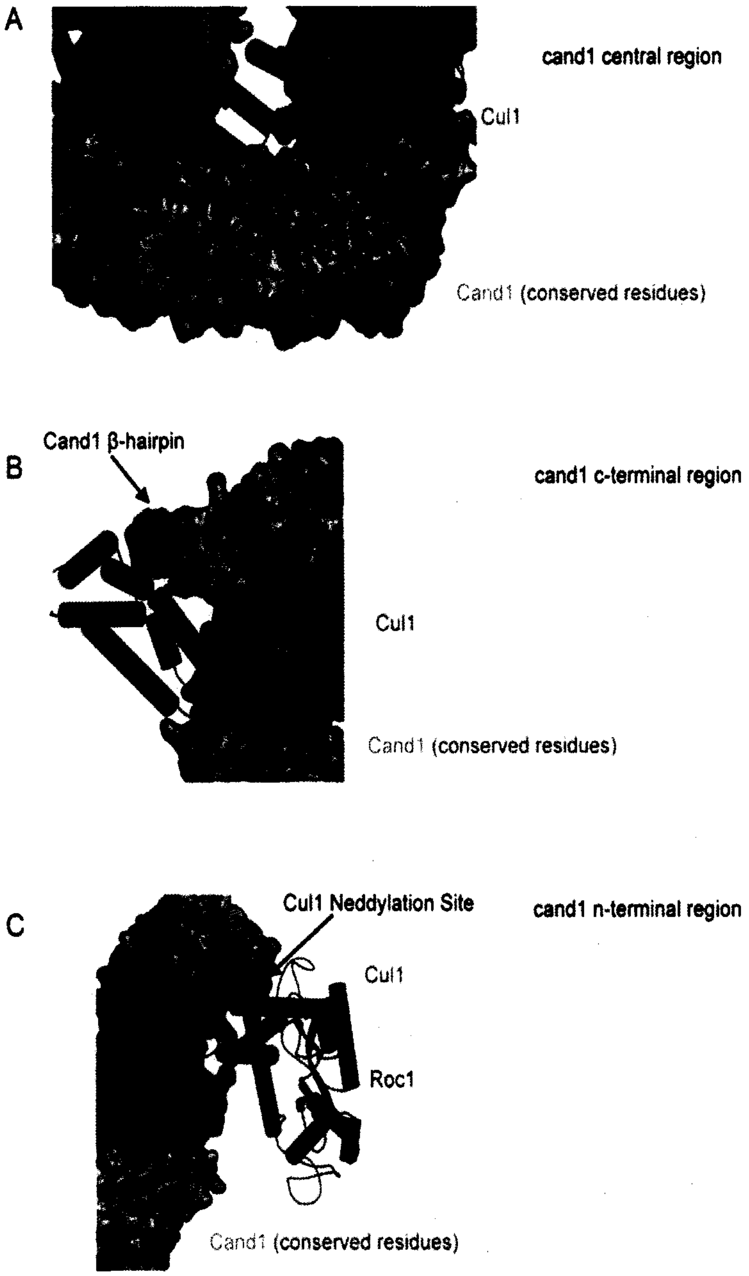

Figure 2.3. Analysis of Cand1-Cul1 interface. (A) The main pocket of Cand1 interacts with the bulk of Cul1's N-terminal stalk. The lack of conserved residues in this region indicates other cullins may also be able to interact with Cand1. (B) Cand1 C-terminal interactions with Cul1's N-terminus. The interaction between Skp1 and Cul1 occurs in this region and disruption occurs by the highly conserved region on Cand1's β-hairpin. (C) Interaction of Cand1 with Cul1 neddylation site occurs in this region. The covalent attachment of the 8kd Nedd8 protein to Cul1 would disrupt the extensive interactions between Cul1 and Cand1.

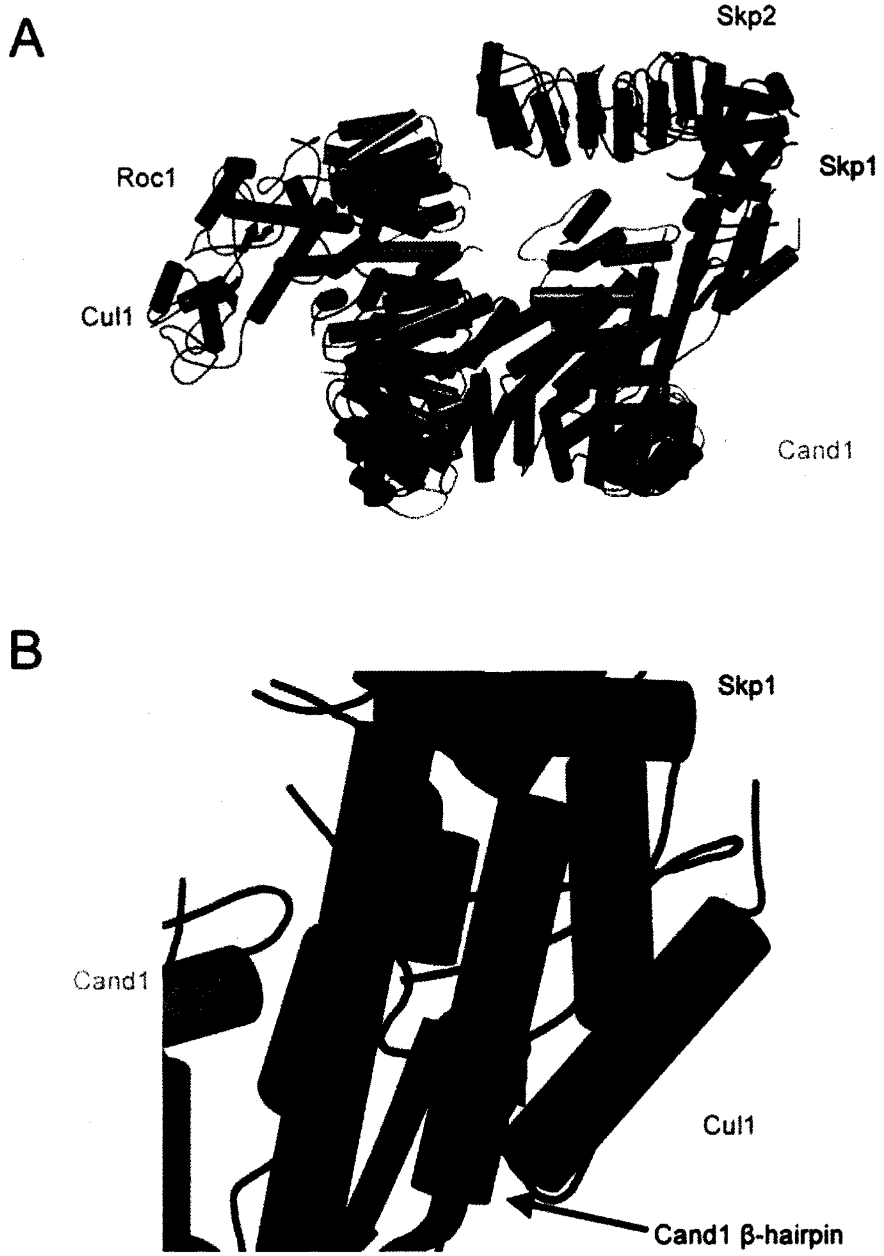

Figure 2.4. Cand1 β-hairpin collides with Skp1 in superimposition model. (A) Superimposition of Cand1-Cul1-Roc1, F-box^{Skp2}-Skp1-Cul1-Roc1, and Skp2-Skp1 crystal structures. (B) Close-up view of Cand1's β-hairpin with Cul1 and Skp1, collision between Skp1 and Cand1's β-hairpin is seen.

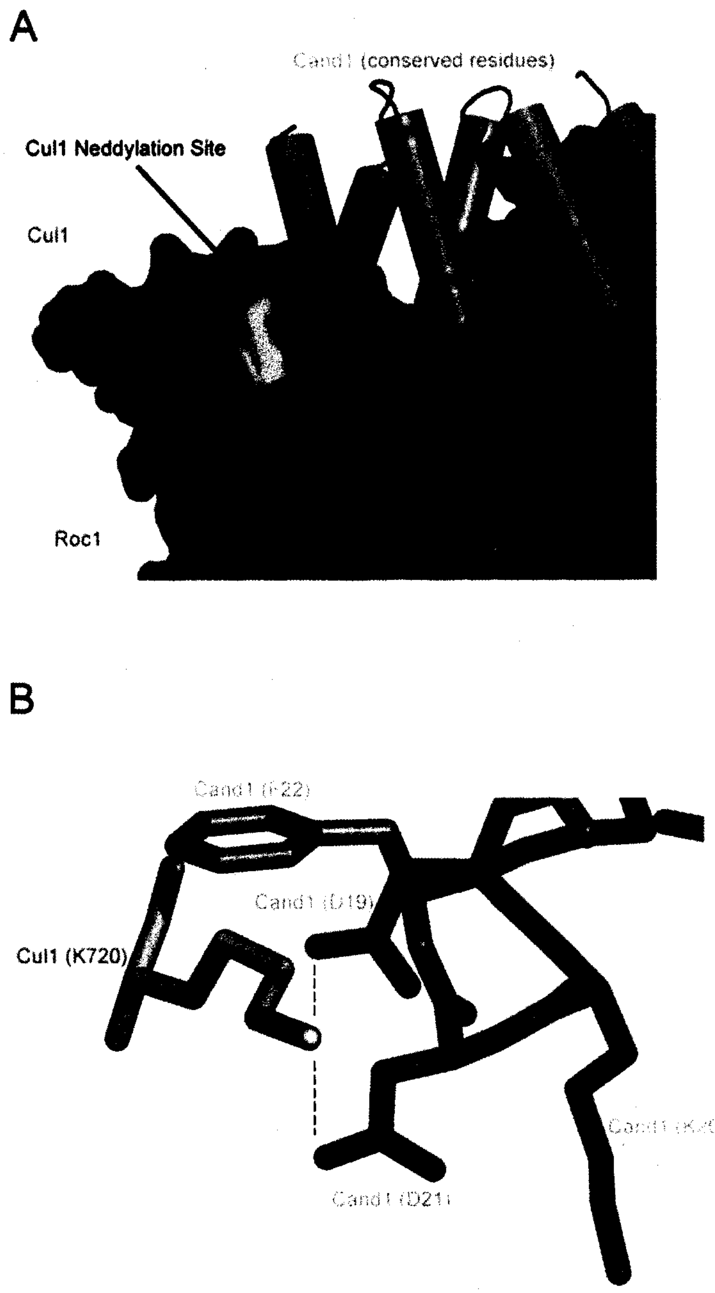

Figure 2.5. Cand1 interaction with Cul1 neddylation site, evidence for Cand1 as an Universal regulator of Cullin-UBL ligases. (A) Surface view of Cul1-Roc1 with first two highly conserved HEAT repeats of Cand1. (B) Interaction between the completely conserved Asp19 and Asp21, of the conserved DKD (cyan) motif on Cand1, with Lys720 (yellow). The Salt bridges formed between Cand1's DKD motif and Lys720 leave Lys720 not exposed to solvent.

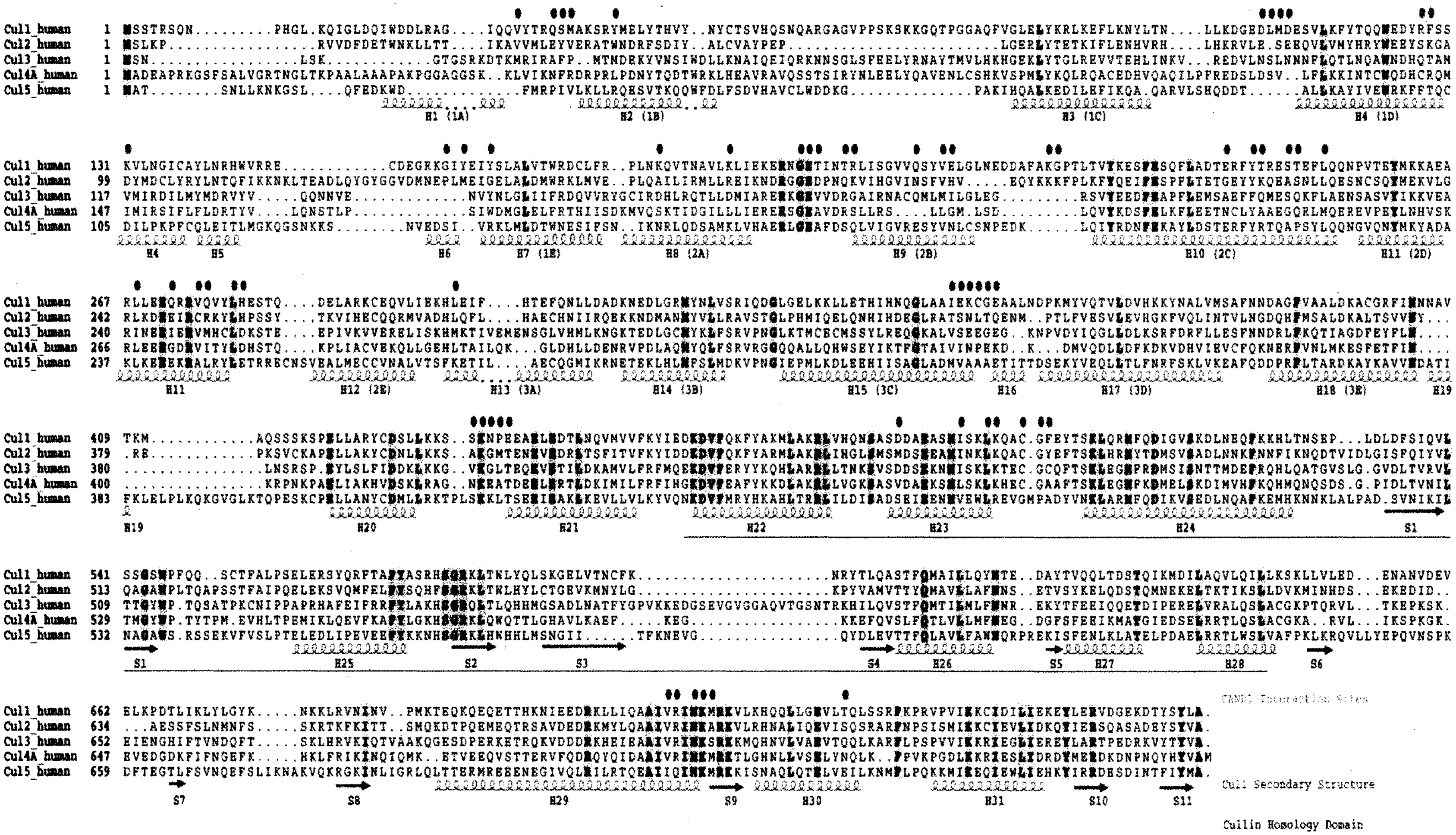

Figure 2.6. Alignment of Cullin Paralogues and residues of Cul1 that interact with CAND1. Larger version of this figure can be found online (http://www.cell.com/cgi/content/full/119/4/517/DC1/). The alignment shows that many of the interactions that occur between Cul1's c-terminus and Cand1 are conserved. This helps explain how Cand1 can bind to many members of the Cullin family.

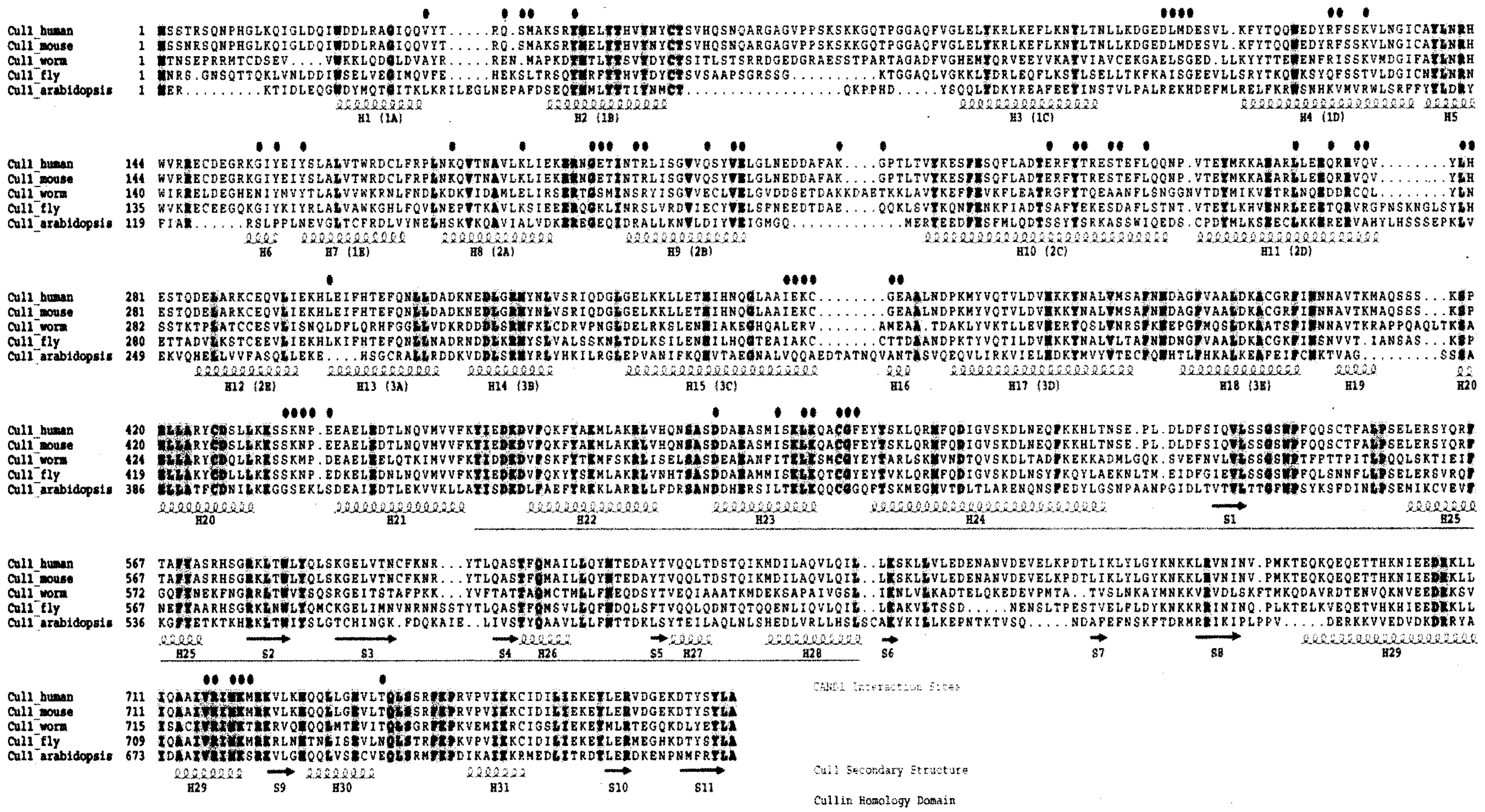

Figure 2.7. Alignment of Cullin Orthologues and residues of Cul1 that interact with CAND1. Larger version of this figure can be found online (http://www.cell.com/cgi/content/full/119/4/517/DC1/). The alignment shows that many of the interactions that occur between Cul1's c-terminus and Cand1 are conserved. This helps explain how Cand1 can bind to many members of the Cullin family.

