Regulation of SCF Ubiquitin Ligases by Jab1/Csn5 and the Cop9 Signalosome

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Abstract

SCF ubiquitin ligases regulate the ubiquitin-dependent proteolysis of a myriad of substrate proteins, including p27, Cyclin E, and IκBα. To further gain insight into SCF regulation and function, we purified SCF from mammalian cells and found the Cop9 Signalosome associated with SCF. Interestingly, deletion of the CSN in *S. pombe* resulted in the hypermodification of Cul1 with Nedd8 *in vivo*. Furthermore, we found that the CSN can promote the removal of Nedd8 from Cul1 *in vitro*, suggesting CSN regulates SCF through deneddylation. To investigate the basis of CSN-dependent deneddylation activity, we analyzed the CSN and the 26S proteasome for conserved sequences that could be representative of a catalytic motif. We identified the JAMM motif in Csn5 and Rpn11 of the proteasome. Mutations in JAMM eliminated CSN-dependent deneddylating activity. Moreover, mutations in JAMM reduce the restrictive temperature of several SCF temperature sensitive mutants, suggesting that CSN acts positively on SCF activity. Finally, a JAMM mutant failed to rescue Csn5Δ defects in drosophila. To investigate the biological effect due to loss of deneddylating activity, we constructed a stable cell line that expresses an inducible siRNA sequence toward CSN5. We found that knock-down of Csn5 results in a dramatic decrease in F-box protein levels. Moreover, this loss correlates with F-box protein substrate accumulation and hyperneddylation of Cul1. We propose an autocatalytic mechanism for the turnover of F-box proteins that is dependent upon the deneddylating activity of CSN.

Table of Contents

List of Figures and Tables

Figures

Tables

Chapter 1: Introduction

Ubiquitin-Proteasome System

The 76 amino acid protein ubiquitin belongs to one of the most powerful systems within a eukaryotic cell, the ubiquitin proteasome system (UPS). This power is evident from the system's function: the UPS manages the bulk of intracellular proteolysis and can destroy any protein fed into its cogs. Given this ability, the UPS has far-reaching effects and has been shown to act in the regulation of almost every facet of eukaryotic cell biology, from transcription, to cell cycle regulation, to development, to immunology. Furthermore, this system has been shown to be essential for life in all eukaryotes tested. However despite our current knowledge, we have only just begun to shed light on how this system can manipulate cellular process, as well as the molecular mechanisms hidden behind its shroud. Indeed, it is hard to study and define a system when many of the components have yet to be discovered.

Given the vast influence of the UPS, the ramifications of its failure can be destructive. Loss of UPS function has been implicated in numerous diseases, including neurodegenerative, autoimmune, and viral diseases as well as cancer. Despite the wealth of knowledge that has been formed on the link between the UPS with certain diseases, we have yet to determine the pathways leading to UPS malfunction in many of these maladies. Moreover, we are just beginning to harness this knowledge in disease prevention.

On the opposite extreme, exploiting the power of this system can be extremely useful in the treatment of diseases (reviewed in Goldberg and Rock 2002). One of the most striking examples of this can be found in the recent advances in cancer therapy. A

boom in UPS research for cancer therapy has led to drugs that target the UPS, the most notable of which is Velcade—an inhibitor of the terminal step of the UPS—which is currently used to treat patients with multiple myeloma. In addition to the monumental undertaking to treat cancer, attempts are being made within the biomedical industry to treat diseases such as HIV, Parkinson's, and inflammation using chemicals that effect UPS function.

The complicated nature of the UPS makes this system difficult to dissect. However, intracellular proteolysis catalyzed by the UPS can be naively simplified into two discrete phases requiring an ensemble of players: ubiquitin conjugation (ubiquitination) and degradation.

Ubiquitination

Ubiquitination involves a three-step cascade ultimately resulting in the conjugation of the protein ubiquitin onto a target protein. In the first step, which requires the hydrolysis of ATP to AMP, the 76 amino acid protein ubiquitin is 'activated' by an ubiquitin-conjugating enzyme (E1). This step results in the formation of a covalent thioester intermediate between the C-terminal glycine of ubiquitin and the catalytic cysteine of the E1 enzyme. This high-energy thioester is preserved in step two by the transfer of ubiquitin onto a catalytic cysteine of one of several ubiquitin-conjugating enzyme (E2) to form a subsequent thioester intermediate. Ultimately, either alone or in collaboration with an ubiquitin-ligating enzyme (E3), the ubiquitin-conjugating enzyme can release its cargo onto lysines of the substrate protein forming an isopeptide bond between the terminal amino group of the target lysine and the C-terminal glycine of

ubiquitin. A caveat to this final step is that not only can a lysine of the substrate protein act as an acceptor residue for ubiquitin, but also lysines within ubiquitin, leading to the formation of high molecular weight chains of ubiquitin attached to the target protein.

The number of players in this ubiquitination ensemble allows for a remarkable degree of variation in this pathway. For example, in yeast there is only one E1 enzyme, eleven E2 enzymes, and each of these E2 enzymes can interact with a multitude of E3 ligases (reviewed in Pickart 2001). Given that E3 ligases are thought to provide substrate specificity to this system, this multitude of E3s can target an enormous array of substrates for ubiquitination. Moreover, the E2 and E3 enzymes can catalyze a wide assortment of different ubiquitin modifications—each with a remarkably different fate.

The attachment of a single ubiquitin molecule (monoubiquitination) onto a substrate has been attributed to lysosomal sorting and trafficking (reviewed in Schnell and Hicke 2003). Monoubiquitination can act as a signal for internalization of cell surface proteins into the endocytic pathway and has also been found to alter the activity of certain endocytic enzymes. Moreover, monoubiquitination of histones can contribute to gene regulation and silencing (reviewed in Schnell and Hicke 2003).

Given that ubiquitin contains seven potential acceptor lysines, the formation of an ubiquitin-ubiquitin isopeptide can yield a great many flavors of modification (reviewed in Pickart and Fushman 2004). Two lysines within ubiquitin have been extensively studied, lysine 48 and lysine 63. Lysine 48 (K48) linkages are thought to contribute to protein destabilization, as these linkages are recognized by the 26S proteasome for degradation (see below). Mutations of K48 leads to lethality in budding yeast, and K48 mutants fail to form high molecular weight conjugates on many target proteins studied (reviewed in

Pickart and Fushman 2004). Alternatively, mutation of K63 in budding yeast results in UV sensitivity, an altered DNA damage response, and a defect in stress response (Arnason and Ellison 1994; Spence et al. 1995), suggesting lysine 63 linkage (K63) is involved in multiple pathways, including DNA damage, protein trafficking, ribosomal function, and inflammation response (reviewed in Pickart and Fushman 2004). In addition, K63 linkages have been found to participate in the regulation of several proteins—including the DNA polymerase factor PCNA (Hoege et al. 2002) and the immune response factor NEMO (Zhou et al. 2004).

Degradation

Polyubiquitination of a target protein via lysine 48 of ubiquitin targets the protein conjugate for degradation by a large, highly conserved multiprotein complex—the 26S proteasome. The proteasome can be dissected into two large sub-complexes, each with a defining function: the core regulates substrate proteolysis while the cap regulates substrate selection and translocation (reviewed in Pickart and Cohen 2004).

The proteasome cap sub-complex can be further separated into two components—the lid and base (reviewed in Pickart and Cohen 2004). The lid is thought to play two main roles in target degradation. First, through a mechanism aided by the delivery of substrates through at least one of several multiubiquitin chain receptors, the lid can recognize and bind to polyubiquitinated proteins (Verma et al. 2004). This first step is then followed by the deubiquitination of the substrate utilizing a metalloenzyme present in the Rpn11 protein (see Chapter 3 and Verma et al. 2002). These two steps above, through a mechanism that is not fully understood, work in concert with the second

lid sub-complex, the base, which plays a role in the unfolding of the substrate to facilitate translocation into the core (reviewed in Pickart and Cohen 2004).

The core sub-complex of the proteasome resembles a barrel in shape, the center of which contains the catalytic protease sites that hydrolyzes substrates fed though its ends (reviewed in Pickart and Cohen 2004). The ends of this barrel are closed by the 19S sub-complex—one on both ends—which are thought to 'gate' both substrate translocation to and peptide release from the core. This 'gating' mechanism is controlled, at least in part, by the base protein Rpt2, which somehow regulates entry and exit to and from the core (Kohler et al. 2001). Interestingly, proteolytic cleavage of substrate proteins within the core participates in antigen presentation in the immune system (reviewed in Kloetzel 2004), and the protease sites within the core are able to splice peptides to yield novel antigenic peptides (Vigneron et al. 2004). Despite the enormous amount of research on the mechanism of the 26S proteasome, it is still unclear how substrate recognition, unfolding, deubiquitination, translocation and proteolysis function either alone or together to control substrate proteolysis.

E3 Ubiquitin Ligases

The E3 ligase family of proteins is enormously diverse and is characterized by two separable activities: substrate recognition and poly-ubiquitin chain formation utilizing the E2 enzyme. Given the diversity of the substrate recognition domain, most E3 ligases are identified through the more conserved domains that catalyze poly-ubiquitin chain formation. Structurally speaking, two prominent domains represent this activity: the RING (really interesting new gene) and HECT (homologous to E6-AP carboxy terminus)

domains. At least one of these domains, or a variation thereof, is present in all known E3 ligases, and it is currently not known if other domains, in addition to these two, possess E3 ligase activity.

The HECT ubiquitin ligase domain was originally characterized during studies with the human papillomavirus (HPV) (reviewed in Pickart 2001). Infection with oncogenic forms of HPV causes the tumor suppressor protein p53 to be down-regulated in a 26S proteasome dependent manner (Scheffner et al. 1990). Biochemically, p53 ubiquitination was dependent upon the E6 protein of HPV and an associated E6 protein, E6-AP (Scheffner et al. 1993). Ubiquitination of p53 depended upon the highly conserved C-terminus of E6-AP, later termed the HECT domain, and homologs containing this domain were found in several different proteins (Huibregtse et al. 1995). All HECT domains contain a conserved catalytic cysteine, and ubiquitination of substrates catalyzed by the HECT domain is dependent upon thioester formation between this conserved cysteine and ubiquitin (Scheffner et al. 1995).

The RING domain is a zinc-binding domain similar to a zinc finger (Freemont 1993). In contrast to the HECT domain, the RING does not form a catalytic thio-ester attachment between itself and ubiquitin, rather it is thought to somehow activate the E2 enzyme (Seol et al. 1999). Variations of the RING domain have recently been identified, and these also contain ubiquitin ligase activity (Aravind and Koonin 2000; Aravind et al. 2003). The most notable RING-containing E3 ligases characterized to date are the anaphase-promoting complex (APC) and SCF ubiquitin ligases.

APC Ubiquitin Ligase

The APC is a highly conserved, multi-protein complex essential for mitotic progression (reviewed in Peters 2002). Composed of at least 11 subunits, its activity is dependent upon the binding of a single substrate adapter protein (Peters 2002). Among these, Cdc20 and Cdh1 both control the ubiquitination of a myriad of substrates and, whereas Cdc20 appears to regulate substrates in mitosis, Cdh1 regulates substrate ubiquitination in G1/S (Peters 2002).

