CAND-1 and regulation of Cullin Ring Ubiquitin ligases in *C. elegans*

by

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ABSTRACT

Cullins function in multisubunit ubiquitin ligase (E3) complexes to promote the ubiquitin-mediated proteolysis of substrates and regulate a wide range of cellular processes. CAND1 is a HEAT repeat protein that binds to cullins and regulates their ubiquitin ligase activity by inhibiting the formation of full E3 complexes. All cullins are modified by the covalent linkage of Nedd8, which is a ubiquitin-like protein that is required for full cullin activity. CAND1 cannot bind a cullin that is neddylated but it forms tight complexes with the unneddylated form of cullins. It was originally believed that neddylation of CUL1 bound to CAND1 could dissociate CAND1 and trigger active SCF complex formation (Jidong Liu, Molecular Cell, December 2002). However, more recent biochemical analysis and the crystal structure of CAND1 bound to CUL1 revealed that neddylation can not decouple CAND1 from CUL1 (Goldenberg, Cell, November 2004). Currently, it is not known how CAND1 that is bound to a cullin can be dissociated, or the physiological significance of CAND1 binding to cullin proteins *in vivo*.

In this study we determined the roles of CAND-1 in modulating the functions of the *C. elegans* cullins. *C. elegans* has only a single CAND1 ortholog: *cand-1(Y102A5A.1)*. We have identified CAND-1 as a major component of CUL-2 and CUL-4 complexes. Analysis with the yeast two-hybrid system indicates that both the N- and C-terminal domains of CAND-1 interact with all six *C. elegans* cullin proteins. The mutant phenotype of *cand-1* does not show any major cullin loss of function phenotypes. We have determined the neddylation level of CUL-2 and CUL-4 in *cand-1* mutant and found that the level of neddylation increased substantially for both cullins, indicating that CAND-1 negatively regulates neddylation i*n vivo*. We also carried out a genomic screen for *cand-1* enhancers, and found 18 enhancers that are potential regulator(s) of CAND-1 and the CRLs (**C**ullin **R**ing ubiquitin **L**igase) activation cycle.

INDEX WORDS: *Caenorhabditis elegans*; CAND1; CUL-1; CUL-2; CUL-4; Skp1; F-box protein; Rbx1; Nedd8; *cand*-1; Y102A5A*.1*; cell cycle; cullin; ubiquitin; ubiquitin ligase and CRL.

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DEDICATION

Dedicated to my children: Rusha, Suvra and Debesh

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CHAPTER I

Background and significance

Ubiquitin-mediated proteolysis

Protein degradation is critical for the regulation of a large number of diverse cellular processes. The majority of protein degradation in cells occurs via the ubiquitin-mediated proteolytic pathway (Ciechanover et al., 1984; Rock et al., 1994). Alteration in ubiquitination reactions has been implicated in pathogenesis of multiple human diseases (Ciechanover and Schwartz, 2004). Ubiquitin is an evolutionarily conserved 76 amino acid polypeptide that is covalently attached to target proteins by the concerted actions of three classes of enzymes (Glickman and Ciechanover, 2002; Pickart, 2001). A ubiquitin-activating enzyme (E1) utilizes one ATP to activate ubiquitin. The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). E2s interact with ubiquitin-protein ligases (E3s), which also bind the substrate. The E3 brings the E2 and the substrate together. The E2 can then either directly conjugate ubiquitin to the substrate or, in the case of HECT-domain E3s, transfer the ubiquitin as a high-energy thiol intermediate to the E3, which then transfers it to the substrate.

The attachment of a single ubiquitin to a substrate can alter protein function or localization (Hicke, 2001). The tandem attachment of multiple ubiquitins to form a polyubiquitin chain can also alter function or localization, or mark the substrate for degradation by the 26S proteasome, depending on the type of linkage within the polyubiquitin chain (Pickart and Fushman, 2004). In the most cases, protein is targeted for degradation when ubiquitin is

covalently linked to the target protein through specific lysine residues and forms a polyubiquitinated chain through K48-G76 isopeptide bonds between ubiquitin monomers (Hochstrasser, 1996). In some cases, poly-ubiquitin chains can form by alternate covalent linkages between K63 and G76 residues of ubiquitin monomers, leading to subcellular compartment sorting of target proteins instead of degradation (Pickart, 2001).

Ubiquitin-activating enzyme (E1)

The ubiquitin-activating enzyme (E1) initiates the ubiquitin-mediated proteolysis pathway by activating ubiquitin (Fig. 1.1). Usually there is a single E1 enzyme in an organism (McGrath et al., 1991). E1 activates the C-terminal Gly residue of ubiquitin in an ATP dependent manner. This biochemical reaction has two intermediate steps; ubiquitin interacts with ATP to form ubiquitin adenylate with the release of PPi and then the activated ubiquitin is transferred to E1 from ubiquitin adenylate. Ubiquitin then forms an intermolecular thioester bond between C-terminal Gly residue of ubiquitin and Cys residue of E1 and releases AMP (Hershko and Ciechanover, 1998). This activated ubiquitin is now ready to transfer to a ubiquitin-conjugating enzyme (E2).

Ubiquitin-conjugating enzymes (E2s)

The second step of the ubiquitination reaction is to transfer a ubiquitin from the E1 to the ubiquitin-conjugating enzyme (E2). E2 conjugates with ubiquitin by forming a thioester linkage through a Cys residue (Fig. 1.1) (Hershko and Ciechanover, 1998). E2 enzymes can transfer ubiquitin to substrates with the help of an E3 ubiquitin ligase in most of the cases but in vitro E2s have been shown to directly bind to substrates without the presence of an E3 (Goebl et al., 1988; Hershko and Ciechanover, 1998; Kalchman et al., 1996). Unlike E1, there are thirteen E2-like proteins in budding yeast and at least 50 E2s have been found in humans (Glickman and Ciechanover, 2002; Hochstrasser, 1996; Pickart, 2001).

Ubiquitin-ligases (E3s)

A ubiquitin-ligase or E3 ubiquitin ligase is required in the final step of the ubiquitination reaction pathway (Figure 1.1) (Hershko and Ciechanover, 1998). With the help of ubiquitin ligase, ubiquitin molecule is covalently bound to the substrate via an amide isopeptide bond which is located between the c-terminal glycine residue of ubiquitin and an £-amino group of a lysine residue of a target protein (Hershko and Ciechanover, 1998). Among the three enzymes (E1, E2 and E3), the E3 ubiquitin ligase plays a central role in determining the specificity of the substrates. There are many different E3s involved in the recognition of different target proteins. In humans, several percent of the genome are associated with E3s or E3 complex components (Semple, 2003).

E3 enzymes can be largely classified into two major classes on the basis of the mechanism to ligate ubiquitin to substrates: HECT-domain E3s; and RING-domain E3s (Pickart, 2004).

HECT-domain E3s

The HECT-domain E3s contain an approximately 350 amino acid long HECT (Homology to the E6-AP Carboxyl Terminus) domain (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Pickart, 2001). The HECT-type of E3s are the only known E3 ubiquitin ligases which form a thioester bond with ubiquitin at the HECT-domain at the C-terminal site before the final attachment of ubiquitin to the substrate. The ubiquitin is then transfered to the substrates that are already recruited to the E3 via N-terminal unique domain (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Pickart, 2001). E6-AP (E6-associating protein) was originally identified as a HECT domain E3 that targets p53 for degradation in the presence of the HPV oncoprotein E6 (Scheffner et al., 1993; Scheffner et al., 1995).

RING-domain E3s

The RING (Really Interesting New Gene)-domain family represents the largest group of E3s known to date which contain a RING-finger motif (Pickart, 2001). The RING-finger motif consist of a Cys-rich consensus sequence and capable of binding to E2 (Borden, 2000; Saurin et al., 1996). RING-domain E3s can be further classified into three sub-classes based on the type of their RING domain: RING-HC (C3HC4), RING-H2 (C3H2C3), and RING-IBR-RING (Jackson et al., 2000; Tanaka et al., 2004). A majority of RING-domain E3s form multi-protein complexes in which the RING domain recruits E2 to the complex (Lorick et al., 1999; Seol et al., 1999).

CULLIN/RING-H2 Ubiquitin ligases (CRLs)

Ubiquitin ligases provide the substrate specificity for ubiquitination (ubiquitylation) reactions. The largest known class of ubiquitin ligases are cullin-RING ubiquitin ligases (CRLs) (Petroski and Deshaies, 2005). Anaphase promoting complex (APC) also designated as another class of E3s. The APC2 subunit of APC/C (anaphase promoting complex/cyclosome) also has a 'cullin-homology' domain (Yu et al., 1998; Zachariae et al., 1998). The APC/C is active from the metaphase-to-anaphase transition to the beginning of S phase and ubiquitinates cell cycle regulators (Koepp et al., 1999). However the APC/C complex is clearly distinct from other cullin-based E3 complexes in its structure and regulation (Petroski and Deshaies, 2005).

CRLs regulate diverse cellular processes, including multiple aspects of the cell cycle, transcription, signal transduction, and development (Petroski and Deshaies, 2005). CRLs are multisubunit complexes that include a cullin, a RING H2 finger protein, a substrate-recognition subunit (SRS), and with the exception of CUL3-based CRLs, an adaptor subunit that links the SRS to the complex. There are five major categories of cullins in metazoa (CUL1 through CUL5) (Kipreos et al., 1996; Nayak et al., 2002b), and an additional, potentially vertebrate-specific class

containing CUL7 and PARC (Parkin-like cytoplasmic protein) (Skaar et al., 2007). CRLs are activated by the covalent attachment of the ubiquitin-like protein Nedd8 to the cullin, and are inhibited by binding to the CAND1 inhibitor (Petroski and Deshaies, 2005). Recently, it has become apparent that many CRLs function as dimers, which is another potential source of regulation.

The structure of CRL complexes

The most intensively studied cullin is metazoan CUL1 and its budding yeast ortholog Cdc53. CUL1 and Cdc53-based CRLs are called SCF complexes, and contain four subunits: Skp1; CUL1 (Cdc53); an F-box protein; and the RING H2 finger protein Rbx1/Roc1/Hrt1 (Petroski and Deshaies, 2005). The crystal structure of the SCF complex reveals that the cullin acts as a rigid backbone for the assembly of the complex (Wu et al., 2003; Zheng et al., 2002b) (Fig. 1.2A). The CUL1 C-terminus binds Rbx1 and the N-terminus binds the adaptor Skp1. Rbx1 facilitates the recruitment of the E2 to the complex (Kawakami et al., 2001). The adaptor Skp1 binds the SRS, which is an F-box protein that links to Skp1 through the F-box motif. The F-box protein binds and positions the substrate for ubiquitination by the E2. The combination of distinct F-box proteins with the core components creates unique SCF complexes that bind distinct sets of substrates. Metazoan genomes contain a relatively large number of genes encoding F-box proteins, e.g., humans have ~70 F-box proteins, while *C. elegans* has over 300 (Jin et al., 2004; Kipreos and Pagano, 2000). Many uncharacterized yeast and mammalian F-box proteins are capable of forming SCF complexes *in vitro*, suggesting the existence of a large number of SCF complexes (Cenciarelli et al., 1999; Kus et al., 2004). F-box proteins generally bind to phosphorylated residues on substrates, and therefore, substrate degradation by SCF complexes is regulated by phosphorylation (Petroski and Deshaies, 2005).

CUL2-based CRL complexes have a structure similar to that of the SCF complex (Fig. 1.2 B). Rbx1 similarly binds to the C-terminus of CUL2, and the adaptor Elongin C binds to the N-terminus (Kamura et al., 1999b; Pause et al., 1999). Elongin C is a Skp1-related protein that binds the complex as a heterodimer with the ubiquitin-related protein Elongin B (Conaway and Conaway, 2002). SRSs bind to Elongin C through a VHL-box protein motif in the SRS (Kamura et al., 2004).

CUL5 is the closest paralog to CUL2 (Nayak et al., 2002b), and CUL5 CRLs have a structure similar to that of CUL2 CRLs (Kamura et al., 2004; Yu et al., 2004) (Fig. 1.2C). Both CUL-2 and CUL-5 CRLs employ Elongin C as the adaptor protein. Despite containing the same adaptor protein, CUL2 and CUL5 complexes bind different classes of SRSs. CUL5 complex SRSs utilize the SOCS-box motif to bind to Elongin C. The SOCS-box motif is similar to the VHL-box motif of CUL2 complex SRSs. Both motifs have an N-terminal subdomain (the BC-box) that binds Elongin C. However, the C-terminal regions of the motifs are distinct: the SOCS-box has a CUL5-box subdomain; and the VHL-box has a CUL2-box subdomain. These C-terminal subdomains are proposed to bind to the relevant cullin and thereby provide specificity (Kamura et al., 2004; Mahrour et al., 2008). CUL5 CRL complexes also utilize the RING H2 finger protein Rbx2/Roc2 rather than the related Rbx1, which is present in the other classes of CRLs (Kohroki et al., 2005).

CUL3 CRL complexes contain Rbx1, but differ from other CRL classes in that there is no adaptor protein (Fig. 1.2D). Instead, the SRS binds directly to the N-terminus of CUL3 using a BTB/POZ domain (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003b; Xu et al., 2003). There are hundreds of BTB proteins in metazoan species, suggesting **a** large number of distinct CUL3 complexes (Petroski and Deshaies, 2005).

CUL4 CRL complexes contain Rbx1 and the adaptor protein DDB1 (Groisman et al., 2003; Wertz et al., 2004) (Fig. 1.2E). DDB1 binds to SRSs that contain WD-repeats of a subclass called 'WDXR' or 'DXR', which mediate interaction with DDB1 (Angers et al., 2006; He et al., 2006; Higa et al., 2006b; Jin et al., 2006). In at least one case, DDB1 has been reported to bind a substrate directly, providing the possibility that DDB1 can function as both an adaptor and an SRS (Hu et al., 2004).

Cellular functions of cullins in *C. elegans*

A broad range of cellular and developmental processes are regulated by Cullin complexes. In *C. elegans* the loss of function of each cullin is well characterized. Lack of *cul-1* results in failure in cell cycle exit which causes hyperplasia in all larval tissues (Kipreos et al., 1996). CUL-1 also has additional role in DTC (distal tip cell) migration, regulation of *C. elegans* nervous system and life span (Ding et al., 2007; Fielenbach et al., 2007). The RNAi of the other component of SCF complex such as F box proteins (*lin-23* and *skp2*) and Skp1 homologs (*skr-1* and *skr-2*) also show subsets of *cul-1* phenotypes (Kipreos et al., 1996; Nayak et al., 2002a).

CUL-2 is required for the G1-to-S phase transition, mitotic chromosome condensation, promote meiotic anaphase II, sex determination and proper placement of anterior posterior axiin *C. elegans*. CUL-2 also negatively regulates CKI-1, degrades cyclin B and CCCH-finger polarity proteins (DeRenzo et al., 2003; Feng et al., 1999; Liu et al., 2004; Starostina et al., 2007). *cul-2* deletion mutant has fewer and larger germ cells than wild-type animal and *cul-2* homozygous hermaphrodites generate 24 cell staged arrested embryos. *cul-2* homozygous males are feminized with incomplete male tail and make oocyte like cells inside the gonad (Feng et al., 1999). ZIF-1, FEM-1 and ZYG-11 are the recognized SRSs for CUL-2 complex in *C. elegans.* ZIF-1 degrades CCCH-finger polarity proteins, FEM-1 degrades the inhibitor of male

development TRA-1, while ZYG-11 is required for meiotic anaphase II, anterior posterior polarity of the animal and degradation of cyclin B1 in zygote (DeRenzo et al., 2003; Liu et al., 2004; Starostina et al., 2007).

CUL-3 is required for the meiosis-mitosis transition and targets MEI-1 a Katanin like protein for degradation (Pintard, L., Kurz, T., *et. al.,* 2003). In *C. elegans,* MEI-1 has the microtubule severing activity which is required for the meiotic spindle organization. Inactivation of CUL-3 causes failure in MEI-1 degradation, resulting in disorganized mitotic spindle positioning, elongation and cytokinesis defect at the first mitotic division in zygotes. The loss-of-function of the BTB containing protein, *mel-26* also shows the same phenotype and its physical interaction with CUL-3 suggests MEL-26 functions in a complex with CUL-3 to target MEI-1 (Pintard *et. al.*, 2003; Xu L *et. al.* 2003).

CUL-4 is required to maintain genome stability by restraining DNA replication licensing factor CDT-1. RNAi inactivation of *cul-4* causes CDT-1 to stabilized and re-replication in the seam cells and distal tip cell. Additionally *cul-4* mutant worms are arrested at L2~ 4 larval stages (Kim and Kipreos, 2007; Zhong et al., 2003). CUL-4 also required for the exportation of the replication licensing factor CDC-6 after origin firing to maintain genome stability (Kim et al., 2007).

CUL-5 and CUL-6 are required to prevent spontaneous mutations in germ cells but do not have any growth or developmental role in *C. elegans* (Kamath et al., 2003; Pothof et al., 2003; Tijsterman et al., 2002).

Dimerization of CRLs

A number of CRL complexes function as dimers. CUL1, CUL3 and CUL4-based (Chew and Hagen, 2007) CRL complexes have been observed to form dimers or multimers *in vivo* (Chew et al., 2007; Tang et al., 2007; Wimuttisuk and Singer, 2006b). In contrast, CUL2 and CUL5 CRL complexes have only been observed as monomers (Chew et al., 2007). There are two potential mechanisms of dimerization: SRS-mediated dimerization (which has been demonstrated for SCF complexes); and a Nedd8-cullin linkage (which has been demonstrated for CUL3 CRL complexes). SRS-mediated dimerization relies on binding between SRS proteins to link together two CRL complexes. Multiple F-box proteins have been observed to form dimers *in vivo*, including Fbw7, Pop1 & Pop2, Cdc4, Met30, Skp2, and bTrcp1 & bTrcp2 (Dixon et al., 2003; Hao et al., 2007; Kominami et al., 1998; Suzuki et al., 2000; Welcker and Clurman, 2007; Wolf et al., 1999; Zhang and Koepp, 2006). Dimerization of F-box proteins is initiated through a conserved D-domain located immediately N-terminal of the F-box motif (Hao et al., 2007; Tang et al., 2007; Wolf et al., 1999). Analysis of SCF^{Cdc4} complexes by small angle X-ray scatter analysis indicates that the two substrate-binding sites of the SRSs and the two E2-binding sites form a coplanar surface in a suprafacial orientation (Tang et al., 2007) (Fig. 1.3A).

The bivalent geometry of the dimeric SCF structure provides different distances between a substrate-binding site and the two E2 docking sites (Tang et al., 2007) (Fig. 1.4A). These distinct catalytic site-to-substrate distances can allow an SCF complex to target different-sized substrates and accommodate changes in the length of the elongating polyubiquitin chain (Tang et al., 2007) (Fig. 1.4). For the SCF^{Cdc4} complex, dimerization does not affect its affinity for the substrate Sic1, but is required for optimal ubiquitin chain initiation and elongation (Hao et al., 2007; Tang et al., 2007). The *in vitro* ubiquitination of three of four tested SCF^{Cdc4} substrates is more efficiently ubiquitinated by dimeric SCF^{Cdc4} (Tang et al., 2007). Similarly, dimeric mammalian $SCF^{Fbw7/hCdc4}$ can more efficiently ubiquitinate its substrate cyclin E than can

monomeric $SCF^{Fbw7/hCdc4}$ (Hao et al., 2007). Dimerization also provides the potential for the two SRSs in the complex to work together to bind one substrate so that it is optimally tethered for ubiquitination, as has been proposed for the dimeric $CRL3^{Keap1}$ complex binding to its substrate Nrf2 (McMahon et al., 2006).

SRSs can bind to SCF complexes as both homodimers and heterodimers. The F-box proteins □TrCP1 and □TrCP2 form both homo and heterodimeric complexes, but only the homodimeric forms of each can ubiquitinate the substrate IκBα (Suzuki et al., 2000). In contrast, the fission yeast F-box proteins Pop1 and Pop2 target the degradation of the substrates Cdc18p and Rum1p as a heterodimeric Pop1/Pop2 complex even though both Pop1 and Pop2 can also form homodimers (Kominami et al., 1998; Wolf et al., 1999). Thus both homodimers and heterodimers can form active SCF complexes, thereby providing the possibility for combinatorial regulation of SCF activity.

Many CUL3 complex SRSs form homodimers, including Keap1, MEL-26, RhoBTB2, and SPOP (Hernandez-Munoz et al., 2005; McMahon et al., 2006; Pintard et al., 2003b; Wilkins et al., 2004). Nevertheless, the dimerization mechanism that has been reported for CUL3 complexes does not require SRS dimerization, but rather involves physical interaction between an unneddylated CUL3 and a Nedd8 that is covalently bound to another CUL3 (Wimuttisuk and Singer, 2006b) (Fig. 1.3B). The winged-helix B (WH-B) domain in the C-terminus of the unneddylated CUL3 binds to Nedd8. As Nedd8 is conjugated to a lysine residue within the WH-B domain, the same region of both CUL3 proteins is involved in the interaction. (Pintard et al., 2003b).