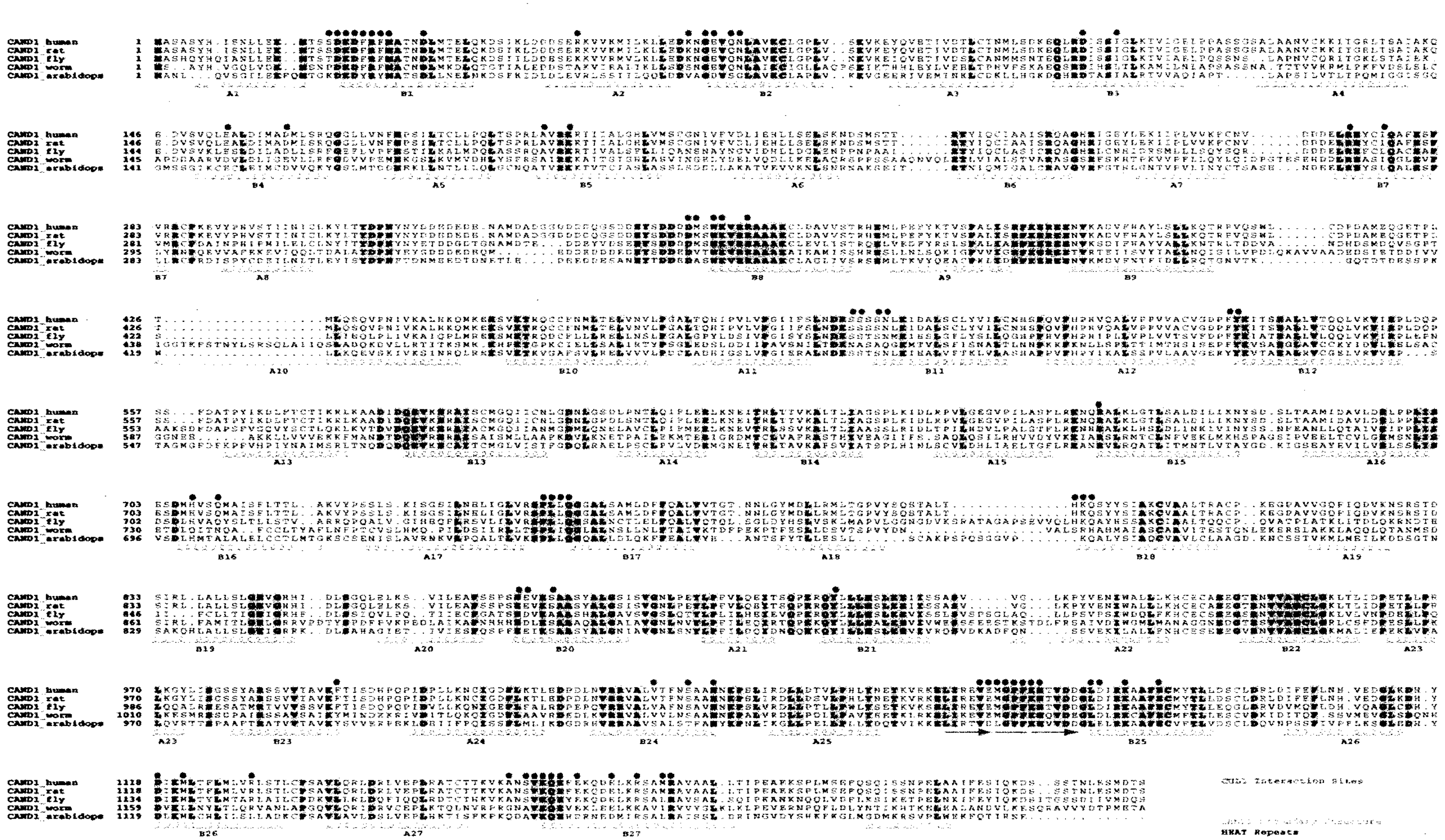

Figure 2.8. Alignment of CAND1 Orthologues and residues that interact with Cul1. Larger version of this figure can be found online (http://www.cell.com/cgi/content/full/119/4/517/DC1/). This alignment shows that most of the interactions between Cul1 and Cand1 are localized to regions that are conserved on Cand1.

Figure 2.9. Confirmation changes between Cand1 bound Cul1 and unbound Cul. Cand1-bound Cul1 (purple) and unbound Cul1 (green) have some slight structural changes, with Cul1 becoming slightly flattened at the N-terminus in the presence of Cand1. The C-terminus of Cul1 is not drastically changed. As Cand1 bound Cul1 does not undergo any major structural changes the mode of Cand1 inhibition of SCF formation is not by inducing a conformational change on Cul1. Alignment of Cul1 structures was done in Pymol (Delano, 2002)

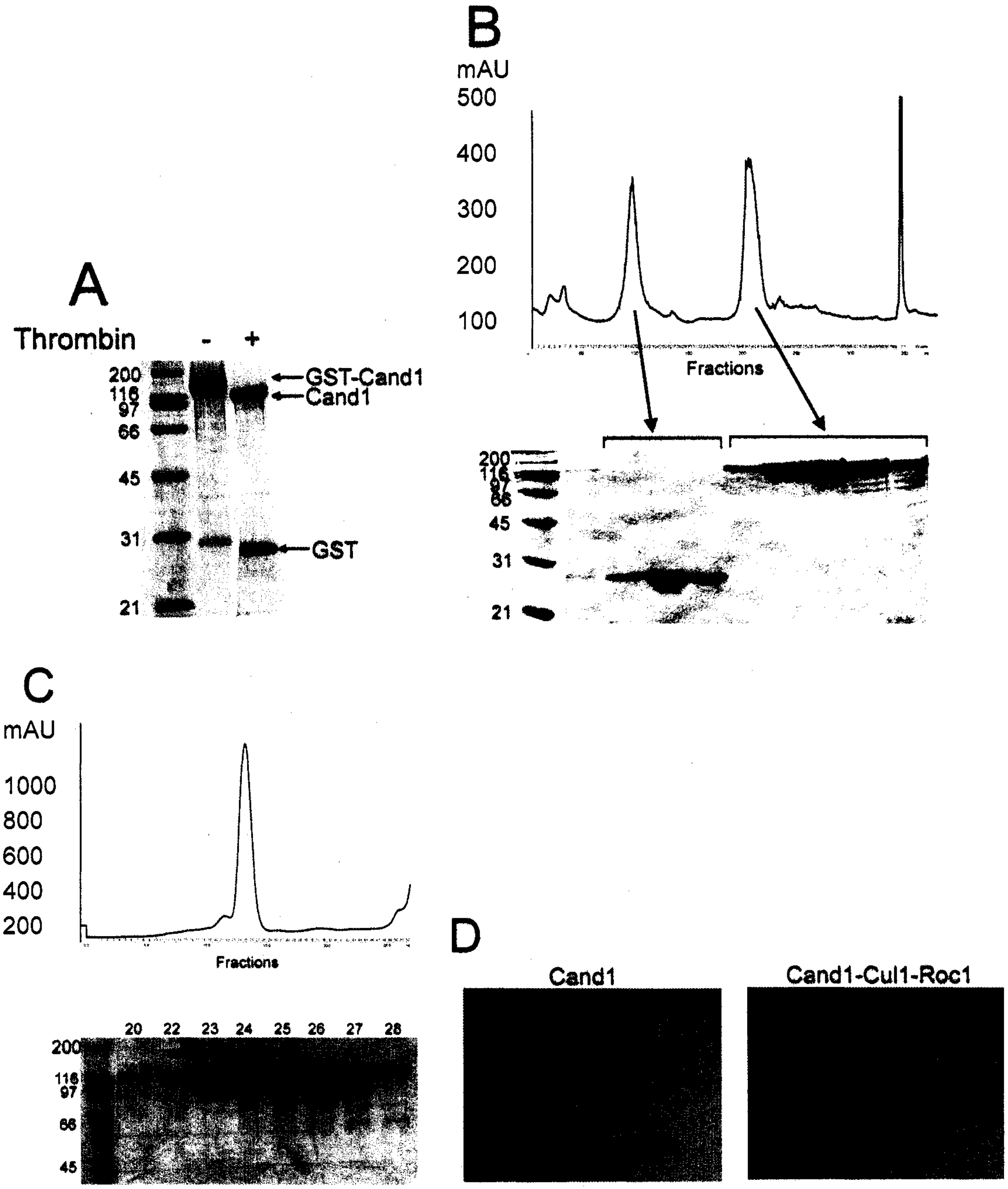

Figure 2.10. Purification of Cand1 from E. Coli. (A) GST-Cand1 purified by affinity purification and the GST-tag was removed by thrombin digestion. (B) The cleaved GST and Cand1 were separated by anion exchange chromatography and (C) size exclusion chromatography. (D) The resulting Cand1 protein was concentrated to 13mg/ml and the Cul1-Roc1 protein was concentrated to 8.8mg/ml and mixed 1:1 for the Cand1-Cul1-Roc1 crystals (3.0Å x-ray diffraction). The Cand1 crystals were achieved with 13mg/ml protein (9.0Å x-ray diffraction).

Chapter 3

Skp1-Fbox Proteins and Nedd8 System relieve Cand1 inhibition of SCF Formation

Introduction

Ubiquitin-dependent proteolysis controls protein stability and thus plays a crucial role in numerous biological processes. The successive action of the ubiquitin-activating enzyme (UAE), ubiquitin-conjugating enzyme (UBC), and ubiquitin-ligase (UBL) result in targeted ubiquitination of a substrate (Hershko & Ciechanover, 1998). These enzymes covalently link a poly-ubiquitin chain to a protein, allowing that protein to be recognized by the proteasome for destruction. The last step in the cascade, the UBL ligases, are the most significant constituent of the cascade as they bring all the components together to allow for ubiquitination to occur (Pickart, 2001).

The SCF (Skp1-Cul1-F-box protein) complex is the best-characterized multi-subunit UBL ligase. It is involved in ubiquitination of several key cellular regulators (Deshaies, 1999). The complex is made up of the invariant Skp1, Cul1, and Roc1 proteins that form the core of the scaffold. Roc1 binds the UBC conjugating enzyme via its conserved zinc finger domain while Skp1 binds to variable F-box proteins, each of which binds a substrate or set of substrates. Both of these proteins are held at opposite ends of Cul1 with the F-box proteins traversing the 100Å distance along Cul1, bringing the substrate close to the UBC as seen in Figure 1.2 (Zheng *et al*, 2002). There are over 60 F-box proteins in humans, which allows for many protein substrates to be targeted by this model. In addition to Cul1 there are many other members of the cullin family (Cul2, -3, -4A. -4B. and -5) that all work in a similar manner, binding Roc1 and forming ubiquitin ligases with substrate-adaptor proteins.

The main focus of our research into cullin-ligases has been focused on substrate recruitment by F-box proteins. However, the exact manner in which these multi-subunit complexes are assembled and dissembled is not clear. The regulation of these complexes is crucial due to their involvement in regulation of key cellular proteins. If they were constantly active then a substrate would always be degraded, without it ever being allowed carry out its prescribed function. The converse is equally troubling. Therefore, it is extremely important that we understand how these UBL ligases are regulated. One manner of regulation is by auto-ubiquitination of the F-box proteins, where the F-box proteins themselves become ubiquitinated and degraded by the proteasome. No longer allowing their associated substrates to be degraded. (Zhow and Howley, 1998).

Nedd8 is an 8kd ubiquitin like molecule that is attached to substrates in a similar manner to ubiquitin, formation of a peptide bond with a lysine residue. However, unlike ubiquitin Nedd8 does not form chains and only a single Nedd8 is attached. All cullins can be modified by Nedd8 on a completely conserved lysine residue at the C-terminus (Zheng et al., 2002) and is removed by the CSN5 subunit of the COP9 signalsome (Cope and Deshaies, 2003). Like ubiquitin, Nedd8 modification also requires an UAE and UBC enzyme that are specific for Nedd8. The significance of neddylation has been shown in fission yeast, C. elegans, and mice where the Nedd8 gene was crucial for cell viability (Dharmasiri et al., 2003; Kurz et al., 2002; Osaka et al., 2000; Tateishi et al., 2001). Furthermore, disruption of the Nedd8 system results in an increase in SCF substrates, supporting the idea that Nedd8 is required for SCF function. While it is easy to suppose that disruption of deneddylation would result in hyper-activation of SCF and an increase in substrate degradation, the opposite is true; instead, substrates accumulate upon an increase in Nedd8 modified cullins. This evidence strongly suggests that a cycle of neddylation/deneddylation is crucial for proper SCF function. Removal of Nedd8 from cullin is done by the COP9 signalsome, deneddylation, specifically the CSN5 subunit (Cope and Deshaies, 2003). While it is easy to suppose that disruption of deneddylation would result in hyper-activation of SCF and an increase in substrate degradation, the opposite is true in this case, where substrates are accumulated upon deneddylation inhibition. This evidence strongly suggests that a cycle of neddylation/deneddylation is crucial for proper SCF function. It has also been shown that CSN5 is required for F-box proteins to remain stable, and not become degraded. This is most likely due to the co-localization of the F-box proteins with CSN and the CSN-associated Usp12, which de-ubiquitinates the F-box proteins (Cope & Deshaies, 2006).

The biochemical evidence for this model was unclear until Cand1 was identified. It was shown that non-Nedd8 bound Cul1 could bind Cand1 however, upon neddylation of Cul1 a Cand1-Cul1 complex could no longer be detected. Also, in the presence of Cand1 a functional SCF complex could not be formed (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Yogosawa et al., 1996; Zheng et al., 2002). These lines of evidence provided the key information for one of Nedd8's role in Cul1 regulation, dissociating an inhibitor of the SCF complex. Nedd8 has also been noted to aid in ubiquitin transfer to substrates and association of the SCF complex with the proteasome (Tanji *et al*, 2005; Wu *et al*, 2000). This information did not address directly the role of CSN nor how deneddylation was required but it does allow for a $\text{SCF}^{\text{Nedd8}}$ model to be proposed of $\text{SCF}^{\text{Nedd8}}$ dependent substrate-ubiquitination, SCF deneddylation, disruption of SCF by Cand1, followed by Cullin neddylation and re-assembly of the SCF complex.

The structural evidence presented in chapter 2 and published recently (Goldenberg *et al*, 2004) provides further evidence that this model is correct. The structure also provided specific evidence for how Cand1's β-hairpin disrupts Cul1-Skp1 binding and how the Cand1 binding at Cul1's neddylation site might block Nedd8 being covalently attached to Cul1. While the structure was insightful, biochemical evidence to support these conclusions was required. It was also unclear how Neddylation of Cul1 once Cand1 is bound could occur. Here biochemical evidence confirming the model proposed by the Cand1-Cul1-Roc1 structure is presented, as well as evidence showing that Skp1-Skp2 and the neddylation system components are required to relieve Cand1's inhibition of Cul1, thus allowing Cul1 to form a functional SCF complex.

Materials and Methods

Protein Overexpression and Purification

Full length human Cand1 was overexpressed as a Glutathione-S-Transferase (GST)-fusion protein in E. coli. Full length Cul1 and full length Roc1 were overexpressed in both E. coli and insect cells as previously reported (Zheng et al., 2002b). For bacterial expression of the Cul1-Roc1 complex, a "Split-and-Coexpress" scheme was used, where Cul1's N-terminus was expressed in a His-tagged chloremphenicol resistant vector and Cul1's C-terminus and Roc1 were expressed with Roc1-GST tagged in an ampicillin resistant vector. Both vectors were co-expressed and affinity purification was done against Roc1's GST tag. The resulted protein complex retains its full function and intact structure (Zheng et al., 2002b). Cul3 was purified as a GST-fused full-length protein from bacteria, as was Keap1. All proteins were isolated from the soluble cell lysate by glutathione affinity chromatography. After cleavage by thrombin, the Cand1 protein was further purified by anion exchange and gel filtration chromatography and concentrated by ultrafiltration. The Cul1-Roc1 complex, Skp1-F-box^{Skp2} complex, Skp1-Skp2, and the Nedd8 UAE were purified following previously reported procedures (Schulman et al., 2000; Walden et al., 2003; Zheng et al., 2002b). The Nedd8 UBC (Ubc12) and Nedd8 were both produced in E. coli as GST-fusion proteins and further purified by anion exchange and gel filtration chromatography. β-TRCP was purified from insect cells as a GST-fusion protein.

GST pull down assay

GST-fused wild type and mutant Cand1 proteins (150 μg) bound to glutathione sepharose 4B beads (100 μl) were incubatedwith Cul1-Roc1 (100 μg) and variable amounts of Skp1 bound to F-box^{Skp2} or Skp2 for 3 hours at 4°C. Supernatant, containing unbound proteins, was removed after centrifugation. The beads were then washed three times with 500 μl of wash buffer containing 20mM Tris-HCl pH=8.0, 200mM NaCl, 5mM DTT. The resin bound proteins were eluted with 50mM Tris-HCl pH=8.0, 200mM NaCl, and 10mM reduced glutathione. A fraction of the eluted proteins was separated by SDS-PAGE and visualized by Coomassie staining.