The key role the APC plays in mitotic progression involves chromosome segregation: the APC ubiquitinates an inhibitor of the enzyme Separase—Pds1—in mitosis (Ciosk et al. 1998). This ubiquitination and subsequent degradation triggers the activity of Separase—a protease that cleaves the protein glue holding mitotic chromosomes together in metaphase—allowing for chromosome separation (Ciosk et al. 1998). In addition, the APC can ubiquitinate the mitotic B type Cyclins, whose activity impedes exit from mitosis (Peters 2002). Recent roles for the APC have been identified in G0/G1, and the APC has been shown to regulate the abundance of numerous proteins in this cell cycle phase, including Cdc6 (Petersen et al. 2000), Skp2 (Bashir et al. 2004; Wei et al. 2004), Cyclin A, and UbcH10 (Rape and Kirschner 2004).

SCF Ubiquitin Ligases

Another class of RING E3 ligases that has been extensively studied is that of the SCF family of ubiquitin ligases. SCF consists of four components: Skp1, Cul1, a variable F-box protein, and Hrt1/Rbx1/Roc1 which harbors the RING domain. Three of these four subunits were identified in the budding yeast *S. cerevisiae*, where deletion or mutation of

Cul1 (Cdc53), Skp1, or Cdc4—an F-box protein—results in cell cycle arrest due to the accumulation of the Cyclin CDK inhibitor Sic1 (Schwob et al. 1994; Bai et al. 1996). Subsequently, the fourth subunit, Hrt1/Roc1/Rbx, was identified by multiple groups (Ohta et al. 1999; Seol et al. 1999; Skowyra et al. 1999; Tan et al. 1999).

Each of these four subunits contributes to SCF function in a distinct way. Both Cul1 and Hrt1 are thought to contain the core polyubiquitination activity, as these two proteins alone are sufficient to activate the E2 and catalyze substrate independent poly-ubiquitin chain formation (Ohta et al. 1999; Seol et al. 1999; Skowyra et al. 1999). Alternatively, the F-box protein is thought to bind specifically to substrates, thereby recruiting them to the catalytic core for ubiquitination. Skp1 bridges the F-box protein and the Cullin subunit, and Skp1 can bind to several F-box proteins each with a potentially unique cargo that is primed for ubiquitination (Bai et al. 1996).

The structure of SCF has lead to a key question into how this complex functions (Zheng et al. 2002). Cul1 forms a very elongated and rigid structure, which binds Hrt1 on one end and Skp1 on the other (Zheng et al. 2002). Interestingly, the F-box protein Skp2 in this structure binds solely to Skp1, and modeling of both the E2 enzyme and substrate reveals a huge gap between the E2 active site and lysines of a substrate. Thus a key question remains as to how this gap is bridged by the SCF enzyme (Zheng et al. 2002).

Given the number of substrate-recognizing F-box proteins identified to date (in yeast there have been 18 identified F-box proteins; in Arabidopsis there have been 400; and in humans there have been over 70), the number of potential SCF 'flavors' is astounding (Petroski and Deshaies 2005). This vast number of different F-box proteins suggests that SCF has the potential to catalyze the destruction of an enormous range of

proteins, in each case utilizing the same Cul1/Hrt1/Skp1 core (Petroski and Deshaies 2005). Indeed, a large set of SCF substrates and their opposing F-box protein set has been identified in several organisms (reviewed in Petroski and Deshaies 2005). These substrate/F-box protein pairs play roles in diverse processes such as cell cycle progression, immune response, signal transduction, and transcription. In almost all of these cases, post-translational covalent modification targets recognition of the substrate by the F-box protein, and phosphorylation appears to be the most predominant covalent modification. However, oligosaccharide modifications have been found to target substrates to SCF^{Fbw2} (Yoshida et al. 2002; Petroski and Deshaies 2005).

In the yeast *S. cerevisiae*, the most well-studied SCF substrate is the Cyclin dependent kinase inhibitor Sic1. Sic1 binds to and restricts the G1 Cyclin/CDK complex activity, preventing entry into S phase (Mendenhall 1993). Upon phosphorylation by Cyclin/CDK in late G1, Sic1 is rapidly ubiquitinated by SCF^{CDC4} and degraded by the proteasome, releasing active Cyclin/CDK complex to promote entry into S phase of the cell cycle (reviewed in Deshaies 1999).

In humans, several substrates have been identified and characterized, including p27, IκBα, and Cyclin E. p27, similar to Sic1 in yeast, binds to and inhibits the G1/S phase kinase Cyclin E/Cdk2 and is thought to be ubiquitinated by SCF^{Skp2} (Carrano et al. 1999). Cyclin E has been found to be ubiquitinated by SCF^{Fbw7} (Koepp et al. 2001; Strohmaier et al. 2001), and this ubiquitination suppresses chromosome instability caused by hyperactivity of Cyclin E (Rajagopalan et al. 2004). Lastly, IκBα is ubiquitinated by $SCF^{\beta-TrCP}$, and this ubiquitination causes the release of the transcription factor NFκB from the cytoplasm into the nucleus (Yaron et al. 1998).

Given the diverse nature of SCF substrates, it is of critical importance to regulate SCF activity. There are two methods through which regulation is accomplished to control spurious ubiquitination by SCF. First, as discussed earlier, regulation occurs at the level of substrate recognition as almost all substrates examined to date require a post-translational modification to target them to SCF (Petroski and Deshaies 2005). Second, SCF activity is controlled by the post-translational modification of Cul1 with the Ubiquitin-like protein Nedd8 (see below).

Cullin-Based Ubiquitin Ligases

In addition to the SCF ubiquitin ligases, other Cullin-based ligases are thought to play a similar role in the UPS. In humans, seven different Cullin proteins have been identified, and each Cullin is thought to assemble into a distinct SCF-like complex with multiple co-factors playing the roles of Skp1 and the F-box protein (Petroski and Deshaies 2005).

Cul2 assembles with the Elongin B/C, VHL, and SOCS box proteins. The most notable example of Cul2 activity stems from studies involving the substrate adaptor module von Hippel-Lindau tumor suppressor (VHL). Cul2, Elongin B/C, and VHL can catalyze the ubiquitination of the transcription factor HIF-1α, which regulates a slurry of genes involved in hypoxia and angiogenesis (Maxwell et al. 1999). During hypoxic (low oxygen) conditions HIF-1α is extremely stable, causing it to promote the transcription of an array of genes involved in hypoxia. Alternatively, during normoxic (normal oxygen) conditions, HIF-1α is rapidly degraded in a proteasome/VHL dependent manner.

Interestingly, conversion of a specific proline into a hydroxyproline residue targets HIF-1α to VHL, promoting its ubiquitination under normoxic conditions (Ivan et al. 2001).

Recently, Cul3 was found to associate with the 'Broad complex/Tramtrack/Bric-a-Brac' (BTB) family of proteins, and several of these BTB proteins have been found to play roles in a wide array of processes (Furukawa et al. 2003; Geyer et al. 2003; Pintard et al. 2003b; Xu et al. 2003). The BTB protein MEL-26 was recently found to negatively regulate the oocyte spindle assembly factor MEI-1 (Pintard et al. 2003b). Failure to degrade MEI-1 results in defects in microtubule dependent processes, and the negative regulation of MEI-1 depends upon Cul3 (Pintard et al. 2003a). Another substrate of the Cul3 ligase is the oxidative stress protein Nrf2 (Kwak et al. 2003; McMahon et al. 2003; Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa and Xiong 2005). The number of BTB proteins currently identified indicates that more substrates will be identified in the near future (Pintard et al. 2004).

Although the exact topology of the Cul4 complex is still unresolved, it appears to assemble with the protein DNA Damage Binding Protein 1 (DDB1) (Dornan et al. 2004; Hu et al. 2004). This complex can target the tumor suppressor gene p53, the transcription factor c-jun, and the replication licensing factor Cdt1 for ubiquitination (Dornan et al. 2004; Hu et al. 2004; Wertz et al. 2004). Interestingly, DDB1 and Cul4 have been found to assemble with the proteins Cop1 and Det1, both of which appear intimately tied in with ubiquitination (Hu et al. 2004; Wertz et al. 2004) and were originally identified in plants as regulators of photomorphogenesis (see Cop9 Signalosome below).

Similar to Cul2, Cul5 can assemble with Elongins B/C and SOCS box proteins (Kamura et al. 2004). Whereas the VHL protein specifically interacts with the Cul2

complex, SOCS box proteins interact with the Cul5 complex and specificity appears to be determined, at least in part, by the Cullin protein (Kamura et al. 2004).

The Cul7 protein can assemble with Skp1 and at least one F-box protein, Fbx29 (Dias et al. 2002). Deletion of Cul7 results in abnormal vascular development (Arai et al. 2003), and Cul7 has been found to be associated with the viral oncoprotein Simian Virus 40 Large T-Antigen (Ali et al. 2004). However, it is still unclear whether Cul7 behaves similarly to Cul1, or Cul7 orchestrates the ubiquitination of distinct F-box protein substrates.

Ubiquitin-Like Protein Nedd8

The ubiquitin-like protein Nedd8 (Neural precursor cell-Expressed Developmentally Down-regulated 8) was originally identified within a set of genes that are developmentally down-regulated (Kumar et al. 1992). Cloning of Nedd8 showed it to be 60% identical and 80% homologous to ubiquitin (Kamitani et al. 1997), and further research led to the discovery that all members of the Cullin protein family in human cells were covalently modified with Nedd8 (Hori et al. 1999). This modification, similar to monoubiquitination, occurs on a substrate lysine through the C-terminal glycine of Nedd8 (Kamitani et al. 1997) and requires a heterodimeric E1 enzyme and an E2 enzyme (Ula1 (also known as ABP-BP1) and Ubc12, respectively (Osaka et al. 1998)). However, in contrast to ubiquitin, Nedd8 is not thought to confer instability. Rather, modification of Cul1 with Nedd8 increases SCF activity *in vitro* both in a substrate independent and substrate dependent manner (Podust et al. 2000; Wu et al. 2000). The mechanism of this

activation has been shown to be based upon E2 affinity for the E3, as neddylation of Cul1 enhances the recruitment of the E2 Ubc4 (Kawakami et al. 2001).

Compromising global neddylation *in vivo* is lethal in fission yeast *S. pombe*, mammalian, and plant model systems (Osaka et al. 2000; Tateishi et al. 2001; Ohh et al. 2002; Dharmasiri et al. 2003). In addition, the direct neddylation of Cul1 is essential, as a mutant lacking the neddylated lysine in fission yeast Cul1 fails to complement Cul1 null strains (Osaka et al. 2000). Interestingly, neddylation is not essential in budding yeast *S. cerevisiae*; however, mutations within the Nedd8 pathway have synthetic interactions with SCF temperature sensitive mutations (Lammer et al. 1998), suggesting neddylation influences SCF activity in budding yeast as well.

Recently, other proteins modified by Nedd8 have emerged from the shadows. VHL, p53, and MDM2 all have been shown to be modified with Nedd8 (Stickle et al. 2004; Xirodimas et al. 2004). Not surprisingly, each of these proteins plays a role in the UPS: VHL is a substrate adaptor protein for the Cul2 ligase, and MDM2 is a ubiquitin ligase that targets p53 for ubiquitination. It will be exciting to see if other proteins will be identified to be neddylated in the near future (see Chapter 5).