There is, however, conflicting data on the prevalence of Nedd8-cullin-based dimerization. While Wimuttisuk and Singer found that CUL3 with a mutated SRS-binding site still forms dimers

in vivo (thereby providing evidence for Nedd8-cullin-based interaction) (Wimuttisuk and Singer, 2006a), Chew et al. found that CUL3 with a mutated SRS-binding site does not form dimers *in vivo* (Chew et al., 2007). Both groups used the same experimental strategy and cell line. The divergent results imply either that Nedd8-cullin-based interaction is the dominant method of dimerization, or that it has at most a minor role in CUL3 dimerization (and that SRS-based dimerization is predominant). Thus the importance of the Nedd8-cullin binding mechanism is currently unresolved.

Do other cullins besides CUL3 form Nedd8-cullin dimers? It has been observed that human CUL1 in which the adaptor-binding region has been mutated can still form dimers or multimers *in vivo*, suggesting an SRS-independent interaction mechanism (Chew et al., 2007). In contrast, the dimerization of budding yeast SCF^{Cdc4} occurs exclusively through an SRS-mediated mechanism (Tang et al., 2007). Moreover, in budding yeast, Nedd8-cullin interaction is unlikely to be an important dimerization pathway, as Rub1 (Nedd8) is not required for viability in budding yeast and so cannot be essential for cullin functions (Lammer et al., 1998; Liakopoulos et al., 1998). It should be noted that budding yeast do not have a clear CUL3 ortholog (Nayak et al., 2002b), and it is possible that Nedd8-cullin dimerization is specific for CUL3.

One of the characteristics of the Nedd8-cullin dimerization mechanism is that the dimeric CRL complex must have equal levels of neddylated and unneddylated cullins. Immunoprecipitation of the CUL3 substrate cyclin E pulls down roughly equivalent levels of neddylated and unneddylated CUL3 (Wimuttisuk and Singer, 2006a). In contrast, immunoprecipitation of the substrates of $CRL2^{VHL}$ or $SCF^{\beta TrCP}$ pulls down predominantly neddylated cullins, implying that $SCF^{\beta TrCP}$ and $CRL2^{VHL}$ do not function as Nedd8-cullin dimers (Kawakami et al., 2001; Read et al., 2000; Sufan and Ohh, 2006). These results suggest that

Nedd8-cullin dimerization is not widespread among other (non-CRL3) classes of CRL complexes.

It has been reported that the CRL2 SRS VHL is a dimer *in vivo* and that the dimerization is required for $CRL2^{VHL}$ activity *in vivo* (Chung et al., 2006). However, it has also been reported that CUL2 is not present as a dimer or multimer in cells (Chew et al., 2007). A model that incorporates both of these results is that a monomeric CRL2 complex binds to dimeric VHL (Fig. 1.3C). Experimental results that directly test this model are not yet available.

The turnover of substrate-recognition subunits

SRSs recognize and recruit substrates to the CRL complex. Genetic evidence from yeast suggests that F-box proteins compete with each other for binding to the core CRL complex (Patton et al., 1998; Zhou and Howley, 1998). Therefore the regulation of SRS levels (through synthesis or turnover) can directly influence the relative proportion of different CRL complexes.

In both yeast and mammals, F-box proteins are often unstable and undergo proteasome-mediated degradation as a result of autoubiquitination when linked to the SCF complex (Galan and Peter, 1999; Li et al., 2004; Mathias et al., 1999; Smothers et al., 2000; Wirbelauer et al., 2000; Zhou and Howley, 1998). The overexpression of substrates can stabilize F-box proteins because the bound substrate protects the F-box protein from autoubiquitination (Galan and Peter, 1999; Li et al., 2004). Autoubiquitination of SRSs is potentially a broadly based mechanism among CRLs, as it is also observed for the CUL3 complex SRSs RhoBTB2 and Keap1 in mammals, and Btb3 in fission yeast (Geyer et al., 2003; Lo and Hannink, 2006; Wilkins et al., 2004).

In contrast to SCF SRSs, which are often destabilized after binding the SCF complex, the CUL2 complex SRS VHL is stabilized by its association with the CRL2 complex (Kamura et al., 2002; Schoenfeld et al., 2000). In the absence of binding the CRL2 complex, VHL is degraded through a proteasome-dependent mechanism, presumably via the activity of another E3 (Schoenfeld et al., 2000). Many other SRS proteins are also degraded through the activity of other E3s. For example, the APC/C (anaphase promoting complex/cyclosome) ubiquitin ligase targets the degradation of the SCF SRSs Skp2 and Tome1, and $SCF^{\beta TrCP}$ targets the degradation of the SCF SRS Emi1 (Ayad et al., 2003; Bashir et al., 2004; Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003; Wei et al., 2004).

As we shall see in the following sections, a central role of two major CRL regulators (CSN and CAND1) is to regulate the autoubiquitination of SRSs. Uncontrolled autoubiquitination leads to the inactivation of CRLs due to a loss of SRSs. On the other hand, SRS turnover is essential to allow the switching of SRSs among core CRL complexes so that the relative proportions of different CRLs reflect changes in SRS levels.

Regulation of CRLs by Nedd8 conjugation

Cullins are post-translationally modified by the covalent attachment of the ubiquitin-like protein Nedd8 to a conserved lysine residue in a process termed neddylation (Pan et al., 2004). Nedd8 conjugation increases CRL ubiquitin ligase activity *in vitro* (Morimoto et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000) by promoting the recruitment of the E2 through direct interaction between Nedd8 and the E2 (Kawakami et al., 2001; Saha and Deshaies, 2008; Sakata et al., 2007). Based on the interaction of E2s with RING finger domains (such as is found in Rbx1) (Zheng et al., 2000), it has been proposed that both Nedd8 and Rbx1 form a common interface for loading the E2 (Sakata et al., 2007). But the crystal structure of CRL5 reveals that

Nedd8 conjugation induces a major conformational changes to the C-terminal domain of the cullin which causes RING domain of the Rbx1 to escape from its binding pocket in the cullin and remain flexibly tethered to the cullin by an extended β-sheet similar to a balloon on a string (Duda et al., 2008). This structural modification upon neddylation allows Rbx1 bound to charged E2~Ub to move closer to substrates and adopt multiple orientations to accommodate a growing polyubiquitin chain (Duda et al., 2008). In this context, it is clear that the E2 cannot bind to both Nedd8 and Rbx1 simultaneously. Interestingly, CUL1 is able to self-conjugate a ubiquitin to the neddylation site in vitro to activate its ubiquitin ligase activity similar to the activation of neddylation at the same site, suggesting an alternate pathway for CRL activation (Duda et al., 2008).

Nedd8 conjugation is required for the *in vivo* function of CUL1, CUL2, and CUL3 in a number of metazoan species and fission yeast (Ohh et al., 2002; Osaka et al., 2000; Ou et al., 2002; Pintard et al., 2003a). However, in budding yeast, Nedd8 is not essential for SCF-mediated processes, although it does enhance SCF activity (Lammer et al., 1998; Liakopoulos et al., 1998).

The neddylation reaction is similar to the ubiquitination reaction, and involves a heterodimeric E1 (APP-BP-1/Uba3) that activates Nedd8, the E2 UBC12 that conjugates Nedd8 to the cullin, and DCN1 (defective in cullin neddylation) and Rbx1 as E3s (Furukawa et al., 2000; Gong and Yeh, 1999; Kamura et al., 1999a; Kurz et al., 2008; Kurz et al., 2005; Liakopoulos et al., 1998; Megumi et al., 2005; Sufan and Ohh, 2006). DCN1 was identified as a protein that promotes the neddylation of CUL-3 in *C. elegans* and Cdc53 in budding yeast (Kurz et al., 2005). DCN1 binds to the cullin and the neddylation E2 UBC12 to facilitate UBC12 loading onto the cullin (Kurz et al., 2008). While DCN1 promotes neddylation, it is not essential for the neddylation reaction *in vivo* (Kurz et al., 2005). The CRL component Rbx1 also plays a central

role in neddylation. *In vivo*, only cullins that are complexed with Rbx1 undergo neddylation (Furukawa et al., 2000; Kamura et al., 1999a; Megumi et al., 2005; Sufan and Ohh, 2006), and mutation of the RING finger motif of Rbx1 abolishes neddylation *in vitro* (Kamura et al., 1999a). Rbx1 can promote neddylation *in vitro* in the absence of DCN1 if there are sufficiently high levels of E2, while the presence of DCN1 allows neddylation at lower E2 levels (Kurz et al., 2008). Based on the observation that DCN1 can physically bind to Rbx1 (Yang et al., 2007), it is likely that the two proteins form a multisubunit E3 for the neddylation reaction, although it is possible that Rbx1 is the predominant E3 and DCN1 is a cofactor.

In *C. elegans*, loss of DCN-1 causes embryonic arrest due to loss of CUL-3 activity; while in budding yeast, a *DCN1* null mutant is viable, consistent with the observation that Rub1 (Nedd8) is not essential in budding yeast (Kurz et al., 2005). A loss-of-function mutant of an *Arabidopsis Dcn1* homolog had no effect on SCF^{TIR1}-regulated pathways, however, there may be redundancy as there are three *Dcn1*-related genes in *Arabidopsis* (Biswas et al., 2007). The mammalian DCN1 ortholog (SCCRO, squamous cell carcinoma-related oncogene) is amplified in several human tumors, and functions as an oncogene when overexpressed (Sarkaria et al., 2006), however there are currently no reports on its role in regulating neddylation.

Regulation of CRLs by the CSN complex

The COP9 Signalosome (CSN) is a conserved eight-subunit complex that was originally identified in *Arabidopsis* (Wei et al., 1994; Wei and Deng, 1992). The eight subunits of the CSN complex are homologous to eight subunits of the 19S proteasome lid complex and to three subunits of the eIF3 translation initiation factor complex, suggesting a common origin for these three protein complexes (Schwechheimer, 2004). CSN physically associates with the 26S proteasome, and may function as an alternate lid for the proteasome (Huang et al., 2005; Peng et al., 2003). CSN has been implicated in wide range of biological processes including plant

photomorphogenesis, yeast mating pathways, signal transduction, the regulation of DNA repair, and cell cycle regulation (Cope and Deshaies, 2003; Wolf et al., 2003). Biochemically CSN is associated with three activities, phosphorylation, deneddylation, and deubiquitination, with the latter two activities directly regulating CRLs (Cope and Deshaies, 2003; Wolf et al., 2003).

Nedd8 conjugates are removed from cullins (in a process termed deneddylation) by the isopeptidase activity of the metalloprotease CSN5/Jab1 subunit of CSN (Cope et al., 2002; Lyapina et al., 2001). Inactivation of CSN increases the levels of neddylated cullins *in vivo* (Lyapina et al., 2001; Menon et al., 2007; Pintard et al., 2003a; Schwechheimer et al., 2001). Counterintuitively, CSN inactivation reduces the activity of CUL1, CUL3, and CUL4-based CRL complexes in cells despite increased neddylation levels (Cope et al., 2002; Doronkin et al., 2003; Feng et al., 2003; Groisman et al., 2003; Liu et al., 2003; Pintard et al., 2003a; Schwechheimer et al., 2001; Wang et al., 2003; Zhou et al., 2003). The loss of CRL activity can be attributed to significantly lower SRS levels due to increased autoubiquitination of SRSs (as shown in yeast, humans, *Drosophila*, and *Neurospora*) (Chew et al., 2007; Cope and Deshaies, 2006; He et al., 2005; Wee et al., 2005; Wu et al., 2005; Zhou et al., 2003). The deneddylation activity of CSN is primarily responsible for preventing the autoubiquitination of SRSs (Cope and Deshaies, 2006).

The deubiquitinase activity of CSN contributes to the stabilization of CUL1 and CUL3 SRSs in fission yeast, presumably by removing ubiquitin that is conjugated to the SRSs (Wee et al., 2005; Zhou et al., 2003). CSN deubiquitinase activity also stabilizes Rbx1 in humans (Hetfeld et al., 2005; Peth et al., 2007). In addition to stabilizing SRSs and Rbx1, CSN is also required for the stability of the cullins CUL1 and CUL3 in *Drosophila*, and CUL1 in *Neurospora* (He et al., 2005; Wu et al., 2005). In humans, inactivation of CSN does not affect cullin levels, except for a modest reduction in CUL2 (Cope and Deshaies, 2006).

How the interaction of CSN with CRLs is regulated is unknown. However, the interaction can clearly be subject to active regulation as shown by the rapid release of the $CRL4^{DDB2}$ complex from CSN upon UV irradiation, and conversely, the rapid binding of the $CRL4^{CSA}$ complex to CSN upon UV irradiation (both $CRL4^{DDB2}$ and $CRL4^{CSA}$ are involved in aspects of DNA damage repair) (Groisman et al., 2003). More generally, substrate binding has been implicated in the regulation of neddylation and deneddylation. Substrate binding increases the neddylation levels of human CUL1, CUL2, CUL3, and CUL4 *in vivo* (Bornstein et al., 2006; Chew and Hagen, 2007; Sufan and Ohh, 2006). *In vitro* experiments indicate that substrate binding increases neddylation levels by preventing the deneddylation of cullins by CSN (Bornstein et al., 2006). Substrate binding presumably blocks deneddylation either by inhibiting the deneddylation of CRLs that are bound to CSN or by preventing the association of CRLs with CSN. In contrast to the *in vitro* results, *in vivo* experiments indicate that substrate binding to CUL1 can increase neddylation levels independently of CSN, suggesting that substrate binding promotes the neddylation reaction in cells (Chew and Hagen, 2007).

Regulation of CRLs by the inhibitor CAND1

CAND1 (cullin-associated and neddylation-dissociated) is an inhibitor that binds to cullin-Rbx complexes that lack both neddylation and adaptors (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Zheng et al., 2002a). In humans, CAND1 consists of 27 HEAT (huntingtin-elongation-A subunit-TOR) repeats. The crystal structure of CAND1-CUL1-ROC1/RBX1 demonstrated that C-terminal 23-27 HEAT repeats of CAND1 interact with the N-terminal domain of CUL1 and make contact with all three cullin repeats. The first two HEAT repeats of the N-terminal arch of CAND1 wrap around the C-terminal domain of CUL1, also with the Ring domain of RBX1. In the active SCF complex SKP1 binds to the first cullin repeat of CUL1 at N-terminus and Nedd8 conjugation occurs at the 720 lysine residue on the CUL1 C-terminal domain. It is obvious that when CAND1 binds to form CAND1-CUL1-ROC1/RBX1

complex it replaces SKP1 from binding site on the N-terminal region of CUL-1 and buries the lysine residue for Nedd8 conjugation at the CUL1 C-terminal domain (Goldenberg et al., 2004) (Fig. 1.5).

CAND1 is capable of binding to all cullins in human cells (Liu et al., 2002; Min et al., 2003). However, in certain cells, CAND1 preferentially associates with a subset of cullins. In human HEK293T cells, CAND1 associates primarily with CUL1 (Chew and Hagen, 2007; Oshikawa et al., 2003). CAND1 can also bind to CUL4A and CUL5 in HEK293T cells, but there is no observed interaction with CUL2 or CUL3 (Liu et al., 2002). In contrast, in human HeLa cells, CAND1 interacts with CUL1, CUL2, CUL3, and CUL4A (Min et al., 2003). The reason for these differences (either based on cell lines or experimental conditions) is not understood. In *C. elegans*, CAND1 binds at high level to CUL-2, but does not have detectable binding to CUL-3 (Luke-Glaser et al., 2007; Starostina et al., 2007).

CAND1 binding to cullin-Rbx is incompatible with neddylation. The presence of Nedd8 on the cullin blocks CAND1 binding, suggesting that CAND1 binds to cullin-Rbx only after CSN has removed Nedd8 (Liu et al., 2002; Zheng et al., 2002a). CAND1 can dissociate the adaptor Skp1 from unneddylated CUL1 *in vitro*, suggesting that once Nedd8 has been removed, CAND1 is capable of stripping off the adaptor and binding the cullin (Liu et al., 2002) (Fig. 1.5).

Counterintuitively, inactivation of CAND1 leads to the inactivation of SCF complexes in humans and *Arabidopsis*, and CUL3 complexes in humans (Cheng et al., 2004; Chew et al., 2007; Chuang et al., 2004; Feng et al., 2004; Lo and Hannink, 2006; Zheng et al., 2002a). In the case of human SCF^{Skp2}, the inactivation of CAND1 is correlated with reduced levels of the SRS Skp2, which is proposed to result from autoubiquitination (Chew et al., 2007; Zheng et al., 2002a). In contrast, the activity of the $CRL3^{Keap1}$ complex is inhibited upon CAND1 inactivation

even though increased levels of Keap1 bind to CUL3, and Keap1 interaction with its substrate is increased, suggesting that the presence of CAND1 is required for $CRL3^{Keap1}$ activity independently of SRS stabilization (Lo and Hannink, 2006).

CRL activation cycles

CRLs transit through different stages of assembly, sequestration, and neddylation. These changes can be considered an activation cycle, with CRL components switching from an inactive form (lacking Nedd8 and/or adaptor or SRS, and potentially sequestered by CAND1) to an active form (with attached SRS and Nedd8 conjugation). An outline of a proposed CRL activation cycle is presented in (Fig. 1.5).

CSN-mediated CRL protection

There appear to be two pathways by which CRLs can switch between active and inactive forms. One pathway involves CRL docking with CSN (Fig. 1.5, top). CSN can bind to completely assembled CUL1 and CUL4 CRL complexes, based on the observation that all CRL components, including SRSs, are found to associate with CSN (Feng et al., 2003; Groisman et al., 2003; Higa et al., 2006a; Liu et al., 2005; Lyapina et al., 2001; Schwechheimer et al., 2001; Wang et al., 2003). The deneddylation and deubiquitination activities of CSN can stabilize SRSs by preventing autoubiquitination (Chew et al., 2007; Cope and Deshaies, 2006; He et al., 2005; Wee et al., 2005; Wu et al., 2005; Zhou et al., 2003). CSN therefore keeps CRL complexes in a protected, inactive state. What regulates CRL binding to CSN is not fully understood. Substrate binding to SCF complexes is incompatible with CSN-mediated deneddylation (Bornstein et al., 2006), and it is possible that substrate binding leads to the dissociation of CRL complexes from CSN or inhibits the association of CRLs with CSN. Once CRL complexes are released from

CSN, they can become neddylated and fully active. The depletion of substrates may lead to the re-association of CRLs with CSN, although this has not yet been experimentally demonstrated.

CAND1-mediated CRL sequestration

The second pathway to modulate CRL activity is initiated by the degradation of the SRS (Fig. 1.5). In the absence of substrate, SRSs can undergo autoubiquitination (Galan and Peter, 1999; Li et al., 2004). Additionally, other E3 ligases can induce SRS degradation. Once the SRS is degraded, the core CRL components can associate with CSN and undergo deneddylation. CAND1 can presumably dissociate adaptors from the unneddylated cullin-Rbx complex *in vivo*, as CAND1 has been shown capable of doing so *in vitro* (Liu et al., 2002). The mechanism by which cullin-Rbx complexes are released from CAND1 sequestration has not yet been resolved. However, once released, the binding of cullin-Rbx to adaptor and SRS will reconstitute the CRL complex. The binding of substrate then induces neddylation and full activity (Chew and Hagen, 2007).

The purposes of the activation cycle

What is the purpose of the activation cycle for CRLs? There are three major possibilities. The first purpose appears to be to allow CRLs to efficiently switch between different SRSs. SRS degradation frees the core CRL components to reassemble with new SRSs. A dynamic CRL activation cycle allows adjustments in the proportions of specific CRL complexes in order to reflect changes in the cellular levels of SRSs. It is currently unclear whether CAND1 sequestration is a common aspect of SRS switching or if CRL components sans-SRS generally bypass this step (Fig. 1.5). The observation that only certain cullins interact appreciably with CAND1 in certain mammalian cell lines suggests that CAND1 sequestration is not a requirement for SRS switching.

The second purpose of the activation cycle is to stabilize CRL complexes. Loss of either CSN or CAND1 produces a loss of CRL activity that is attributable, in large part, to the autodegradation of SRSs (Chew et al., 2007; Cope and Deshaies, 2006; He et al., 2005; Wee et al., 2005; Wu et al., 2005; Zheng et al., 2002a; Zhou et al., 2003). This suggests that both CSN and CAND1 are essential to dampen uncontrolled CRL ubiquitin ligase activity in order to prevent CRLs from "burning out" by autoubiquitination of the available pool of SRSs.