In vitro neddylation assay

The purified Cul1-Roc1 complex (5μg) was mixed with the Nedd8 UAE (50ng), Nedd8 (0.8μg), and variable amounts of the Nedd8 UBC, Cand1 and Skp1 in a buffer system containing 20mM Tris-HCl pH=8.0, 200mM NaCl, 5mM dithiothretiol, 1mM MgCl2, and 5mM ATP. After one-hour incbuation at room temperature or 37°C, SDS-PAGE loading buffer was added to stop the reaction. The sample was subsequently analyzed by SDS-PAGE.

Results and Discussion

Competition of Skp1 vs. Cand1 for Cul1 binding

The Cand1-Cul1-Roc1 structure gives many clues towards Cand1's regulation of SCF formation (Goldenberg et al, 2004). As the structure is just one state of the protein complex it is necessary to use biochemical studies to confirm the hypothesis derived from the structure. The first step was to re-confirm previous results showing that Cand1 can block Skp1 binding to Cul1. To show this a GST-Cand1 protein was immobilized on beads and used as bait to pull down Cul1 and possibly Skp1 (Figure 3.1). Cul1 was added to Cand1 with two times the number of moles of Cand1, to insure saturation of the bait (GST-Cand1), with the excess Cul1 then washed off. Skp1 was then added in increasing amounts, up to 50x excess the molar ratio of Cand1, and incubated together for 30 minutes before the unbound proteins were washed off and remaining proteins on the beads were eluted. Despite the extreme excess of Skp1, Cul1 was not dissociated from Cand1 binding. This confirms previous results from other laboratories and collaborates the tight interaction seen the Cand1-Cul1-Roc1 structure where ~8900Å^2 of surface area were buried between the two proteins.

Cand1 regulation of SCF formation

The Cand1-Cul1-Roc1 structure suggests that out of the ~1300 residues of Cand1 only the β-hairpin, which is found at the intra-repeat region of HEAT repeat 25, is responsible for blocking Skp1 binding to Cul1 and therefore is responsible for SCF formation. As the region indicated on Cand1 for Skp1 regulation is so small, it is important to determine if this result is correct or if it is an artifact of the crystallization process, with crystallization locking the β-hairpin of Cand1 in a non-native position. To confirm this a GST-Cand1 pull down assay was carried out with wild-type Cand1 as well as a mutant Cand1 protein with the β-hairpin residues indicated in Skp1 binding being deleted (Figure 3.2). The GST-Cand1Δ β construct was still capable of binding Cul1. However, unlike the wild-type protein that does not bind to Skp1 and Cul1 simultaneously, the mutant Cand1 protein was able to form interactions with Cul1 and Skp1 concurrently. This result confirms that out of the ~1300 residues on Cand1 only the β-hairpin is responsible for blocking Skp1 binding to Cul1. It is also likely that the remainder of the Cand1 is required for holding the highly conserved β-hairpin in place at this low-homology region of Cul1. The interaction of Cul1 and Cand1 is most likely regulated at the mutually conserved Cul1 neddylation site and Cand1's first two HEAT-repeats, thus allowing for disruption of numerous members of the Cullin family based multi-subunit UBL ligases, with only one inhibitor, Cand1.

Regulation of Cand1 by Neddylation of Cul1

The Cand1-Cul1-Roc1 structure shows that the Cul1 neddylation site, Lys720, is completely buried in the structure, which is in opposition to the Cul1-Roc1 structure where Lys720 is exposed to solvent. Previous evidence has shown that upon neddylation of Cul1, Cand1 can no longer bind. However, how this occurs with Cand1 bound to Cul1 is unclear. It is possible that the Nedd8 UBC binding to Roc1 causes a conformational change in the Cand1-Cul1 structure, partially relieving Cand1's inhibition of Cul1 in a manner sufficient to allow for Cul1-neddylation to occur and completely dissociate Cand1. It is also possible that the equilibrium between free Cul1 and Cand1-bound Cul1 might allow for neddylation to occur or other cellular factors may be involved. To test if Cul1-neddylation is possible in the presence of Cand1 a reconstituted neddylation assay was carried out using all recombinant proteins made in bacteria. Without Cand1 pre-incubation with Cul1-Roc1, neddylation of Cul1 could occur (Figure 3.3). However, when Cand1 was added in equal molar ratios to Cul1, neddylation of Cul1 was completely blocked. This rules out that equilibrium between Cand1 bound and Cand1 free Cul1 allows for Cul1 neddylation in the presence of Cand1. However, it is possible to that the Nedd8 UBC is causing a conformational change. To test this increasing amounts of the Nedd8 UBC were added to the reaction, but even when 10 times excess Nedd8 UBC was added to the reaction (1/100th this amount was required for efficient Cul1 neddylation) no neddylated Cul1 was seen in the presence of Cand1. It was also possible that Skp1 was disrupting the Cand1-Cul1 interaction, again even with 20 times excess Skp1 no neddylated Cul1 was produced in the presence of Cand1. With these results and results from previous groups (Liu et al, 2002), whom carried out assays using whole cell lysate that produced Cul1 neddylation in the presence of Cand1, the best remaining alternative from the structural evidence was that other cellular factors might be involved in relieving Cand1's inhibition of Cul1 neddylation.

Isolation of novel SCF regulators from cell lysate

With the possibility of other cellular factors being involved in neddylation of Cand1-bound Cul1, cell lysate fractionation was carried out with the resulting fractions being added to the neddylation assay described above. If factors responsible for Cul1 neddylation were present in these cell lysate fractions then neddylated Cul1 could be detected, the lysate fraction would be purified further and additional neddylation assays carried out until homogeneity of a particular factor(s) was achieved. Pellets from HEK293 cell lysate were fractionated based on charge and size. While preliminary results were promising no additional factors that enhanced Cand1-bound Cul1 neddylation could be purified to homogeneity (preliminary results shown in Appendix B). Therefore, the initial positive fractions from a Q-sepharose column were used for mass spectrometry analysis, but only previously identified components of the SCF complex were isolated, such as Skp1 (data not shown).

Comparison of Neddylation in an immobilized system and a resin-free system

There are key differences between the published results indicating that neddylation of Cul1 can occur (Liu et al., 2002) and our assay showing inhibition of Cul1-neddylation by Cand1. As mentioned above one of those differences is the use of immuno-precipitated Cul1 by Lieu et al (2002), while our assays used all recombinant proteins. Another important difference is the assays contained proteins immobilized on resin vs. proteins free in solution.

To address this discrepancy we attempted to neddylate Cul1 with both immobilized Cul1-Roc1 and free Cul1-Roc1 in the presence of Cand1 (Figure 3.4). We re-capitulated our previous results that Cand1 completely inhibits Cul1 neddylation with the proteins free in solution. However, we could not repeat this result with immobilized Cul1 and Cand1 could not block neddylation of immobilized Cul1. This result brings into question the previous results where Cul1 was neddylated and released from Cand1 as these assays were carried out in an immobilized system (Liu et al., 2002). As Cul1 acts as its own Nedd8-ligase we know it must be active and folded correctly in the immobilized form. While it is still possible that there are additional factors involved in releasing Cul1 from Cand1, thus allowing neddylation of Cul1 to occur and a function SCF complex to form, it brings into question previous data suggesting this possibility as the immobilization of the proteins produces a false positive. This false positive could be due to improperly folded Cul1-Roc1 being bound by the resin while in the case of the free system it has been purified or being locked into a non-native confirmation due to being bound to the glutathione-4B resin. In either case this assay shows a discrepancy between the two systems and brings into question previous results of Cul1 neddylation during immobilization on beads.

Effect of Skp2 on Cul1-Neddylation in the presence of Cand1

Since the structure was published (Goldenberg *et al*, 2004) several other papers have come out describing Cand1's role in regulation of cullins (Hu *et al*, 2004; Feng *et al*, 2004; Chuang *et al*, 2004; Chen *et al*, 2006; Lo & Hannink, 2006; Bornstein *et al*, 2006). The most recent of these at the time of this writing is the description by Bornstein et al. In their publication they demonstrate that full-length Skp2, not just the F-box domain, is required to allow neddylation of Cul1 in the presence of Cand1.

Based on our structure as well as others of the SCF complex (Zheng *et al*, 2002) and corresponding model (Figure 2.4) this result was unexpected. In the structure of Cul1-Roc1-Skp1-F-box^{Skp2} and the corresponding model using the Skp1-Skp2 (Schulman *et al*, 2000) structure to complete the full-length complex no interactions between the LRR of Skp2 and Cand1 were predicted. The only interactions between Skp1 and Cand1 that were predicted occurred at the β-hairpin of Cand1, as discussed above. However, Skp1 was found in the cell lysate fractions that gave enhancement of Cul1 neddylation in the presence of Cand1.

With the publication by Bornstein et al, it was suggested that full-length Skp2, not just its F-box domain, is required for release of Cul1 from Cand1. To take a closer look at this interaction we first examined if full-length Skp1-Skp2 could bind to Cul1 in the presence of Cand1. As seen in Figure 3.5A Skp1-Skp2 did not bind to Cul1 in the presence of Cand1 in a pull-down assay. This assay was carried out with GST-Cand1 immobilized on glutathione resin and loaded with Cul1, which then underwent three washes to remove unbound Cul1. Skp1-Skp2 was then added to this complex, underwent three more washes, and remaining proteins were eluted. Skp1-Skp2 did not bind to Cul1 in the presence of Cand1 nor did it interact directly with Cand1. This corresponds with our previous results showing Skp1 alone does not to Cul1 in the presence of Cand1 in a manner robust enough to be detected in a pull-down. These results show that full-length Skp2 does not greatly improve Skp1-Skp2's binding to Cul1.

However, in direct opposition to our previous results showing that Skp1 does not help Cul1 undergo Nedd8 conjugation in the presence of Cand1 Skp1, we and others (Bornstein *et al*, 2006) have shown that Skp1-Skp2, specifically full-length Skp2 that includes its LRR substrate binding motif, is able to cause a conformational change in Cand1 that is sufficient to allow neddylation of Cul1 to occur (Figure 3.4B). These results show that despite the fact that Skp1-Skp2 does not bind to Cul1 in a manner strong enough to be seen in a binding assay, it does allow for Cand1-bound Cul1 to be neddylated. This suggests that the interaction between Skp1-Skp2 and Cand1-bound Cul1 is transient and causes a conformational change in Cul1 exposing K720, which allows for neddylation to occur. How the Nedd8 UBC gains access to the Cul1 neddylation site, which is mostly buried in the Cand1-Cul1-Roc1 complex, remains to be clarified. However, as we do have a Cand1 mutant that is capable of binding Cul1 and Skp1-Skp2 we could use these proteins, along with Nedd8 UBC, in structural studies to trap the intermediate of this complex and reveal clues towards the mechanism. It poses a very interesting structural question as to how a protein-protein interaction that accounts for only a small percentage of the total protein can cause a conformational change on the opposite side of the protein.

Substrate adaptor proteins are required for Cullin neddylation in the presence of Cand1

The reconstitution of Cul1 neddylation in presence of Cand1 by Skp1-Skp2 is intriguing. However, this result alone does not demonstrate that it is common for all Cullins or Fbox/Substrate-adaptor proteins. To expand this model we tested β-TRCP to see if it could also restore Cul1 neddylation once Cand1 bound it. Figure 3.6 shows that β-TRCP can also allow for Neddylation of Cand1-bound Cul1. The F-box domain of β-TRCP and Skp2 are similar; however, the substrate-binding domain of Skp2 is a helical Leucine-Rich-Repeat (LRR) structure and β-TRCP has a WD40 β-propeller structure (Figure 1.3). Showing that two structurally different F-box proteins can both allow for Cul1 neddylation in the presence of Cand1.

Cand1 enhances de-neddylation of Cul1 by the COP9 signalsome

While we have shown through structural and biochemical studies how Cand1 plays a role in regulation of the SCF complex formation and how it's inhibition of Cul1 neddylation is relieved, there may be other roles Cand1 is playing in the cell. One role that Cand1 could play is competition for non-Nedd8 associated Cul1. This hypothesis brings us to COP9 and its ability to de-neddylate Cul1 with the CSN5 subunit. Results in our laboratory using recombinant COP9 to de-neddylate Cul1 require long incubation periods and suggest that COP9 alone is very inefficient. After four-hour incubation periods of COP9 with Cul1-Nedd8, complete de-neddylation is not seen. One reason for this could be the Nedd8-free-Cul1 binds to COP9 in competition with Cul1-Nedd8.

To test this hypothesis de-neddylation of Nedd8-bound-Cul1 was carried out in the presence of increasing amounts of CAND1 (Figure 3.8). While a molar ratio of CAND1 to Cul1 at 1:10 did not have a significant impact on de-neddylation, increasing the ratio to 1:2 or 1:1 resulted in almost complete de-neddylation by COP9. This occurs without COP9 and Cul1 interacting directly (Figure 3.7A). However, it does coincide with the strength of Cand1-Cul1 binding versus Cul1-COP9 binding (Figure 3.7). COP9 does not interact strongly with Cul1-GST on glutathione resin while Cand1 can stay associated with Cul1-GST through 6-washes with 200mM NaCl. While COP9-Cul1 interaction can be detected on gel filtration columns or native gels (data not shown) a pull-down assay most likely requires a stronger interaction.

Conclusions

While the structure gave excellent clues to how Cand1 and Nedd8 interaction with Cul1 are mutually exclusive, the biochemical data provides evidence for a clearer model of SCF regulation. Cand1 blocks SCF formation through its conserved β-hairpin residues interact at Cul1's N-terminal Skp1 binding site. Simultaneously the Cand1-Cul1 interface is regulated by neddylation at Cul1's C-terminal neddylation site. A substrate-adaptor (e.g. Skp2 or βTRCP) is required for Cul1 neddylation. The exact mechanism for this is unclear, but it does produce novel questions that could be answered by further structural studies. Cand1 also increases the efficiency of Cul1 de-neddylation by COP9, which has been shown to be a key step in substrate-adaptor recycling. These data have introduced novel roles for Cand1 in SCF regulation.

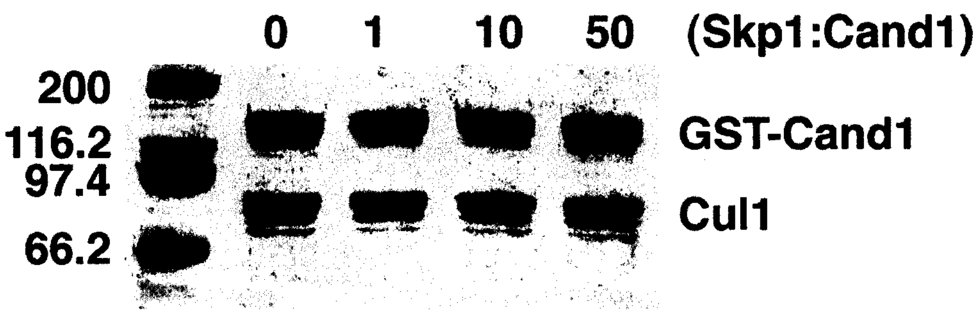

Figure 3.1. Skp1 Is Unable to Compete with Cand1 for Cul1 Binding
An in vitro pull-down assay showing excess Skp1 is unable to compete off Cand1 from Cul1-Roc1. 11ug of GST-Cand1 was purified and bound to glutathione-4B resin. The GST-Cand1 was incubated with 15ug Cul1 and increasing amounts of Skp1, up to 50x molar ratio of Cand1. The proteins were incubated together for one hour at room temperature and unbound proteins were removed by three washes. The bound proteins were then eluted and visualized with coomasie staining on a 10% acrylamide gel.

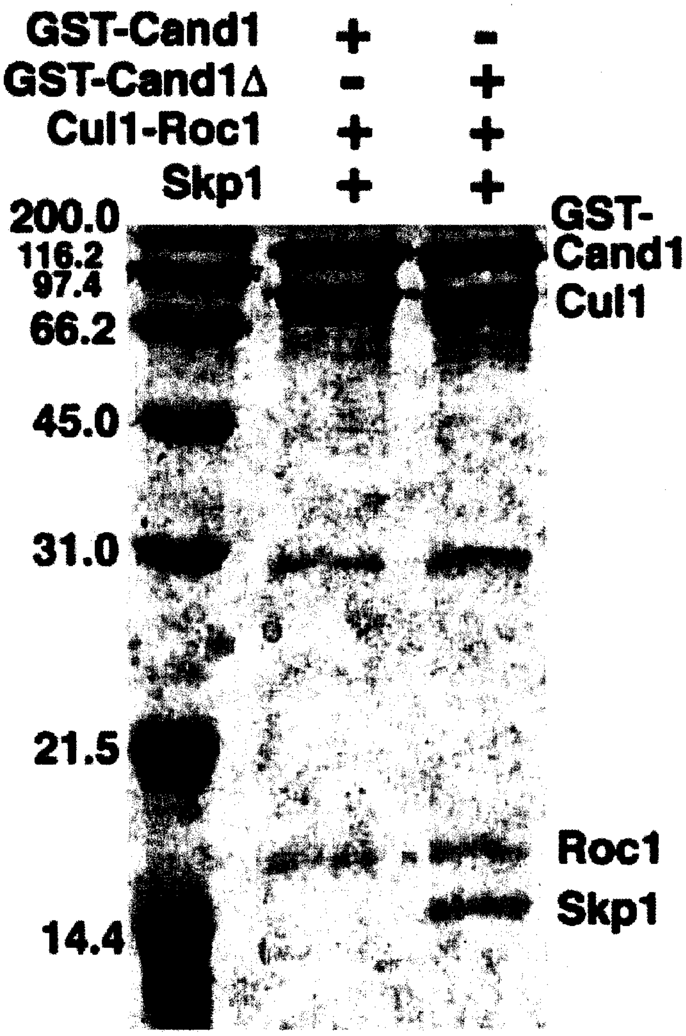

Figure 3.2. Deletion of Cand1's β-Hairpin allows Cand1-Cul1 complex to interact with Skp1. An in vitro pull down assay showing the Cand1 β-hairpin is solely responsible for disrupting Skp1-Cul1 interactions. Both wild type and a mutant Cand1 lacking 12 β-hairpin residues (Arg1064 to Val 1075) were purified as GST-fusion proteins and bound to the glutathione-4B resins, which were subsequently incubated with a mixture of purified Cul1-Roc1 and Skp1-F-box^{Skp2} complexes. Following an extensive wash, the proteins were eluted by glutathione and analyzed by SDS-PAGE.