Cop9 Signalosome

The Cop9 signalosome (CSN) was originally found in a screen in the plant *A. thaliana* for mutants that display a constitutive photomorphogenic phenotype—a phenotype where plants grow in the dark as if they were cultivated in light (reviewed in Wei and Deng 1999). Subsequently, all the genes involved in this process were cloned and found to be extremely conserved from fission yeast to humans (Wei and Deng 1999).

Interestingly, all eight subunits are highly homologous to the eight subunits of the 26S

proteasome lid and more distantly to the eukaryotic translation initiation factor 3 (eIF3),

and the gene products of the CSN are found to associate in a hetero-octomeric complex

(Wei and Deng 1999).

Two domains are found in the CSN, 26S proteasome and eIF3, which characterize

much of the homology between these distinct complexes: the PCI domain and the MPN

domain (reviewed in Schwechheimer and Deng 2001). The PCI domain is found in Csn1,

Csn2, Csn3, Csn4, Csn7, and Csn8. Named for the complexes this motif is found in

(Proteasome, Cop9 Signalosome, eIF3), no biological function has been attributed to the

domain—the PCI domain is predicted to be strictly a-helical, ~200 amino acids in length,

and does not contain any conserved residues suggestive of catalytic activity (Hofmann

and Bucher 1998).

The MPN domain, named after the N-terminal region of two proteins it was

identified in—Mpr1 and Pad1, is present in Csn5 and Csn6 subunits of the CSN and their

corresponding homologs in the Proteasome and eIF3 (Aravind and Ponting 1998).

Predicted to have an α/β structure, this motif spans ~140 amino acids, typically in the

amino terminus (Hofmann and Bucher 1998). More detailed analysis of the MPN domain

will be presented in Chapter 3.

Molecular dissection of CSN in plants revealed an intricate pathway whereby

CSN regulates photomorphogenesis (reviewed in Cope and Deshaies 2003). A key

regulator of photomorphogenic development, HY5 is a transcription factor regulated by

the UPS. In the dark, HY5 is degraded rapidly by the UPS in a process requiring the

RING finger protein Cop1. In contrast, when plants are grown in the light, HY5

accumulates causing the transcription of an array of genes required for photomorphogenic development. Interestingly, the accumulation of HY5 in the light requires the translocation of Cop1 from the nucleus to the cytoplasm—physically occluding Cop1 from its target HY5. In the dark, Cop1 is exclusively nuclear, thereby resulting in the ubiquitination of HY5 and loss of photomorphogenic development.

Mutants in CSN result in a constitutive cytoplasmic localization for Cop1 (reviewed in Cope and Deshaies 2003). Therefore when CSN is mutated, HY5 accumulates to high levels, resulting in the expression of the photomorphogenic program regardless of light conditions. Despite considerable progress made over the last several years, an exact mechanism as to how CSN regulates Cop1 localization has yet to be determined.

In other organisms, CSN has been implicated in a wide array of processes. In the fruit fly *drosophila*, mutations in either Csn4 or Csn5 cause lethality during larval development (Suh et al. 2002). Moreover, these mutations result in a failure to differentiate photoreceptor neurons (Suh et al. 2002) and activation of a meiotic checkpoint (Doronkin et al. 2002; Oron et al. 2002). Given that Cyclin E and CSN mutations suppress each other, it has been proposed that the effects of CSN mutations are dependent upon Cyclin E turnover (Doronkin et al. 2003).

In fission yeast the deletion of CSN subunits tested to date does not affect viability (this is in direct contrast to all multicellular organisms currently examined, in which deletion of CSN components results in large developmental defects culminating in death). Csn1 and Csn2 were discovered in a screen for proteins that rescue cell cycle arrest due to a DNA damage checkpoint (Mundt et al. 1999). Csn1 and Csn2 deletions

alone result in slower cell cycle progression and a heterogenous cell length (Mundt et al.

1999); however deletions of other CSN components do not display this phenotype

(Mundt et al. 2002). Csn1 and Csn2 were later identified as regulators of ribonucleotide

reductase (RNR)—the fission yeast homolog of RNR, Suc22, is a multicopy suppressor

of the Csn1/Csn2 phenotype (Liu et al. 2003). Of note, Liu and colleagues discovered

that Csn1 and Csn2 are essential to properly regulate the RNR/Suc22 inhibitor Spd1, as

Spd1 accumulates to high levels in Csn1 and Csn2 mutants resulting from loss of Cul4

dependent UPS turnover of Spd1 (Liu et al. 2003).

In *C. elegans*, RNAi of Csn5 results in sterile worms and blocks Mei-1 down-

regulation resulting in an array of defects, including defects in nuclear positioning,

anaphase, and cytokinesis (Pintard et al. 2003a). Mei-1 is a substrate of the BTB protein

Mel-26, and BTB proteins assemble with Cul3 components, suggesting Csn5 plays a role

in BTB protein substrate ubiquitination (see Chapters 3 and 4 for a more detailed

mechanism)(Pintard et al. 2003b).

Csn5 has been linked to numerous processes through protein-protein interactions

based on two-hybrid screens. The first discovery of Csn5 in a two-hybrid screen was

reported by Claret and colleagues when they found Csn5 bound to the transcription factor

c-jun (Claret et al. 1996). Csn5 overexpression enhanced c-jun/AP-1 transcriptional

activity *in vivo*, suggesting that Csn5 was a positive regulator of c-jun (Claret et al. 1996).

Subsequent to this report, Csn5 emerged in a study looking for proteins that bound the

Cyclin/CDK inhibitor p27 (Tomoda et al. 1999). Overexpression of Csn5 resulted in an

increased turnover of p27, presumably by increasing p27 translocation from the nucleus

to the cytoplasm (Tomoda et al. 1999). The cytokine MIF (macrophage inhibitory factor)

also binds to Csn5, and MIF was found to antagonize Csn5-dependent p27 regulation (Kleemann et al. 2000). Csn5/MIF interaction is dependent upon the MPN domain of Csn5 (Burger-Kentischer et al. 2005). A fourth interaction was discovered recently between Csn5 and the Glucose-regulated destruction domain (GRDD) of Topoisomerase II (Yun et al. 2004). Csn5 can promote the turnover of Topoisomerase II, and this activity is dependent upon the GRDD of Topoisomerase II and the MPN domain of Csn5 (Yun et al. 2004). The transcriptional regulators Id1 and Id3 both bind to Csn5, and CSN appears to regulate the turnover of these two proteins (Berse et al. 2004). Lastly, Csn5 was found to bind to the Estrogen Receptor (ER), and this interaction interplays with the turnover of ER (Callige et al. 2005). It remains to be seen how Csn5 can regulate this wide array of processes; nevertheless these interactions support a major role for the CSN in a wide array of processes.

SCF and the Cop9 Signalosome

To further gain insight into SCF regulation and function, we purified SCF from mammalian cells. The Cop9 signalosome was found to associate with SCF in both humans and fission yeast. Interestingly, deletion of the CSN resulted in the hypermodification of Cul1 with Nedd8. We found that, *in vitro* and *in vivo*, CSN can promote the removal of Nedd8 from Cul1 (Chapter 2). These data suggest that CSN contains an intrinsic or tightly associated deneddylating enzyme.

To investigate the basis of CSN-dependent deneddylation activity, we analyzed the CSN and the 26S proteasome for conserved sequences that could be representative of a catalytic motif. We identified the JAMM motif in Csn5 and Rpn11 of the proteasome.

Mutations in JAMM eliminated CSN deneddylating activity. Moreover, mutations in JAMM reduce the restrictive temperature of several SCF temperature-sensitive mutants, suggesting that CSN acts positively on SCF activity. Finally, a JAMM mutant failed to rescue Csn5Δ defects in drosophila. This suggests that Csn5 is a metalloprotease specific for Cullin-Nedd8 conjugates (Chapter 3).

To investigate the biological effect due to loss of deneddylating activity, we constructed a stable cell expressing an inducible siRNA for Csn5. Loss of Csn5 results in a dramatic decrease in F-box protein levels. Moreover, this loss correlates with F-box protein substrate accumulation and hyperneddylation of Cullin proteins. We propose an autocatalytic mechanism for the turnover of F-box proteins that is modulated by the deneddylating activity of CSN (Chapter 4).

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Chapter 2: Cop9 Signalosome Promotes Cleavage of Nedd8 From Cul1

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Summary

SCF ubiquitin ligases participate in a wide array of regulatory processes, including cell cycle progression and signal transduction. In an attempt to identify additional SCF subunits and/or SCF regulators, we purified SCF and any associated proteins from mammalian cells. Mass spectrometry of purified components identified all eight subunits of the highly conserved complex known as the Cop9 signalosome (CSN). Deletion of CSN components in fission yeast *S. pombe* resulted in the hyper-modification of Cul1 with the ubiquitin-like protein Nedd8. Moreover, *in vitro* and *in vivo* data showed that CSN can promote the cleavage of Nedd8 from Cul1. Given that Nedd8-Cul1 conjugates have a higher *in vitro* ubiquitin ligase activity, we propose that CSN acts as a negative regulator of SCF and this regulation underlies the functions previously attributed to CSN.

Introduction

Ubiquitin dependent proteolysis is triggered by the addition of ubiquitin chains onto the substrate proteins, targeting the substrate for degradation by the 26S proteasome

(reviewed in Pickart 2001). This ubiquitin conjugation to substrate proteins occurs through the coordination of multiple steps. First, ubiquitin is activated in a step that requires ATP hydrolysis and an ubiquitin-activating enzyme (E1) to produce a high-energy thioester intermediate between the C-terminal glycine of ubiquitin and the active site cysteine of the E1. Next, ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2) creating a subsequent thioester between ubiquitin and the active site cysteine of the E2 enzyme. Finally, in concert with an ubiquitin-ligating enzyme (E3) ubiquitin is attached onto lysine residues of the substrate protein to form an isopeptide bond between the C-terminal glycine of ubiquitin and the terminal amino group of the target lysine residue. This final step results in the polyubiquitination of the substrate protein, which is subsequently recognized by the 26S proteasome for degradation.

Numerous E3 enzymes have been identified to date. These enzymes are characterized by the presence of one of two distinct E3 domains: HECT and RING. The HECT domain has been identified in multiple proteins, most notably in the E6-AP protein that targets the tumor suppressor p53 for ubiquitination (reviewed in Pickart 2001). The RING domain is a domain related to the zinc finger domain (Freemont 1993) and modifications of the RING domain have been identified to contain ubiquitin ligase activity (Aravind and Koonin 2000; Aravind et al. 2003). One notable RING containing E3 ligases the SCF ubiquitin ligase.

SCF is highly conserved and can be found in organisms ranging from budding yeast to humans. SCF is composed of four subunits: Cul1, Hrt1/Rbx1/Roc1, Skp1, and a variable F-box protein (reviewed in Deshaies 1999). Whereas ubiquitin ligase activity is thought to reside in the Cul1 and RING-domain-containing Hrt1 subunits, substrate

specificity is conferred by the F-box protein and, given the vast number of F-box proteins, it is thought that SCF ligases can be reprogrammed simply by exchange of the F-box protein (reviewed in Kipreos and Pagano 2000). Moreover, several F-box proteins have been found to play roles in such diverse processes as Auxin response in plants (reviewed in Dharmasiri and Estelle 2002), immune response (reviewed in Karin and Ben-Neriah 2000) and cell cycle progression (reviewed in Ang and Wade Harper 2005) suggesting that SCF dependent ubiquitination underlies these processes.