The third potential purpose is that cycles of neddylation and deneddylation are directly required for CRL ubiquitin ligase activity. This model is based largely on studies of CUL-3 in *C. elegans* (Pintard et al., 2003a). *C. elegans* CUL-3 is inactive when either neddylation or deneddylation pathways are compromised, yet combining compromised neddylation and deneddylation pathways restores CUL-3 function (Pintard et al., 2003a). This suggested that balanced (but slower) cycling between neddylated and unneddylated states allows CUL-3 activity, while unchecked neddylation or deneddylation (which eliminates cycling) is incompatible with CUL-3 activity. However, an alternative interpretation of the results has been proposed that casts doubt on this model (Wimuttisuk and Singer, 2006b). CUL-3 dimers created by Nedd8-cullin interaction require both neddylated and unneddylated CUL3 in equal proportion (Wimuttisuk and Singer, 2006b) (Fig. 1.3B). Inactivation of either the neddylation or deneddylation pathways by themselves would produce predominantly unneddylated or neddylated CUL-3, respectively. In such a situation, the absence of sufficient levels of both neddylated and unneddylated CUL-3 would reduce the formation of active Nedd8-cullin dimers. Therefore, until there is additional evidence, it is not possible to conclude that neddylation/deneddylation cycles are inherently required for CRL activity.

Unresolved Questions

There are unresolved questions about multiple aspects of the global regulation of CRLs. Dimerization has only recently been recognized as an essential characteristic of many CRLs. It is not yet known to what extent the different dimerization mechanisms are utilized. The SRS-based dimerization mechanism has been well substantiated for SCF complexes, but has not yet been rigorously tested for other cullin-based CRLs. Conversely, the Nedd8-cullin dimerization mechanism has so far only been reported for CUL3 CRL complexes, and the structure has not been fully determined. Finally, the possibility of monomeric CRL cores binding to dimeric SRSs has not yet been rigorously tested.

While the biochemistry of cullin neddylation has been determined, it is not yet clear how neddylation is regulated *in vivo*. There is evidence that substrate binding promotes neddylation, yet how substrate binding mechanistically induces neddylation is not apparent. There is also evidence that substrate binding inhibits the deneddylation activity of CSN. It is possible that substrate binding is linked to the dissociation of CRLs from CSN, but this has not yet been demonstrated. Moreover, it is likely that there will be other undiscovered mechanisms that regulate CSN – CRL interactions potentially in response to intracellular signals

The functional role of CAND1 in sequestering cullins is still mysterious. If CSN is capable of binding to CRLs to prevent autoubiquitination, why is CAND1 also required? Additionally, multiple aspects of CAND1 activity are also unknown. CAND1 only binds unneddylated cullins, but it is not known whether CAND1 binding is actively coupled to CSN deneddylation, as is suggested by *in vitro* experiments. It is not known how CAND1 is released from cullin-Rbx in cells. The observation that endogenous cullins can be released from CAND1 while recombinant cullins cannot, suggests either that the cullin must be post-translationally modified or that a

'dissociation factor' is required to release CAND1. Finally, it is unclear why CAND1 exhibits preferential binding to particular classes of cullins in different cell lines and organisms.

The activation cycle is not fully understood. It would be helpful to know which stages of the cycle are rate limiting and accumulate CRL components during steady-state conditions. It also remains to be determined whether different classes of CRLs employ inherently different activation cycles. In this study we characterized the *C. elegans* ortholog of mammalian CAND-1 and its *in vivo* function in relation to cullin regulation. We also extend our study to a genetic screen to identify possible factor(s) required for CAND-1 function regulation and/or the regulation of the CRL activation cycle.

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Figure 1.1. Ubiquitin mediated proteolysis

Ubiquitin is activated by a ubiquitin activating enzyme (E1). The activated ubiquitin is transferred to a ubiquitin conjugating enzyme (E2), which transfers it to a target protein with the help of a ubiquitin ligase (E3). Once the substrate protein is ubiquitinated it is recognized and degraded by the 26S proteasome. (Diagram provided by E.T. Kipreos).

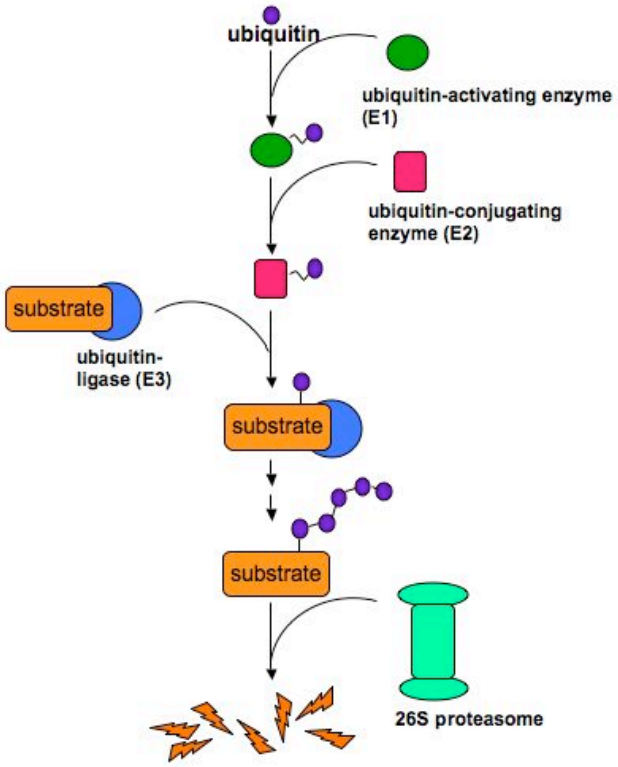

ubiquitin
ubiquitin-activating enzyme (E1)
ubiquitin-conjugating enzyme (E2)
substrate
ubiquitin-ligase (E3)
substrate
substrate
26S proteasome

Figure 1.2. Structures of multisubunit CRL complexes

Diagrams of the CUL1 (A), CUL2 (B), CUL5 (C), CUL3 (D), and CUL4 (E) CRL complexes. Proteins in the complexes are labeled. The structures are described in the text. Figure from (Bosu and Kipreos, 2008)

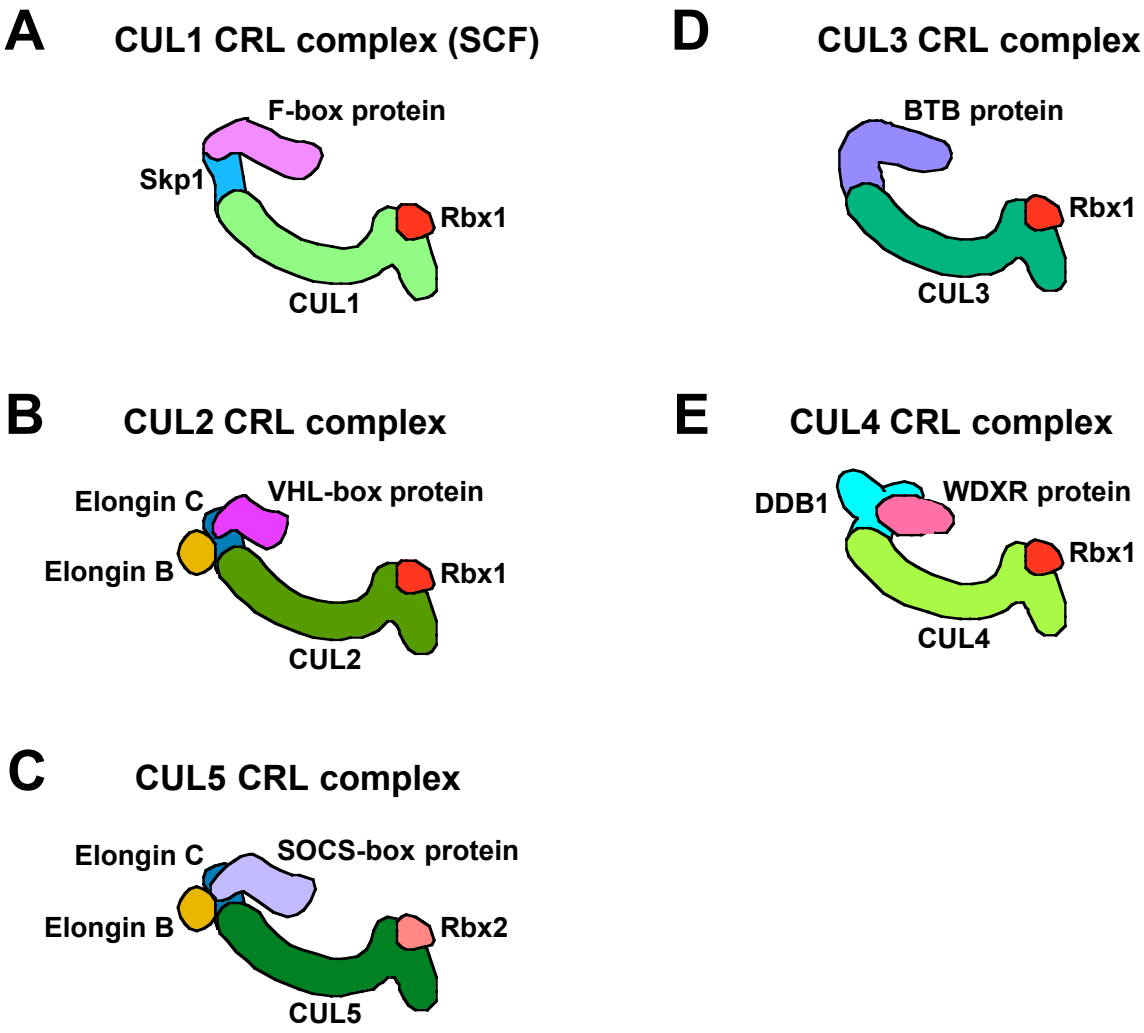

A
CUL1 CRL complex (SCF)
F-box protein
Skp1
Rbx1
CUL1
B
CUL2 CRL complex
Elongin C
VHL-box protein
Elongin B
Rbx1
CUL2
C
CUL5 CRL complex
Elongin C
SOCS-box protein
Elongin B
Rbx2
CUL5
D
CUL3 CRL complex
BTB protein
Rbx1
CUL3
E
CUL4 CRL complex
DDB1
WDXR protein
Rbx1
CUL4

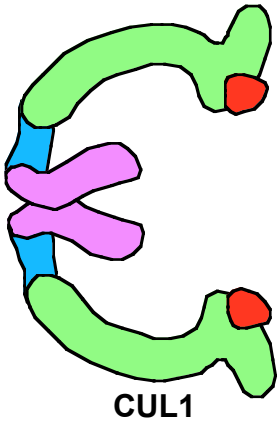
Figure 1.3. Proposed models for dimerization of CRL complexes

(A) Diagram of an SRS-mediated dimeric SCF complex. Dimerization is mediated by interactions between the SRSs in each CRL. This structure has been experimentally confirmed (Tang et al., 2007).

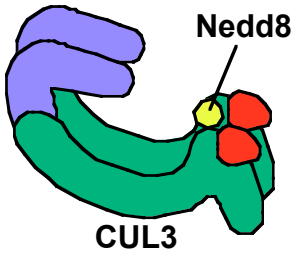
(B) Diagram of a Nedd8-cullin-based dimeric CRL3 complex. Dimerization is mediated by interaction between Nedd8, which is covalently linked to one CUL3 protein, and the WH-B domain of an unneddylated CUL3 (Wimuttisuk and Singer, 2006b). The overall structure of the Nedd8-cullin-based dimer has not been determined. The dimer is drawn in a head-to-head conformation to accommodate the binding of a dimeric SRS to the two CUL3 N-termini (as many CRL3 SRSs are constitutively dimeric *in vivo*).

(C) Diagram of a monomeric CRL2 complex binding a dimeric SRS. The existence of such a structure has not yet been directly confirmed by experiments (see text). Proteins are labeled as in Fig. 1.3. Figure from (Bosu and Kipreos, 2008)

A SRS-based CRL dimer



B Nedd8-cullin-based CRL dimer



C SRS dimer with CRL monomer

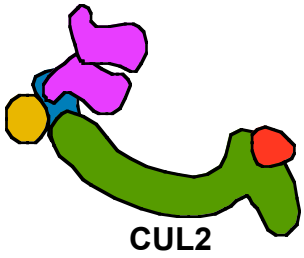


Figure 1.4. Poly-ubiquitination reactions by monomeric and dimeric SCF complexes

Diagram of poly-ubiquitin conjugation to a substrate (rectangle) by monomeric (A) and dimeric (B) SCF complexes. Top panels, E2 with activated ubiquitin prior to binding. Middle panels, E2 with activated ubiquitin loaded onto E3 but prior to transfer of ubiquitin to substrate. Bottom panels, the substrate has a three-ubiquitin chain and a new E2 with activated ubiquitin has docked. Note how the ability of E2s to load onto both sites of the dimeric SCF complex facilitates the addition of ubiquitin onto the growing polyubiquitin chain. In the diagram, the addition of the first ubiquitin is more sterically favorable from the E2 docking site that is closer to the substrate, while additions to the elongated polyubiquitin chain are more favorable from the more distant E2 docking site. Proteins are labeled as in Fig. 1.3. Figure from (Bosu and Kipreos, 2008)

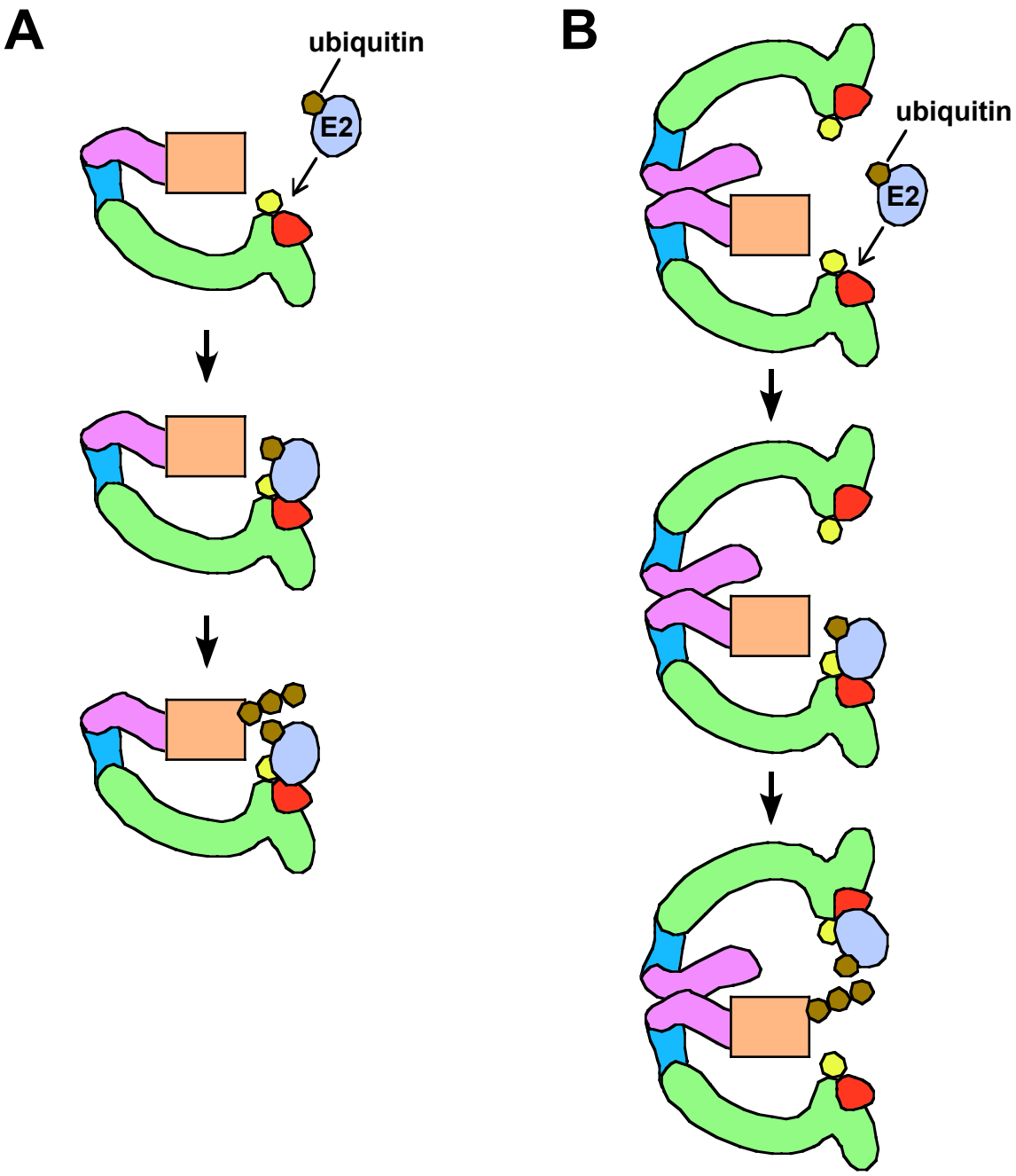
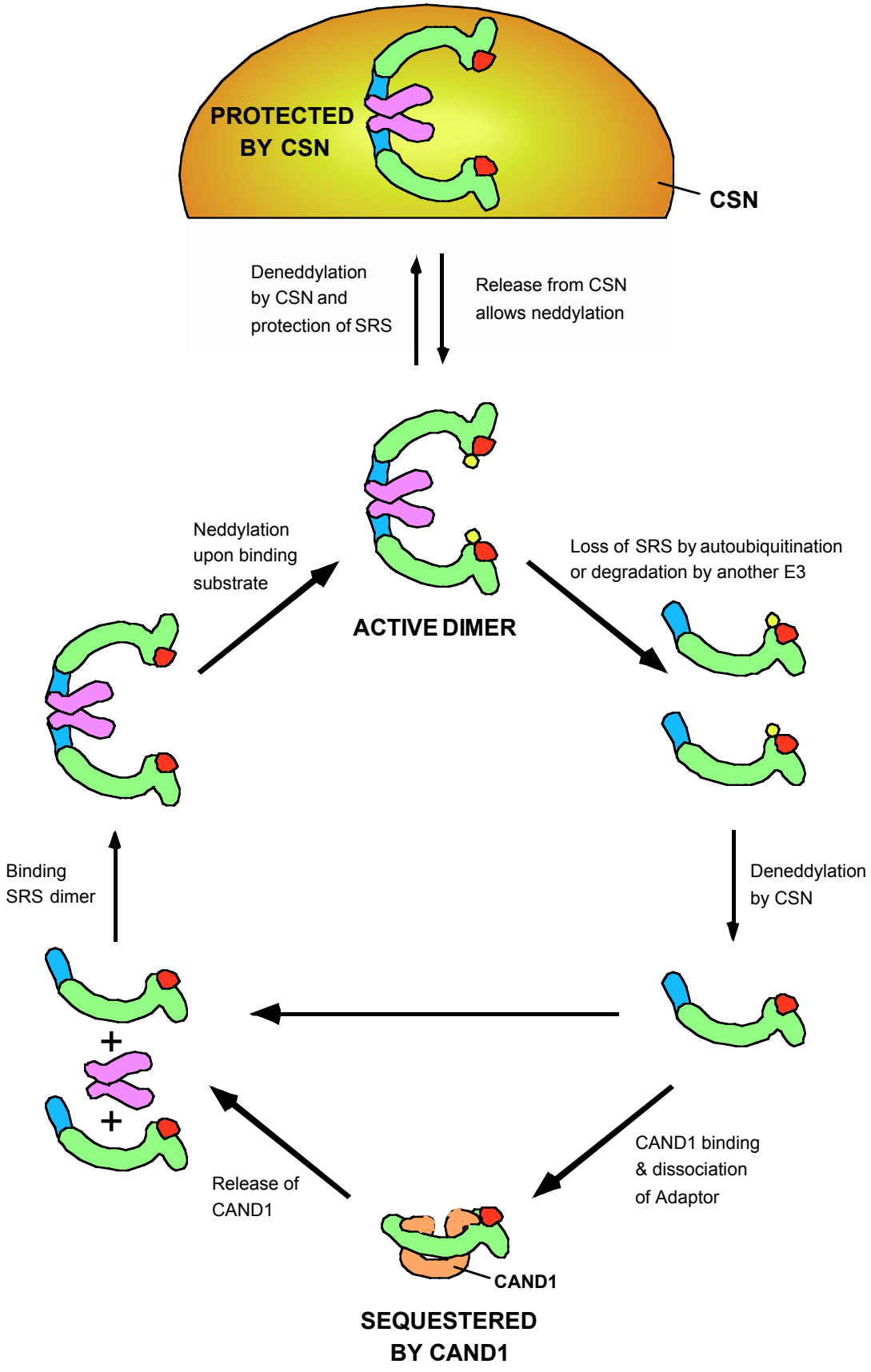

A
ubiquitin
E2
B
ubiquitin
E2

Figure 1.5. Proposed activation cycle for an SCF complex

Diagram of a proposed SCF activation cycle. The SCF complex can shift between an active dimeric complex and a CSN-bound state in which the cullin is deneddylated and the SRS is protected from autoubiquitination (top). The mechanisms that regulate SCF interaction with CSN are not fully understood, but substrate binding may be associated with either releasing SCF from CSN or preventing SCF binding to CSN. When substrate is lacking, SCF complexes can either rebind CSN or lose their SRS due to autodegradation. Loss of the SRS (by autoubiquitination or the activity of other E3 ligases) allows deneddylation by the CSN complex. The deneddylated adaptor-cullin-Rbx1 complex can then either rebind an SRS to reform an SCF complex (horizontal arrow) or undergo sequestration by CAND1 (bottom), in which the adaptor is stripped away from cullin-Rbx1 in the process of CAND1 binding. CAND1 is released via an as yet undefined mechanism that involves cullin-Rbx1 binding either to the adaptor (shown) or an adaptor-SRS complex (not shown). The adaptor-cullin-Rbx1 complex binds an SRS dimer to form a dimeric SCF complex. Substrate binding promotes cullin neddylation to allow full activation of the SCF complex. Proteins are labeled as in Figs 1.3 and 1.4. Figure from (Bosu and Kipreos, 2008)


PROTECTED
BY CSN
CSN
Deneddylation
by CSN and
protection of SRS
Release from CSN
allows neddylation
Neddylation
upon binding
substrate
ACTIVE DIMER
Loss of SRS by autoubiquitination
or degradation by another E3
Binding
SRS dimer
Deneddylation
by CSN
Release of
CAND1
CAND1 binding
& dissociation
of Adaptor
CAND1
SEQUESTERED
BY CAND1

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CHAPTER II

C. elegans CAND-1 regulates cullin neddylation but is dispensable for essential cullin functions

Introduction

A wide range of cellular processes are controlled by regulated protein degradation. The ubiquitin proteasome system degrades the majority of cellular proteins (Ciechanover et al., 1984; Rock et al., 1994). Ubiquitin is an evolutionarily conserved 76 amino acid polypeptide that can be covalently linked to substrates to mark them for degradation by the 26S proteasome or to affect their activity or subcellular localization (Hicke, 2001; Pickart and Fushman, 2004). For proteasome mediated degradation, a substrate should be modified by the attachment of multiple ubiquitins in a tandem array. The ubiquitination reaction involves a multi-enzymatic pathway mediated by a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (Glickman and Ciechanover, 2002; Pickart, 2001). In this process, E3s provide specificity as they bind to specific substrates and to an E2 to facilitate the transfer of ubiquitin either directly from the E2 to the substrate, or indirectly via intermediate transfer of the ubiquitin to the E3 in the case of HECT-domain E3s (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Pickart, 2001).