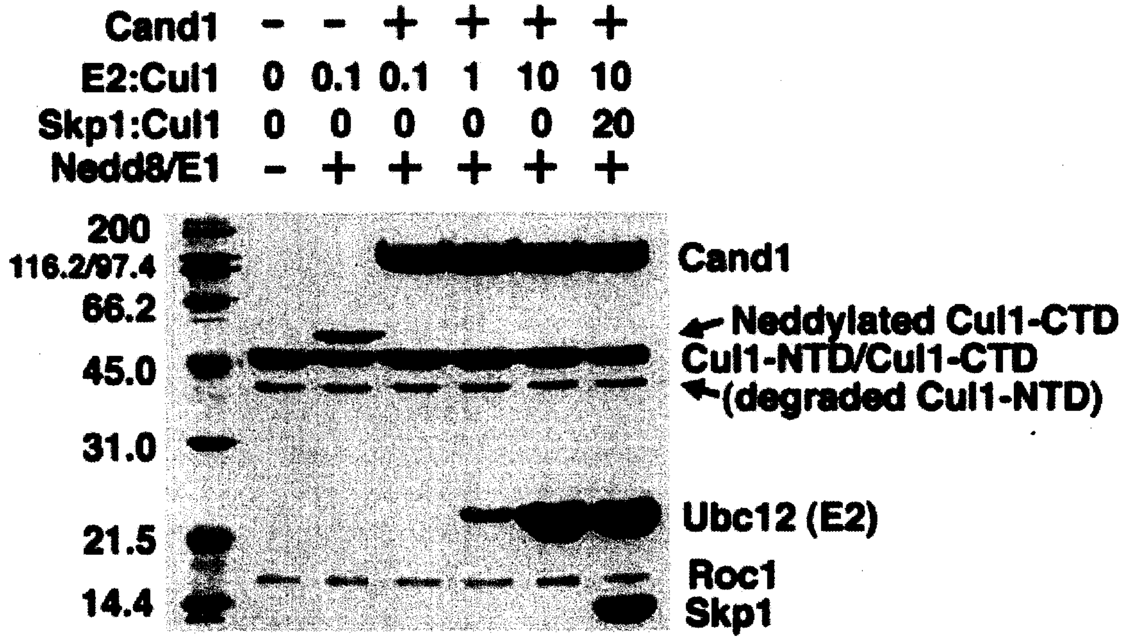

Figure 3.3. The Neddylation Enzymatic System Is Not Sufficient to Decouple Cand1 From the SCF catalytic core. The first lane shows the Cul1 NTD and CTD in the spliced form of Cul1 co-migrated on the gel. In the second lane neddylation of Lys720 on the Cul1 CTD up-shifted the Cul1 CTD band by about 8 kD. Cul1 neddylation was effectively blocked when an equal molar amount of Cand1 was present. All proteins were overexpressed and purified from E. coli. The neddylation enzymatic system failed to modify Cand1-bound Cul1, even when the Nedd8 E2 enzyme (Ubc12) is in excess amount and in the presence of excess Skp1 and Skp2^{Fbox}. The Skp2^{Fbox} is not seen as it runs at ~7kd.

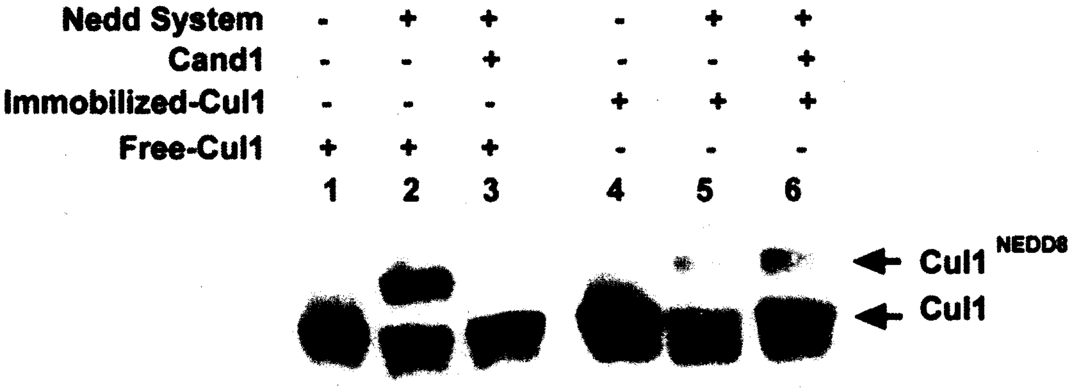

Figure 3.4. Neddylation of free-Cul1 and immobilized-Cul1 in the presence and absence of Cand1. The first three lanes were carried out in the exact same manner as in Figure 3.3, lanes 1-3. In lane 2,3,5, and 6 the neddylation reactions were carried out for 30 minutes at room temperature. In lanes 3 and 6 Cul1 and Cand1 were incubated together for 15 minutes prior to the addition of the neddylation system components. In lane 3 with all proteins in solution inhibition of Cul1-neddylation by Cand1 is complete. The assays carried out in lanes 4-6 used GST-Roc1-Cul1 immobilized on glutathione-4B resin in the same fashion as Lieu et al (2006). Lane 6 shows that Cul1 was neddylated with Cand1 present, in contrast to lane 3 where Cul1 was not neddylated.

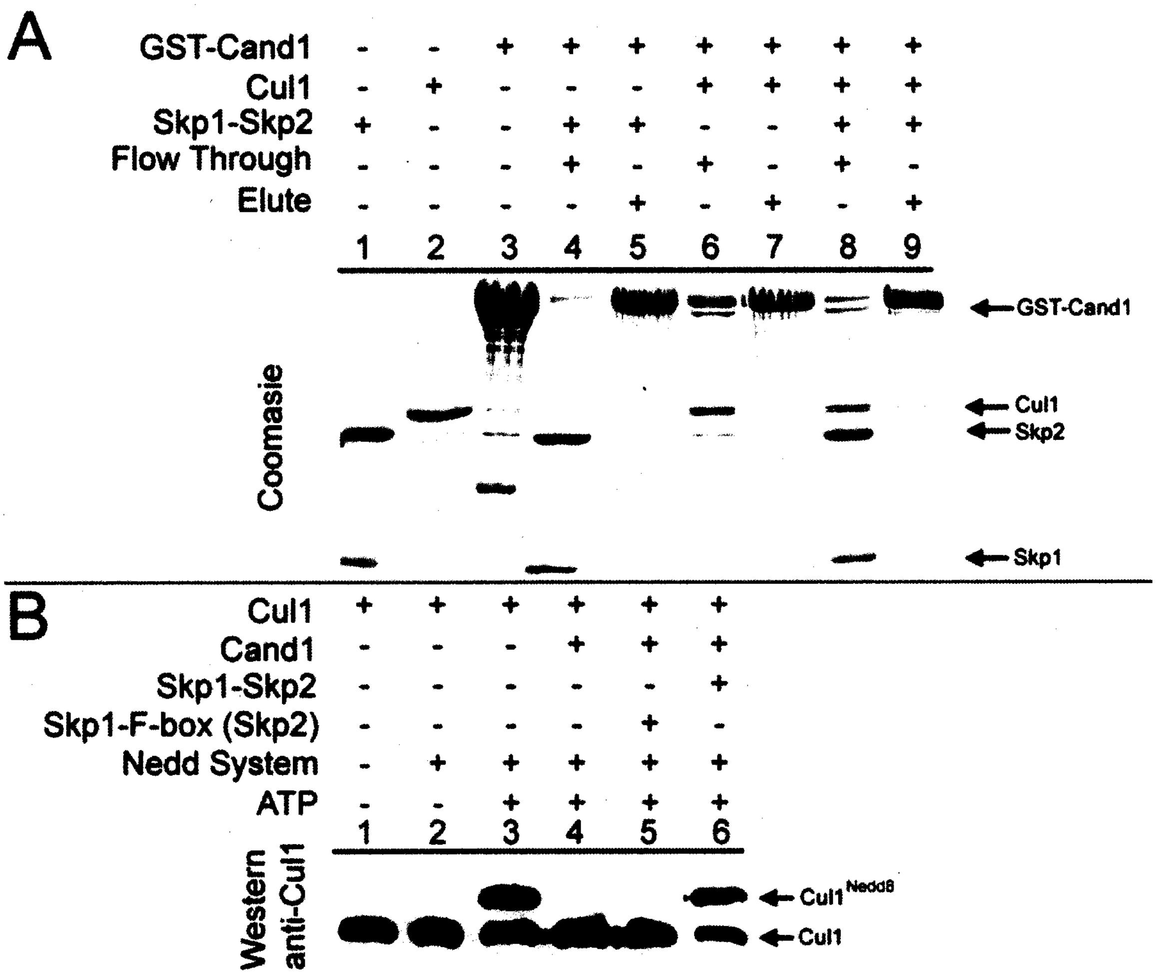

Figure 3.5. Interaction of Skp1-Skp2 with Cul1 in the presence of Cand1 and relief of Cand1 Inhibition on Cul1 Neddylation by Skp1-Skp2. (A) Binding of Skp1-Skp2 to Cul1 pre-loaded onto GST-Cand1 immobilized on glutathione resin. Skp1-Skp2 did not bind directly to Cand1 nor were they able to bind to Cand1 bound Cul1. (B) Effect of Skp1-Skp2 and Skp1-F-box^{Skp2} on Cul1-neddylation in the presence of Cand1. The neddylation reaction was carried out at room temperature for 30 minutes with Cand1 and Cul1 being incubated together for 15 minutes prior addition of the other components, lanes 4-6. Despite no interaction being seen in the GST-pull down assay in panel A, there is a clear effect seen on Cul1 neddylation. Typically a positive result in a GST-pull down requires a strong protein-protein interaction, but it does not mean that no interaction is present.

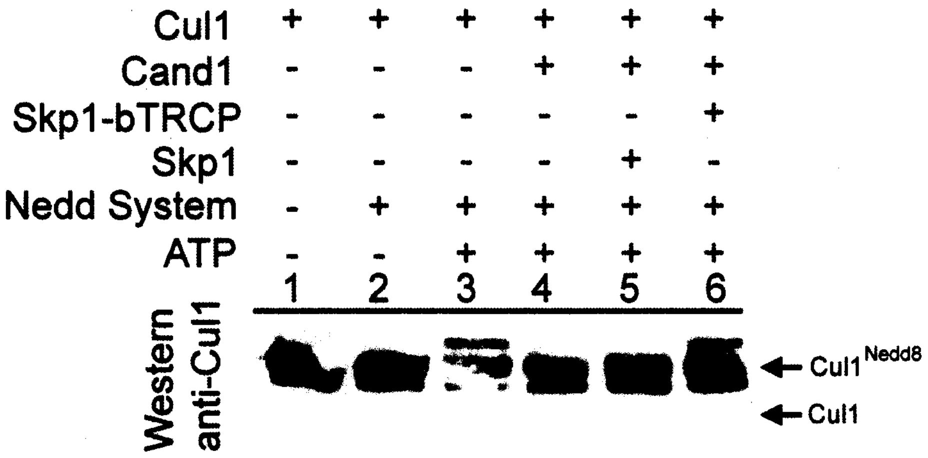

Figure 3.6. Cullin-substrate adaptor proteins are required for relief of Cand1 inhibition on Cullins, analysis Cul1-neddylation in the presence of β-TRCP. β -TRCP1 restored neddylation of full-length Cul1, bacterially expressed, in the presence of Cand1. The neddylation reaction was carried out at room temperature for 30 minutes with Cand1 and Cul1 being incubated together for 15 minutes prior addition of the other components, lanes 4-6.

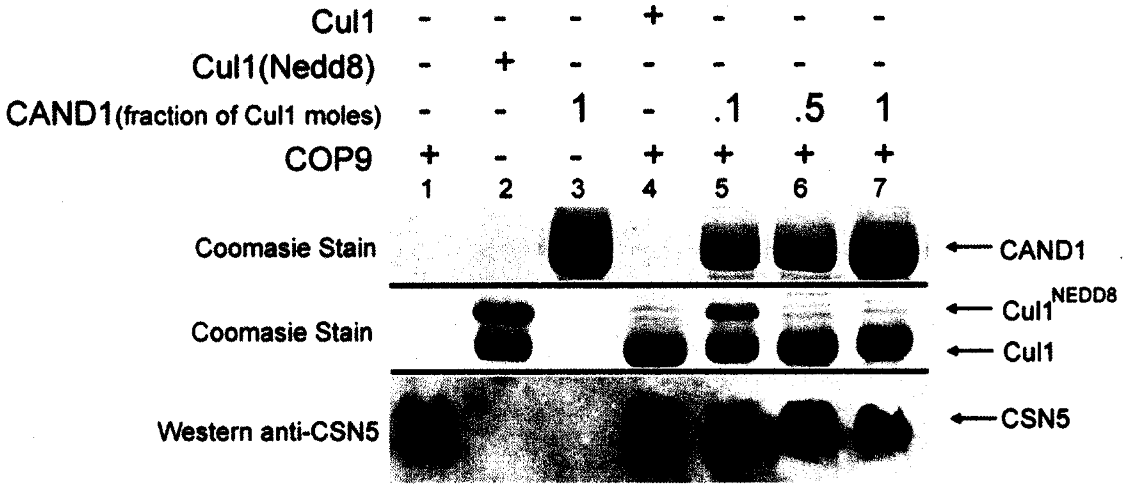

Figure 3.7. De-neddylation of Cul1-Nedd8 by the CSN5 subunit of COP9 in the presence and absence of CAND1. Lane 1 contains purified COP9 alone, as shown by western against the CSN5 component responsible for removal of Nedd8 from Cul1. Lane 2 contains Neddylated-Cul1 that has been purified by anion exchange and size exclusion chromatography to remove all neddylation system components or other contaminating proteins. Lane 3 contains purified Cand1. Lane 4 shows that COP9 has no effect on Cul1 protein that has not been neddylated. COP9 does not remove Nedd8 from Cul1 efficiently with low molar rations of Cand1-Cul1, as seen in Lane 5. The assays in lanes 6 and 7 were carried out in the same manner as the assay in lane 5 with increasing amounts of Cand1. The removal of Nedd8 from Cul1 is clearly seen with the 8kd drop in Cul1 protein. All de-neddylation reactions were carried out for 30 minutes at room temperature.

Chapter 4

Identification of Novel Factors in Cul3 based Ubiquitination

Introduction

Ubiquitin-dependent proteolysis controls protein stability and thus plays a crucial role in numerous biological processes. Targeting a protein for degradation by the ubiquitin pathway requires the successive action of the ubiquitin-activating enzyme (UAE), ubiquitin-conjugating enzyme (UBC), and ubiquitin-ligase (UBL) (Hershko & Ciechanover, 1998). Together these enzymes work together to covalently link a poly-ubiquitin chain to a protein, which then allows for that protein to be recognized by the proteasome for destruction. The last step in the cascade, the UBL ligases, are the most significant constituent of the cascade as they bring all the components together to allow for ubiquitination to occur (Pickart, 2001).

The best-characterized multi-subunit UBL-ligase is the SCF (Skp1-Cul1-F-box protein) complex. It is involved in the ubiquitination of several key cellular regulators (Deshaies, 1999). Another member of the Cullin UBL family is Cul3, which was recently identified to be associated with BTB-domain containing proteins that act as substrate adaptors. The BTB-domain is structurally homologous to Skp1, while sharing low sequence homology. In contrast to the SCF system, the BTB-domain containing proteins fulfill the role of Skp1 and the F-box proteins simultaneously. The best characterized role for this Cul3-BTB system is the degradation of the transcription factor Nrf2.

Molecular mechanism activating Nrf2

The blocking of genotoxic damage from outside sources is the first step in prevention of cancer. This can be achieved either by reducing the formation of reactive carcinogenic species or stimulating their detoxification. Many xenobiotic metabolizing enzymes are involved in both phase-I (oxidation and reduction) and phase-II biotransformation (conjugation) reactions (Itoh *et al*, 1999a; Kensler *et al*, 2000; Kong *et al*, 2001; Kwak *et al*, 2001; Owuor & Kong, 2002). In general, carcinogen activation takes place primarily during phase I metabolism that cytochrome P450 mono-oxygenases are responsible for. The phase II reactions counter the harmful actions of phase-I enzymes by reducing the electrophilicity of reactive carcinogens through enzymatic conjugation with endogenous ligands such as glutathione and glucuronic acid.

Genomic analysis have revealed that the cis-acting ARE [5′-(G/A)TGA(G/C)nnnGC(G/A)-3′], a specific DNA-promoter-binding region that can be transcriptionally activated by numerous antioxidants and/or electrophiles, exists in the 5′ flanking region of genes encoding phase-II detoxifying enzymes (Motohashi *et al*, 1997; Chen & Kong, 2004; Xu *et al*, 2005). The transcriptional activation of ARE is induced by various chemical compounds, including Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, mercaptans, trivalent arsenicals, heavy metals, and dithiolethiones. Since structurally diverse groups of chemicals are able to activate ARE, it seems unlikely that a receptor-ligand binding mechanism would be involved in bridging the chemical inducers to ARE. In the course of unraveling molecular mechanisms underlying ARE activation, a nuclear transcription factor Nrf2 and the

Kelch-like ECH-associated protein 1 (Keap1), were identified to be involved in transmitting the inducer-generated signals to ARE through oxidation and covalent modification of the thiol groups of Keap1 (Itoh *et al*, 1999a; Itoh *et al*, 1999b; T'Jampens *et al*, 2002; Itoh *et al*, 2003; McMahon *et al*, 2003).