SCF has been most thoroughly studied in the yeast *S. cerevisiae*. Deletion or mutation of any SCF component can cause cell cycle arrest due to the accumulation of the Cyclin CDK inhibitor Sic1 (Schwob et al. 1994; Bai et al. 1996). Sic1 is phosphorylated at the end of the G1 phase of the cell cycle by the G1 Cyclin/CDK complex, and phosphorylated Sic1 binds strongly to the Cdc4 F-box protein (Verma et al. 1997). Upon assembly of an active SCF complex, Sic1 is rapidly ubiquitinated in a process that requires the E2 Cdc34 binding to Cul1/Cdc53 (Willems et al. 1996).

In mammalian cells, SCF is thought to play a role in the ubiquitination of several proteins. Most notably SCF plays a role in the regulation of the transcription factor NFκB (reviewed in Ben-Neriah 2002). NFκB is sequestered in the cytoplasm by the negative regulator IκBα. Upon stimuli to induce NFκB-dependent transcription, IκBα is phosphorylated and ubiquitinated by the $SCFB^{\beta\text{-TrCP}}$ ubiquitin ligase. This ubiquitination targets IκBα for proteasome dependent degradation, releasing NFκB into the nucleus where it can exert its power.

Although little is known about the regulation of SCF activity, the Cul1 subunit is covalently modified with the ubiquitin-like protein Nedd8 (Hori et al. 1999). Nedd8 is

60% identical and 80% homologous to ubiquitin (Kamitani et al. 1997). In contrast to ubiquitination, neddylation does not seem to render its conjugates unstable, rather neddylation of Cul1 appears to increase the recruitment of the E2 enzyme (Kawakami et al. 2001) resulting in an enhanced SCF ubiquitination activity in both substrate independent and substrate dependent assays (Podust et al. 2000; Wu et al. 2000). Moreover, global neddylation is essential in fission yeast, plants, and mammalian cells (Osaka et al. 2000; Tateishi et al. 2001; Ohh et al. 2002; Dharmasiri et al. 2003) and direct neddylation of Cul1 is essential in fission yeast (Osaka et al. 2000). Although the neddylation of Cul1 requires a pathway similar to ubiquitination—it requires a distinct E1 and E2 enzyme—and the mechanism of the removal of Nedd8 (deneddylation) has not been thoroughly established.

Here we find a highly conserved eight-subunit complex, the Cop9 Signalosome (CSN), to be tightly associated with SCF. Deletion of CSN components in *S. pombe* severely alters the neddylation state of Cul1. Moreover, *in vitro* analysis points to a role of deneddylation for CSN, as the lack of deneddylation in CSN deficient extracts can be restored with both wild-type extracts and highly purified CSN from pig spleen. Deletion of Csn5 in budding yeast shows that CSN plays a similar role in *S. cerevisiae*, indicating the high conservation of this process. CSN has been found to be involved in multiple processes—including cell cycle progression and development—however the molecular basis of these processes has not been established. We propose that CSN regulates SCF through deneddylation and that deneddylation underlies the activities previously attributed to CSN.

Results

Identification of SCF associated proteins

To attempt to identify either regulators or additional subunits of SCF, we sought

to purify SCF and associated proteins from mammalian cells. Retroviruses were made

that expressed a C-terminal truncation of human Cul1 with a C-terminal myc9 epitope tag

and used to infect NIH3T3 cells. Complexes were enriched on anti-myc (9E10) beads,

washed, and eluted with SDS, and eluates were fractionated by SDS-polyacrylamide Gel

electrophoresis (SDS-PAGE). Silver staining of isolated complexes revealed several

protein bands found in hCul1 IPs but not in controls (Figure 1). Mass spectrometry

analysis revealed that, in addition to the SCF subunits Cul1, Hrt1/Roc1/Rbx1, Skp1, and

several F-box proteins, eight proteins were identified that corresponded to the entire

complex of the Cop9 Signalosome (CSN).

Cop9 Signalosome regulates Cul1 Neddylation

To investigate whether the CSN plays a role in SCF regulation, we utilized the

high conservation of CSN and SCF and analyzed SCF components in fission yeast

deleted for the largest subunit of the CSN, CSN1. Interestingly, Cul1 migrated in a

slower form when CSN1 was deleted (Figure 2A). Although this slower migrating form

was present in wild-type cells, its abundance was much lower. We therefore reasoned that

this slower migrating form of Cul1 was the Nedd8 conjugated (neddylated) form of Cul1.

To investigate whether the slower migrating form of Cul1 was the neddylated

form of Cul1, we mutated the lysine in Cul1 on which Nedd8 is attached to arginine

(K719R). Over-expression of the Cul1 (K719R) form completely obliterated the slower

migrating form of Cul1 (Figure 2B). Moreover, expression of an HA-tagged form of

Nedd8 showed that deletion of CSN1 caused a higher abundance of Nedd8 to be

immunoprecipitated with Cul1 (Figure 2C). These data confirm that deletion of CSN1

results in a hyper-neddylation of Cul1.

SCF composition in csn1Δ S. pombe

To investigate whether deletion of CSN1 perturbs SCF localization or assembly

indirectly leading to altered neddylation of Cul1, we analyzed SCF composition,

localization, and activity in *csn1Δ S. pombe*. Immunoprecipitation of SCF using Cul1-

myc13 showed that SCF still assembled appropriately with the proteins Hrt1 and Skp1

(Figure 3A). Sub-cellular fractionation revealed that no change in SCF localization could

be found when CSN1 was deleted (Figure 3B). Moreover, immunoprecipitation of Cul1

from either wild-type or *csn1Δ* revealed that the altered neddylation in *csn1Δ* increased

SCF activity (Lyapina et al. 2001). These data indicate that SCF composition and

localization is unaltered in *csn1Δ S.pombe*.

CSN promotes the deneddylation of Cul1

Given that CSN does not appear to change SCF localization and assembly, we

reasoned that CSN alters the neddylation of Cul1 directly. Two models may account for

the increased neddylation due to loss of Csn1—1) CSN directly governs the neddylation

state of Cul1 by regulating an isopeptidase specific for Cul1-Nedd8 conjugates or 2) loss

of CSN results in the accumulation of a deneddylation inhibitor. In an effort to

discriminate between these two models, we created an *in vitro* system to test

deneddylation activity in the presence or absence of CSN. Lysates were made from CSN1

Cul1-myc13 strain or *csn1Δ* cells and were incubated either alone or together for 30

minutes. Incubation of *csn1Δ* lysates alone resulted in no change in the neddylation status

of Cul1 (Figure 4B, lane 1). Upon addition of wild-type extracts to *csn1Δ* lysates, Cul1

was completely deneddylated (Figure 4B). These data suggest that deletion of CSN1

results in an ablation of deneddylating activity. Surprisingly, addition of purified CSN

isolated from Pig Spleen (psCSN, Figure 4A) caused a complete and rapid deneddylation

of Cul1 (Figure 4B).

To address these results further, we purified Cul1-Nedd8 conjugates from *csn1Δ*

lysates and analyzed the ability of CSN to deneddylate this purified substrate. Addition of

purified psCSN caused rapid deneddylation of Cul1-Nedd8 conjugates, suggesting that

either CSN has intrinsic deneddylating activity or was tightly associated with a

deneddylating activity (Figure 4C).

CSN5 is the only CSN homolog readily found in *S. cerevisiae,* and deletion of

CSN5 resulted in the hyper-neddylation of the budding yeast Cul1, Cdc53 (Figure 4D).

These data signify the conservation of this mechanism. Recently, several distant budding

yeast homologs of CSN have been identified, and deletion of these components resulted

in the hyper-neddylation of Cul1 (Wee et al. 2002).

Discussion

We report the characterization of a novel activity associated with the Cop9

Signalosome. This activity can promote the removal of Nedd8 from Cul1 *in vivo* and *in*

vitro. Loss of CSN does not appear to affect the assembly or localization of SCF; however, Cul1 immunoprecipitates from *csn1Δ* strains have a higher ubiquitination activity, supporting a role for CSN in the deneddylation of Cul1 (Lyapina et al. 2001). Although hyperneddylated Cul1 has a higher activity *in vitro* (Podust et al. 2000; Wu et al. 2000; Lyapina et al. 2001), diminished CSN activity results in the impaired turnover of an SCF substrate in *A. thaliana* (Schwechheimer et al. 2001). This contradiction supports a model whereby cycles of neddylation and deneddylation are important for optimal SCF activity.

Interestingly, the phenotypes resulting from the loss of neddylation or deneddylation do not mimic each other. Deletion of Nedd8 and its corresponding activating and conjugating enzymes is lethal in *S. pombe* (Osaka et al. 2000) and produces lethal effects in mammalian and plant systems (Tateishi et al. 2001; Dharmasiri et al. 2003). Moreover these phenotypes are, at least in part, due to the neddylation of Cul1 as a mutation of the neddylated lysine on Cul1, which is lethal in *S. pombe* (Osaka et al. 2000). In contrast, only deletion of CSN1 or CSN2 has cellular defects in fission yeast, and these defects do not appear to be due to loss of deneddylation, as other CSN deletion mutants lose deneddylation but do not show this phenotype (Zhou et al. 2001). What may account for this discrepancy? One can imagine a scenario where neddylation affects the assembly of SCF—improper assembly from the loss of neddylation may result in lethality due to the absence of SCF activity. In contrast, the disassembly of SCF via deneddylation may be as important to SCF activity (for example disassembly may be needed for F-box protein exchange). However, constant translation and assembly may overcome this deficiency. Although our data and others, show that proper neddylation or

deneddylation is not essential for Skp1, Cul1, and Hrt1 assembly in *S. pombe* (Figure 3a and Osaka et al. 2000), F-box protein assembly was never examined in these two scenarios. Moreover, the identification of the protein CAND1—a protein that only binds to unmodified Cul1, is displaced by neddylation, and when bound to Cul1 occludes Skp1 from binding SCF—supports a model whereby neddylation and deneddylation effect SCF assembly (Liu et al. 2002; Zheng et al. 2002; Oshikawa et al. 2003).

The CSN has been implicated in numerous cellular processes, including development, cell cycle progression, and transcription. Despite numerous efforts, a molecular basis has yet to be tied into the genetic phenotypes found for the CSN. We propose that deneddylation is the molecular basis that underlies these numerous processes CSN has been linked to.

Currently, there are eight proteins known to be neddylated in human cells: Cul1 to Cul5, VHL, MDM2, and p53 (Hori et al. 1999; Stickle et al. 2004; Xirodimas et al. 2004). It is still unclear whether all these proteins are regulated by CSN. However, deletion of CSN subunits in *S. pombe* results in the hyperneddylation of Cul3, suggesting that the deneddylation mechanism is conserved between Cullins (Zhou et al. 2001).

Although we have identified the CSN to promote the deneddylation of Cul1, the enzyme responsible for this activity is a mystery (see Chapter 3). Is this activity intrinsic to CSN, or is CSN tightly associated with a deneddylating enzyme? What are the repercussions to SCF activity when deneddylation is lost? These questions will be addressed in the following chapters.