Cullin-RING ligases (CRLs) are multisubunit E3 ubiquitin ligases that comprise the largest family of E3s in metazoa (Bosu and Kipreos, 2008; Petroski and Deshaies, 2005). There are five major classes of cullins in metazoa, and each forms a distinct type of CRL complex. The best-studied class of CRL complex contains the cullin CUL1, and is given the name SCF in reference to three of its four subunits: Skp1, CUL1 (or Cdc53), an F-box protein, and the RING H2 finger

protein Rbx1/Roc1/Hrt1. The cullin functions as a rigid platform for the assembly of the complex. CUL1 binds the adaptor Skp1 at its N-terminus and the RING H2 finger protein Rbx1 at its C-terminus (Petroski and Deshaies, 2005). Rbx1 recruits E2s charged with ubiquitin to the complex (Kawakami et al., 2001). Skp1 is the adaptor protein that binds to different F-box proteins. F-box proteins serve as substrate-recognition subunits (SRSs) that bind substrates to the complex. There can be tens or hundreds of F-box proteins in eukaryotic organisms, and the binding of distinct F-box proteins to the core SCF complex forms distinct E3 complexes (Cenciarelli et al., 1999; Jin et al., 2004; Kipreos and Pagano, 2000; Kus et al., 2004).

The CRL complexes formed with the other cullins have a similar structure to that of SCF complexes (Petroski and Deshaies, 2005). All classes of CRL complexes contain the RING H2 finger protein Rbx1, while some CRL5 complexes alternatively contain the related Rbx2 protein (Kamura et al., 2004). CUL2- and CUL5-based CRL2 and CRL5 complexes share the same adaptor protein elongin C (a Skp1-related protein), which is bound to the ubiquitin-related protein elongin B. CRL2 complexes contain SRSs with the VHL-box motif, while CRL5 complexes contain SRSs with the related SOCS-box motif. CUL4-based CRL4 complexes contain the adaptor protein DDB1, and an SRS that generally contains WD-repeat proteins of the 'WDXR/DXR' subfamily (Angers et al., 2006; Groisman et al., 2003; He et al., 2006; Higa et al., 2006; Jin et al., 2006; Wertz et al., 2004). Only CUL3-based CRLs show a major difference in the structural pattern relative to SCF complexes, as they employ BTB/POZ domain proteins as SRSs that bind directly to the cullin and the substrate, thereby obviating the need for a separate adaptor protein (Furukawa, He et al. 2003; Geyer, Wee et al. 2003; Pintard, Willis et al. 2003; Xu, Wei et al. 2003).

CRLs are activated by Nedd8 (neural precursor cell-expressed developmentally down regulated 8), which is a ubiquitin-like protein that is conjugated to cullins at a conserved lysine

residue in the C-terminal region in a process termed neddylation (Pan et al., 2004; Petroski and Deshaies, 2005). The neddylation reaction is similar to the ubiquitination reaction and involves a heterodimeric E1 (APP-BP-1/Uba3), the E2 (UBC12), and DCN1 (defective in cullin neddylation) and Rbx1 as E3s (Furukawa et al., 2000; Gong and Yeh, 1999; Kamura et al., 1999; Kurz et al., 2008a; Kurz et al., 2005; Liakopoulos et al., 1998; Megumi et al., 2005; Sufan and Ohh, 2006). In *C. elegans* and budding yeast, DCN1 was identified as a protein that enhances the neddylation of CUL-3 and Cdc53 by facilitating the loading of the neddylation E2 UBC12 onto the cullin (Kurz et al., 2008b; Kurz et al., 2005). While Rbx1 is required for the neddylation as only cullin coupled with Rbx1 can undergo neddylation process (Furukawa et al., 2000; Kamura et al., 1999; Megumi et al., 2005; Sufan and Ohh, 2006). DCN1 can physically bind to Rbx1 and forms a multisubunit E3 or act as a cofactor for the neddylation reaction (Bosu and Kipreos, 2008; Yang et al., 2007).

Neddylation is required for the function of CUL1, CUL2, and CUL3 in a number of species including fission yeast *in vivo* and also increases CRL ubiquitin ligase activity *in vitro* (Morimoto et al., 2000; Ohh et al., 2002; Osaka et al., 2000; Ou et al., 2002; Pintard et al., 2003a; Podust et al., 2000; Read et al., 2000; Wu et al., 2000). Nedd8 can directly interact with the E2, and Nedd8 conjugation to the cullin potentiates the recruitment of the E2 to the CRL complex (Kawakami et al., 2001; Saha and Deshaies, 2008; Sakata et al., 2007). Crystal structure studies indicate that Nedd8 conjugation induces a major conformational change to the C-terminal domain of the cullin which causes the RING domain of the Rbx1 to escape from its binding pocket in the cullin and remain flexibly tethered to the cullin by an extended b-sheet, similar to a balloon on a string (Duda et al., 2008). This structural modification allows Rbx1 bound to charged E2~Ub to move closer to substrates and adopt multiple orientations to accommodate a growing polyubiquitin chain (Duda et al., 2008). Interestingly, CUL1 is able to self-conjugate a ubiquitin to the neddylation site in vitro to activate its ubiquitin ligase activity similar to the activation of

neddylation at the same site, suggesting an alternate pathway for CRL activation (Duda et al., 2008). In budding yeast Nedd8 is not required for essential SCF functions although it enhances SCF activity (Lammer et al., 1998; Liakopoulos et al., 1998).

The COP9 signalosome (CSN) contains a Nedd8 isopeptidase activity, and plays a critical role in Nedd8 dissociation (deneddylation) (Cope et al., 2002; Lyapina et al., 2001). The CSN5 subunit of COP9 signalosome contains a JAMM metalloenzyme domain that mediates the deneddylation activity (Cope, Suh et al. 2002). Inactivation of CSN increases the levels of neddylated cullins *in vivo* (Lyapina et al., 2001; Menon et al., 2007; Pintard et al., 2003a; Schwechheimer et al., 2001), but loss of CSN activity reduces the activity of SCF, CRL3, and CRL4 complexes *in vivo* (Cope et al., 2002; Doronkin et al., 2003; Feng et al., 2003; Groisman et al., 2003; Liu et al., 2003; Pintard et al., 2003a; Schwechheimer et al., 2001; Wang et al., 2003; Zhou et al., 2003). The loss of CRL activity upon inactivation of CSN can be attributed to a significant reduction in SRS levels caused by increased autoubiquitination of the SRSs (Chew et al., 2007; Cope and Deshaies, 2006; He et al., 2005; Wee et al., 2005; Wu et al., 2005; Zhou et al., 2003). In the cell, autoubiquitination of SRSs is inhibited by the deubiquitinase activity associated with CSN that is mediated by Ubp12p in yeast or its ortholog USP15 in humans (Wee et al., 2005; Zhou et al., 2003). The deubiquitinase activity of CSN contributes to the stabilization of CRL1 and CRL3 complexes presumably by removing ubiquitin that is conjugated to the SRSs (Wee et al., 2005; Zhou et al., 2003).

Human CAND1 (cullin-associated and neddylation-dissociated) is a 120kDa protein that contains multiple HEAT repeats (Goldenberg et al., 2004). CAND1 directly binds to unneddylated cullin-Rbx1 complexes to inhibit the formation of the larger active multisubunit CRL complex. CAND1 binds to the unneddylated form of cullins but cannot bind to neddylated cullins (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Zheng et al., 2002). In certain human

cell lines, CAND1 appears to preferentially associate with CUL1, but in other cell lines, it has been found to interact with all cullins (Chew and Hagen, 2007; Oshikawa et al., 2003) (Liu et al., 2002; Min et al., 2003). In *C. elegans*, CAND1 has been shown to bind at high levels to CUL-2, but does not have any detectable binding to CUL-3 (Luke-Glaser et al., 2007; Starostina et al., 2007).

The crystal structure of human CAND1-CUL1-Rbx1 reveals that the CAND1 N-terminus binds to the cullin C-terminus, and the CAND1 C-terminus interacts with the cullin N-terminus (Goldenberg et al., 2004). CAND1 binding to CUL1 prohibits CUL1 from interacting with the adapter Skp1, and blocks access to the lysine residue of CUL1 to which Nedd8 is conjugated, thus inhibiting SCF formation and activation (Liu et al., 2002; Zheng et al., 2002). Inactivation of CAND1 leads to the inactivation of SCF complexes in humans and *Arabidopsis*, and CUL3 complexes in human (Cheng et al., 2004; Chew et al., 2007; Chuang et al., 2004; Feng et al., 2004; Lo and Hannink, 2006; Zheng et al., 2002). Similar to loss of CSN, loss of CAND1 in humans causes a reduction in the levels of the SRS Skp2 through autoubiquitination (Chew et al., 2007; Zheng et al., 2002). In contrast, the activity of the $CRL3^{Keap1}$ complex is inhibited upon CAND1 inactivation even though increased levels of the SRS Keap1 bind to CUL3, suggesting that CAND1 is required for CUL3 activity independently of SRS stabilization (Banks et al., 2006).

Here we have analyzed the *C. elegans* CAND1 ortholog, CAND-1. We observe that CAND-1 is a major component of CUL-2 and CUL-4 complexes and that CAND1 modulates their deneddylation state *in vivo*. In *cand-1* mutants, CUL-2 and CUL-4 neddylation levels increase at the expense of the unneddylated forms. However, unlike in humans and *Arabidopsis*, CAND-1 is not required for the majority of *C. elegans* cullin functions.

Materials and Methods:

Strains and RNAi

The following *C. elegans* strains were used: N2, wild type; ET327, *cand-1(tm1683)*/*unc-76(e911)*; JR667, *unc-119(e2498::Tc1)*, wIs51[seam cell::GFP marker]; ET342, *him-8(e1489)*, ekEx19[*Pcul-2*::*cul-2*::FLAG plus the pRF4 plasmid containing *rol-6(su1006)*]; ET361, *unc-119(ed3)*, ekIs9[pPD49.26/*Pcul-4*::*cul-4*::FLAG]; EU626, *rfl-1(or198)*; JR667, *unc-119(e2498)*, wIs51[seam cell::GFP marker plus *unc-119(+)*]; ET365, *cand-1(tm1683)*, wIs51; ET271, ekEx13[*Pwrt-2*::CDC-6::tdTomato plus pRF4] into H1::GFP integrated line; ET363, *cand-1(tm1683)*, ekEx13; ET113, *unc-119(ed3)*, ekIs2[pPD3.01b/*cyb-1 = Ppie-1*::GFP::CYB-1 + *unc-119(+)*]; ET364, *cand-1*, ekIs2. RNAi was performed by feeding bacteria that express dsRNA for specific genes to L4 stage larvae, as described (Kamath and Ahringer, 2003). *cand-1* feeding bacteria was made by transforming plasmid pPD129.36/*cand-1* into *E. coli* strain HT115.

Two-hybrid assay

Two-hybrid analysis was performed with the full-length and truncated *cand-1* genes in the pACT2 (Gal4 activation domain) vector and with full-length cullin genes in the pAS2 (Gal4 DNA binding domain) vector (Clontech). Transformation of the *S. cerevisiae* strain pJ69-4A (James et al., 1996) and the liquid-based lacZ enzymatic assay were performed as described (Janssen, 1995). Both histidine- and adenine-deficient selective media were use to test interaction in the two-hybrid system.

Antibody production and immunofluorescence

Antisera to CAND-1 was produced in rabbits by immunization with a fusion protein comprising the C-terminal 374 amino acids of CAND-1 linked to a histidine tag in the pET15b vector (Novagen). The HIS-CAND-1 fusion protein was isolated under denaturing conditions

using Ni-NTA agarose (Qiagen) according to the manufacturers instructions. Anti-CAND-1 antibodies were affinity purified against the recombinant protein linked to PVDF membrane as described (Harlow and Lane, 1988). Anti-rabbit Alexa Fluor 488 (Molecular probe) and anti-mouse rhodamine (Cappel) were used as secondary antibodies. DNA was stained with 1 µg/ml Hoechst 33258 dye. Immunofluorescence was performed on animals fixed using the "freeze-crack" method as described (Miller and Shakes, 1995).

Microscopy

Animals were observed by differential contrast interference (DIC) and immunofluorescence microscopy using a Zeiss Axioskop microscope. Images were taken with a Hamamatsu ORCA-ER digital camera with Openlab 4.0.2 software (Improvision). Images were processed with Adobe Photoshop 7.0. Matched images were taken with the same exposure time and processed identically. Matched images of anti-CAND-1, and DAPI staining were deconvolved to equivalent extents to minimize background fluorescence using the multineighbor deconvolution program of Openlab.

Co-Immunoprecipitation, western blots, and mass spectrometry

Worms were lysed with NP-40 buffer containing 10 mM HEPES (pH 7.2), 150 mM NaCl, 1% NP-40, 2 mM EDTA, complete protease inhibitors cocktail (Roche), and 50 µM N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL; Sigma-Aldrich). The primary antibodies used in immunoprecipitation and western detection were monoclonal anti-Flag (M2; Sigma), rabbit polyclonal anti-CUL-2 (Miller and Shakes, 1995; Zhong et al., 2003), anti-CAND-1, and anti-NEDD-8 (Zymed). Anti-rabbit-HRP (Pierce) and anti-mouse-HRP (Pierce) were used as secondary antibodies for western blots that were visualized using the Advanced ECL

chemiluminescence system (GE healthcare). In gel digestion and MS/MS analysis were carried out by the University of Georgia Proteomic Center.

RT-PCR and isolation of *cand-1* cDNA

RT-PCR (reverse transcription-polymerase chain reaction) method was applied for the detection of *cand-1* mRNA expression levels. Total RNA was isolated from whole worm lysate using TRIzol reagent (Invitrogen), according to the manufacturers instructions. RNA was first reversely transcribed into cDNA using a reverse transcriptase with RT-PCR kit from Promega, the resulting cDNA was used as templates for subsequent PCR amplification using following primer pair: gtccatgggtAGTGCTTATCATGTCGGGC and tcaggatccTTATGCAGTTTCCATTGGAGT. The PCR product was sequencing using the following primers: gtagatcacttgtattcattccgt; atcactttcaacgatcctttg; atcggtccagtagtgattgga; cgtgagctgtcggcttgtggt; ctattgcacgcgttgaaagag; and gacatcactcaattctcgtca.

Results

C. elegans has one CAND1 ortholog, CAND-1 (Y102A5A.1), which is located on chromosome V at genetic position +11.2. The gene spans 11.7 kb of genomic DNA; it includes 12 exons and is predicted to encode a 1274 amino acid polypeptide (Fig. 2.1D). The CAND-1 protein has significant sequence identity with orthologous CAND1 proteins: 37% identity with *H. sapiens* CAND1; 37% for *X. laevis*; 36% for *D. melanogaster*; and 22% for *S. pombe* CAND1.

CAND-1 interacts with all cullins and is associated with CUL-2 and CUL-4 *in vivo*

CAND1 was identified by mass spectrometry from co-immunoprecipitations with CUL-2::FLAG and Cul-4::FLAG, and is one of the most abundant proteins associated with the two cullins (Fig. 2.1A,B). To determine if CAND-1 can physically associate with all *C. elegans* cullins,

we tested interactions with the two-hybrid system. Full-length and truncated forms of CAND1 were placed in the two-hybrid pACT2 vector to produce translational fusions with the Gal4 activation domain. CAND-1 is able to interact with all six *C. elegans* cullins in the two-hybrid system (Fig. 2.1C). Deletion of the N-terminal 415 amino acids of CAND-1 decreases interaction significantly, while deletion of the C-terminal 519 or 847 amino acids had less effect on cullin binding (Fig. 2.1C), indicating that the N-terminal region is more important for interaction.

We obtained a *cand-1* deletion allele, *tm1683*, from the National Bioresource Project for *C. elegans* (Japan). The *tm1683* deletion removes 23 base pairs of exon 2, intron 1, and 118 bps of exon 3 (Fig. 2.1D). We sequenced *cand-1* cDNA from the *tm1683* deletion strain and found that the deletion produces an in-frame fusion of exon 1 and exon 3 (data not shown). The *cand-1(tm1683)* allele is predicted to encode a protein of 132 kDa, similar in size to the wild-type CAND-1 protein of 141 kDa. The deletion includes part of the N-terminal region that makes direct contact with cullins (Goldenberg et al., 2004), and that we found is important for interaction with *C. elegans* cullins. CAND-1 mutant protein containing the *tm1683* deletion had significantly reduced interactions with all cullins in the two-hybrid system, similar to a deletion of the entire N-terminal region (Fig. 2.1C). We generated affinity purified anti-CAND-1 antibody against the bacterially expressed C-terminal 374 amino acids of CAND-1. Western blot analysis of whole worm lysate using the anti-CAND-1 antibody revealed a single protein band. The protein level of the CAND-1(tm1683) mutant protein is 12-fold lower than wild-type CAND-1 protein (11.9 ± 3.2; n = 3), suggesting that the mutant protein is unstable (Fig. 2.1E). Treatment of *cand-1(tm1683)* mutants with *cand-1* RNAi reduced the level of CAND-1 protein to essentially undetectable levels (Fig. 2.1E).