Besides direct oxidation or covalent modification of thiol groups of Keap1, the Nrf2–Keap1–ARE signaling can be modulated by post-transcriptional modification of Nrf2. Phosphorylation of Nrf2 by phosphatidylinositol 3-kinase (PI3K) (Nakaso *et al*, 2003), protein kinase C (PKC) (Huang *et al*, 2000; Bloom & Jaiswal, 2003), and c-Jun NH2-terminal kinase (JNK) facilitate the release of Nrf2 from Keap1 and subsequent translocation (Keum *et al*, 2003). PKC directly phosphorylates Nrf2 at Serine 40 thereby promoting its dissociation from Keap1 (Huang *et al*, 2002). In response to oxidative stress, the activation of signaling cascades mediated by PI3K results in depolymerization of actin microfilaments thereby facilitating Nrf2 translocation to the nucleus (Nakaso *et al*, 2003; Pischke *et al*, 2005), which can be pharmacologically inhibited by targeting PI3K. MAP kinases, such as ERK, JNK, and p38, are also considered to be involved in the ARE activation with some evidence of activation via Nrf2–ARE (Nakaso *et al*, 2003; Zipper & Mulcahy, 2000).

Keap1 enhances Nrf2 degradation by the proteasome via Cullin3-based UBL

The treatment of cells with ARE inducers causes dissociation of Nrf2 from Keap1, allowing Nrf2 to translocate to the nucleus, heterodimerize with small Maf, and bind to ARE, eventually resulting in transcriptional regulation of target genes. It is noticeable that murine Keap1 contains 25 cysteine residues that are conserved in human and rat homologues, and murine Nrf2 contains 7 cysteine residues (Hayes & McMahon, 2001; Itoh *et al*, 2003). Some phase II enzyme inducers may interact with these thiol groups to oxidize or covalently modify them (Dinkova-Kostova *et al*, 2005b). Such modification of cysteine thiols of Keap1, Nrf2, or both may cause disruption of the Nrf2–Keap1 complex leading to dissociation of Nrf2 from Keap1. According to a model of the Nrf2–Keap1 interaction, Keap1 binds to the actin cytoskeleton via Kelch repeats in the C-terminus of the protein and dimerizes with another Keap1 molecule through their respective BTB domains (Dinkova-Kostova *et al*, 2005a). Nrf2 is then anchored in the cytoplasm through interaction between its Neh2 domain and Kelch regions of the dimerized Keap1 proteins. Furthermore, a critical role of two cysteine (C273 and C288) residues of Keap1 for the stability of the Nrf2–Keap1 complex has been demonstrated (Zhang & Hannink, 2003). Inducers of the ARE-driven genes covalently modify or oxidize cysteine thiol groups in the IVR region of Keap1. This results in conformational changes of Keap1, which then becomes unable to bind to Nrf2, thereby facilitating nuclear translocation of Nrf2. The sequestered Nrf2 is subjected to degradation through the ubiquitin–proteasome pathway as observed in the cases of other transcriptional regulators (Sekhar *et al*, 2002; Itoh *et al*, 2003). Once phase-II enzyme inducers stimulate cells, Nrf2 sequestered in the cytosol compartment is liberated and becomes stabilized against degradation. The liberated Nrf2 also undergoes proteasomal degradation, but the turnover rate is significantly reduced. Therefore, the Nrf2 protein turnover is regulated through Keap1-mediated subcellular compartmentalization.

The exact manner in which BTB-proteins interact with Cul3 is unknown; given the number of BTB proteins predicted to interact with Cul3 a crystal structure would serve as a crucial model for all other BTB proteins as they are shown to be Cul3 adaptor proteins. Also, as this system is new within the ubiquitin field new factors are being identified at a rapid pace. Having a full-recombinant and functional Cul3-based ubiquitination would allow for biochemical confirmation of the role for novel factors identified via genetic or proteomic approaches.

Cul3-based ubiquitination in the Wnt-β-catenin Pathway

Due to recent work between others and myself (Angers et al, 2006) Cul3 based-ubiquitination was shown to negatively regulate the Wnt-β-catenin. The Wnt-β-catenin is crucial signaling pathway for early development and in adults. Loss of regulation within the Wnt-β-catenin has been seen in several cancers and developmental diseases (Wong *et al*, 2001). Wnt is an extracellular signal that interacts with Frizzled receptors. Under non-frizzle stimulated conditions β-catenin is constitutively degraded by the multi-subunit ubiquitin ligase made up of Skp1, Cul1, and the F-box protein β-TRCP. When WNT interacts with Frizzled receptors it activates Dishevelled (Dsh) and β-catenin is no longer destroyed. β-catenin can then go into the nucleus and carry out its role as a transcription factor (Wodarz et al, 1998). Work carried out in collaboration with Stephane Angers (Angers et al, 2006) helped address the mechanism of Dishevelled's function of β-catenin regulation.

Materials and Methods

Protein Overexpression and Purification

Full-length murine Nrf2 and the Keap1 binding domain Neh2 were over-expressed as a Glutathione-S-Transferase (GST)-fusion protein in E. coli in the pCool expression vector, which has a thrombin site between the GST-tag and the protein. Full-length murine Nrf2 was also over-expressed in a 6x-Histidine-Taged-expression vector pAL. The PCR products were digested with NdeI and NotI and ligated into the NdeI–NotI-digested pCool or pAL. Keap1 was initially over-expressed in pCool as well, but was then seen to have an internal thrombin site so was then put into the pGev expression vector, which has a TEV site between the GST-tag and protein. The PCR products were digested with EcoRI and NotI and ligated into the EcoRI–NotI-digested pGEV. All proteins were isolated from the soluble cell lysate by glutathione affinity chromatography. Nrf2 and Neh2's tags were cleaved by thrombin while Keap1's GST tag was cleaved by TEV. After cleavage the proteins were further purified by anion exchange and gel filtration chromatography and concentrated by ultra-filtration. Cul3 was over-expressed as a split protein, in a similar fashion to Cul1 as previously described (Zheng *et al*, 2002). FLJ was over-expressed in pGEX ampicillin resistant vector and purified as a GST-fusion protein.

Human Dishevelled was purified from Hi-5 insect cells on 15cm plates using a baculovirus expression vector with a His-tag (Wenqing Xu, unpublished). Dishevelled was isolated from the soluble cell lysate by nickel affinity chromatography and dialyzed overnight against 50mM Tris 8.0 and 200mM NaCl to remove the imidiazole from 6x-histidine tag purification that may disrupt protein-protein interactions in future assays. Human ubiquitin UAE activating enzyme and UBC (UbcH5b) were purified by affinity chromatography, anion exchange, and gel filtration.

Human Kelch12 was over-expressed as a 6-Histidine-fusion protein under chloramphenicol resistance. To make the protein soluble it was co-expressed with Cul3's N-terminus, which was over-expressed in pCool. Both proteins were isolated from the soluble cell lysate by glutathione affinity chromatography.

Gel Filtration Shift Assay

In order to determine if proteins interact, the elution profile of a protein on a gel filtration column can be examined. If the proteins interact they will migrate to a different elution position on the column indicative of a larger molecular weight. Previously purified Keap1 and Neh2 were added to gel filtration column (22ml) alone or together. Elutions were done in elution buffer (20mM Tris, 200mM NaCl, 5mM DTT) at a rate of 0.5ml/min. Proteins were detected by UV absorbance and visualized with Coomasie.

GST-pull down assay

GST-fused wild type Cul3 proteins were co-purified with mutant and wild-type Kelch12 (100 μg) and bound to glutathione sepharose 4B beads (100 μl). They were incubatedwith Disheveled (100 μg) 1 hour at 4°C. Supernatant, containing unbound proteins, was removed after centrifugation. The beads were then washed three times with

500 μl of wash buffer containing 20mM Tris-HCl pH=8.0, 200mM NaCl, 5mM DTT. The resin bound proteins were eluted with 50mM Tris-HCl pH=8.0, 200mM NaCl, and 10mM reduced glutathione. A fraction of the eluted proteins was separated by SDS-PAGE and visualized by Coomassie staining. GST-FLJ bound to glutathione sepharose 4B was also used to bind Keap1 in a similar fashion.

In vitro ubiquitination assay

Purified Cul3-Roc1-Keap1 complex (8μg) was mixed with UAE (10ng), UbcH5b (0.8μg), ubiquitin, and Nrf2 in a buffer system containing 20mM Tris-HCl pH=8.0, 200mM NaCl, 5mM dithiothretiol, 1mM MgCl2, and 5mM ATP. After one-hour incubation, SDS-PAGE loading buffer was added to stop the reaction. The sample was subsequently analyzed by SDS-PAGE.

Results and Discussion

Reconstitution of Cul3 based Nrf2-Ubiquitination

In order to better study Cul3-based ubiquitination it is crucial to re-constitute ubiquitination of a Cul3 substrate. As Nrf2 ubiquitination is the best-understood substrate of Cul3 to date this target was chosen. Cul3 was expressed in a similar fashion to Cul1 (Zheng *et al*, 2002), with two fragments being co-expressed one of which is GST-tagged, both fragments are then isolated by affinity purification with Cul3 re-assembling due to hydrophobic interactions between helical-motifs.

Keap1 localizes to the soluble portion of the cell lysate only when the first 50 amino acids were not included in the final construct, making the Keap1(N2C1) construct. As seen in Figure 4.1 the first 50 residues are not conserved nor do they contain predicted structure. However, to ensure that this truncated protein still interacted with Nrf2 a gel filtration shift assay was carried out.

Proteins elution profile on a gel filtration column can be examined in order to determine if proteins interact. If the proteins interact they will migrate to a different elution position on the column indicative of a larger molecular weight, if they do not interact then they will come down at elution positions as when they are run alone. The elution profiles of Keap1, Nrf2's Keap1 binding domain (Neh2) (Itoh *et al*, 1999b), and Keap1with Neh2 were examined. Alone, Keap1 and Neh2 are eluted in range corresponding to their predicted molecular weights. However, when combined and passed over the gel filtration column together they elute at a molecular weight larger than either one alone (Figure 4.2).

Once assuring that the Keap1 N-terminal truncation mutant can interact with Nrf2 it I attempted to ubiquitinate full-length Nrf2 fused to a 6x-His tag. To ensure that Cul3 and Keap1 were assembled in their native confirmation they were mixed together and run over anion exchange columns and gel filtration columns. Only those fractions that contained both proteins were used for the Nrf2 ubiquitination assay. All components for the ubiquitination system (Cul3-Keap1, Nrf2, Ubch5b, UAE) were incubated at room temperature for 30min and analyzed via western blot against the 6x-His tag on Nrf2 (Figure 4.3). The change in molecular weight of Nrf2, due to ubiquitination, required UAE, UBC, and UBL. If any component of the ubiquitination cascade was missing then modification of Nrf2 did not occur. This result confirmed previous results showing that Nrf2 ubiquitination is dependent on a Cul3-Keap1 multi-subunit ubiquitin ligase.

Degradation of DSH by Cul3 based ubiquitination via BTB-adaptor proteins

With a functional Cul3-based ubiquitination assay we were now able to determine if Cul3 could degrade other substrates and if any novel proteins were mediators of Cul3-based ubiquitination. As part of a collaboration where novel Cul3 proteins were identified using unique proteomics approaches (Angers *et al*, 2006) we demonstrated that Cul3 in conjunction with a novel BTB-adaptor protein, Kelch12, could degrade Disheveled.

The first step in this process was to show that Disheveled could interact with Cul3 via the adaptor protein Kelch12. To do this the N-terminus of Cul3 was co-expressed with a wild type Kelch12 and a mutant that lacked the last Kelch repeat (Figure 4.4). Disheveled clearly interacts with full-length Kelch12 bound to Cul3-NTD as evaluated by GST-pull down assay. Without the complete Kelch repeat motif binding with Disheveled is abrogated. Once having established that can bind to Kelch12-Cul3 directly, an ubiquitination assay was carried out with full-length Cul3-Kelch12 being pulled down from stable HEK293 cells over-expressing Flag-Cul3-Kelch12. Recombinant Disheveled, UAE, and Ubch5B (UBC) were added to these components and ubiquitination of Disheveled was seen (Figure 5a Angers *et al*, 2006).

Identification of Potential Modulators on Cul3 dependent Ubiquitination

Through a continued collaboration additional components of ubiquitination pathways were identified. Tandem-affinity purification (TAP) of known ubiquitin targets by our collaborator Ben Major using the TAP method used by Angers et al (2006) revealed many new targets. One of these proteins, FLJ, was shown to interact with machinery of unique cullin-ubiquitination pathways. Overexpression of FLJ in HEK293 cells and isolating associated proteins with the two-tag system, which limits non-specific results in the following mass spectrometry assay, showed interesting results. These mass spec results identified β-catenin, β-TRCP, and Nrf2 as interactors of FLJ. These proteins are associated with Cul1-based UBLs and Cul3-based UBLs. These preliminary results were interesting, but it was possible that they were interacting via a common component or non-specific interactions. To confirm this we purified recombinant FLJ as a GST-fusion protein and immobilized it as bait protein on glutathione-sepharose beads. Recombinant Cul1, Cul3, β-catenin, β-TRCP, Nrf2, and Keap1 were then mixed with the immobilized FLJ, washed to remove unbound proteins, and detected via western blot (Figure 4.5). In both cases the substrate and substrate-adaptor proteins directly interacted with FLJ. However, the cullin proteins did not interact directly with FLJ. Cul1 and Cul3 were not detected in the eluted samples, while they were present in the inputs samples. Keap1, Nrf2, β-catenin, and β-TRCP were detected in both the inputs and elutes of the FLJ pull-downs. Equal loading of samples was shown by western blot against the vsv tag on FLJ. This showed that the eluted samples all contained equivalent amounts of FLJ. These recombinant protein results confirm the TAP-tag/mass spectrometry data and indicate that FLJ may be a novel factor of Cullin-based ubiquitination.

Conclusion

The modular nature of cullin based UBLs allows them to regulate numerous substrates. It is also crucial that the degradation of these substrates is tightly controlled. Nrf2 is degraded by a novel cullin substrate-adaptor protein, Keap1, which now serves as the prototype for all BTB-domain containing proteins that interact with Cul3. Reconstitution of this assay using all E. Coli derived protein confirms previous genetic results. The number of BTB-domain containing proteins identified by sequence now exceeds 60 in humans and will likely continue to be identified in the future. The complexity of cullin based ubiquitination is also shown by the continued identification of direct interactors that span different cullin systems. While the exact role of FLJ has yet to be shown in recombinant assays, the fact that direct interactors of Cullin-based ubiquitination are still being identified demonstrates the complexity of this system as well as how much is left to be learned.

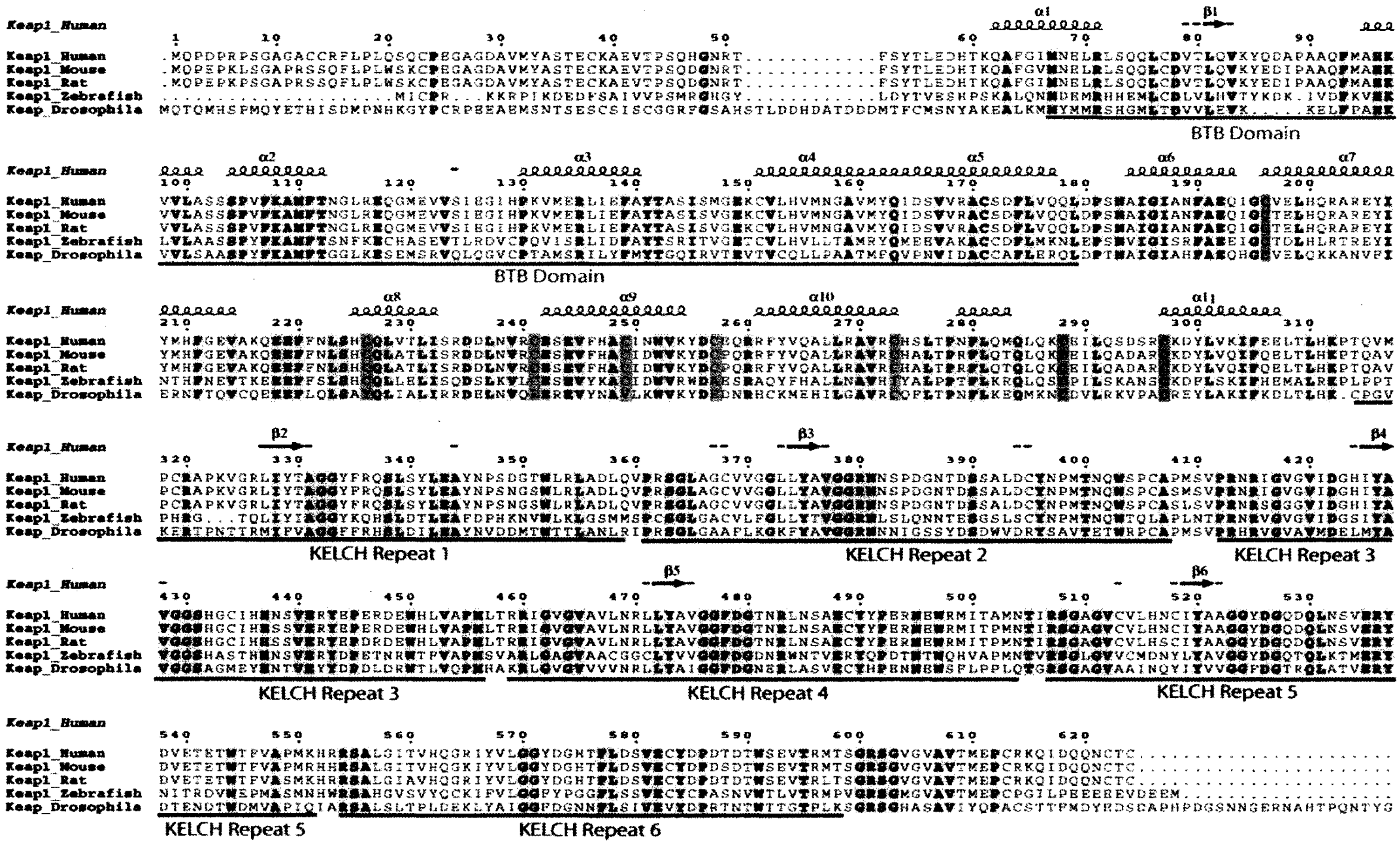

Figure 4.1. Alignment of Keap1 paralogues with BTB domain and KELCH repeats. Is important to note that not only are the BTB domain and Kelch domains are conserved in this family of proteins, but so is the domain between the BTB and Kelch repeats. This region has been implicated as being sensitive to oxiditation, which then causes changes in structure of the protein.