Materials and Methods

Yeast Methods, Plasmids, and Strains: csn1Δ, csn2Δ, Csn1-myc13 strains, and pRep41-Csn2-HA plasmid were a gift from A. Carr (Mundt et al. 1999). Cul1-myc13 strain and anti-Pcu1 (Cul1) antibody were gifts from D. Wolf (Lyapina et al. 2001; Zhou et al. 2001). pRep41-Myc-Cul1(K713R) was a gift from S. Kato (Osaka et al. 2000).

Immunoprecipitation of SCF Interacting Proteins: Cul1-ΔC was tagged on its C-terminus with two Tobacco Etch Virus (TEV) protease sites followed by the Myc9 epitope. Cell extracts were prepared in lysis buffer (20mM HEPES (pH 7.2), 150mM NaCl, 0.2% triton X-100, 10mM EDTA, 50mM NaF, 1mM DTT, 1mM PMSF, 1mM benzamidine, 0.25ug/mL pepstatin, and 5ug/mL each of leupeptin, aprotinin, and chymotrypsin). Anti-Myc beads were added to 0.5g of extract for 2hrs at 4C. Beads were washed 4 times in lysis buffer and eluted with 66Units/mL of TEV protease (Gibco) for 14hrs, and eluted proteins were analyzed by SDS-PAGE and silver staining.

Subcellular Fractionation of S. pombe Lysates: Yeast were grown in YES media until OD595 = 0.5, harvested, and washed in ice cold STOP buffer (50mM NaF, 10mM TRIS (7.5), and 0.02% NaAzide). Yeast were resuspended in 600uL of Buffer S (1.4M Sorbitol, 40mM HEPES (7.2), 0.5mM $MgCl_2$). Spheroplasts were made by incubating for 40 minutes at 30°C in the presence of 100ug/mL zymolyase (ICN). Cells were washed in ice cold Buffer F (20mM HEPES (7.2), 18% Ficoll 400 w/v, 0.5mM $MgCl_2$ supplemented with 1mM PMSF, 0.25ug/mL pepstatin, and 5ug/mL each of leupeptin,

aprotinin, and chymotrypsin). Cells were lysed in 200uL using a stainless steel pestle.

Unlysed cells were removed by centrifuging in a micro-centrifuge at 2000Krpm. Nuclei

were layered by isolating the supernatant and adding to the top of a cushion (buffer F

supplemented with 7% ficoll 400 and 20% Glycerol) and centrifuging 4000Krpm. Nuclei

(pellet) were lysed in 200uL of 20mM HEPES (7.2), 500mM NaCL, 1% triton X-100,

1mM EDTA supplemented with 1mM PMSF, 0.25ug/mL pepstatin, and 5ug/mL each of

leupeptin, aprotinin, and chymotrypsin. Nuclear and cytosolic fractions were analyzed by

SDS-PAGE and western blot. Protein localization percentages were gathered using NIH

Image Software (v 1.62) (error +/- 5%).

S. pombe Lysates for Immunoblotting and Deneddylation Assays: Strains were grown in

50mL of YES media until OD595 = 0.5. Cells were harvested and washed in ice cold

STOP buffer (50mM NaF, 10mM TRIS (7.5), and 0.02% NaAzide), pelleted, and

resuspended in an equal volume of lysis buffer (20mM HEPES (pH 7.2), 150mM NaCl,

0.2% triton X-100, 10mM EDTA, 50mM NaF, and 1mM DTT supplemented with 1mM

PMSF, 0.25ug/mL pepstatin, and 5ug/mL each of leupeptin, aprotinin, and

chymotrypsin). An equal volume of glass beads was added (0.5um, Sigma), and cells

were vortexed at maximum speed 6 X 30 seconds. Extracts were cleared by

microcentrifugation (14Krpm) and protein concentration was determined by Bio-Rad

protein assay (BioRad). For crude lysate deneddylation assays, lysates were normalized

for equal protein concentration in lysis buffer and combined in equal amounts. Reactions

were incubated at $30\,^{\circ}\mathrm{C}$ for 30 minutes in a volume of 20uL. For immunoprecipitation,

lysates were incubated with anti-myc resin for 1 hour at $4\,^{\circ}\mathrm{C}$. Beads were washed 4 times

in lysis buffer. For purified deneddylation reactions, beads were incubated either alone or in the presence of 3ug of purified CSN for 60 minutes at 30°C.

Purification of Pig Spleen CSN: Purification of mammalian Pig Spleen CSN has been described elsewhere (Wei and Deng 1998). Briefly, 2-2.5X volume/weight of buffer A (50mM TRIS (7.0), 1.5mM $MgCl_2$, 10mM Kcl, 0.2mM EDTA, 5% Glycerol, 5mM DTT and 2mM PMSF supplemented with 0.25ug/mL pepstatin, and 5ug/mL each of leupeptin, aprotinin, and chymotrypsin) was added to thawed Pig Spleen and homogenized in a blender. Extract was filtered through cheese cloth, centrifuged at 8Kg for 15 min. Supernatant was centrifuged again at 12Kg for 15min, and 1/4 volume of PEG solution (60% Polyethylene Glycol (MW 3350) in buffer A) was added and incubated on ice for 30 minutes. Suspension was centrifuged 10Kg for 10 minutes and supernatant discarded. Pellet was resuspended in buffer B (50mM Bis-TRIS (6.4), 1.5mM $MgCl_2$, 10mM KCl, 0.2mM EDTA, 0.01% NP-40, 100mM NaCl, 10% Glycerol, 5mM DTT, and 2mM PMSF supplemented with 0.25ug/mL pepstatin and 5ug/mL each of leupeptin, aprotinin, and chymotrypsin) and centrifuged 15Kg for 30 minutes. Supernantant was loaded onto a Q Sepharose Fast Flow column equilibrated with EB (20mM Bis-tris (6.4) and 10% Glycerol). Column was washed with EB + 0.2M NaCl and eluted with EB + 0.4M NaCl. Eluate was loaded onto a Phenyl Sepharose High Performance column equilibrated with EB (10mM Bis-TRIS propane (7.0), 10% Glycerol, 0.3M NaCl, 0.15 M Na_2SO_4). Column was washed with EB and eluted with 10mM Bis-TRIS propane (7.0) and 10% Glycerol. Immediately after elution, buffer B was added to eluate, and eluate was loaded onto a Heparin Sepharose Affinity Column equilibrated with 10mM Bis-TRIS propane

(7.0), 10% Glycerol, and 100mM NaCl. Column was washed with equilibration buffer and eluted with 0.1M Sodium Phosphate (7.0) and 0.2M NaCl. Eluate was loaded onto a Mono Q Ion Exchange Column equilibrated in 20mM Bis-TRIS propane (7.0) and 10% Glycerol. A gradient was run using 0.2M to 0.4M NaCl and fractions were collected. Fractions were analyzed by SDS-PAGE, and fractions containing CSN were pooled and loaded onto a Superose 6 Gel Filtration Column equilibrated with 20mM TRIS (7.2), 150mM NaCl, 10% Glycerol, and 2mM $MgCl_2$. Fractions were collected and analyzed for CSN, and fractions containing CSN were pooled and stored at -80C.

Acknowledgements

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Figures

Figure 2-1: Cop9 Signalosome purifies with SCF

(A) Identification of SCF associated proteins. Myc9-TEV-tagged human Cul1 were purified from NIH 3T3 cell extracts, separated by SDS-PAGE and silver stained, and specific bands were excised and protein identities determined by mass spectrometry. Negative control is extracted alone with no tagged protein. Molecular weights are indicated on the left.
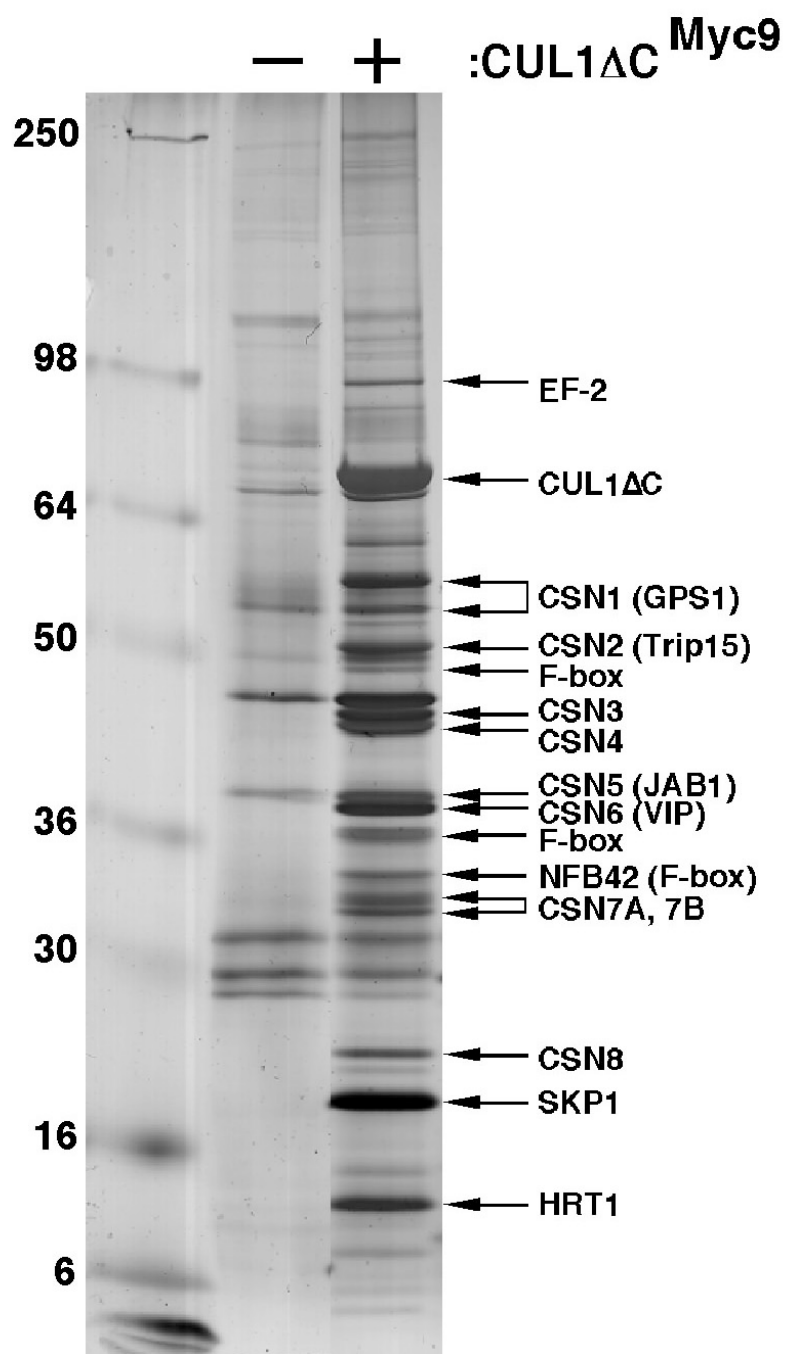
Figure 2-1

Figure 2-2: Deletion of CSN components alters the neddylation of Cul1

(A) Deletion of Csn1 results in a slower migration of Cul1. Crude lysates from exponentially growing cells of the cell lines indicated were analyzed by western blot for Cul1.