CAND-1 developmental expression pattern

We performed immunofluorescence with anti-CAND-1 antibody to determine the developmental expression pattern of CAND-1. In early embryos, CAND-1 is expressed in all cells, predominantly in the nucleus. Embryonic staining is strongest during early embryonic stages and is reduced as embryos progress to later stages (Fig. 2.2). The observation of CAND-1 protein in the one-cell stage zygote indicates that CAND-1 protein is provided as maternal product by the hermaphrodite parent. During larval stages, CAND-1 is observed in proliferative cell lineages, including the seam cells, P-cells, somatic gonad, and germline. CAND-1 expression is also observed in a subset of non-proliferative tissues, including hypodermal cells, rectal gland, and neuronal cells in the head and tail (data not shown). In adults, anti-CAND-1 staining is restricted to the intestine and germline, with the strongest staining in oocyte nuclei (Fig. 2.2). In younger adults, faint vulval cell staining is observed, presumably reflecting CAND-1 protein that perdures from the L4 larval stage (data not shown). The absence of CAND-1 expression in the majority of adult somatic tissues suggests that CAND-1 does not function in tissue homeostasis in the adult. The absence of adult staining mirrors the lack of CUL-2 and CUL-4 expression in adult somatic cells (Feng et al., 1999; Zhong et al., 2003). Overall, our results indicate that CAND-1 is expressed primarily in proliferating cells of the embryo and larvae, but also has expression in a subset of non-proliferating larval cells.

cand-1 mutants do not exhibit major cullin loss-of-function phenotypes

We have characterized the *cand-1(tm1683)* deletion mutant, which can be maintained as a homozygous strain. Approximately 22% of *cand-1(tm1683)* mutant embryos in late embryonic stage (39/180), and 12% of the progeny of *cand-1(tm1683)* homozygotes arrest at the L2 stage (22/180). The remaining 66% become adults, but the adult hermaphrodites lay ~70% less eggs than wild type (83 ± 10 vs. 262 ± 15; n = 10). Adult *cand-1* mutants also show low penetrant phenotypes: protruding vulva (39%; 70/180); and defective tail morphology, including tail bobs

(12%; 21/180) (Fig. 2.3). Developmental timing is slower for *cand-1(tm1683)* mutants than for wild type (85.3 ± 11.2 hr vs. 64.8 ± 3.9 hrs at 20° C for laid egg to adult stage; n = 30 each).

The *tm1683* allele appears to be a hypomorph because the phenotypes of *cand-1* mutants become worse after *cand-1* RNAi (Table 2.1). The finding that *cand-1* RNAi reduces the level of CAND-1 protein in *cand-1(tm1683)* mutants to almost undetectable levels suggests that combining the mutant with RNAi results in almost complete loss of function (Fig. 2.1E). In this regard, it should be noted that *cand-1(tm1683)*, *cand-1(RNAi)* animals are still viable (Table 2.1). As described above, loss of CAND1 in *Arabidopsis* and mammalian cells leads to a loss of cullin function. In *C. elegans*, the loss of function for any of four cullins (CUL-1, CUL-2, CUL-3, or CUL-4) causes death with severe cellular defects. We examined *cand-1* mutants for the major phenotypes associated with specific cullin inactivations in order to determine if CAND-1 is required for these cullin functions. *cul-1* mutants exhibit extensive hyperplasia of multiple tissues resulting from a failure of cells to exit the cell cycle after proliferation (Kipreos et al., 1996). *cand-1* mutants do not exhibit hyperplasia in postembryonic tissues, with the exception of mild hyperplasia observed in seam cells (described below). *cul-2* mutants exhibit a number of distinct phenotypes including a block in meiotic progression, defective G1-to-S phase progression in germ cells, a failure of chromosome condensation, defective anterior-posterior polarity, and mitotic prometaphase delay in the early embryo (Feng et al., 1999; Liu et al., 2004; Sonneville and Gonczy, 2004). These phenotypes, which are each potentially lethal, are not observed in *cand-1* mutants (data not shown). Similarly, *cul-3* mutant phenotypes affecting meiosis and the initial mitosis of the early embryo (Pintard et al., 2003b) are not observed in *cand-1* mutants (data not shown). *cul-4* mutants exhibit a fully-penetrant L2-stage arrest that is associated with DNA re-replication in blast cells (Zhong et al., 2003). *cand-1* mutants exhibit an impenetrant L2-stage arrest similar to *cul-4* mutants, but do not exhibit DNA re-replication (Table 2.1 and data not shown). Therefore, the majority of severe cullin phenotypes are not observed in *cand-1*

mutants with *cand-1* RNAi. These observations imply that *C. elegans* CRLs can adequately provide their cellular functions in the absence of CAND-1.

cand-1 mutants have increased seam cell numbers and defective alae

cand-1 mutant adult hermaphrodites exhibit protruding vulvae with abnormal morphology, yet vulva cell numbers are normal in *cand-1* mutants (22 ± 2 vs. 22 ± 0 in wild type; n = 40). A defect in seam cells, which later become hypodermal cells, can lead to vulval eversion due to the loss of structural connection between the vulva and the differentiated lateral seam cells (Bettinger et al., 1997; Euling et al., 1999; Newman et al., 1996). To analyze seam cell numbers, we used a seam cell::GFP marker, which is expressed exclusively in seam cells. We created a *cand-1(tm1683);* scm::GFP strain and used GFP expression to follow the seam cells. There was no difference in the level of scm::GFP expression per cell, but we found that adult *cand-1* mutants have increased seam cell numbers (18.5 ± 1.4, n = 19) compared to wild type (16 ± 0, n = 10) (Fig. 2.4A).

Differentiated seam cells produce alae in the adult stage. Adult alae comprise cuticular ridges that run the length of the nematode on each of the lateral sides (Sulston and Horvitz, 1977). We examined alae in *cand-1* mutant adults. About 65% of *cand-1* mutants show gaps in alae, as well as irregular alae patterns (n = 20), similar to the disrupted alae observed in mutants of the SCF complex SRS *lin-23*, which have extra seam cells and discontinuous alae (Fig. 2.4B, data not shown). These observations suggest that CAND-1 is required to negatively regulate seam cell divisions.

cand-1 and cullin genetic analysis

To probe the interaction of CAND-1 and cullins further, we addressed whether loss of one copy of *cul-2* or *cul-4* genes would affect the *cand-1* mutant phenotype. Both *cul-2* and *cul-4* null

mutants are recessive and heterozygotes do not exhibit obvious mutant phenotypes (Feng et al., 1999; Zhong et al., 2003). *cand-1(tm1683)* homozygote, *cul-4(gk434)*/+ heterozygote double mutants have increased levels of L2 stage arrest relative to *cand-1* mutants alone (21% vs. 12%; n = 43 and 40) and increased levels of protruding vulva (59% vs. 39% for *cand-1* alone, n=40). The L2-stage arrest is a *cul-4* mutant phenotype, and protruding vulvae are associated with cul-4 and cul-1 inactivations, although in those mutants, the vulvas have altered cell numbers (Kim and Kipreos, 2007;Kipreos et al., 1996). In a *cand-1(tm1683)* homozygote, *cul-2(ek1)*/+ heterozygote strain, several germ cells per gonad arm (3-5) undergo G1 arrest with increased cell size and 2n DNA content (4.2 $\pm$ 0.78, n=10) that is similar to, but much less penetrant than the 100% G1 arrest seen for *cul-2* mutant germ cells (neither *cand-1* mutants nor *cul-2*(ek1)/+ animals exhibit any G1 arrested germ cells). Additionally, *cand-1* mutants are hypersensitive to *cul-1*, *cul-2*, *cul-3*, and *cul-4* RNAi treatments (Table 2.2). In general, these observations suggest that loss of CAND-1 reduces cullin functions. We also analyzed two proteins that are regulated by cullins: cyclin B1, whose protein level is negatively regulated by CUL-2; and CDC-6, whose nuclear export requires CUL-4 activity (Kim et al., 2007; Liu et al., 2004). In *cul-2* mutants, cyclin B1 is not degraded during meiosis, while in *cul-4* mutants, CDC-6::GFP is not exported to the nucleus as seam cells enter S phase. In *cand-1* mutants, similar to wild type, cyclin B1 levels do not perdure in zygotes, and CDC-6 remains cytoplasmic in seam cells after entry into S phase (180-210 minutes post hatch). We conclude that CAND-1 is required for optimal cullin function, but that in the absence of CAND-1, cullins are sufficiently active to maintain their normal functions that are required for viability.

Loss of CAND-1 increases the proportion of neddylated CUL-2 and CUL-4

All cullins are modified by Nedd8 to form active E3 complexes and CAND-1 forms complexes only with cullins that lack the Nedd8 modification (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Zheng et al., 2002). Endogenous CUL-2 and transgenic CUL-2::FLAG

and CUL-4::FLAG, exhibit a slower mobility protein band on SDS-PAGE. This slower mobility protein corresponds to the neddylated isoform, as determined by staining of immunoprecipitated protein with anti-Nedd8 antibody (Fig. 2.5A; data not shown).

To determine the effect of loss of CAND-1 on CUL-2 and CUL-4 neddylation levels, we analyzed CUL-2 and CUL-4::FLAG protein in *cand-1* mutants and in *cand-1* mutants exposed to *cand-1* RNAi, which lack detectable CAND-1 protein. In *cand-1* mutants (with or without *cand-1* RNAi), the proportion of total CUL-2 that is neddylated was significantly increased relative to that observed in wild-type animals (Fig. 2.5B,C,F). This was attributable to an increase in the level of neddylated CUL-2 and a decrease in the level of unneddylated CUL-2. For CUL-4::FLAG, we also observed a larger proportion of neddylated protein (Fig. 2.5D,E). Overall, these results indicate that CAND-1 is a negative regulator of the neddylation of CUL-2 and CUL-4 in *C. elegans*. In this respect it is notable that loss of CAND1 in mammals and *Arabidopsis* does not produce noticeable changes in CUL1 neddylated levels (Chew and Hagen, 2007; Chuang et al., 2004; Feng et al., 2004; Zheng et al., 2002).

ubc-12 RNAi can counteract the effect of *cand-1* loss on cullin neddylation

UBC-12 and RFL-1 are required for CUL-3 neddylation (Jones and Candido, 2000; Kurz et al., 2005; Luke-Glaser et al., 2005). We determined the effect of inactivating both *ubc-12* and *cand-1* on cullin neddylation. RNAi depletion of *ubc-12* in *cand-1* mutants reversed the increase in cullin neddylation that is observed in *cand-1* mutants alone to levels similar to that in wild type. Conversely, neddylation levels were higher in the double mutant than in *ubc-12(RNAi)* animals (Fig. 2.6). However, this reversal of neddylation defects (upon combining *cand-1* and *ubc-12* loss-of-function) did not rescue either the *cand-1* phenotype or the *ubc-12* phenotype. In fact, *ubc-12(RNAi) cand-1* mutants showed an enhanced embryonic lethality compared to either single inactivation (sterile F1 progeny for the double inactivation vs. F1 progeny that produce

eggs for which 50% die for *ubc-12* RNAi and 22% die for *cand-1* mutants). Therefore, inactivating *ubc-12* in the *cand-1* mutant causes synthetic lethality despite the fact that cullin neddylation levels are much more similar to wild type. This suggests that CAND-1 is important beyond merely altering cullin neddylation levels.

Discussion

In this study, we report the expression pattern and mutant phenotype of the *C. elegans* ortholog of mammalian CAND1, as well as its role in the regulation of neddylation levels of *C. elegans* cullins. CAND-1 is expressed in mitotically-dividing germ cells and postembryonic blast cells, including seam cells, P cells, and the somatic gonad. In embryos, CAND-1 protein is provided as maternal product and overall CAND-1 expression is stronger in early embryonic stages, which correlates with the maximal proliferation rate during embryogenesis (Sulston, 1983). CAND-1 is also expressed in a subset of non-proliferative tissues, including the rectal gland and neuronal cells in the head and tail regions, suggesting that CAND-1 has non-cell cycle related functions as well. The expression pattern of CAND-1 correlates with the expression of CUL-2 and CRL4 components (Feng et al., 1999; Kim and Kipreos, 2007; Zhong et al., 2003). This matches the expectation that CAND-1 would be present in all cells that express cullins.

CAND-1 can interact with all cullins and is required for proper neddylation levels

CAND-1 can form a tight complex with unneddylated cullin/Rbx1 (Liu et al., 2002; Min et al., 2003; Oshikawa et al., 2003; Zheng et al., 2002). We found that CAND-1 can interact with all *C. elegans* cullins in the yeast two-hybrid system. In this system, the N-terminal domain of CAND-1 is the most important region for interaction with cullins. The CAND-1(tm1683) mutant protein which has a small in-frame deletion in the N-terminal region has significantly reduced ability to

interact with cullins, which is consistent with the observation that the N-terminal region is critical for the interaction.

The modification of cullins by Nedd8 is required to form active E3 complexes. Neddylation and deneddylation are crucial events for proper cullin functions (Jones and Candido, 2000; Morimoto et al., 2000; Ohh et al., 2002; Osaka et al., 2000; Ou et al., 2002; Pintard et al., 2003a; Podust et al., 2000; Read et al., 2000; Wee et al., 2005; Wu et al., 2005; Wu et al., 2000). In mammals and *Arabidopsis*, loss of CAND1 does not alter the level of neddylated or unneddylated CUL1 (Chuang et al., 2004; Feng et al., 2004; Zheng et al., 2002). However, in this study we have shown that loss of *cand-1* increases the neddylated forms of CUL-2 and CUL-4 with commensurate decreases in the unneddylated forms. CAND-1 could regulate the neddylation levels by reducing the rate of deneddylation or by sequestering cullins to prevent their interaction with the neddylation machinery. Mammalian CAND1 has been shown to enhance the rate of deneddylation of CUL1 *in vitro*, although the mechanism for this action is not fully understood (Min et al., 2005).

In *Drosophila* and *Neurospora*, increased neddylation of CUL1 and CUL3 induces their degradation (He et al., 2005; Wu et al., 2005). Our data indicates that the increased levels of neddylated CUL-2 and CUL-4 do not alter their total protein levels. This result is similar to that observed in mammals where cullins are not destabilized by increased neddylation (Cope and Deshaies, 2006). Overall our results indicate that in *C. elegans*, CAND-1 is required to limit the extent of CUL-2 and CUL-4 neddylation *in vivo*, but does not regulate CUL-2 or CUL-4 protein levels.

Cullin-dependent cellular functions are largely unaffected in *cand-1* mutants

cand-1(tm1683) mutant homozygotes exhibit a range of impenetrant phenotypes, including: late-stage arrested embryos, L2 larval stage arrest, protruding vulva, longer postembryonic development timing, and lower progeny numbers. Nevertheless, *cand-1(tm1683)* mutants are viable. The *cand-1(tm1683)* mutation results in greatly reduced CAND-1 protein levels, and these can be reduced further by *cand-1* RNAi depletion. Despite *cand-1(tm1683)* with *cand-1(RNAi)* animals exhibiting virtually no CAND-1 protein, these animals are viable, although the penetrance of the phenotypes associated with the *cand-1(tm1683)* mutation alone increase. Therefore, CAND-1 is not required for viability in *C. elegans*.

cand-1 mutants have only limited overlap of phenotypes with cullin mutants. For the mutant phenotypes associated with loss of CRL4, *cand-1* mutants share an L2-stage arrest, although it occurs at lower penetrance and it is not clear that the arrest arises from the same underlying defect. *cand-1* mutants do not show the CRL4 defects of DNA re-replication or germ cell degeneration (Kim and Kipreos, 2007; Zhong et al., 2003). For the mutant phenotypes associated with loss of CUL-1, we observe that *cand-1* mutants show limited hyperplasia of seam cells, while *cul-1* mutants have more robust hyperplasia in all postembryonic somatic blast cell lineages (Kipreos et al., 1996). *cand-1* mutants do not show a number of distinct phenotypes that are specific for *cul-2* and *cul-3* inactivations (Feng et al., 1999; Liu et al., 2004; Pintard et al., 2003a; Sonneville and Gonczy, 2004). As loss of CAND-1 does not show many of the severe cellular defects that are associated with loss of CUL-1, CUL-2, CUL-3, or CUL-4, we conclude that loss of CAND-1 is not essential for many (if not most) cullin functions in *C. elegans*.

CAND-1 is a positive regulator of CRL activity in *C. elegans*

The biochemical function of CAND-1 is to inhibit CRL complex formation, yet in humans and plants, loss of CAND1 has been shown to inhibit CRL activities. To address whether *C.*

elegans CAND-1 negatively or positively regulates CRL function *in vivo*, we asked what effect loss of CAND-1 had on partial cullin inactivations. If CAND-1 negatively regulates CRL functions in vivo, then we would expect that loss of CAND-1 would suppress partial loss-of-function phenotypes for the cullins. Conversely, if CAND-1 positively regulates CRL functions in vivo, then loss of CAND-1 would enhance hypomorphic cullin phenotypes. To address this question, we asked how the *cand-1* mutant affected heterozygous strains of *cul-2* and *cul-4* mutants, and the partial inactivating effects of *cul-1*, *cul-2*, and *cul-4* RNAi. In *cand-1* homozygous, *cul-2* heterozygous double mutants, we observed a few enlarged germ cells with 2n DNA content per gonad arm, which is the hallmark of a G1 arrest. While *cul-2* homozygous adults exhibit 100% G1 arrested mitotic germ cells, we do not observe the G1 arrest phenotype with either *cul-2* heterozygotes or *cand-1* mutants alone, suggesting that the phenotype in the double mutant resulted from a genetic interaction of *cand-1* mutant with heterozygous *cul-2* mutant, presumably due to a degradation of the half-level of CUL-2 function when CAND-1 is inactivated. Similarly, *cul-4* heterozygote, *cand-1* homozygotes have increased levels of L2-stage arrested progeny and increased levels of protruding vulva, both of which are *cul-4* mutant phenotypes. Finally, *cand-1* mutants are hypersensitive to *cul-1*, *cul-2*, and *cul-4* RNAi treatment in comparism to wild type animals, suggesting that the reduced levels of these cullins arising from the RNAi cannot function as effectively in *cand-1* mutants as in wild-type. Based on this genetic evidence we infer that CAND-1 is required for optimal cullin functions in *C. elegans*, which is apparent when cullin activity is reduced.

CAND1 regulates CUL-2 and CUL-4 neddylation levels in *C. elegans*

Inactivation of CAND-1 leads to an increase in the level of neddylated CUL-2 and CUL-4 in *C. elegans*. This increased CUL-2 neddylation can be countered by co-inactivation of the Nedd8 E2 UBC-12 with CAND-1. Conversely, co-inactivation of the CSN deneddylase and

CAND-1 leads to an even higher level of CUL-2 neddylation, suggesting that CAND-1 inhibits neddylation independently of the neddylation and deneddylation enzymes.

It has been proposed that the cycling of CRLs between active and inactive states is required for full activity (Cope and Deshaies, 2003; Pintard et al., 2003a; Wolf et al., 2003). One observation that supports this cycling hypothesis is that *C. elegans* CUL-3 is inactive when either neddylation or deneddylation is reduced, but co-reduction of both neddylation and deneddylation restores CUL-3 activity (Pintard et al., 2003a). These observations are also consistent with the hypothesis that the most important function of cullin neddylation and deneddylation regulatory enzymes is to ensure a proper level of neddylated to unneddylated cullin. Based on our observations on cullin neddylation it is reasonable to consider that the major role of CAND-1 could be to maintain the proper level of cullin neddylation *in vivo.* However, when we inactivate the Nedd8 E2 UBC-12 in *cand-1* mutants, CUL-2 and CUL-4 neddylation level was restored to that of wild type but the *ubc-12* RNAi treatment did not suppress the *cand-1* mutant. On the contrary, *cand-1* mutants with *ubc-12* RNAi treatment exhibited a more severe 100% sterility phenotype not observed with inactivation of *cand-1* or *ubc-12* alone. This suggests that the cycling hypothesis may not apply to all cullins and simply maintaining the proper cullin neddylation levels is not sufficient to suppress *cand-1* mutants. CAND-1 may have essential functions beyond regulating cullin neddylation.

Our results suggest that CAND-1 is a positive regulator of cullins in *C. elegans*, whose activity is required for optimal viability and reproductive success. CAND-1 inhibits cullin neddylation, but CAND-1 is not essential for CRL function, although it promotes full CRL activity. Many questions on how CAND-1 regulates CRL function remain to be explored, but future analysis of CAND-1 in the powerful *C. elegans* genetic system is likely to provide unique insights.

Acknowledgements

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Figure 2.1. Interaction of CAND-1 with *C. elegans* cullins

CAND-1 co-immunoprecipitates with CUL-2::FLAG (panel A) and CUL-4::FLAG (B). Silver-stained SDS-PAGE gels are shown for anti-FLAG purifications from strains containing CUL-2::FLAG or CUL-4::FLAG and from control wild-type animals. The CAND1 protein band was identified by mass spectrometry and is labeled. (C) Two-hybrid analysis of interaction between CAND-1 and the *C. elegans* cullins. On the left are diagrams of full-length, truncated, and *tm1683* mutant CAND-1 proteins (the region of protein remaining in the truncation is given in the name). On the right is a graph of the results from a two-hybrid lacZ expression assay that provides quantitation of interactions between the six cullin proteins and the CAND-1 proteins. CAND-1 was expressed from the pACT2 vector, which provides fusion with the Gal4 activation domain vector; and cullins (or the negative control LIN-23 protein; Kipreos et al., 2000) are in the pAS1-CYH2 vector, which provides a fusion to the Gal4 DNA binding domain. (D) Schematic of the *cand-1* genomic region on chromosome V for wild-type and the tm1683 deletion mutant. Exons are represented as boxes and lines represent intron. An arrow indicates the start point and direction of translation. The region deleted in the *tm1683* mutant allele is shown in the lower diagram as the missing regions encompassed by a 'V-shaped' lower line. (E) Effect of RNAi on CAND-1 protein levels. Wild type animals and *cand-1* mutants were treated with *cand-1* feeding RNAi (+ lanes) or control OP50 bacteria (- lanes). Total worm lysate was probed with anti CAND-1 antibody. Note that *cand-1* mutants have lower CAND-1 levels, and that *cand-1* RNAi reduces CAND-1 levels in both the wild-type and *cand-1* mutants.