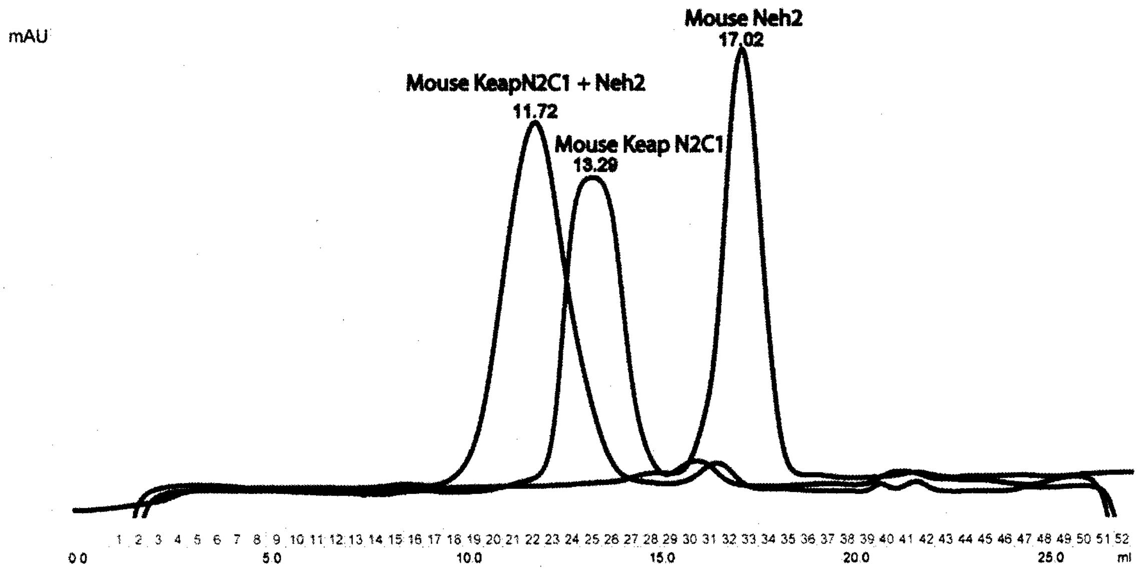

Figure 4.2. Gel Filtration of Keap1, Neh2, and Keap1-Neh2. The elution profile of Keap1(N2C1) alone and Neh2 alone were both in accordance with proteins of their predicted molecular weight. Together they shifted into earlier fractions, indicating that they are interacting. Proteins were run alone or together following a 30min incubation at 4°C. A superdex 200 gel filtration column was used with running buffer of 20mM Tris pH=8.0, 200mM NaCl, and 5mM DTT.

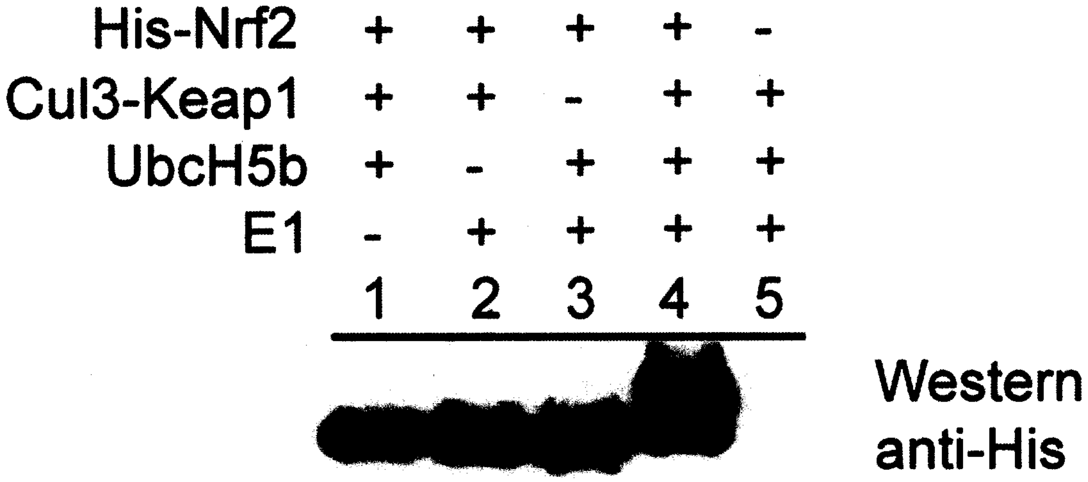

Figure 4.3. Ubiquitination of Nrf2 is Cul3 and Keap1 dependent. The ubiquitination of Nrf2 requires Cul3 and Keap1. Removing any component of the ubiquitin system abolishes the ubiquitination of Nrf2 and the antibody is specific to Nrf2 alone as seen in lane 5 where Nrf2 is not present.

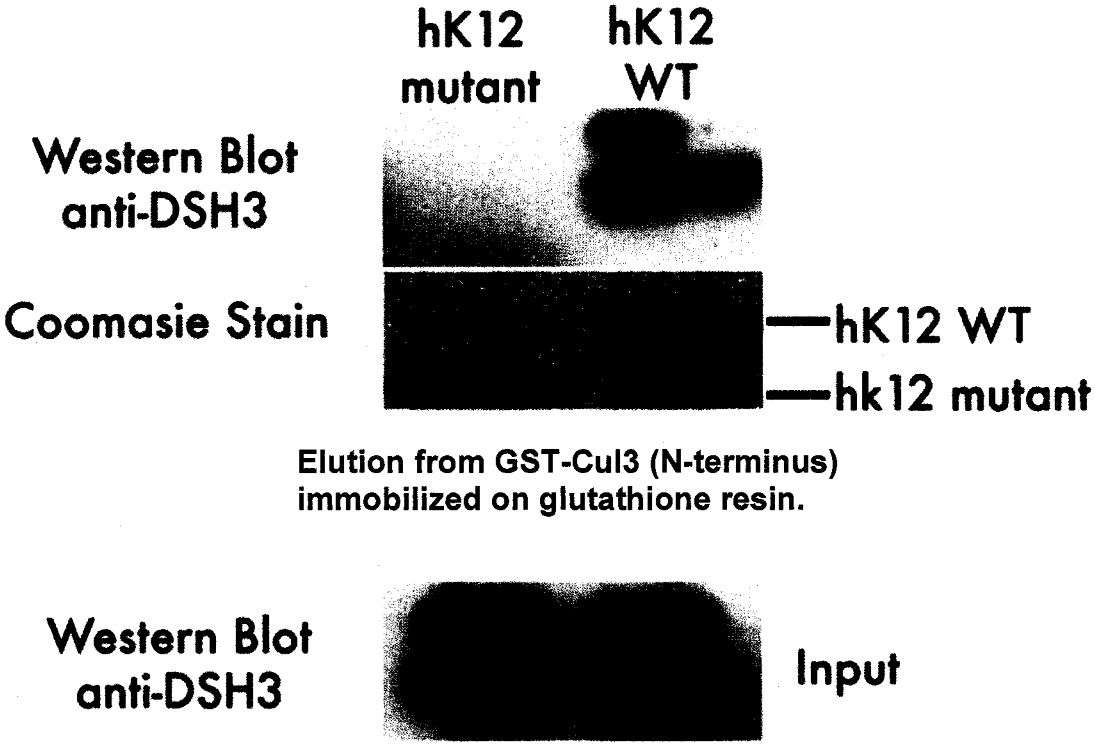

Figure 4.4. Binding of recombinant Disheveled-3 (DSH3) to wild-type Kelch12. Kelch12 was visualized with coomasie and the lower molecular weight of the Kelch repeat deletion mutation are clear. DSH3 is only associated with the full-length Kelch12.

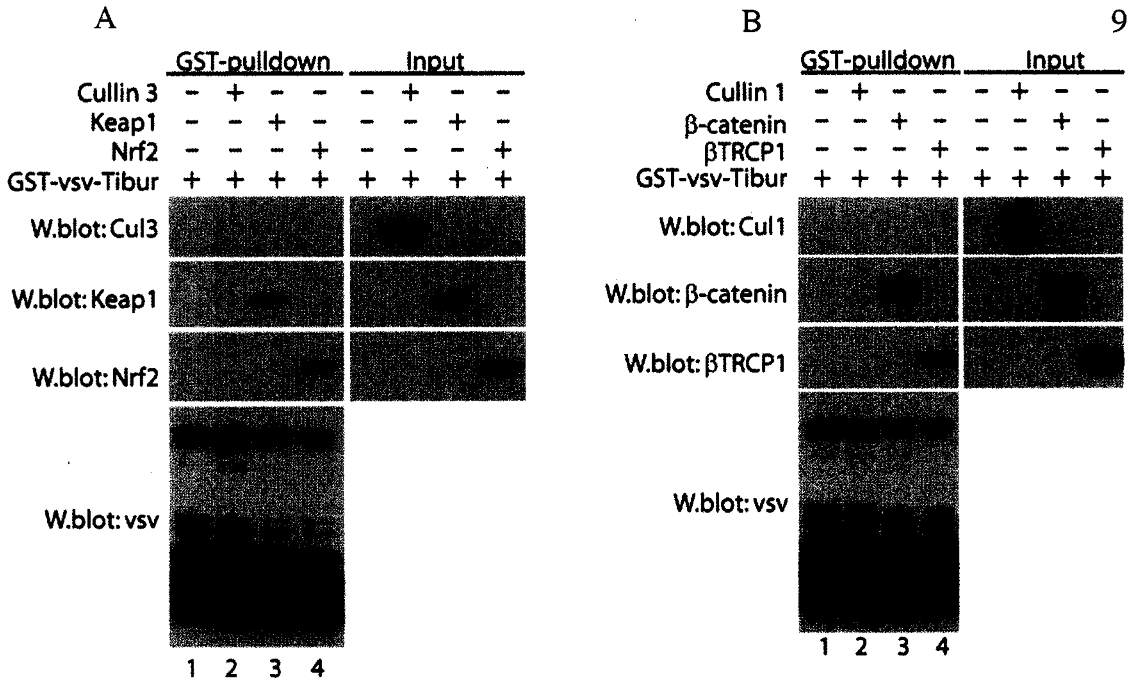

Figure 4.5. Interaction of FLJ with cullin-UBL machinery. GST-tagged FLJ based-pull-downs were done to determine the direct interactors of cullin-UBL machinery. Western blots were done against the specific proteins as well as the vsv-tag on FLJ. (A) Cullin-3 UBL components Keap1, Nrf2, and Cul3 were incubated with immobilized GST-FLJ. Unbound proteins were washed off and remaining proteins were eluted. Cul3 does not interact directly while Keap1 and Nrf2 do interact directly with FLJ. (B) Cullin-1 UBL components β-catenin, β-TRCP, and Cul1 were incubated with immobilized GST-FLJ. Unbound proteins were washed off and remaining proteins were eluted. Cul1 does not interact directly with FLJ while β-catenin and β-TRCP do. This was produced in collaboration with Ben Majors.

BIBLIOGRAPHY

Angers S, Thorpe CJ, Biechele TL, Goldenberg SJ, Zheng N, Maccoss MJ, Moon RT(2006) The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. *Nat Cell Biol* **8**: 348-357.
Blackwell TS, Christman JW(1997) The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* **17**: 3-9.

Bloom DA, Jaiswal AK(2003) Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J Biol Chem* **278**: 44675-44682.

Bordallo J, Plemper RK, Finger A, Wolf DH(1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9**: 209-222.

Borden KL(2000) RING domains: master builders of molecular scaffolds? *J Mol Biol* **295**: 1103-1112.

Bornstein G, Ganoth D, Hershko A(2006) Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. *Proc Natl Acad Sci U S A* .

Brooks CL, Gu W(2006) p53 Ubiquitination: Mdm2 and Beyond. *Mol Cell* **21**: 307-315.
Cardozo T, Pagano M(2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* **5**: 739-751.

Chen C, Kong AN(2004) Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radic Biol Med* **36**: 1505-1516.

Chen HY, Sun JM, Zhang Y, Davie JR, Meistrich ML(1998) Ubiquitination of histone H3 in elongating spermatids of rat testes. *J Biol Chem* **273**: 13165-13169.

Chen X, Zhang J, Lee J, Lin PS, Ford JM, Zheng N, Zhou P(2006) A Kinase-Independent Function of c-Abl in Promoting Proteolytic Destruction of Damaged DNA Binding Proteins. *Mol Cell* **22**: 489-499.

Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T(1995) Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev* **9**: 1586-1597.

Chuang HW, Zhang W, Gray WM(2004) Arabidopsis ETA2, an Apparent Ortholog of the Human Cullin-Interacting Protein CAND1, Is Required for Auxin Responses Mediated by the SCFTIR1 Ubiquitin Ligase. *Plant Cell*.

Ciechanover A(1998) The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J* **17**: 7151-7160.

Conaway RC, Brower CS, Conaway JW(2002) Emerging roles of ubiquitin in transcription regulation. *Science* **296**: 1254-1258.

Cope GA, Deshaies RJ(2006) Targeted silencing of Jab1/Csn5 in human cells downregulates SCF activity through reduction of F-box protein levels. *BMC Biochem* **7**: 1.
Cope GA, Suh GS, Aravind L, Schwarz SE, Zipursky SL, Koonin EV, Deshaies RJ(2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**: 608-611.

Deshaies RJ(1999) SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* **15**: 435-467.

Deshaies RJ, Chau V, Kirschner M(1995) Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *EMBO J* **14**: 303-312.

Dhananjayan SC, Ismail A, Nawaz Z(2005) Ubiquitin and control of transcription. *Essays Biochem* **41**: 69-80.

Dieterle M, Thomann A, Renou JP, Parmentier Y, Cognat V, Lemonnier G, Müller R, Shen WH, Kretsch T, Genschik P(2005) Molecular and functional characterization of Arabidopsis Cullin 3A. *Plant J* **41**: 386-399.

Dinkova-Kostova AT, Holtzclaw WD, Kensler TW(2005) The Role of Keap1 in Cellular Protective Responses. *Chem Res Toxicol* **18**: 1779-1791.

Dinkova-Kostova AT, Holtzclaw WD, Wakabayashi N(2005) Keap1, the Sensor for Electrophiles and Oxidants that Regulates the Phase 2 Response, Is a Zinc Metalloprotein. *Biochemistry* **44**: 6889-6899.

Duan L, Reddi AL, Ghosh A, Dimri M, Band H(2004) The Cbl family and other ubiquitin ligases: destructive forces in control of antigen receptor signaling. *Immunity* **21**: 7-17.

Elder RT, Song XQ, Chen M, Hopkins KM, Lieberman HB, Zhao Y(2002) Involvement of rhp23, a Schizosaccharomyces pombe homolog of the human HHR23A and Saccharomyces cerevisiae RAD23 nucleotide excision repair genes, in cell cycle control and protein ubiquitination. *Nucleic Acids Res* **30**: 581-591.

Feldman RM, Correll CC, Kaplan KB, Deshaies RJ(1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91**: 221-230.

Feng S, Shen Y, Sullivan JA, Rubio V, Xiong Y, Sun TP, Deng XW(2004) Arabidopsis CAND1, an Unmodified CUL1-Interacting Protein, Is Involved in Multiple Developmental Pathways Controlled by Ubiquitin/Proteasome-Mediated Protein Degradation. *Plant Cell* **16**: 1870-1882.

Fuller W, Cuthbert AW(2000) Post-translational disruption of the delta F508 cystic fibrosis transmembrane conductance regulator (CFTR)-molecular chaperone complex with geldanamycin stabilizes delta F508 CFTR in the rabbit reticulocyte lysate. *J Biol Chem* **275**: 37462-37468.

Geyer R, Wee S, Anderson S, Yates J, Wolf DA(2003) BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. *Mol Cell* **12**: 783-790.

Gmachl M, Gieffers C, Podtelejnikov AV, Mann M, Peters JM(2000) The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. *Proc Natl Acad Sci U S A* **97**: 8973-8978.

Goldenberg SJ, Cascio TC, Shumway SD, Garbutt KC, Liu J, Xiong Y, Zheng N(2004) Structure of the cand1-cul1-roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell* **119**: 517-528.

Gray WM, Hellmann H, Dharmasiri S, Estelle M(2002) Role of the Arabidopsis RING-H2 protein RBX1 in RUB modification and SCF function. *Plant Cell* **14**: 2137-2144.
Haas AL, Rose IA(1982) The Mechanism of Ubiquitin Activating Enzyme. *JBC* **257**: 10329-10337.

Haas AL, Warms JVB, Hershko A, Rose IA(1982) Ubiquitin Activating Enzyme. *JBC* **257**: 2543-2548.

Haldeman MT, Xia G, Kasperek EM, Pickart CM(1997) Structure and function of ubiquitin conjugating enzyme E2-25K: the tail is a core-dependent activity element. *Biochemistry* **36**: 10526-10537.

Hayes JD, McMahon M(2001) Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett* **174**: 103-113.

Hein C, André B(1997) A C-terminal di-leucine motif and nearby sequences are required for NH4(+)-induced inactivation and degradation of the general amino acid permease, Gap1p, of Saccharomyces cerevisiae. *Mol Microbiol* **24**: 607-616.

Hershko A, Ciechanover A(1998) The ubiquitin system. *Annu Rev Biochem* **67**: 425-479.
Hicke L(1997) Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J* **11**: 1215-1226.

Hipp MS, Raasi S, Groettrup M, Schmidtke G(2004) NEDD8 ultimate buster-1L interacts with the ubiquitin-like protein FAT10 and accelerates its degradation. *J Biol Chem* **279**: 16503-16510.

Hochstrasser M(1995) Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr Opin Cell Biol* **7**: 215-223.

Hu J, McCall CM, Ohta T, Xiong Y(2004) Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage. *Nat Cell Biol* **6**: 1003-1009.

Huang DT, Paydar A, Zhuang M, Waddell MB, Holton JM, Schulman BA(2005) Structural basis for recruitment of Ubc12 by an E2 binding domain in NEDD8's E1. *Mol Cell* **17**: 341-350.