(B) Altered migration of Cul1 in *csn1Δ* strains is dependent upon the neddylated lysine residue in Cul1. Wild-type Cul1 of Cul1 (K713R) were ectopically expressed in wild-type or *csn1Δ* strains. Crude lysates from these strains were analyzed by western blot against myc to detect Cul1.

(C) Deletion of Csn1 results in the hyperneddylation of Cul1. Nedd8 tagged on its N-terminus with an HA epitope was ectopically expressed in wild-type or *csn1Δ* strains. Cul1 was purified by immunoprecipitation using Cul1 antibodies, and immunoprecipitates were analyzed by western blot against either the HA epitope on Nedd8 or Cul1.
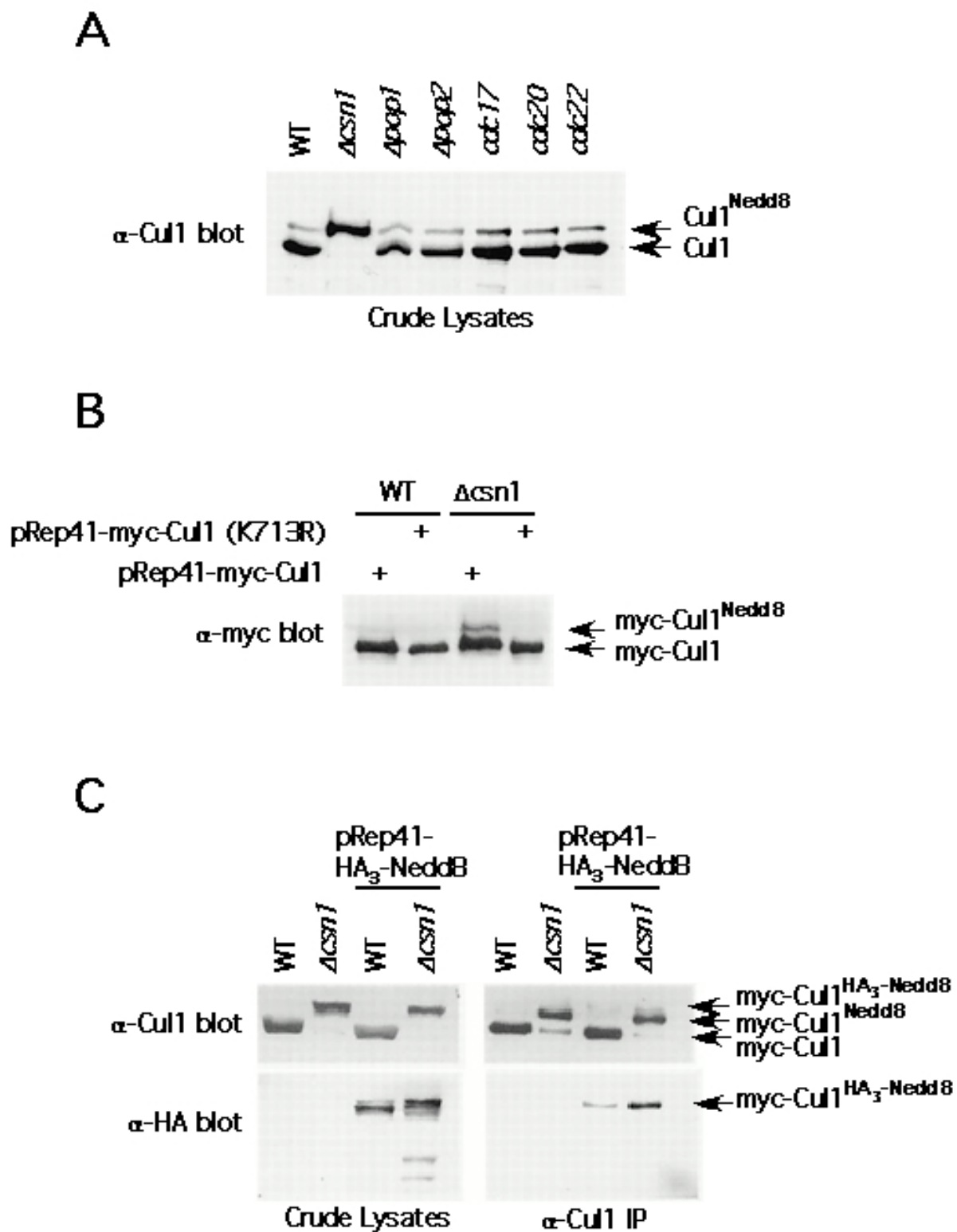
Figure 2-2

Figure 2-3: SCF composition in *csn1Δ* strains

(A) Both steady-state levels and assembly of SCF components in wild-type versus *csn1Δ* strains. Lysates from exponentially growing cells containing myc tagged Cul1 were analyzed immediately or immunoprecipitated with anti-myc resin. Lysates and immunoprecipitates were analyzed by western blot against myc, Skp1, and Hrt1.

(B) Pcu1 localization in wild-type or mutant cells. Nuclear and cytoplasmic extracts were analyzed by western blot with antibodies against Cul1. Purity of nuclear and cytoplasmic fractions were analyzed by Mcm5 and Pyruvate Kinase, respectively. Percentages represent the amount of Cul1 in each fraction.

A
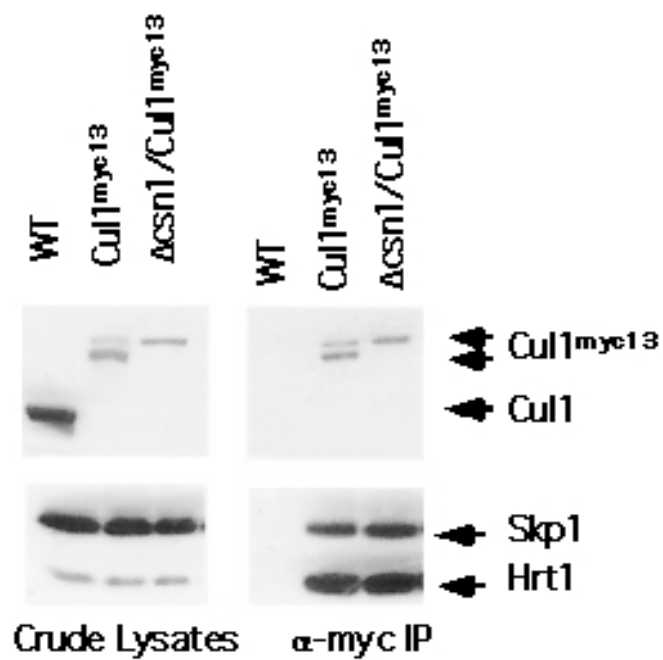
	WT	*Δcsn1*	*Δpop2*	*cdc20*
Cyoplasmic	31%	18%	42%	51%
Nuclear	69%	82%	58%	49%

Figure 2-3

Figure 2-4: Cop9 Signalosome has deneddylating activity

(A) Coomassie stain of purified pig spleen CSN.

(B) *csn1Δ S.pombe* extracts lack deneddylating activity. Extracts were made from wild-type, Cul1-myc13, or *csn1Δ* strains. *csn1Δ* extracts were incubated either alone (lane 1) or together with wild-type, Cul1-myc13 (lane 2), or purified pig spleen CSN (lane 3) to test for deneddylating activity. Addition of the alkylating agent NEM (10mM) completely inhibited deneddylation activity.

(C) Purified Cul1-Nedd8 conjugates are deneddylated by pig spleen CSN (psCSN). Nedd8-Cul1-myc13 conjugates were purified from *csn1Δ* strains by immunoprecipitation and incubated either alone or together with 3ug of psCSN for 60 minutes at 30ºC.

(D) Deletion of the Csn5 homolog in *S. cerevisiae* causes accumulation of a modified form of Cul1. Total lysates of the strains indicated were analyze by western blot against Cul1 (Cdc53).
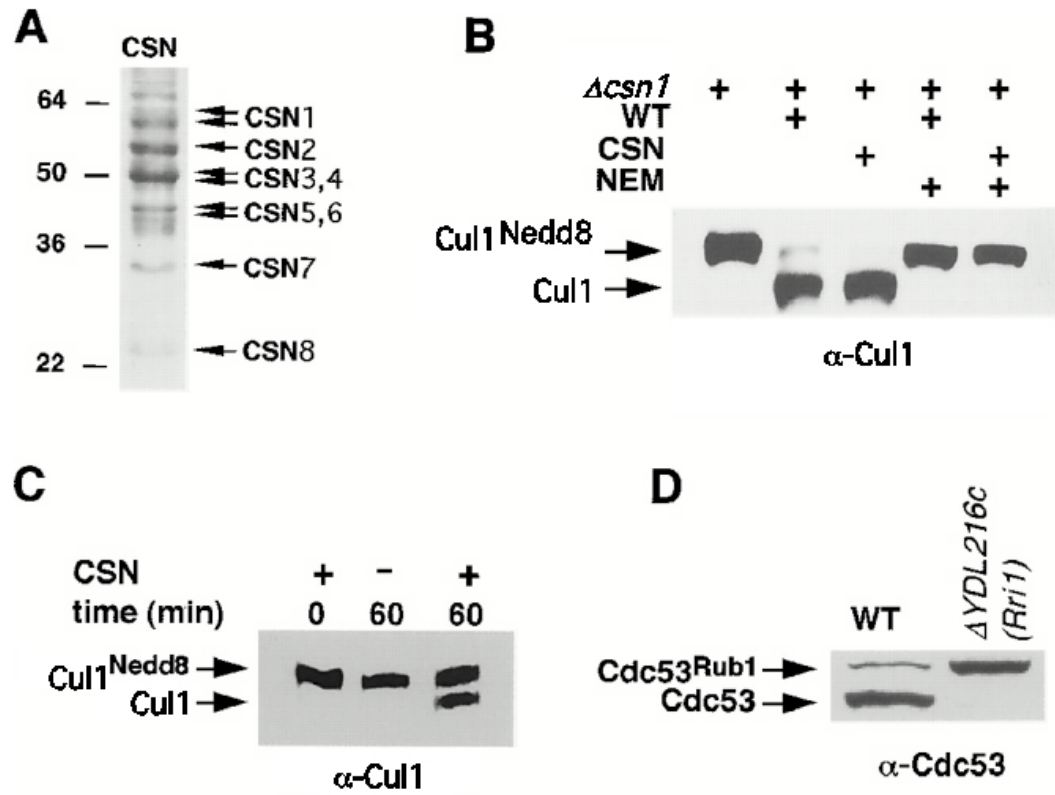
Figure 2-4

Chapter 3: Identification of a Novel Deneddylating Enzyme in Jab1/Csn5

Greg A. Cope, Greg S. B. Suh, L. Aravind, Sylvia Schwarz, S. Lawrence Zipursky, Eugene V. Koonin, and Raymond J. Deshaies. Published in Science, 298, 608-611, 2002. (Contribution from GS Figure 5a-5h; SS Figure 1a, 3b, and 3c; LA and EK Figure 1)

Summary

The Cop9 Signalosome (CSN) can promote the removal of the ubiquitin-like protein Nedd8 from Cul1; however the molecular basis behind this activity is unknown. Here, we define a metalloprotease motif present within the Jab1/Csn5 subunit of CSN that is conserved in archaea, bacteria, and eukaryotic homologs of Csn5. This motif, which we term JAMM, was found to underlie CSN-dependent deneddylation. Metal chelators and mutations within JAMM abolished CSN-dependent activity with no effect on CSN assembly. Residues of JAMM were required to sustain optimal SCF activity *in vivo*, and JAMM mutants failed to rescue proper development in *Drosophila melanogaster*. We propose that JAMM underlies the physiological roles previously attributed to CSN and that other JAMM containing proteins are metalloproteases.