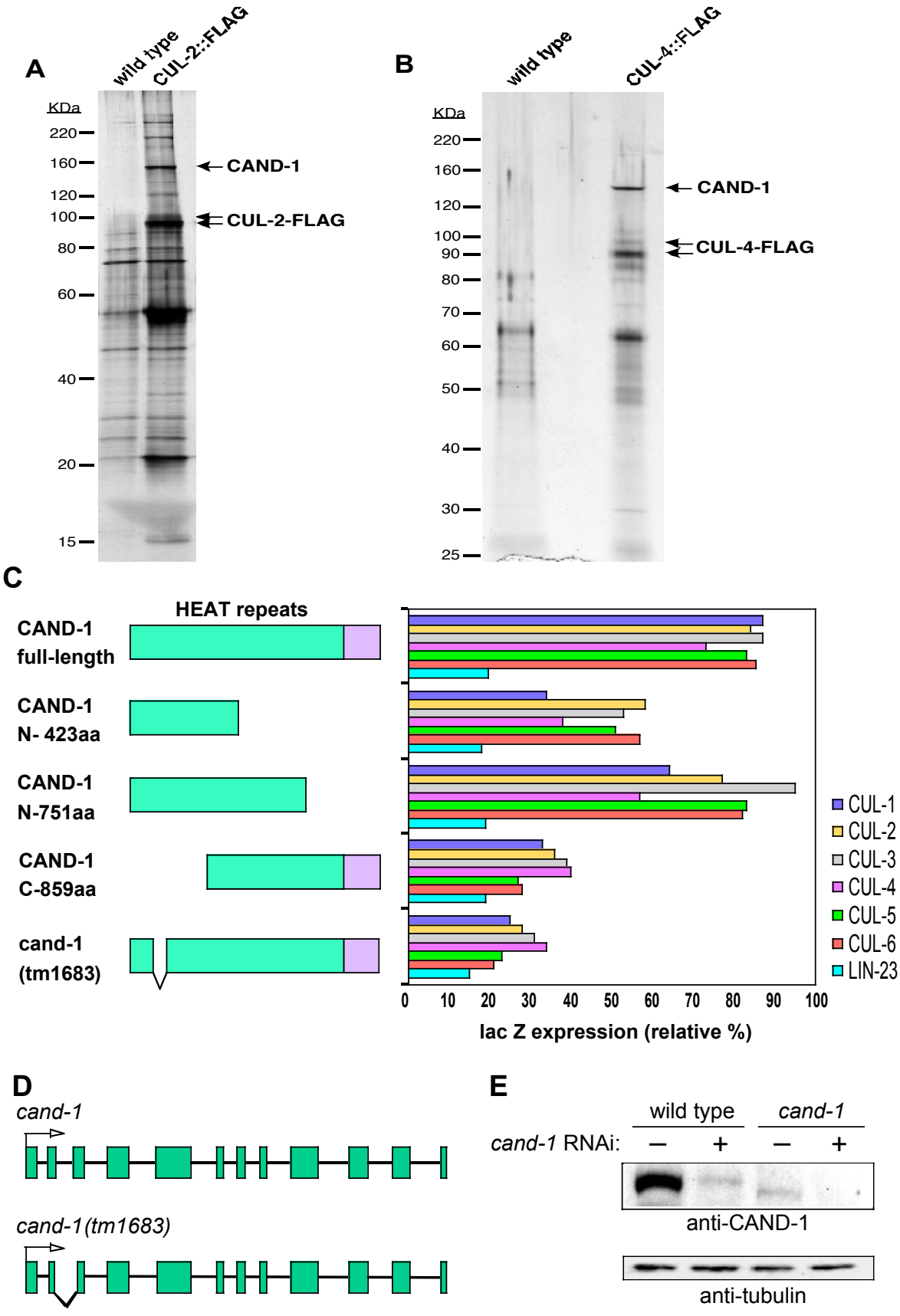

A
wild type
CUL-2::FLAG
KDa
220
160
120
100
80
60
40
20
15
CAND-1
CUL-2-FLAG
B
wild type
CUL-4::FLAG
KDa
220
160
120
100
90
80
70
60
50
40
30
25
CAND-1
CUL-4-FLAG
C
HEAT repeats
CAND-1
full-length
CAND-1
N- 423aa
CAND-1
N-751aa
CAND-1
C-859aa
cand-1
(tm1683)
0
10
20
30
40
50
60
70
80
90
100
lac Z expression (relative %)
CUL-1
CUL-2
CUL-3
CUL-4
CUL-5
CUL-6
LIN-23
D
cand-1
cand-1(tm1683)
E
wild type
cand-1
cand-1 RNAi:
–
+
–
+
anti-CAND-1
anti-tubulin

Figure 2.2. CAND-1 expression pattern

DIC images of wild type and *cand-1* mutants, stained with anti-CAND-1 and DAPI. Intestinal cell (I), vulval precursor cells (VPC). In embryos, CAND-1 is expressed predominantly in the nucleus. In larvae, CAND-1 is expressed in tissues that undergo proliferation, including the germline, seam cells, P-cells, somatic gonad, and germline. In adults, staining is restricted to the intestine and germline. Scale bar = 10 μm.

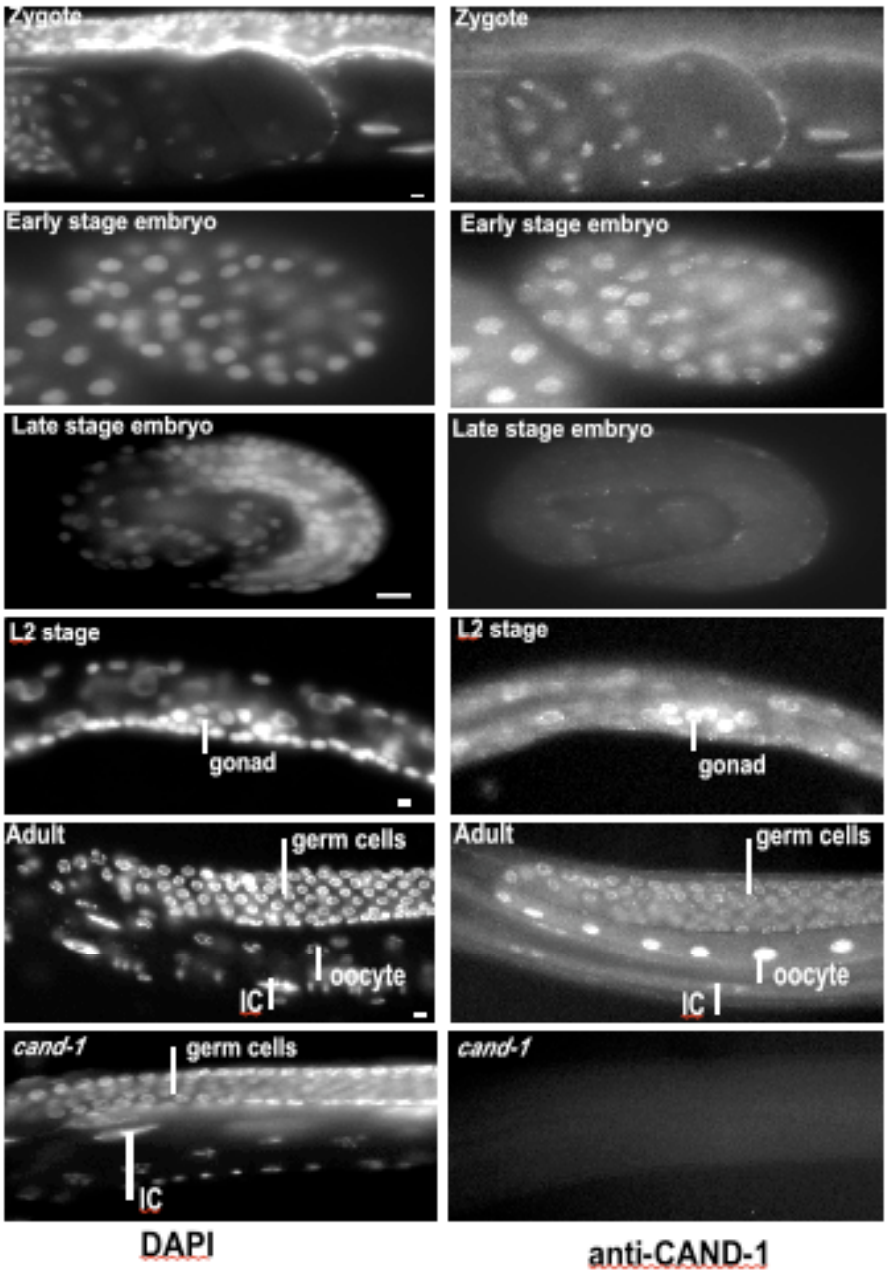

Zygote
Zygote
Early stage embryo
Early stage embryo
Late stage embryo
Late stage embryo
L2 stage
gonad
L2 stage
gonad
Adult
germ cells
oocyte
IC
Adult
germ cells
oocyte
IC
cand-1
germ cells
IC
cand-1
DAPI
anti-CAND-1

Figure 2.3. *cand-1* mutant phenotypes

DIC images of *cand-1* mutants and wild-type animals are presented. The top panels show a late-stage pretzel embryo for wild-type and an arrested *cand-1* embryo. Scale bar = 10 μm.

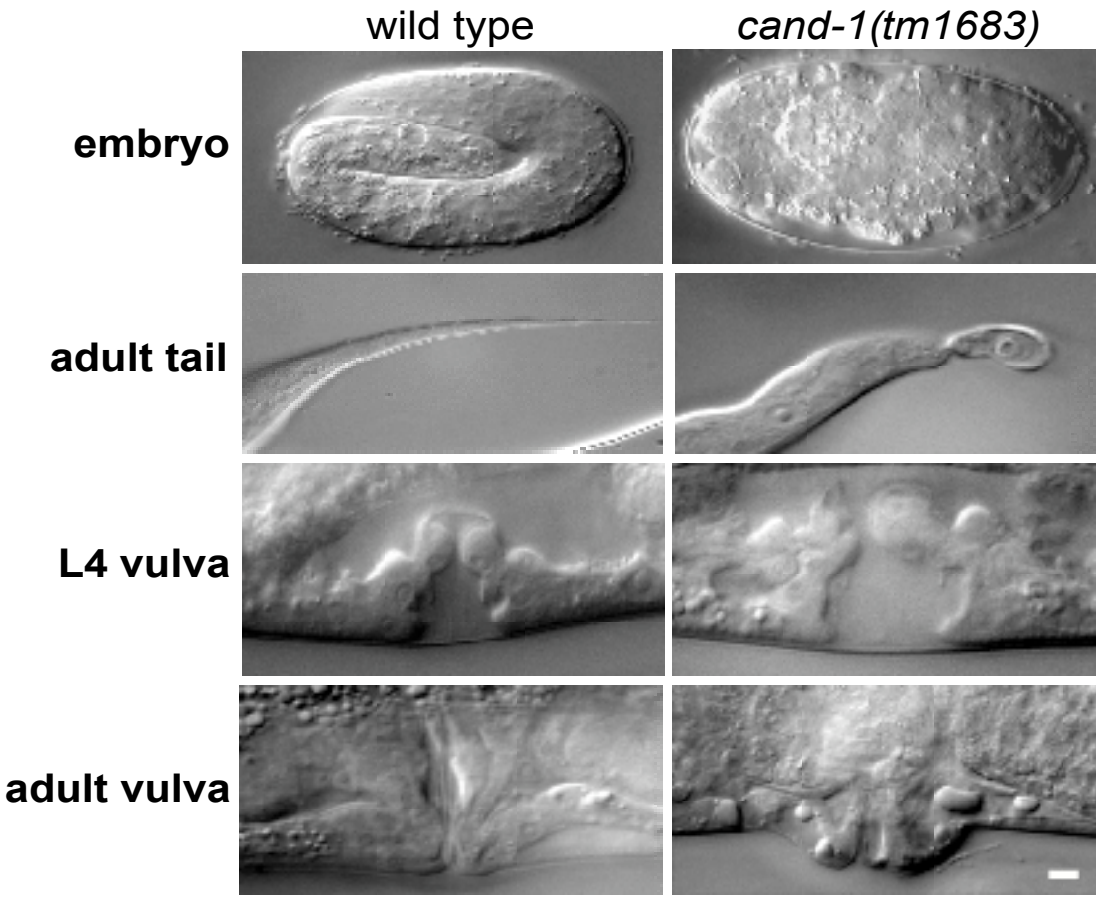
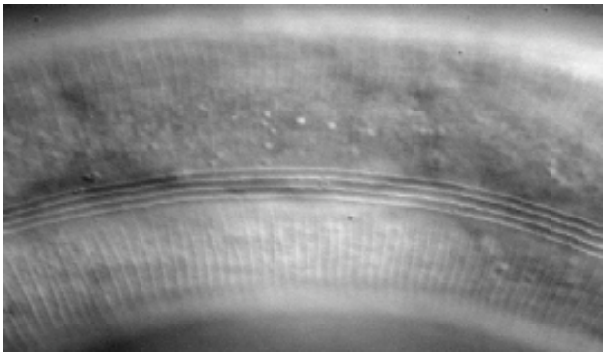

wild type
cand-1(tm1683)
embryo
adult tail
L4 vulva
adult vulva

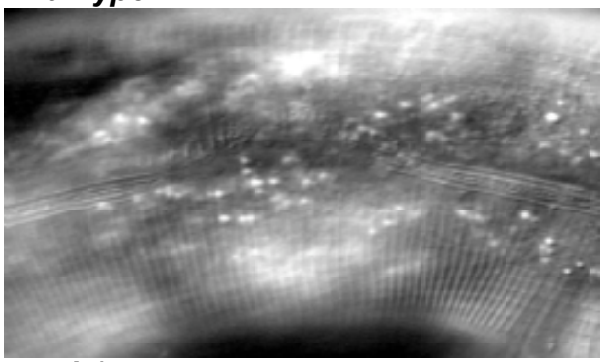
Figure 2.4. *cand-1* mutant has more seam cells and defective alae

(A) Epifluorescence images of scm::GFP signal in seam cells from wild type (top) or *cand-1* mutants (bottom). In these images, the wild type adult has 15 seam cells on its lateral side, and the *cand-1* adult has 21 seam cells. (B) DIC images of alae on wild-type (top), *cand-1* mutant (middle two images), and lin-23 mutant (bottom) adults. Note that wild type adults have four alae ridges. In *cand-1* mutants, these alae are often missing from sections (second panel) or have defective morphology (third panel). The lin-23 mutant, which also exhibits excessive seam cell numbers, has similar defects in alae formation (bottom panel and data not shown). Scale bar = 10 μm.

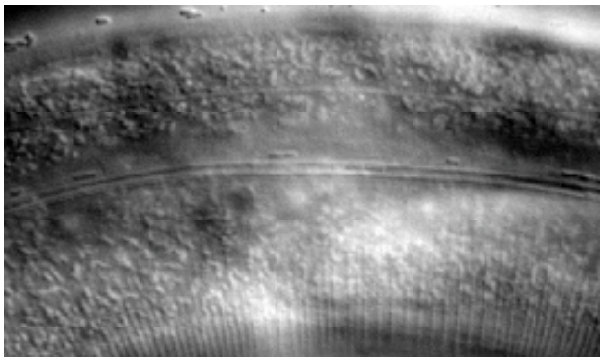
A



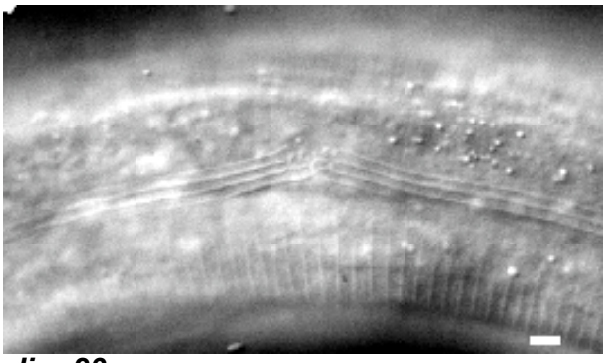
Wild-Type



cand-1

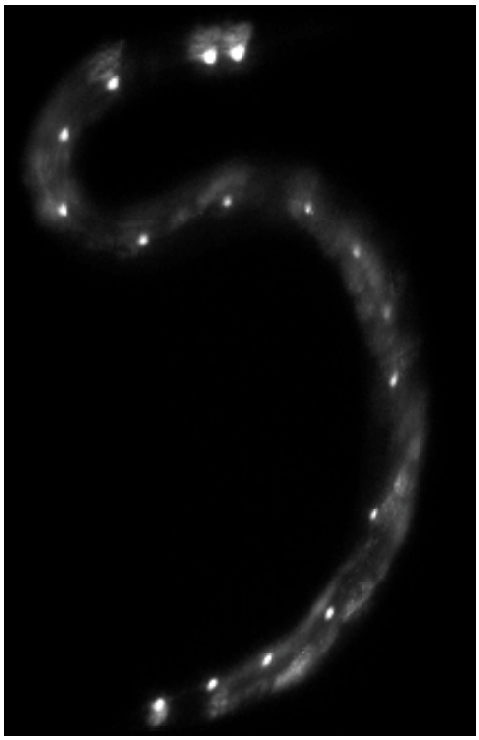


cand-1

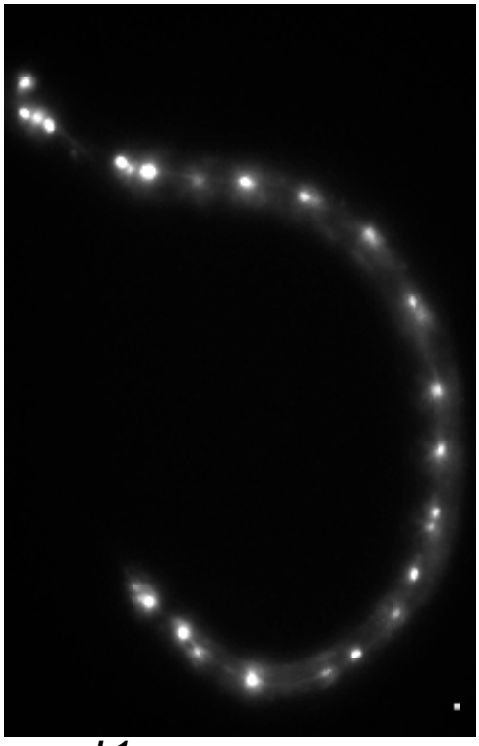


lin -23

B



Wild-Type



cand-1

Figure 2.5. The effect of CAND-1 on CUL-2 and CUL-4 neddylation

(A) Co-immunoprecipitation with anti-FLAG antibody in wild type and in animals ectopically expressing CUL-2::FLAG followed by Western blot with anti-FLAG and anti-nedd8 antibody. (B,C) Whole-worm lysates from wild type and *cand-1* mutants blotted with anti-CUL-2 antibody. (D,E) Total worm lysates prepared from ectopically expressed CUL-4::FLAG in *cand-1* mutant, wild-type and CUL-4::FLAG-expressing animals. The effect of neddylation on CUL-4::FLAG was detected by blotting with anti-FLAG antibody. (F) Whole-worm lysates from wild type and *cand-1* mutants with or without *cand-1* RNAi blotted with anti-CUL-2 antibody. Scale bar = 10 μm.