Huang HC, Nguyen T, Pickett CB(2000) Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc Natl Acad Sci U S A* **97**: 12475-12480.
Huang HC, Nguyen T, Pickett CB(2002) Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem* **277**: 42769-42774.

Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM, Pavletich NP(1999) Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**: 1321-1326.

Huibregtse JM, Scheffner M, Howley PM(1993) Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Mol Cell Biol* **13**: 4918-4927.

Hwang JW, Min KW, Tamura TA, Yoon JB(2003) TIP120A associates with unneddylated cullin 1 and regulates its neddylation. *FEBS Lett* **541**: 102-108.
Itoh K, Ishii T, Wakabayashi N, Yamamoto M(1999) Regulatory mechanisms of cellular response to oxidative stress. *Free Radic Res* **31**: 319-324.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M(1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* **13**: 76-86.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, Yamamoto M(2003) Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* **8**: 379-391.

Jackson PK, Eldridge AG(2002) The SCF ubiquitin ligase: an extended look. *Mol Cell* **9**: 923-925.

Kamitani T, Kito K, Fukuda-Kamitani T, Yeh ET(2001) Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1. *J Biol Chem* **276**: 46655-46660.

Kamura T, Conrad MN, Yan Q, Conaway RC, Conaway JW(1999) The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev* **13**: 2928-2933.

Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, Iliopoulos O, Lane WS, Kaelin WG, Elledge SJ, Conaway RC, Harper JW, Conaway JW(1999) Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* **284**: 657-661.

Karin M, Ben-Neriah Y(2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18**: 621-663.

Kensler TW, Curphey TJ, Maxiutenko Y, Roebuck BD(2000) Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiins. *Drug Metabol Drug Interact* **17**: 3-22.

Keum YS, Owuor ED, Kim BR, Hu R, Kong AN(2003) Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element (ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC). *Pharm Res* **20**: 1351-1356.

Kim I, Rao H(2006) What's Ub Chain Linkage Got to Do with It? *Sci STKE* **2006**: pe18.
Kitagawa M, Hatakeyama S, Shirane M, Matsumoto M, Ishida N, Hattori K, Nakamichi I, Kikuchi A, Nakayama K(1999) An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J* **18**: 2401-2410.

Kito K, Yeh ET, Kamitani T(2001) NUB1, a NEDD8-interacting protein, is induced by interferon and down-regulates the NEDD8 expression. *J Biol Chem* **276**: 20603-20609.
Klapisz E, Sorokina I, Lemeer S, Pijnenburg M, Verkleij AJ, van Bergen en Henegouwen PM(2002) A ubiquitin-interacting motif (UIM) is essential for Eps15 and Eps15R ubiquitination. *J Biol Chem* **277**: 30746-30753.

Kominami K, Ochotorena I, Toda T(1998) Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero- and homo-complexes together with cullin-1 in the fission yeast SCF (Skp1-Cullin-1-F-box) ubiquitin ligase. *Genes Cells* **3**: 721-735.

Kong AN, Yu R, Hebbar V, Chen C, Owuor E, Hu R, Ee R, Mandlekar S(2001) Signal transduction events elicited by cancer prevention compounds. *Mutat Res* **480-481**: 231-241.

Kong SK, Chock PB(1992) Protein ubiquitination is regulated by phosphorylation. An in vitro study. *J Biol Chem* **267**: 14189-14192.

Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K, Yamamoto M, Kensler TW(2001) Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat Res* **480-481**: 305-315.

Layfield R, Tooth D, Landon M, Dawson S, Mayer J, Alban A(2001) Purification of poly-ubiquitinated proteins by S5a-affinity chromatography. *Proteomics* **1**: 773-777.

Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B, Yarden Y(1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12**: 3663-3674.

Liakopoulos D, Doenges G, Matuschewski K, Jentsch S(1998) A novel protein modification pathway related to the ubiquitin system. *EMBO J* **17**: 2208-2214.
Lin D, Tatham MH, Yu B, Kim S, Hay RT, Chen Y(2002) Identification of a substrate recognition site on Ubc9. *J Biol Chem* **277**: 21740-21748.

Lo SC, Hannink M(2006) CAND1-Mediated Substrate Adaptor Recycling Is Required for Efficient Repression of Nrf2 by Keap1. *Mol Cell Biol* **26**: 1235-1244.
Lord JM, Roberts LM, Stirling CJ(2005) Quality Control: Another Player Joins the ERAD Cast. *Curr Biol* **15**: R963-R964.

Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Wei N, Deshaies RJ(2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**: 1382-1385.

McMahon M, Itoh K, Yamamoto M, Hayes JD(2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* **278**: 21592-21600.

Meek DW(2004) The p53 response to DNA damage. *DNA Repair (Amst)* **3**: 1049-1056.

Meusser B, Hirsch C, Jarosch E, Sommer T(2005) ERAD: the long road to destruction. *Nat Cell Biol* **7**: 766-772.

Min KW, Hwang JW, Lee JS, Park Y, Tamura TA, Yoon JB(2003) TIP120A associates with cullins and modulates ubiquitin ligase activity. *J Biol Chem* **278**: 15905-15910.

Miyake S, Lupher ML, Druker B, Band H(1998) The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. *Proc Natl Acad Sci U S A* **95**: 7927-7932.

Mori S, Claesson-Welsh L, Okuyama Y, Saito Y(1995) Ligand-induced polyubiquitination of receptor tyrosine kinases. *Biochem Biophys Res Commun* **213**: 32-39.

Mori S, Heldin CH, Claesson-Welsh L(1992) Ligand-induced polyubiquitination of the platelet-derived growth factor beta-receptor. *J Biol Chem* **267**: 6429-6434.

Mori S, Heldin CH, Claesson-Welsh L(1993) Ligand-induced ubiquitination of the platelet-derived growth factor beta-receptor plays a negative regulatory role in its mitogenic signaling. *J Biol Chem* **268**: 577-583.

Morimoto M, Nishida T, Honda R, Yasuda H(2000) Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). *Biochem Biophys Res Commun* **270**: 1093-1096.

Morimoto M, Nishida T, Nagayama Y, Yasuda H(2003) Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability. *Biochem Biophys Res Commun* **301**: 392-398.

Motohashi H, Shavit JA, Igarashi K, Yamamoto M, Engel JD(1997) The world according to Maf. *Nucleic Acids Res* **25**: 2953-2959.

Murray A(1995) Cyclin ubiquitination: the destructive end of mitosis. *Cell* **81**: 149-152.
Nakaso K, Yano H, Fukuhara Y, Takeshima T, Wada-Isoe K, Nakashima K(2003) PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. *FEBS Lett* **546**: 181-184.

Nakayama K, Nagahama H, Minamishima YA, Matsumoto M, Nakamichi I, Kitagawa K, Shirane M, Tsunematsu R, Tsukiyama T, Ishida N, Kitagawa M, Hatakeyama S(2000) Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J* **19**: 2069-2081.

Nakayama KI, Nakayama K(2006) Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6**: 369-381.

Ortolan TG, Tongaonkar P, Lambertson D, Chen L, Schauber C, Madura K(2000) The DNA repair protein rad23 is a negative regulator of multi-ubiquitin chain assembly. *Nat Cell Biol* **2**: 601-608.

Osaka F, Kawasaki H, Aida N, Saeki M, Chiba T, Kawashima S, Tanaka K, Kato S(1998) A new NEDD8-ligating system for cullin-4A. *Genes Dev* **12**: 2263-2268.

Osaka F, Saeki M, Katayama S, Aida N, Toh-E A, Kominami K, Toda T, Suzuki T, Chiba T, Tanaka K, Kato S(2000) Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *EMBO J* **19**: 3475-3484.

Oshikawa K, Matsumoto M, Yada M, Kamura T, Hatakeyama S, Nakayama KI(2003) Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. *Biochem Biophys Res Commun* **303**: 1209-1216.

Owuor ED, Kong AN(2002) Antioxidants and oxidants regulated signal transduction pathways. *Biochem Pharmacol* **64**: 765-770.

Page AM, Hieter P(1999) The anaphase-promoting complex: new subunits and regulators. *Annu Rev Biochem* **68**: 583-609.

Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, Park M(2001) Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell* **8**: 995-1004.

Peters JM(1998) SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr Opin Cell Biol* **10**: 759-768.

Peters JM(2005) Cyclin degradation: don't mes(s) with meiosis. *Curr Biol* **15**: R461-R463.

Pickart CM(1997) Targeting of substrates to the 26S proteasome. *FASEB J* **11**: 1055-1066.

Pickart CM(2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**: 503-533.

Pintard L, Kurz T, Glaser S, Willis JH, Peter M, Bowerman B(2003) Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in C. elegans. *Curr Biol* **13**: 911-921.

Pintard L, Willems A, Peter M(2004) Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family. *EMBO J* **23**: 1681-1687.

Pintard L, Willis JH, Willems A, Johnson JL, Srayko M, Kurz T, Glaser S, Mains PE, Tyers M, Bowerman B, Peter M(2003) The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature* **425**: 311-316.

Pischke SE, Zhou Z, Song R, Ning W, Alam J, Ryter SW, Choi AM(2005) Phosphatidylinositol 3-kinase/akt pathway mediates heme oxygenase-1 regulation by lipopolysaccharide. *Cell Mol Biol (Noisy-le-grand)* **51**: 461-470.

Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW, Branton PE(2001) Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev* **15**: 3104-3117.

Read MA, Brownell JE, Gladysheva TB, Hottelet M, Parent LA, Coggins MB, Pierce JW, Podust VN, Luo RS, Chau V, Palombella VJ(2000) Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. *Mol Cell Biol* **20**: 2326-2333.

Sawasdikosol S, Pratt JC, Meng W, Eck MJ, Burakoff SJ(2000) Adapting to multiple personalities: Cbl is also a RING finger ubiquitin ligase. *Biochim Biophys Acta* **1471**: M1-M12.

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM(1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75**: 495-505.

Scheffner M, Nuber U, Huibregtse JM(1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**: 81-83.

Schulman BA, Carrano AC, Jeffrey PD, Bowen Z, Kinnucan ER, Finnin MS, Elledge SJ, Harper JW, Pagano M, Pavletich NP(2000) Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature* **408**: 381-386.

Schwechheimer C, Villalobos LI(2004) Cullin-containing E3 ubiquitin ligases in plant development. *Curr Opin Plant Biol* **7**: 677-686.

Sekhar KR, Yan XX, Freeman ML(2002) Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2. *Oncogene* **21**: 6829-6834.

Sigismund S, Polo S, Di Fiore PP(2004) Signaling through monoubiquitination. *Curr Top Microbiol Immunol* **286**: 149-185.

Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW(1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91**: 209-219.

Sommer T, Wolf DH(1997) Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J* **11**: 1227-1233.

Spence J, Gali RR, Dittmar G, Sherman F, Karin M, Finley D(2000) Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* **102**: 67-76.

Stebbins CE, Kaelin WG, Pavletich NP(1999) Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science* **284**: 455-461.

Stogios PJ, Downs GS, Jauhal JJ, Nandra SK, Privé GG(2005) Sequence and structural analysis of BTB domain proteins. *Genome Biol* **6**: R82.

Stogios PJ, Privé GG(2004) The BACK domain in BTB-kelch proteins. *Trends Biochem Sci* **29**: 634-637.

Swaminathan G, Tsygankov AY(2006) The Cbl family proteins: Ring leaders in regulation of cell signaling. *J Cell Physiol* .
T'Jampens D, Devriendt L, De Corte V, Vandekerckhove J, Gettemans J(2002) Selected BTB/POZ-kelch proteins bind ATP. *FEBS Lett* **516**: 20-26.

Tanaka K, Suzuki T, Chiba T(1998) The ligation systems for ubiquitin and ubiquitin-like proteins. *Mol Cells* **8**: 503-512.

Tanji K, Tanaka T, Kamitani T(2005) Interaction of NUB1 with the proteasome subunit S5a. *Biochem Biophys Res Commun* **337**: 116-120.

Tanji K, Tanaka T, Mori F, Kito K, Takahashi H, Wakabayashi K, Kamitani T(2006) NUB1 Suppresses the Formation of Lewy Body-Like Inclusions by Proteasomal Degradation of Synphilin-1. *Am J Pathol* **169**: 553-565.

Thien CB, Langdon WY(2005) c-Cbl and Cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses. *Biochem J* **391**: 153-166.
Urbé S(2005) Ubiquitin and endocytic protein sorting. *Essays Biochem* **41**: 81-98.
van Delft S, Govers R, Strous GJ, Verkleij AJ, van Bergen en Henegouwen PM(1997) Epidermal growth factor induces ubiquitination of Eps15. *J Biol Chem* **272**: 14013-14016.
van den Heuvel S(2004) Protein degradation: CUL-3 and BTB--partners in proteolysis. *Curr Biol* **14**: R59-R61.

Verdecia MA, Joazeiro CA, Wells NJ, Ferrer JL, Bowman ME, Hunter T, Noel JP(2003) Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Mol Cell* **11**: 249-259.

Wada H, Yeh ET, Kamitani T(1999) Identification of NEDD8-conjugation site in human cullin-2. *Biochem Biophys Res Commun* **257**: 100-105.

Wang S, Zheng H, Esaki Y, Kelly F, Yan W(2006) Cullin3 Is a KLHL10-Interacting Protein Preferentially Expressed During Late Spermiogenesis. *Biol Reprod* **74**: 102-108.

Ward CL, Omura S, Kopito RR(1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83**: 121-127.

Weber H, Bernhardt A, Dieterle M, Hano P, Mutlu A, Estelle M, Genschik P, Hellmann H(2005) Arabidopsis AtCUL3a and AtCUL3b form complexes with members of the BTB/POZ-MATH protein family. *Plant Physiol* **137**: 83-93.

Wei N, Deng XW(2003) The COP9 signalosome. *Annu Rev Cell Dev Biol* **19**: 261-286.
Wendland B, Emr SD(1998) Pan1p, yeast eps15, functions as a multivalent adaptor that coordinates protein-protein interactions essential for endocytosis. *J Cell Biol* **141**: 71-84.
Wilkins A, Ping Q, Carpenter CL(2004) RhoBTB2 is a substrate of the mammalian Cul3 ubiquitin ligase complex. *Genes Dev* **18**: 856-861.

Wilkinson KD(1999) Ubiquitin-dependent signaling: the role of ubiquitination in the response of cells to their environment. *J Nutr* **129**: 1933-1936.

Wodarz, A. & Nusse, R. Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14,** 59–88 (1998).

Wolf DA, Jackson PK(1998) Cell cycle: oiling the gears of anaphase. *Curr Biol* **8**: R636-R639.

Wolf DA, McKeon F, Jackson PK(1999) F-box/WD-repeat proteins pop1p and Sud1p/Pop2p form complexes that bind and direct the proteolysis of cdc18p. *Curr Biol* **9**: 373-376.

Wu JT, Lin HC, Hu YC, Chien CT(2005) Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation. *Nat Cell Biol* **7**: 1014-1020.

Xie Y, Varshavsky A(1999) The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. *EMBO J* **18**: 6832-6844.

Xiong X, Chong E, Skach WR(1999) Evidence that endoplasmic reticulum (ER)-associated degradation of cystic fibrosis transmembrane conductance regulator is linked to retrograde translocation from the ER membrane. *J Biol Chem* **274**: 2616-2624.

Xu C, Li CY, Kong AN(2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28**: 249-268.

Xu L, Wei Y, Reboul J, Vaglio P, Shin TH, Vidal M, Elledge SJ, Harper JW(2003) BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. *Nature* **425**: 316-321.

Yamasaki L, Pagano M(2004) Cell cycle, proteolysis and cancer. *Curr Opin Cell Biol* **16**: 623-628.

Yang Y, Li CC, Weissman AM(2004) Regulating the p53 system through ubiquitination. *Oncogene* **23**: 2096-2106.

Yee NS, Hsiau CW, Serve H, Vosseller K, Besmer P(1994) Mechanism of down-regulation of c-kit receptor. Roles of receptor tyrosine kinase, phosphatidylinositol 3'-kinase, and protein kinase C. *J Biol Chem* **269**: 31991-31998.

Zhang DD, Hannink M(2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol* **23**: 8137-8151.

Zheng N, Schulman BA, Song L, Miller JJ, Jeffrey PD, Wang P, Chu C, Koepp DM, Elledge SJ, Pagano M, Conaway RC, Conaway JW, Harper JW, Pavletich NP(2002) Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416**: 703-709.

Zheng N, Wang P, Jeffrey PD, Pavletich NP(2000) Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102**: 533-539.

Zheng N. (2003). Structure (Camb). In *A closer look of the HECTic ubiquitin ligases.*. United States: [UNKNOWN REFERENCE TYPE]

Appendix A

Crystallization Attempts of Keap1-Nrf2

In order to elucidate the mechanism by which Keap1-Nrf2 interacts to allow Nrf2 degradation via a Cul3-based UBL structural studies of these proteins were attempted. All proteins were purified to homogeneity by affinity, ion exchange, and gel filtration chromatography. The proteins were then concentrated to the following concentrations; Neh2 (2.3mg/ml), Keap1 (6.8mg/mg), Keap1-Neh2 (5.8mg/ml), Cul3-Keap1-Neh2 (5.4mg/ml). The protein mixtures were then used in crystallization via the hanging drop vapor diffusion method, in the same manner describe in chapter 2. Numerous commercial and in-house screens were attempted with these proteins as seen in Table A.1. 288 unique conditions were used to in crystallization attempts with most of the proteins at 4°C and room temperature (RT), bringing the unique conditions to 576. With Keap1-Neh2, Keap1, and Neh2 all being used in crystallization experiments as well as optimization based on the initial screens 1824 crystallization drops were attempted. While precipitation was seen in a few drops, further optimization produced no crystals.

The constructs used were all important for Keap1-Nrf2 interactions. Nrf2 interacts with Keap1 via its Neh2 domain, which was used in the crystallization experiments. Its smaller size had a higher probability of forming an ordered lattice crucial for crystallization to occur. The Keap1 protein, while truncated at the N-terminus to allow for solubility, contained both the Cul3 interacting BTB-domain and Neh2 interacting Kelch repeats. While it may have been possible do solve the structure of one of these

Keap1 domains it would not have allowed for interpretation towards its role in Cul3-based ubiquitination; therefore, it was not attempted.