Introduction

The Cop9 Signalosome (CSN) was originally identified in the plant *A. thaliana* in a screen for photomorphogenic mutants—mutants that grow in the dark as if they were

grown in the light (reviewed in Cope and Deshaies 2003). Cloning and analysis of the genes and gene product revealed that these eight proteins form a highly conserved complex present in organisms ranging from fission yeast to humans. Interestingly, the eight subunits of CSN, termed CSN1-CSN8 based on molecular weight, are highly homologous to the 26S proteasome lid and eukaryotic initiation factor 3 (eIF3), although the basis for this homology is not fully understood.

CSN has been found to play a role in a diverse set of processes (reviewed in Cope and Deshaies 2003). In *Drosophila*, mutants of Csn4 or Csn5 result in lethality during larval development (Suh et al. 2002). In fission yeast, CSN1 and CSN2 have been implicated in cell cycle progression via the turnover of a Ribonucleotide Reductase inhibitor Spd1 (Liu et al. 2003). In *C. elegans*, Csn5 was discovered to associate with GLH RNA helicases, and RNAi of Csn5 results in sterile worms, a phenotype that resembles GLH RNAi (Smith et al. 2002).

In addition, CSN subunits have been found to physically associate with many human proteins. Csn5 was found in a two-hybrid to interact with the transcription factor c-jun, and overexpression of Csn5 activates c-jun dependent transcription (Claret et al. 1996). CSN5 was also found to associate with the Cyclin/CDK inhibitor p27, and overexpression of Csn5 results in the translocation of p27 from the nucleus to the cytoplasm where it is degraded (Tomoda et al. 1999). Another binding partner of Csn5, MIF, is a cytokine involved in inflammation, and it has been argued that MIF acts through Csn5 (Kleemann et al. 2000). Indeed, the actions of CSN and its components are diverse; however, a molecular basis to these activities has yet to be described.

CSN was recently identified by us and others to interact with the ubiquitin ligase SCF (Chapter 2, Lyapina et al. 2001; Schwechheimer et al. 2001). SCF is composed of four subunits: Cul1, Hrt1/Rbx1/Roc1, Skp1, and a variable F-box protein (reviewed in Deshaies 1999; Petroski and Deshaies 2005). Two activities characterize the SCF ubiquitin ligase. First, the Cul1 and Hrt1 subunits together can catalyze the formation of poly-ubiquitin chains in a substrate-independent manner, suggesting that these two components harbor the ubiquitination activity of SCF. Second, substrate specificity resides in the F-box protein. F-box proteins provide an added layer of complexity: whereas the other subunits of Cul1 remain unchanged, the F-box protein can be exchanged for each other through Skp1. Given the numerous F-box proteins identified, SCF has the ability to catalyze the ubiquitination of numerous substrates.

Although little is known regarding the regulation of SCF, the Cul1 component is covalently modified by the ubiquitin-like protein Nedd8. Although the steps leading to modification of Cul1 with Nedd8 is similar to ubiquitination, the effect of neddylation does not appear to affect Cul1 stability but increases the affinity of the E2 enzyme for SCF, thus promoting its ubiquitin ligase activity in a substrate-dependent manner.

The removal of Nedd8 from Cul1 has been shown to be promoted by the CSN (Lyapina et al. 2001; Schwechheimer et al. 2001). Deletion of CSN components in *S. pombe* and *S. cerevisiae* results in a hyperneddylation Cul1. Moreover, CSN can promote the removal of Nedd8 from Cul1 *in vitro*, suggesting that CSN is associated with or contains intrinsic deneddylase activity (Lyapina et al. 2001).

All deneddylating and deubiquitinating enzymes characterized to date are based upon a catalytic cysteine within the protease active site (reviewed in Amerik and

Hochstrasser 2004). In *S. cerevisiae*, deletion of all identifiable deubiquitinating enzymes results in mild, if any, phenotypes (Amerik et al. 2000). Alternatively, overexpression of a dual specific protease for ubiquitin and Nedd8 conjugates results in growth inhibition in mammalian cell culture (Gong et al. 2000). However, despite exhaustive research, enzymes responsible for proteasome dependent deubiquitination, as well as Cullin deneddylation, have not been reported.

Here, we undertake a multifaceted approach to identify the enzyme responsible for CSN-dependent deneddylation. Despite mass spectrometry, mutation analysis, and deletion analysis we were unable to identify a deneddylating enzyme that resembles a canonical cysteine-based deubiquitinating-like enzyme. Rather, using a bioinformatics approach we identified a novel metalloprotease motif within Csn5/Jab1. Mutations within this motif abolish CSN activity but do not disrupt complex assembly. Moreover, mutations within this motif exacerbate SCF temperature-sensitive phenotypes in yeast and cannot rescue Csn5-null phenotypes in *Drosophila*. These data suggest that Csn5 is a deneddylating enzyme and that deneddylation underlies at least 1 genetic phenotype previously attributed to the CSN.

Results

CSN is not associated with a stoichiometric deubiquitinating enzyme

Given that purified pig spleen CSN (psCSN) contains deneddylating activity, we performed mass spectrometry on psCSN in an attempt to identify a stoichiometric deubiquitinating-like enzyme. In addition to identifying all eight subunits of the CSN, we

also found proteasome lid components, Cullin proteins, MCM proteins, and PP2A
proteins (Table 1). However, we were unable to conclusively identify any protein that
resembles a deubiquitinating enzyme.

Cys-box within Csn5/Jab1 is not essential for deneddylating activity

Csn5/Jab1 contains a highly conserved 'cys-box' active site that is found in the
active site of all deubiquitinating enzymes. Given that CSN-dependent deneddylating
activity is inhibited by the alkylating agent NEM (Chapter 2 and Lyapina et al. 2001), we
reasoned that Csn5 is a cysteine protease. However, mutation of the proposed active site
cysteine residue did not result in a *csn5Δ* phenotype as expected (Figure 1A). These data
suggest that the cys-box in CSN5 is dispensable for CSN-dependent deneddylating
activity.

Individual deletion of all deubiquitinating enzymes in S. cerevisiae

Budding yeast contains seventeen known deubiquitinating genes, the deletions of
which have been reported elsewhere (Amerik et al. 2000). Of these, sixteen are reported
to be amongst the ubiquitin-specific protease class (UBP) and the last to be a member of
the Ubiquitin C-terminal hydrolase class (UCH) (Amerik et al. 2000). Whereas the UCH
class is thought to contribute to the processing of ubiquitin, less is known regarding the
UBP class. To investigate whether any of the UBP class genes play a role in Cul1
deneddylation, we tested all individual deletion mutants for altered Cul1-Nedd8
conjugates. Analysis of Cul1/cdc53 in these strains revealed that individual deletions of
all these genes had little effect on Cul1 neddylation status (Figure 1B). These data

suggest that either multiple deubiquitinating enzymes contribute to Cul1-Nedd8 de-conjugation, or a novel isopeptidase is responsible for this process.

JAMM: a predicted metalloprotease motif in Jab1/Csn5

To determine the mechanism of Nedd8 removal from Cul1, we bioinformatically analyzed all CSN components for a motif that may represent a catalytic domain and underlie its deneddylating activity. Iterated BLAST search alignment identified a highly conserved motif within Csn5 of the Cop9 Signalosome and Rpn11 of the 26S proteasome, as well as several bacterial and archea proteins. This sequence, which we term JAMM (Figure 2), consisted of four residues: an N-terminal glutamic acid, two histidines separated by one amino acid, and a C-terminal aspartic acid. Given the resemblance of these residues with those found in active sites of numerous metalloproteases (reviewed in Rawlings and Barrett 1995), we postulated that Csn5 is a metalloprotease responsible for deneddylation.

Deneddylation is sensitive to metal chelators

To test whether Csn5 is a metalloprotease, we analyzed the deneddylation of Cul1 in lysates of *csn5Δ* cells by wild-type lysates in the presence or absence of metal ion chelators. Although addition of 1mM EDTA to the reaction caused no inhibition, 20mM of EDTA completely inhibited the reaction (Figure 3A). Moreover, 1mM concentration of the alternative metal chelator 1,10-phenanthroline completely inhibited deneddylation of Cul1 (Figure 3A). Deneddylation of purified Cul1-Nedd8 conjugates was also sensitive to metal chelators, and the deneddylation by purified CSN can be restored with

the addition of excess divalent cations (Figure 3B). Interestingly, addition of excess zinc

caused the inhibition of deneddylation. This inhibition has also been observed with other

well-studied metalloproteases, as a second zinc molecule can occlude active site access to

the substrate (Holland et al. 1995). It should be noted that addition of 1,10-phenanthroline

did not disrupt CSN complex formation, as measured by gel filtration (Figure 3C).

JAMM is essential for deneddylation in vivo

To test the contribution of JAMM residues in the deneddylation of Cul1 *in vivo*,

we mutated three residues of JAMM and tested these mutant CSN5 genes for their ability

to complement a *CSN5Δ* strain. Expression of wild-type CSN5 completely restored Cul1-

Nedd8 levels to wild-type level; however mutation of the two histidines or aspartic acid

to alanine was unable to restore proper neddylation of Cul1 (Figure 4A).

We next wanted to test if these mutants assemble into CSN properly. Co-

expression of Flag tagged Csn5 with myc tagged Csn1 or Csn2 followed by myc IP and

Flag western showed that, despite loss of JAMM, mutant Csn5 could still interact with

both Csn1 and Csn2 (Figure 4B and 4C). These data suggest that JAMM mutation does

not disrupt CSN complex formation.

JAMM is conserved in budding yeast

Given the conservation of CSN deneddylation in budding and fission yeast, we

tested the participation of the JAMM motif in the deneddylation of budding yeast Cul1.

Expression of JAMM mutant Csn5 failed to complement a *csn5Δ* strain, despite

comparable expression levels of the mutants compared to wild-type (Figure 4D). Thus, JAMM is essential for deneddylation in budding yeast.

JAMM is essential for optimal SCF function

Given that hyperneddylated Cul1 results in a higher ubiquitination activity of SCF, we hypothesized that loss of deneddylation would restore, at least in part, SCF activity if SCF activity were compromised in some manner. To test this hypothesis, we used several *S. cerevisiae* strains with temperature sensitive mutations in SCF components. We combined deletion of CSN5 with *cdc53-1*, *cdc34-2*, *skp1-12*, and *cdc4-1* and analyzed the ability of CSN5 deletion to raise the restrictive temperature of these temperature-sensitive mutants. Interestingly, analysis of double mutant strains revealed that deletion of CSN5 lowered the restrictive temperature, in contrast to our initial expectations (Figure 5A). Moreover, this growth restriction is dependent upon the JAMM motif, as ectopic expression of wild-type CSN5 can rescue the growth defect, whereas JAMM mutant CSN5 cannot (Figure 5B).