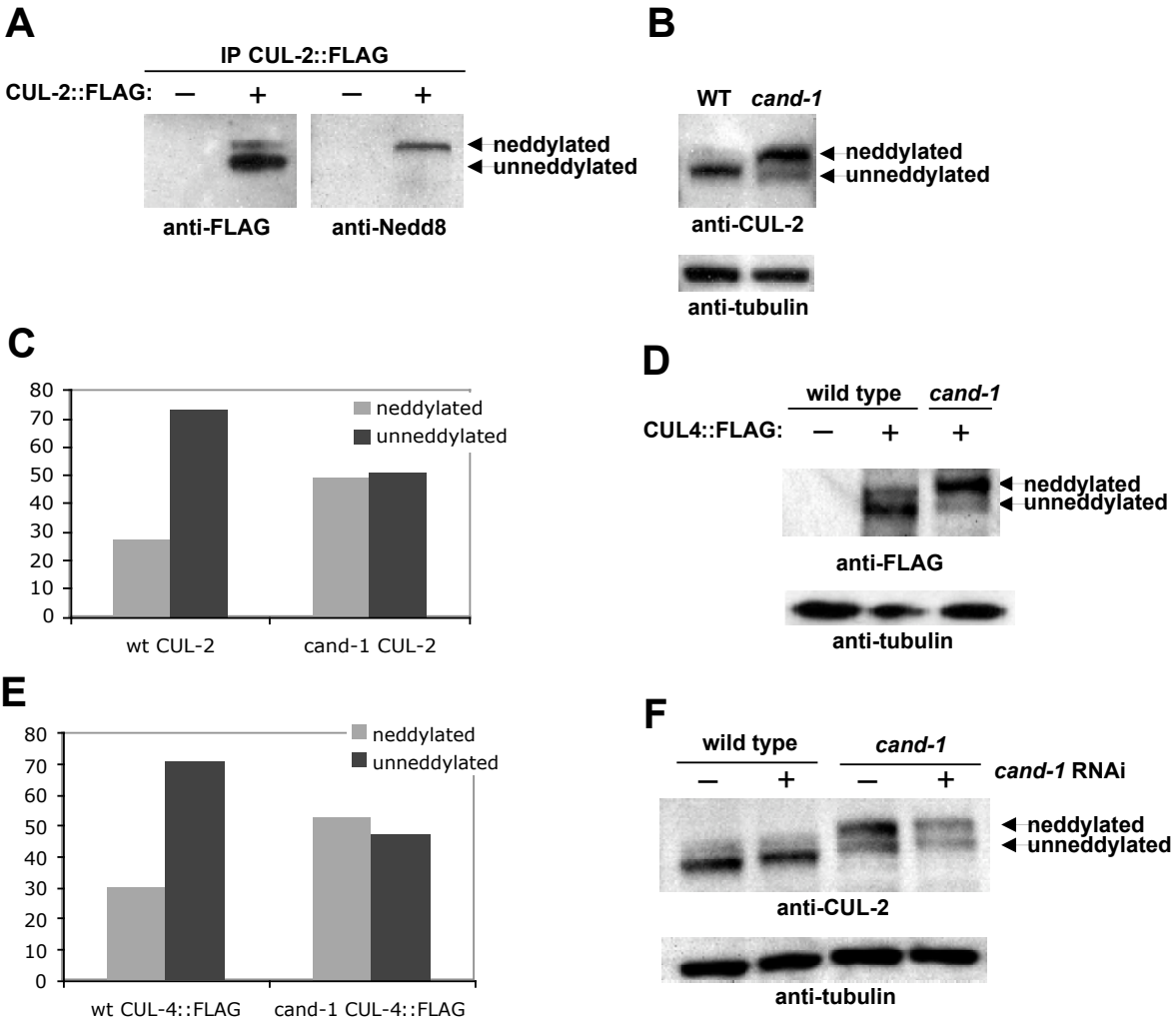

A
IP CUL-2::FLAG
CUL-2::FLAG: – + – +
neddylated
unneddylated
anti-FLAG
anti-Nedd8
B
WT cand-1
neddylated
unneddylated
anti-CUL-2
anti-tubulin
C
80
70
60
50
40
30
20
10
0
neddylated
unneddylated
wt CUL-2
cand-1 CUL-2
D
wild type
cand-1
CUL4::FLAG: – + +
neddylated
unneddylated
anti-FLAG
anti-tubulin
E
80
70
60
50
40
30
20
10
0
neddylated
unneddylated
wt CUL-4::FLAG
cand-1 CUL-4::FLAG
F
wild type
cand-1
– + – +
cand-1 RNAi
neddylated
unneddylated
anti-CUL-2
anti-tubulin

Figure 2.6. ubc-12 RNAi can rescue CAND-1 effect on cullin neddylation

(A) Whole-worm lysates from wild type and cand-1 mutants treated with or without csn-5 and ubc-12 RNAi blotted with anti-CUL-2 antibody. (B) Quantification of the neddylated to unneddylated CUL-2 protein level. (C) Total worm lysates prepared from ectopically expressed CUL-4::FLAG in cand-1 mutant, wild-type and CUL-4::FLAG-expressing animals. Animals were treated with or without csn-5 and ubc-12 RNAi. The effect of neddylation on CUL-4::FLAG was detected by blotting with anti-FLAG antibody. (D) Quantification of the neddylated to unneddylated CUL-4::FLAG protein level.

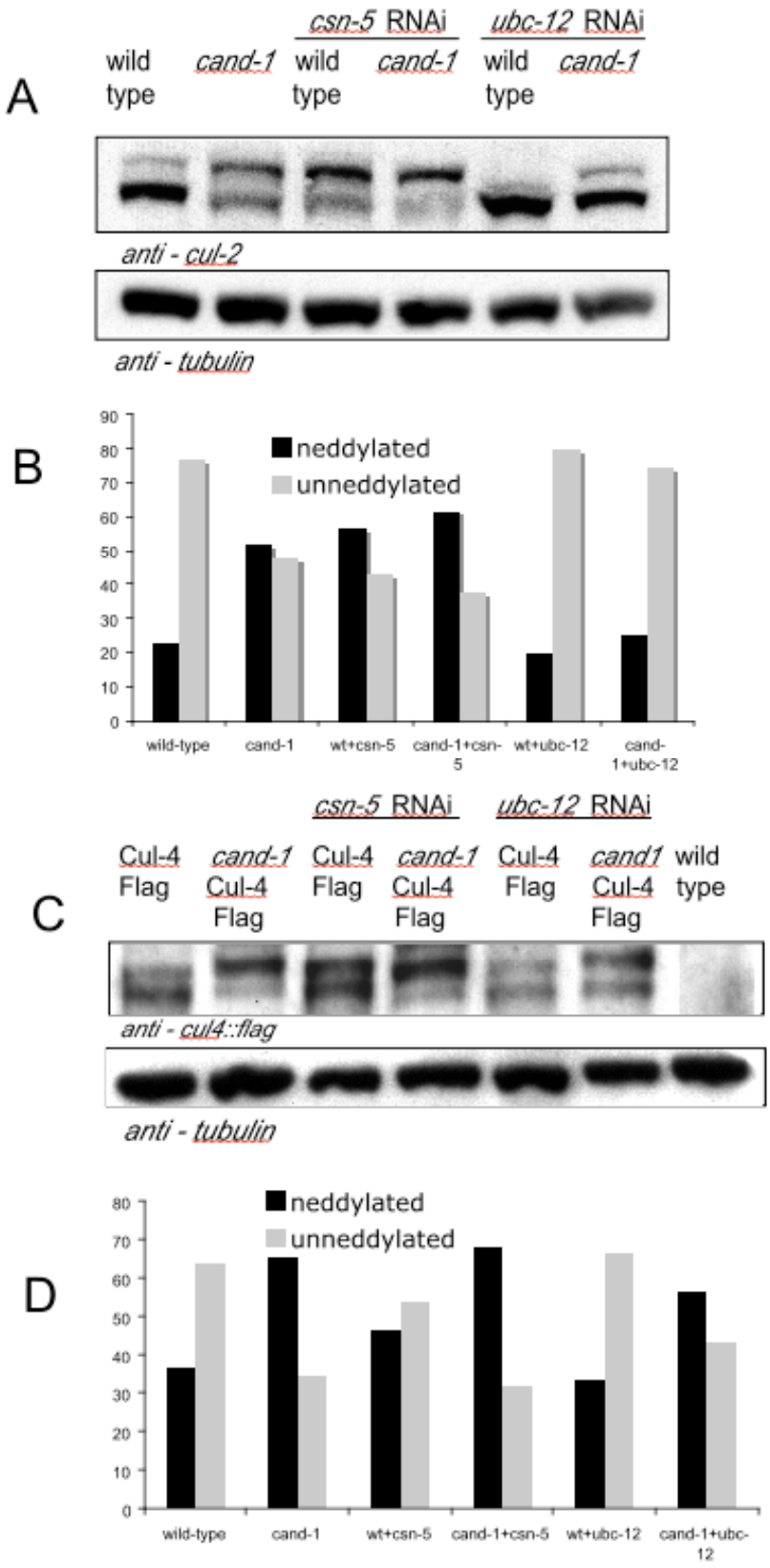

A
csn-5 RNAi
ubc-12 RNAi
wild type
cand-1
wild type
cand-1
wild type
cand-1
anti - cul-2
anti - tubulin
B
neddylated
unneddylated
90
80
70
60
50
40
30
20
10
0
wild-type
cand-1
wt+csn-5
cand-1+csn-5
wt+ubc-12
cand-1+ubc-12
C
csn-5 RNAi
ubc-12 RNAi
Cul-4 Flag
cand-1 Cul-4 Flag
Cul-4 Flag
cand-1 Cul-4 Flag
Cul-4 Flag
cand1 Cul-4 Flag
wild type
anti - cul4::flag
anti - tubulin
D
neddylated
unneddylated
80
70
60
50
40
30
20
10
0
wild-type
cand-1
wt+csn-5
cand-1+csn-5
wt+ubc-12
cand-1+ubc-12

Table 2.1 : *cand-1* mutant phenotype with or without *cand-1* RNAi

Phenotype	N2 (wild-type)	*cand-1*	*cand-1* with *cand-1* RNAi
Total egg count	263 (n =10)	82 (n =10)	60 (n =10)
Dead eggs	0% (n = 100)	22% (n = 180)	35% (n =131)
Protruded vulva	0% (n = 100)	39% (n =180)	48% (n =131)
Tail defect	0% (n = 100)	12% (n =180)	18% (n =131)
sterility	0% (n = 100)	0% (n =180)	7% (n =131)
L2 arrest	0% (n = 100)	12% (n =180)	21% (n =131)

Table 2.2 : Wild-type and *cand-1* mutant phenotype with cullin RNAi

Strain (n = 20)	***cul-1* RNAi**	***cul-2* RNAi**	***cul-3* RNAi**	***cul-4* RNAi**
wt(L4 larvae)	84% Emb	88% Emb	74% Emb	5% Emb, 95%Lva
cand-1(L4 larvae)	100% Emb	96% Emb	97% Emb	68%Emb, 32%Lva
wt(L1 larvae)	Emb, Pvl	Emb	Emb	Emb
cand-1(L1 larvae)	Lva, Ste, Pvl	Emb	Ste	Emb

Phenotypes: Lva, larval arrest; Emb,embryonic lethal; Ste, sterile; Pvl,protruding vulva;
L4 or L1 staged larvae were placed in cullin RNAi plates, n=20 each; F1 progeny were scored for phenotype.

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CHAPTER III

Genomic screening with dsRNAi

Introduction

CRL (Cullin Ring ubiquitin Ligase) regulates diverse cellular processes and their regulation in vivo is not fully understood. The regulation of the interaction of inactive CRL complex with the inhibitor CAND1 and its dissociation from the complex is poorly understood one. The crystal structure of human CAND1 bound to a CUL1-Rbx1 complex indicates that CAND1 wraps around the cullin, with the CAND1 N-terminus bound to the cullin C-terminus and the CAND1 C-terminus bound to the cullin N-terminus (Chew and Hagen, 2007; Goldenberg et al., 2004). CAND1 binding blocks both the adaptor binding site and the Nedd8 conjugation site.

It is reasonable to assume that cells do not produce CAND1 in order to permanently sequester cullin-Rbx complexes, as this would be energetically wasteful. It is therefore pertinent to ask how cullin-Rbx is released from CAND1. There are two potential mechanisms that have been tested to address CAND1 dissociation: 1) neddylation, and 2) the binding of additional CRL components. Neddylation was initially shown to dissociate CAND1 based on in vitro experiments with endogenous human CUL1 that was bound to antibody after immunoprecipitation (Liu et al., 2002). However, studies using soluble, recombinant CUL1 showed that CAND1 is not dissociated by neddylation and instead completely blocks access to the neddylation site (Goldenberg et al., 2004; Hwang et al., 2003). It should be noted that these experiments used different sources of CUL1, endogenous and recombinant (see below).

The second mechanism for CAND1 dissociation is the binding of CRL components. Two groups obtained somewhat different results for this mechanism. Zheng et al. reported that CAND1 could be dissociated from endogenous CUL1 by the addition of the adaptor Skp1 and ATP (Zheng et al., 2002). However, Bornstein et al. indicated that the Skp1-Skp2 complex (but not Skp1 alone) could dissociate CAND1 from endogenous CUL1, and that ATP had no effect on the dissociation (Bornstein et al., 2006). It is currently unclear whether adaptor-SRS or SRS alone is involved in the release of cullin-Rbx. Nevertheless, it is significant that Bornstein et al. showed that Skp1-Skp2 could dissociate CAND1 from endogenous CUL1 but not from recombinant CUL1 (Bornstein et al., 2006). The finding that endogenous CUL1 is more easily released from CAND1 implies a role for either critical post-translationally modification(s) of CUL1 or a 'dissociation factor'.

It has recently been reported that co-inactivation of murine c-Abl and the related c-Arg tyrosine kinase is associated with increased binding between CUL4A and CAND1 (Chen et al., 2006). This suggests that murine c-Abl and c-Arg either promote the dissociation of CAND1 from CUL4A or prevent their association. The mechanistic pathway(s) by which these kinases regulate this interaction has not been resolved.

It has been shown that CAND1 is important to inhibit SRS autoubiquitination but CSN plays an important role for the same function (Chew et al., 2007; Zheng et al., 2002). However, in mammals $CRL3^{Keap1}$ activity requires CAND1 independently of SRS^{Keap1} stability (Lo and Hannink, 2006). As described in chapter II, CAND-1 function is not required for full cullin activity in C. elegans. Currently it is not clear why sequestration of CRL by CAND1 is important, how CAND1 itself is regulated, and the factor(s) required for the CAND-1 dissociation from cullin-Rbx complex in vivo are still unknown. The major advantage of using *C. elegans* is that we can apply forward and reverse genetics to identify genes that are involved in specific cellular functions. In

this study we screened the *C. elegans* genome by RNAi to identify protein(s) that might be involved in decoupling CAND1-Cullin complexes or that are necessary for optimal CAND1 activity.

We found 18 enhancers of *cand-1* mutant from four out of six chromosomes of C. elegans. We also tested the effect of RNAi of the enhancers on the neddylation levels of endogenous CUL-2 to determine their effect on the CRL activation cycle and found two potential genes that may involved in this process.

Materials and method

Genomic screen by RNAi

Double stranded RNA (dsRNA) for the genes of interest were introduced into wild type and *cand-1* mutants by feeding animals with bacteria expressing dsRNA. We used a pre-established RNAi feeding library (Fraser et al., 2000; Kamath and Ahringer, 2003; Kamath et al., 2003) created by Julie Ahringer's group. Their RNAi feeding library contains ~86% of the 19,427 predicted genes of *C. elegans* in double T7 promoter vectors. We followed the liquid culture method for screening as described by the Plasterk and Tijsterman labs (van Haaften et al., 2004). *cand-1(tm1683)* and wild-type homozygous L1 staged larvae were seeded in 96-well plates at 10-20 larvae/well containing the RNAi feeding bacteria. After 7 days in culture, the plates were observed with a dissecting scope for differences between the *cand-1* mutant and wild type wells.

We compared the effect of the inactivation of a particular gene affecting wild type vs. *cand-1* mutants. *cand-1* enhancers will have more severe phenotypes in *cand-1* mutants compared to wild type. An enhancer RNAi would have the seeded wild type larvae becoming adults and then producing hundreds of progeny that cleared the well by eating all of the bacteria.

In contrast, the *cand-1* mutant larvae would arrest as larvae or adults prior to producing progeny and the well would still contain dense bacteria. Genes for which *cand-1* is the suppressor will have less severe phenotypes in *cand-1* mutants than in wild type animals and would have the opposite effect.

Western blot analysis

For the analysis of cullin neddylation, wild-type and *cand-1* mutants L1 larvae were placed on RNAi bacteria plates and allowed to develop until the animals were young adults. Worms were lysed with NP-40 buffer containing 10 mM HEPES (pH 7.2), 150 mM NaCl, 1% NP-40, 2 mM EDTA, complete protease inhibitors cocktail (Roche), and 50 μM N-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL; Sigma-Aldrich). Whole-animal lysate used for western blots with anti-CUL-2 antibody. Control lysate included wild-type without RNAi treatment (a negative control). Rabbit polyclonal anti-CUL-2 (Zhong et al., 2003) was used as primary antibody and anti-rabbit-HRP (Pierce) was used as secondary antibody for western blots that were visualized using the Advanced ECL chemiluminescence system (GE healthcare).

Results

Potential enhancers from C. elegans RNAi library screening

We completed screening four of the six C. elegans chromosome: III ; IV ; V; and X. These four chromosomes cover 9875 genes of the genome. The same genetic screen sought to detect *cand-1* enhancers and genes for which *cand-1* is the suppressor. After initial screening, we identified 88 enhancers that did not have significant effects on wild type animals but severely impacted the viability of *cand-1* mutants, but I did not identify any gene for which *cand-1* is the suppressor (Table 3.1-3.4). We repeated our results with plate-based feeding RNAi for

reproducibility and identified 18 enhancers whose RNAi inactivation affected *cand-1* mutant repeatedly without affecting wild-type animals (Table 3.5).

To identify *cand-1* enhancers that may affect the CRL activation cycle cullin neddylation levels were determined after inactivating enhancers by RNAi in wild-type animals. Our initial screen of chromosome IV identified 26 *cand-1* mutant enhancers (Table 3.2). We have tested the effect of RNAi of these 26 enhancers in wild-type and found that two reproducibly increased the level of neddylated CUL-2 so that it is more abundant than the unneddylated CUL-2 (Fig. 3.1). In wild-type animals, the upper, neddylated CUL-2 band is less abundant. In contrast, RNAi of C49C8.5 and Y73F4A.3 produces higher levels of the upper neddylated CUL-2 band relative to the lower, unneddylated band similar what is observed in the *cand-1* mutant. RNAi of other enhancers had lower but reproducible effects on this ratio (e.g. the equal ratio of bands in F42A9.9 RNAi) (Fig. 3.1).

Discussion

The focus of this study was to identify the factor(s) that are involved in CRL regulation, including CAND-1 binding and dissociation. Our genetic screen can identify genes in the same pathway or separate pathways that do not physically interact. We found 18 enhancers of the *cand-1* mutant and RNAi depletions of these genes specifically enhanced the *cand-1* mutant phenotype. Loss-of-function *cand-1* enhancers are presumed to include the following types of proteins:

1) Proteins that are required for optimal CAND1 activity. As *cand-1(tm1683)* mutant has residual activity, inactivating proteins that promote CAND-1 activity will worsen the mutant phenotypes. These enhancers are expected to exhibit phenotypes in a *cand-1* mutant

background that reflect additional loss of CAND-1 activity, similar to the addition of *cand-1* RNAi to the *cand-1* mutant.

2) Proteins that function in parallel with CAND1 to promote CRL activity. Our results from chapter II suggest that the inactivation of neddylation-promoting genes in combination with CAND-1 inactivation produces enhanced lethality. Therefore, other classes of genes that regulate the activation cycle of cullins may be isolated as *cand-1* enhancers.

3) Proteins that promote the function of specific cullins. In *cand-1* mutant cullin function is reduced which provides a sensitized background for cullin activity. Therefore, proteins that facilitate a particular cullin's activity loss of their function could produce more severe cullin-specific phenotypes in *cand-1* mutants relative to wild-type animals.

It is interesting that the phenotypes associated with 18 enhancers often overlap those of *cand-1* mutants (including embryonic lethality (Emb); protruding vulva/ruptured vulva (Pvl/Rup); slower growth (Gro); larval arrest (Lva); and low brood size), providing the possibility that the full inactivation of the enhancers alone will produce a similar phenotype to that of *cand-1* (Table 3.5). 17 out of 18 enhancers that we identified have been reported to have RNAi phenotypes in large-scale RNAi screens (Table 3.5). *A priori*, one would think that genes with RNAi phenotypes in wild type should not be identified in our screen because they would have effects on the control wells. However, RNAi is often not fully penetrant when provided in feeding constructs. Some of the large-scale RNAi screens that reported these phenotypes used either injection of dsRNA or RNAi-sensitized genetic background to increase the efficacy of feeding RNAi(Gonczy et al., 2000; Piano et al., 2000; Simmer et al., 2003). Further analysis will distinguish whether these superficially similar phenotypes result from the same primary cellular defects.

We also found two enhancers (encode nematode specific protein and not conserved in human) from chromosome IV that affect cullin neddylation levels independently of CAND-1. We were most interested in those enhancers that affect the CRL activation cycle. The major criteria for activated cullin is cullin neddylation. Enhancers that affect cullin neddylation levels independently of CAND-1 could be possible candidates for regulators of the CRL activation cycle.