Table A.1. Conditions and protein concentrations attempted towards Crystallization of Keapl-Nrf2$^{\mathbf{Neh2}}$-Cul3. The table above lists the screens that were carried out on proteins of the Cul3-Keapl-Nrf2^{Neh2} system. Each screen contains 48 unique conditions, unless otherwise noted. All proteins were produced in E. Coli and purified to homogeneity by affinity, ion exchange, and gel filtration chromatography. The protein concentrations were Neh2 (2.3mg/ml), Keapl (6.8mg/mg), Keapl-Neh2 (5.8mg/ml), Cul3-Keapl-Neh2 (5.4mg/ml). Al Keapl-Neh2 proteins were done twice, once by mixing separately purified proteins and once by purifying the proteins together off a gel-filtration column.

Screen	***Neh2***	***Keapl***	***Keapl-Neh2***	***Cul3-Keapl-Neh2***
Hampton Crystal Lite (4°C and RT)	X	X	X	
Hampton Crystal Screen(4°C and RT)	X	X	X	
Hampton Crystal Screen 2(4°C and RT)	X	X	X	
Nikola Screen(4°C and RT)	X	X	X	X
Wizard Screen 1(4°C and RT)	X	X	X	
Wizard Screen 2(4°C and RT)	X	X	X	
Optimizations based on above Screens				
100mM Tris, 200mM Li_2SO_4, 1.5M-3M $(NH_4)_2SO_4$: 24 Conditions			X	
200mM Li_2SO_4, 2M $(NH_4)_2SO_4$, 100mM -MES 6.5, -Hepes 7.0, -BTP 7.0, Tris 8.0: 24 Conditions			X	

Appendix B

Purification of Proteins involved in Neddylation of Cand1 bound Cul1

With the possibility of other cellular factors being involved in neddylation of Cand1-bound Cul1, cell lysate fractionation was carried out with the resulting fractions being added to the neddylation assay described above. If factors responsible for Cul1 neddylation were present in these cell lysate fractions then neddylated Cul1 could be detected, the lysate fraction would be purified further and additional neddylation assays carried out until homogeneity of a particular factor(s) was achieved. Pellets from HEK293 cell lysate were fractionated based on charge and size.

Cell pellets from 5L of HEK293 were re-suspended in 60ml lysis (25mMTris-HCl pH 8.0, 5% glycerol, .1% NP-40, 100mM,Na-Cl, 5mM Mg-Cl2, 1mM Ca-Cl2 with 1mM phenylmethylsulphonyl fluoride and complete protease inhibitors; diluted 1:1000). The cells were lysed via sonication (2x30sec) and spun at 20,000x g for 30 min to remove cellular debris. The supernatant was removed and loaded onto a column (3ml resin /L lysate) that has been pre-equilibrated. Elution was carried out and fractions were used in neddylation assays.

While preliminary results were promising no additional factors that enhanced Cand1-bound Cul1 neddylation could be purified to homogeneity. The first column cell lysates were passed over was a Q-sepharose column with stepwise elutions of increasing NaCl concentrations (Figure B.1). The cell lysate fractions that allowed Cul1 neddylation in the presence of Cand1 were then further purified over Phenyl Sepharose columns, Superdex 200 gel filtration columns, and Source-Q ion exchange columns. While the preliminary results were clear the following columns did not allow purification of any additional factors to homogeneity. Therefore, the initial fractions that allowed Cul1 neddylation in the presence of Cand1 from a Q-sepharose column were used for mass spectrometry analysis. These cell lysate fractions had only undergone a few steps of purification and many proteins were seen., including previously identified components of the SCF complex were isolated, such as Skp1 (data not shown). This led to the hypothesis that previously identified SCF components may be playing a role in Cul1 neddylation once it was bound to Cul1.

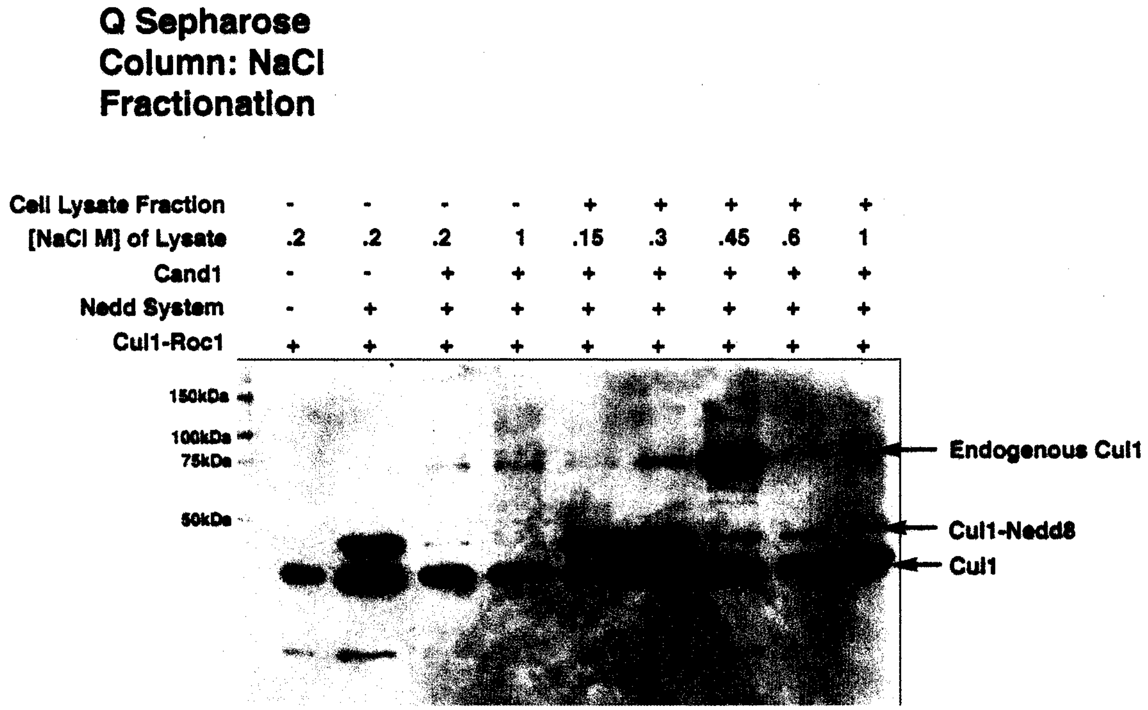

Figure B.1. Isolation of factors that allow Cul1 neddylation in the presence of Cand1. The GST-Cand1 proteins immobilized on glutathione sepharose 4B resin were incubated together for 1 hour on ice and underwent a series of three washes to remove unbound Cul1-Roc1. The GST-Cand1-Cul1-Roc1 was then incubated for 2 hours at 4°C with 1ml of cell lysate fraction from an 8ml Q-sepharose column that cell lysate was passed over. The cell lysate was made from the pellets of 5L of HEK293 cells. The cell lysate and GST-Cand1-Cul1-Roc1 then underwent a series of three washes to remove any unbound proteins and the neddylation system components were added. SDS-PAGE loading buffer was added to stop the reaction prior to western blot analyisis.

CURRIICULUM VITAE

SETH J. GOLDENBERG

EDUCATION

University of Washington ***Ph.D.**, 2006*	Seattle, WA
Drexel University ***MS** June 2002, Biomedical Science* ***BS** June 2002, Bioscience and Biotechnology*	Philadelphia, PA

PUBLICATIONS

Angers S, Thorpe CJ, Biechele TL, **Goldenberg SJ**, Zheng N, Maccoss MJ, Moon RT. (2006) The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. *Nat Cell Biol.* Apr;8(4):348-357.

Goldenberg SJ, Cascio TC, Shumway SD, Garbutt KC, Liu J, Xiong Y, Zheng N. (2004) Structure of the cand1-cul1-roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell.* Nov 12;119(4):517-28.

Soslau G, Wallace B, Vicente C, **Goldenberg SJ**, Tupis T, Spotila J, George R, Paladino F, Whitaker B, Violetta G, Piedra R. (2004) Comparison of functional aspects of the coagulation cascade in human and sea turtle plasmas. *Comp Biochem Physiol B Biochem Mol Biol.* Aug;138(4):399-406.

Soslau G, **Goldenberg SJ**, Class R, Jameson. (2004). Differential activation and inhibition of human platelet thrombin receptors by structurally distinct α-, β-, and γ-thrombin. *Platelets.* May;15(3):155-66.

Drake, DL, Hagerty, MA, Behm, JE, **Goldenberg, SJ**. and Spotila, JR. (2003) Marine turtle nesting at Playa Naranjo, Santa Rosa National Park, Costa Rica, during the 1998-99 season: *Chelonia Conservation and Biology*, v. 4, no. 3, p. 675-678.

Goldenberg SJ, Veriabo, NJ, Soslau, G (2001) A Micromethod To Measure Platelet Aggregation And ATP Release By Impedance. *Thrombosis Research.* July 1;103(1):57-61

Drake, DL, Hagerty, MA, Behm, JE, and **Goldenberg, SJ** (2001). Lepidochelys olivacea (Olive Ridley Sea Turtle) predation. *Herpetological Review.* 32(2): 104

PROFESSIONAL EXPERIENCE (1993-PRESENT)

June 2002-Present University of Washington Seattle, WA

Research Fellow, Graduate Program in Pharmacology

- Solved the x-ray crystal structure of CAND1-Cul1-Roc1. 220kD protein complex, first known inhibitor of Cullin-based E3 ubiquitin ligases. CAND1 is largest HEAT-repeat protein whose crystal structure has been solved to date.

- Collaborated with multiple laboratories around the world on structural and biochemical studies within the ubiquitin field.

- Developed fully recombinant protein modification assay to track isolation of novel factors that are crucial for ubiquitination machinery from human cell lysates.

1996-2002 Drexel Medical School Philadelphia, PA

Master's Research Laboratory, Departments of Biochemistry

- Investigated the signal transduction pathways of platelet aggregation using molecular and biochemical techniques.
- Co-developed novel micro-electrode to detect platelet aggregation in whole blood with Chronolog Corporation.
- Initiated collaborations with Drexel University, the National Aquarium in Baltimore, Virginia Marine Institute, Sea World and the Costa Rican Government to study platelet aggregation in sea-turtles.

1997-1998 Drexel University Philadelphia, PA

Field Research Assistant, Parque Nacional Guanacaste

- Environmental preservation research of nesting sea-turtles. Aided numerous projects on gas-exchange, paternity, predation, and development.

1998-2002 Drexel University Philadelphia, PA

Student Activity Fee Allocation Committee: Chairperson (1999-2001)

- Reviewed proposals from recognized student groups that presented requests for allotments from the Student Activity Fee.

AS CHAIRPERSON:

- Acted as liaison between SAFAC and the student body, administration, and faculty of Drexel University, while supervising annual budgets of approximately $1,000,000.

- Transformed the process through which student groups applied for money relieving the burden of application on student organizations while vastly increasing the quality of information submitted; therefore, the caliber of decisions made for the Drexel Community by SAFAC were considerably enhanced.

- Planned retreats for organization focusing on policies, procedures, team building, significance of decisions made by SAFAC, and how to make ethical decisions.

1993-1996 Arcadia University Glenside, PA

Behavioral Neuroscience Researcher, Department of Psychology

- Performed behavioral assays on rats towards the understanding about the contextual nature of post-traumatic stress disorder.

AREAS OF SCIENTIFIC KNOWLEDGE

- **Pharmacology:** Pharmacokinetics, pharmacodynamics, pharmacogenomics, drug toxicity, CNS, autonomic nervous system, cardiovascular, as well as drugs that affect most physiological systems.

- **Molecular/Cell Biology:** Cloning, PCR, RNA and DNA isolations, and key signal transduction pathways.

- **Biochemistry:** Proficient in biochemistry techniques as well as analysis of biochemical assays. Isothermal Calorimetry, Ca^{2+} mobilization, etc.

- **Ubiquitination:** Up-to-date on current literature in the field. Frequently design novel assays to elucidate functions of ubiquitination pathways. Attended national conference on ubiquitination at Cold Spring Harbor Laboratories, May 2006.

- **Behavioral Neuroscience:** Carry out behavioral assays on rats and transgenic mice to elucidate effects of stress and gene knockouts respectively. Set up new equipment to carry out numerous cognitive measurement assays.

- **Structural Biology:** X-ray crystallography of biological macromolecules. Data collection (user at synchrotron sites ALS & APS) data processing, multiple phase determination procedures, map improvement, model building and graphical representation.

- **Platelet Aggregation and Coagulation:** Carried out blood platelet aggregation and coagulation studies with multiple agonists and inhibitors from humans, sea-turtles, and mice. Trained in Phlebotomy and collected blood samples from donors on a daily basis.

- **Bioengineering:** Collaborated on the development of prototype micro-electrode for use in whole blood platelet aggregation studies.

- **Protein Purification**: *E.Coli* and insect cell expression systems. Affinity and FPLC purification optimization and automation.

- **Protein analysis:** SDS-PAGE, Western blots, immunocytochemistry.

- **Microscopy and immunohistochemistry:** Sectioning of cryo-tissue samples and staining with compound and confocal microscopes.

BUISNESS SKILLS

- **Budget Management:** Required to manage budgets of ~$1 million.

- **Project Management:** Planed and organized groups with members from biology, engineering, and business backgrounds.

- **Scientific Liaison:** Worked with people who have business and general science backgrounds to help them understand significance of specialized scientific work.

- **Policy and Procedure Development:** Create policies and procedures to improve decision-making process while decreasing the burden of time, energy, and cost for those involved.

TEACHING EXPERIENCE

- *Pharmacology.* University of Washington 2003-2004: Teaching Assistant for general pharmacology. Students learned pharmacology related to cardiovascular, nervous, and autonomic systems; as well as pharmacokinetics, pharmacodynamics, pharmacogenomics, drug toxicity and many other topics.

- *Biology, Chemistry, Physics, Math, 1997-2002:* Tutored high school students from high schools in the greater Philadelphia area on numerous topics.

- *General Biology.* Drexel University, Summer 1999: Aided in design and implementation of Philadelphia Gear Up Program at Drexel.

HONORS AND FELLOWSHIPS

- Pre-doctoral Fellow, Cellular and Molecular Biology Training Grant (*Competitive* grant funded by NRSA 2 T32 GM07270-29: June 2003 – June 2006).
- Anthony J. Drexel Scholar, merit based full-tuition scholarship.
- Drexel University Alumni Scholar, award for academic performance.
- Drexel University Student Leader of the Month (October, 2000).

ADDITIONAL SKILLS

- Experienced with UNIX, Mac OSX, and Windows environments.
- Web Design and Development
- Intermediate user of SAS.
- Extensive sailing experience; have been required to live on board for periods up to one month while cruising and raced successfully in multiple classes.

LANGUAGES

- Beginner Italian, French, and Japanese.
- Intermediate Spanish, five years of instruction and six months of immersion in Costa Rica.

ABSTRACTS (POSTERS/TALKS)

Goldenberg SJ and Ning Z (2006). Crystal Structure of Cand1-Cul1-Roc1: Regulatory mechanisms for Cullin-based ubiquitin ligases. *Northwest Crystallography Workshop in Seattle, WA.*

Goldenberg SJ and Ning Z. (2004) Crystal Structure of Cand1-Cul1-Roc1 Complex. 2005 *Molecular Pharmacology Symposium in Leavenworth, WA.*

Soslau G, Morgan DA, Class R, Paladino FV, George R, Whitaker B, Violetta GC, **Goldenberg SJ**, Spotila J. (2004) Coagulation and platelet aggregation in sea turtles. *Proceeding of the 21st Symposium on Sea Turtle Biology and Conservation.*

Soslau G, **Goldenberg SJ**, Navas E, Mattsson C, Nylander S. (2002) (Xi)Melagatran Inhibition of Alpha-Thrombin-Gp Ib and Beta-, Gamma-Thrombin-Par-4 Platelet Aggregation Pathways. *Blood.* 100(11), 255a.

Soslau G, **Goldenberg SJ**, Class R. (2002) Differential Activation and Inhibition of Human Platelet Thrombin Receptors by Structurally Distinct alpha, beta, and gamma-Thrombins. *Blood.* 100(11), 255a.

Goldenberg SJ, Sosalu G. (2002) Inhibition, Isolation, and Sequencing of an Endogenous Platelet Serine Protease. *Fourteenth Annual Drexel/MCPHU Research Day in Philadelphia, PA.* **(Deans Award for Outstanding Research in Biomedical Engineering and Science)**

Soslau G, Morgan DA, Class R, Paladino FV, George R, Whitaker B, Violetta GC, **Goldenberg SJ**, Spotila JR. (2002) Coagulation and Platelet Aggregation in Sea Turtles. *Sea Turtle Symposium, Orlando Fl.*

Solsau G, **Goldenberg SJ**, Class R. (2001) The three thrombin receptors on human platelets respond differentially to alpha-, beta-, and gamma-thrombin. *FAESB Journal.* 15(5), A896.

Goldenberg SJ, Soslau G. (2000) Inhibition of Platelet Aggregation Induced by Non-Proteolytic Ligands by the Serine Protease Inhibitor 4-(2-Aminoethyl)-benzene sulfonyl fluoride (AEBSF). *Twelfth Annual Drexel/MCPHU Research Day in Philadelphia, PA.* **(Drexel Research Award, Honor for Outstanding Research in the Basic and Applied Sciences).**

Goldenberg SJ, Schalles J, Procopio N. (1998) Increase in Rate of Catalase Activity in *Gallus domesticus* Cardiac Muscle Tissue when Compared to *Gallus domesticus* Skeletal Muscle Tissue. *Tenth Annual Drexel Research Day in Philadelphia, PA.*

Goldenberg SJ, Blustein JE. (1997) Withdrawal from Endogenous Opiates Following Forced Swimming in the Rat. *Delaware Valley Undergraduate Psychological Research Symposium.*

Goldenberg SJ, Blustein JE, Adair T. (1996). Contextual Control of Withdrawal from Endogenous Opiates following Extended Swimming in the Rat. *Eastern Psychological Association.* Vol 68(1): 23.