One of the major goals of this screening was to identify factor(s) that dissociate CAND-1 from the cullin complex. This type of factor(s) could be detected from this screen as genes for which *cand-1* is the suppressor. If any gene product is required to decouple CAND-1 from a CAND-1-cullin complex then feeding dsRNAi of that particular gene will decrease the neddylation of cullin as CAND-1 will be always bound to cullin. As a result, cullin functions will be inhibited and RNAi will produce cullin loss-of-function phenotypes. However, inactivating the same gene in *cand-1* mutants will not have any effect because CAND-1 is unavailable to bind with cullin and lack of a factor to dissociate CAND-1 will not have any effect. In our initial screen we were unable to detect candidates for which *cand-1* is the suppressor.

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Table 3.1 Putative *cand-1* enhancers on chromosome III

Cosmid no.	Reported RNAi phenotypes	Proposed function
T24C4.5(phi-53)	Emb, Lva	nucleotidyltransferase activity
T04A8.6	Lvl, Gro, Lva, Sle, stp, Egl	nucleic acid binding
C27F2.8	Lva, dpy, Unc, Emb, Gro, Lvl	integral to membrane
C16A3.6	Lvl, Pvl, Gro, Ste, Emb	Novel
C05D11.11(mel-32)	Emb	glycine hydroxymethyltransferase activity
F11H8.4(cyk-1)	Emb	actin binding
F37C12.1	Emb, Lva, reduced brood size	Novel
F37C12.3	Emb, Lva, Stp	acyl carrier activity
R151.9(pfd-5)	Emb, Unc, Pvl, Stp	unfolded protein binding
B0361.10	Gro, Emb, Stp, Clr, Stp	integral to membrane
C07H6.5(cgh-1)	Emb	ATP dependent helicase activity, RNA helicase activity
ZK783.2(upp-1)	Gro, Emb, Stp, Stp, Lva, lvl	uridine phosphorylase activity
K06H7.6(apc-2)	Emb	ubiquitin protein ligase binding
C02F5.1(knl-1)	Emb, ste, Pvl	kinetochore
F09G8.3	Gro, Emb, Sck	structural constituent of ribosome
C06E1.10(rha-2)	Gro, Emb, Stp	ATP-dependent helicase activity
ZK507.6	Emb	Unknown
Y48A6B.3	Gro	ribonucleoprotein complex
Y48A6B.11(rsa-2)	Gro, Emb, Unc, Stp	protein binding, bridging

Phenotypes: Let, lethal; Lva, larval arrest; Gro, slow growth; Emb,embryonic lethal; Ste, sterile; Bmd,body morphology defective; Pvl,protruding vulva; Rup, ruptured vulva; Unc,uncoordinated; Prl,paralyzed; Sma,small; Dpy,dpy; Adl, adult lethal; Sle, slow embryonic development; and. Phenotypes shared with *cand-1* mutants or RNAi are in bold. RNAi phenotypes are from compilations at WormBase: www.wormbase.org.

Table 3.2. Putative *cand-1* enhancers on chromosome IV

cosmid/gene	Reported RNAi phenotypes	Pvl+*cand-1*	proposed function (protein type)
H04M03.4/glf-1	Let,Unc,Prl, Sma,Dpy	-	Galactofuranose synthesis; UGM, UDP-galactopyranose mutase
C50F7.2/clx-1	none	-	(contains 23 copies of a 15 aa repeat found in collagens)
C33H5.7	Pvl,Adl	Pvl	SWD subunit of histone H3 methyltransferase & RNA cleavage factor II complexes
C07G1.3/pct-1	none	Pvl	PCTAIRE class of cell cycle kinase
K07H8.2	none	-	Homolog of solute carrier family 41 member
T26A8.4	Let,Lva,Gro,Emb,Pvl ,Unc,Sle	Pvl	(CCCH-type zinc finger protein)
F42A9.9	none	-	Novel
C49C8.5	none	-	Fibrinogen alpha chain precursor
D1046.2	Lva,Egl,Rup,Unc	-	(C2H2 type Zn finger protein)
C53D6.2/unc-129	none	-	Bone morphogenetic protein 3 precursor (BMP-3)
C53D6.5	none	Pvl	Novel
Y73F4A.3	none	Pvl	Novel
F23B2.1/tag-60	extended lifespan	Pvl	Na(+)/H(+) exchange regulatory cofactor NHE-RF2
F23B2.9	none	-	Novel
C07D10.3/sre-3	none	-	Serpentine Receptor, class E (epsilon)
F49C12.4	none	Pvl	Novel
F49C12.11	Let,Lva,Emb,Bmd,Unc,Dpy	Pvl	Novel
C33A12.1	none	-	NDUFA5 subunit of the mitochondrial NADH dehydrogenase
C33A12.15	none	-	Novel
R07H5.1/prx-14	Lva,Gro,Emb	-	Peroxisomal membrane protein PEX14
F28D1.10/gex-3	Lva,Gro,Emb,Bmd,Let,Pvl,Ste	Pvl	Homolog of mammalian NAP1; interacts the small GTPase Rac1
C39E9.14/dli-1	Emb,Rup,Pvl,Unc,Muv	Pvl	Dynein light intermediate chain
B0564.1a; B0564.1b.1/tin-9.2	Let,Lva,Ste,low brood,Rup,Unc	Pvl	Exosome ribonuclease complex, subunit Rrp41 Mitochondrial intermembrane space translocase, subunit Tim9
Y41E3.11	Let,Lva,Ste,low brood,Rup,Unc	Pvl	Heterogeneous nuclear ribonucleoprotein U-like protein
Y73F8A.8/pqn-90	none	Pvl	Novel
Y105C5A.7	none	Pvl	non-LTR retrotransposon

Phenotypes: Let, lethal; Lva, larval arrest; Gro, slow growth; Emb,embryonic lethal; Ste, sterile; Bmd,body morphology defective; Pvl,protruding vulva; Rup, ruptured vulva; Unc,uncoordinated; Prl,paralyzed; Sma,small; Dpy,dpy; Adl, adult lethal; Sle, slow embryonic development; and. Phenotypes shared with *cand-1* mutants or RNAi are in bold. RNAi phenotypes are from compilations at WormBase: www.wormbase.org.

Table 3.3. Putative *cand-1* enhancers on chromosome V

cosmid no.	RNAi phenotype	proposed protein function
K06H6.3	no phenotype	nucleotide-sugar transmembrane transporter activity
F41H8.1	no phenotype	integral to membrane
C29G2.2	no phenotype	Novel
F53E10.6	Gro	positive regulation of growth rate
C24B9.10(srg-56)	no phenotype	integral to membrane
F54E2.2	no phenotype	Novel
F59D6.3	no phenotype	aspartic-type endopeptidase activity
C50H11.5(srt-9)	no phenotype	integral to membrane
C36C5.12	no phenotype	integral to membrane
C04E12.7(scrm-3)		Novel
C02A12.5(srbc-32)	no phenotype	integral to membrane
C02A12.6(srbc-36)	no phenotype	integral to membrane
R08F11.6(fpn-1.3)	no phenotype	iron ion transmembrane transporter activity
K07C6.3(cyp-35B2)	no phenotype	electron carrier activity, monooxygenase activity
T09H2.1(cyp-34A4)	no phenotype	electron carrier activity, monooxygenase activity
B0213.15(cyp-34A9)	Age	electron carrier activity, monooxygenase activity
F47D2.9(srh-193)	no phenotype	integral to membrane
C54D10.8	no phenotype	integral to membrane

Phenotypes: Let, lethal; Lva, larval arrest; Gro, slow growth; Emb,embryonic lethal; Ste, sterile; Bmd,body morphology defective; Pvl,protruding vulva; Rup, ruptured vulva; Unc,uncoordinated; Prl,paralyzed; Sma,small; Dpy,dpy; Adl, adult lethal; Sle, slow embryonic development; and. Phenotypes shared with *cand-1* mutants or RNAi are in bold. RNAi phenotypes are from compilations at WormBase: www.wormbase.org.

Table 3.4. Putative *cand-1* enhancers on chromosome X

Cosmid/gene	Reported RNAi phenotype	Proposed function (protein types)
T04G9.1	Unknown	novel
T04G9.4	Emb, Sck, unc	holo-[acyl-carrier-protein] synthase activity
AH9.3	no phenotype	novel
C43H6.7	no phenotype	zinc ion binding
T07D1.4(fox-1)	Egl	nucleic acid binding
F52E4.1(pccb-1)	no phenotype	novel
F20B6.8(hpk-1)	Age	protein kinase activity
F35C8.7(chtl-1)	bli, stp, Lva	integral to membrane
T08A9.1(atg-11)	no phenotype	novel
C10A4.1	no phenotype	novel
R09F10.3	Emb, Ste	Reproduction
D2021.1(Utx-1)	Gro, Unc, Pvl	histone H3 di/trimethyllysine-27 demethylase
F53A9.6	no phenotype	novel
T01C1.2(mbr-1)	fat content increased	DNA binding
F47B10.3	no phenotype	integral to membrane
C05C9.2	no phenotype	novel
F29G6.3	Gro, Clr	Reproduction
C34E11.2	no phenotype	regulation of transcription
H36L18.1	no phenotype	metalloendopeptidase activity
F18H3.3(pab-2)	Emb	nucleic acid binding
Y70D2A.1	no phenotype	novel
F14F4.3(mrp-5)	Gro, Clr	ATPase activity
F31A3.1(abu-3)	no phenotype	novel

Phenotypes: Let, lethal; Lva, larval arrest; Gro, slow growth; Emb,embryonic lethal; Ste, sterile; Bmd,body morphology defective; Pvl,protruding vulva; Rup, ruptured vulva; Unc,uncoordinated; Prl,paralyzed; Sma,small; Dpy,dpy; Adl, adult lethal; Sle, slow embryonic development; and. Phenotypes shared with *cand-1* mutants or RNAi are in bold. RNAi phenotypes are from compilations at WormBase: www.wormbase.org.

Table 3.5. *cand-1* enhancers on chromosomes III, IV, and X

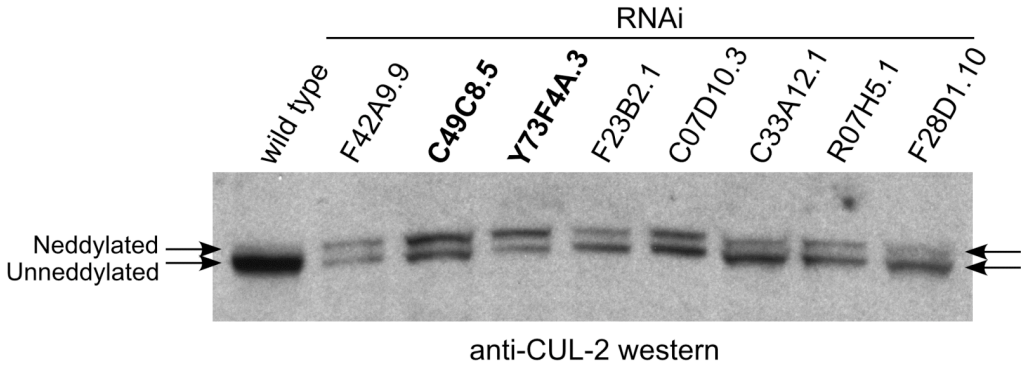
cosmid ID	name	Chr	Reported RNAi phenotypes	human homolog	protein type & function
C27F2.8	-	III	Emb, Lva, Unc, Gro, (none)	RW1; TMEM131	no motifs; unknown function
C05D11.11	mel-32	III	Emb, (none)	SHMT1	serine hydroxymethyltransferase
F11H8.4	cyk-1	III	Emb, Lva, Cyk, Unc, Rup	DIAPH1	formin-homology protein; required for cytokinesis (Ce)(Swan et al., 1998)
F37C12.3	-	III	Emb, Lva, Gro, Stp, (none)	NDUFAB1	NADH-ubiquinone oxidoreductase
R151.9	pfd-5	III	Emb, Lvl, Unc, Pvl, Gro	PFDN5	Prefoldin subunit 5
B0361.10	-	III	Emb, Lva, Gro, Unc, Stp	YKT6	R-SNARE; acyltransferase
K06H7.6	apc-2	III	Emb, Mei, Ste, Pvl, (none)	ANAPC2	APC/C ubiquitin ligase component
Y48A6B.11	rsa-2	III	Emb, Gro, Unc, Pvl, (none)	(not conserved)	no motifs, regulates spindle assembly
C33H5.7	-	IV	Pvl, Adl, (none)	Wdr82	WD-repeats; recruits histone methyl-transferase to transcription sites (Hs)(Lee and Skalnik, 2008)
D1046.2	-	IV	Lva, Rup, Unc, Egl, (none)	(not conserved)	C2H2-type Zn-finger
F49C12.11	-	IV	Emb, Lva, Gro, Bmd, (none)	CCDC72; HSPC016	Coiled-coil protein; unknown function
C33A12.1	-	IV	Emb, Lva, Gro	NDUFA5	NADH dehydrogenase subunit
R07H5.1	prx-14	IV	Emb, Lva, Gro	PEX14	Peroxisomal membrane anchor protein
C39E9.14	dli-1	IV	Emb, Ste, Rup, Pvl, Muv	DYNC1LI2	dynein 1 light intermediate chain 2
Y41E3.11	-	IV	Emb, Lva, Gro, Unc, Rup	HNRPUL1	hnRNP U-like protein 1
F20B6.8	hpk-1	X	Age, (none)	HIPK1	homeodomain-interacting kinase
K03A1.6 & RNAz-515701	his-38 ncRNA	X	Emb, Lva, Gro, Unc, Pvl	HIST1H4C	histone H4 and non-coding RNA
D2021.1	utx-1	X	Emb, Gro, Unc, Pvl, (none)	UTX	histone H3 lysine-27 demethylase

Phenotypes: Emb, embryonic lethal; Lva, larval arrest; Gro, slow growth; Unc, uncoordinated; Bmd, body morphology defective; Pvl, protruding vulva; Rup, ruptured vulva; Muv, multivulvae;

Adl, adult lethal; and Age, shortened lifespan. RNAi phenotypes are from compilations at WormBase: www.wormbase.org. (Ce) = function in C. elegans; (Hs) = function in humans. Chr = chromosome location

Figure 3.1. Effect of enhancers on cul-2 neddylation level

Whole-worm lysates from wild type treated with enhancer RNAi and blotted with anti-CUL-2 antibody.


RNAi
wild type
F42A9.9
C49C8.5
Y73F4A.3
F23B2.1
C07D10.3
C33A12.1
R07H5.1
F28D1.10
Neddylated
Unneddylated
anti-CUL-2 western

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CHAPTER IV

General Discussion

Ubiquitin ligases provide the substrate specificity for ubiquitination reactions. The largest known class of ubiquitin ligases are cullin-RING ubiquitin ligases (CRLs) (Petroski and Deshaies, 2005). CRLs are integral regulators of diverse cellular processes, including the cell cycle, transcription, signal transduction, and development (Petroski and Deshaies, 2005). The majority of dynamic cellular processes are regulated at some level by CRLs. Different cullins form CRLs with different classes of SRSs and usually different adaptor proteins (Petroski and Deshaies, 2005). All CRLs are regulated by binding to an inhibitor called CAND1 (Liu et al., 2002; Zheng et al., 2002). However, there are large gaps in our understanding of the *in vivo* importance of CAND1 and how its binding and dissociation from cullins is regulated. This dissertation demonstrates the importance of CAND-1 for the regulation of cullin neddylation *in vivo* and describes its importance for CRL functions in *C. elegans*. There is also a description of a screen that identifies genetic modifiers of the *cand-1* mutant phenotype.

C. elegans has the five major classes of cullins found in animals, CUL1 through CUL5. Loss of CAND1 in *Arabidopsis* and mammalian cells leads to a loss of CUL1 function, despite CAND1 acting as a cullin inhibitor (Cheng et al., 2004; Chew et al., 2007; Chuang et al., 2004; Feng et al., 2004; Lo and Hannink, 2006; Zheng et al., 2002). In *C. elegans*, the loss of either CUL-1, CUL-2, CUL-3, or CUL-4 lead to death with severe cellular defects. Animals that lack CAND-1 are viable, and we therefore conclude that CAND-1 cannot be essential for CUL-1, CUL-2, CUL-3, or CUL-4 functions. Nevertheless, the presence of CAND-1 has a significant

effect on the neddylation state of the cullins. In *cand-1* mutants, the CUL-2 and CUL-4 are significantly increased, indicating that CAND-1 normally acts to limit their neddylation.

It has been shown that loss of the COP9 signalosome component *csn-5*, which encodes the deneddylase, shows an increase in the neddylation of CUL-3 similar to the increased neddylation observed for CUL-2 and CUL-4 in the *cand-1* mutant (Pintard et al., 2003). However, while *csn-5* mutants exhibit severe *cul-3* mutant phenotypes resulting in embryonic arrest, *cand-1* mutants do not show severe cullin phenotypes. Therefore, it appears that changes in the overall neddylation level do not correlate with cullin function, and that loss of the deneddylase has more severe implications than loss of CAND-1 even though both increase neddylation levels.

Because CAND-1 is a biochemical inhibitor of CRL complexes, the question arises whether loss of CAND-1 inhibits or activates cullin function in *C. elegans*. *A priori*, one would think that loss of CAND-1 would activate CRLs, yet inactivation of CRL1 complexes are observed in mammalian cells and *Arabidopsis* upon inactivation of CAND1. Our results using sensitized genetic backgrounds suggest that *C. elegans* CAND-1 is required for full CRL functions.]We found that loss of CAND-1 increased the penetrance of cullin phenotypes in strains heterozygous for *cul-2* and *cul-4* mutations, and significantly increased the severity of phenotypes associated with the partial loss of function from cullin RNAi. Therefore, it appears that *C. elegans* CAND-1 also functions as a positive regulator of CRL activity *in vivo*, similar to plants and mammals.

Pintard *et al.* presented data that suggested that balanced levels of neddylation and deneddylation are required for CUL-3 activity in degrading the substrate MEI-1. Partial inactivation of neddylation components and partial inactivation of deneddylation components suppress each other's mutant phenotypes (Pintard et al., 2003). Pintard *et al.* proposed that cycles of CUL-3 neddylation and deneddylation were necessary for its ligase activity *in vivo*. Our

results do not support this neddylation cycling hypothesis as a common mechanism to regulate all CRLs. When the increased CUL-2 neddylation levels in *cand-1* mutants are reduced by inactivating the Nedd8 E2 UBC-12, we observe an enhancement of phenotypes rather than a suppression (even though the CUL-2 and CUL-4 neddylation levels are similar to that in wild type). This data suggests that balancing cycling between neddylation and deneddylation states (by getting rid of CAND-1-mediated inhibition of neddylation to counter reduced neddylation in ubc-12 RNAi) do not promote CUL-2 and CUL-4 activity as predicted by the neddylation cycling hypothesis. Rather, the data suggests that CAND-1 has a role in promoting CRL activity beyond merely regulating the level of cullin neddylation.

There are many unresolved issues in understanding CRL regulation that focus on major three processes: **1)** how CRLs shift from an active form to an inactive form; **2)** how inactive cullin-Rbx1 complexes that are sequestered by CAND1 are activated; and **3)** how CRLs are converted to a different CRL with a different SRS. We found 18 enhancers from the genomic screening and further study of them could determine possible factor(s) required for CRL and CAND-1 regulation and functions. Our enhancers could fall into three categories. First, enhancers may regulate the CRL activation cycle independently of CAND-1. This type of enhancers would affect cullin neddylation levels in wild-type animals. We discussed in chapter III that determining the cullin neddylation level in wild-type animals upon enhancer RNAi could identify these regulators of the CRL activation cycle. We can also reveal the level of CUL-2 and CUL-4 proteins in the enhancer RNAi animals to identify enhancers that are required to maintain proper cullin protein levels.

A second class of enhancers is those required for optimal CAND-1 activity. These enhancers could be examined for phenotypes in a *cand-1* mutant background that could reflect additional loss of CAND-1 activity similar to the addition of *cand-1* RNAi to the *cand-1* mutant. As described in chapter III, some of the enhancers have reported RNAi phenotypes that overlap

those of *cand-1* mutants. The full inactivation of these genes may also produce a similar effect on cullin neddylation to that seen in *cand-1* mutants if their function is to promote CAND-1 activity. The inactivation of some enhancers could affect CAND-1 protein levels either in a *cand-1(+)* or *cand-1* mutant background, which would suggest that the enhancer was required to maintain CAND-1 protein levels. Therefore probe for increased cullin neddylation and decreased CAND-1 level upon inactivation of these enhancers in wild-type animals could identify possible interactor(s) or regulator(s) of CAND-1.

A final class of enhancers could be involved in regulation of specific cullin-dependent processes in *C. elegans*. Initial determination of whether enhancers produce phenotypes associated with particular cullins mutants, and that interact with only one or a few cullins, could distinguish these enhancers as well.

The research described in this dissertation has contributed to understanding the extent to which CAND-1 functions as a positive regulator of cullin using the model organism *C. elegans*. The research also provides a framework for analyzing uncharacterized potential regulators of CAND-1 and the CRL activation cycle.

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