The most well-defined substrate for budding yeast SCF is the cell cycle inhibitor Sic1. Mutations in SCF cause Sic1 to accumulate, and thus the growth inhibition was reasoned to be due to csn5Δ and the SCF mutation due to Sic1 accumulation. To test this hypothesis, we combined *skp1-12* and csn5Δ mutations and GAL overexpressed HA tagged Sic1. Induction of Sic1 expression for 1 hour followed by promoter shut-off with dextrose showed that Sic1 is more stable in csn5Δ, suggesting that deneddylation is essential to maintain optimal SCF activity (Figure 5C).

JAMM is essential for Drosophila development

To investigate the role of deneddylation in higher eukaryotes we analyzed the effect of mutation of JAMM on *Drosophila* development. CSN5 was isolated in a screen for proteins involved in *Drosophila* eye disk development. Utilizing previously established assays, we constructed a transgenic fly homozygous for *csnΔ* carrying either wild-type CSN5 or JAMM mutant csn5 (D148N) under the heat shock promoter. Whereas expression of wild-type Csn5 complemented csn5-null phenotype, expression of csn5-D148N failed to rescue both eye disk development in early larval stages and viability at later larval stages despite comparable expression levels (Figure 6A-J). Thus, deneddylation is essential for the development of *D. melanogaster*.

Discussion

We have reported the discovery of a novel isopeptidase activity within the Cop9 Signalosome. The activity of this isopeptidase is sensitive to both metal chelators and mutation of a predicted metalloprotease motif in Csn5, suggesting that Csn5 is a novel metalloprotease. Given the conservation of this motif between Csn5 and Rpn11 of the 26S proteasome, we proposed that a similar activity resides within the proteasome. Indeed, Verma et al has reported that JAMM is essential for the ubiquitin-dependent degradation of Sic1 and that JAMM is responsible for the ATP-dependent hydrolysis of Ubiquitin chains from substrate proteins (Verma et al. 2002).

Subsequent to this work, Ambriggio et al. reported the crystal structure of an Archea homolog of Csn5, termed AfJAMM (Ambroggio et al. 2004). The structure revealed that the four residues in JAMM predicted to coordinate a catalytic metal ion

position both a zinc cation and a nucleophillic water molecule (Ambroggio et al. 2004).

Moreover, the predicted active site structure overlays with several well-studied

metalloproteases (Ambroggio et al. 2004), supporting our hypothesis that Csn5 is a

metalloprotease.

Neddylation of Cul1 is thought to enhance SCF activity through the increase in

binding of the E2 enzyme (Kawakami et al. 2001). Moreover *in vitro* data show that CSN

is capable of suppressing SCF activity (Yang et al. 2002). Thus CSN has been proposed

to act as a negative regulator of SCF activity—deneddylating Cul1 and downregulating

its activity. In contrast to this prediction however, combination of *csn5Δ* and SCF

temperature-sensitive mutations caused a further decrease in the restrictive temperature

(Figure 5A), suggesting that CSN is acting as a positive regulator of SCF. Furthermore,

the degradation of the SCF substrate Sic1 is retarded in the *csn5Δ/skp1-12* double mutant

(Figure 5B). These data are in agreement with genetic interactions of CSN and SCF in

Arabidopsis, *C. elegans*, and *Drosophila*, which indicate that CSN is acting positively on

SCF (Schwechheimer et al. 2001; Cope et al. 2002; Doronkin et al. 2003; Feng et al.

2003; Groisman et al. 2003; Wang et al. 2003). Given the apparent contrast of *in vitro*

and *in vivo* data regarding CSN regulation of SCF, we propose that cycles of

neddylation/deneddylation are required for optimal SCF activity *in vivo* (see below).

Recently, the protein CAND1 was identified to interact with unmodified Cul1

(Liu et al. 2002). Interestingly, CAND1 prevents binding of Skp1 to Cul1 (Liu et al.

2002). In addition, neddylation of Cul1 can displace CAND1 from Cul1, enticing Skp1 to

bind to Cul1 to form a tetrameric active SCF (Liu et al. 2002). These data support a

model whereby neddylation enhances the assembly of SCF. In contrast, deneddylation

must then cause the disassembly of SCF, causing CAND1 to bind and displace Skp1 and the F-box protein.

Disassembly of SCF via deneddylation may be an important factor in SCF regulation. For example, given that F-box proteins have been shown to be unstable in complex with SCF (Zhou and Howley 1998), it is feasible that deneddylation protects F-box proteins from ubiquitination by removing them from the complex entirely. Thus, loss of deneddylation would promote SCF-dependent turnover of F-box proteins, resulting in a pleitropic phenotype due to loss of multiple F-box proteins. Moreover, given that CSN can regulate multiple Cullins (Zhou et al. 2001), perhaps other substrate adaptors of these Cullins are regulated in a similar manner. For further investigation into this theory, see Chapter 4.

Materials and Methods

Yeast strains and plasmids: All yeast strains and plasmids used in this study are listed in table 1, table 2, and table 3. Yeast growth and manipulation were performed as described (Lyapina et al. 2001).

Fission Yeast Native Extracts: Cells were grown to mid-log phase in YES media, and washed in ice cold STOP buffer (50mM Naf, 25mM TRIS ph7.2, 0.02% Sodium Azide). Cell pellets were resuspended in an equal volume of Lysis Buffer (25mM TRIS pH 7.2, 150mM NaCl, 0.3% Triton X-100, 50mM NaF, 1mM EDTA, 1mM PMSF, supplemented with 1X protease inhibitor (5ug/mL of Aprotinin, Chymostatin, and Pepstatin A, 1ug/mL

Leupeptin)) and glass beads (500uM, Sigma). Cells were vortexed for 10 intervals of 40

seconds at maximum, and lysates were cleared by centrifugation (12 Krpm).

Budding Yeast Native Extracts: Cells were grown to mid-log phase in YPD media and

processed as described for fission yeast native extracts. To purify SCF, clarified

Skp1^{myc9}rri1Δ lysates were incubated in the presence of a-myc resin for 1 hour at 4°C.

Beads were washed 4 times in Buffer A (20mM TRIS pH 7.2, 150mM NaCl, and 0.3%

Triton X-100) and 10uL of beads were used per reaction. psCSN was added to the beads,

the volume was brought to 10uL with Buffer A, and beads were incubated for 60 minutes

at 30°C.

Budding Yeast Denatured Extracts: Cells were washed in ice cold STOP buffer (50mM

NaF, 25mM TRIS ph7.2, 0.02% Sodium Azide) and frozen in liquid nitrogen. Pellets

were thawed and an equal volume of glass beads (500uM, Sigma) and SDS lysis buffer

(25mM TRIS pH 7.5, 1% SDS, 1mM EDTA, 1mM PMSF, and 1mM DTT) were added.

Cells were briefly vortexed and boiled for 2 minutes, then vortexed again for 2 minutes.

Lysates were cleared (12 Krpm).

Drosophila Larvae Extracts: Third instar larvae were frozen in liquid nitrogen. Frozen

pellet was ground with a pestle to a powder. 20uL of lysis buffer (25mM TRIS pH 7.2,

150mM NaCL, 5mM DTT, 1mM PMSF, and 2mM MgCl$_2$) was added and mixed.

Extracts were cleared by centrifugation (12 Krpm).

Acknowledgements

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Figures

Figure 3-1: CSN dependent deneddylation is not a classical 'dUb'

(A) Mutation of the 'cys-box' in Csn5 does not effect deneddylation.

(B) Deletion analysis of all known 'dUb's' does not effect the neddylation state of Cul1.

Extracts were made from the *S. cerevisiae* strains indicated and were analyzed by western

blot using antibodies against Cul1 (Cdc53).

A

none
CSN
Flag-Csn5
Flag-Csn5 (C145A)

Cul1^Nedd8
Cul1

α-Cul1

B

ubp1Δ
ubp2Δ
ubp3Δ
ubp4Δ
ubp5Δ
ubp6Δ
ubp7Δ
ubp8Δ
rri1Δ
w/t

α-Cul1

ubp9Δ
ubp10Δ
ubp11Δ
ubp12Δ
ubp13Δ
ubp14Δ
ubp15Δ
ubp16Δ
rri1Δ
w/t

α-Cul1
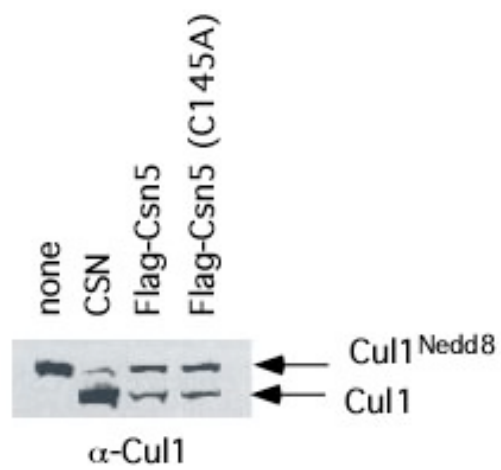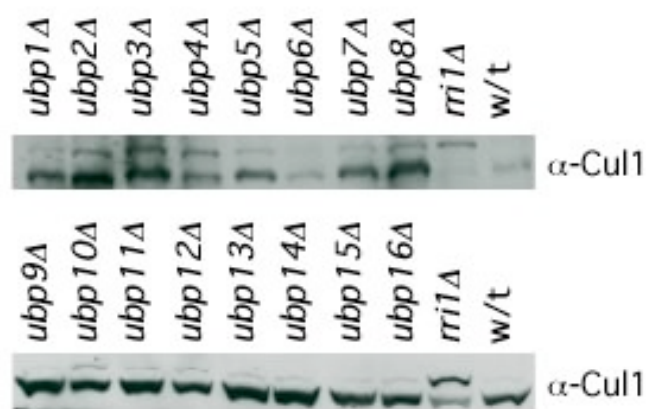
Figure 3-1

Figure 3-2: Identification of the Jab1/MPN domain metalloenzyme (JAMM)

Alignment of predicted JAMM-containing proteins. Sequences were aligned using T-coffee. Eukaryotic proteins are grouped to represent orthologous relationships. Position of the aligned sequence is shown by numbers, and poorly conserved spacers are designated by dashes and numbers. The consensus sequence represents residues conserved in 80% of all proteins. l indicates aliphatic (A, I, L, V), h indicates hydrophobic (F, Y, W, A, I, L, V, M), and s indicates small residues (G, A, C, S, D, N, V, P).

```
12654695_Csn5a_Hsa       74-175   NLEVMGLMLGK-VDGE----TMIIMDSFAL-29-GHLENAIGWYHSHPGYGCWLSGIDVSTQMLNQQFQEPFVAVVIDPTR
7300154_CH5_Dme          71-172   TLEVMGLMLGK-VEDN----TMIVMDAFAL-29-GRMEHAVGWYHSHPGYGCWLSGIDVSTQMLNQTYQEPFVAIVVDPVR
17538322_CE06722_Cel     75-175   NLEIMGLLQGR-IDAN----SFIILDVFAL-29-GRKEKVVGWYHSHPGYGCWLSGIDVSTQTLNQKFQEPWVAIVIDPLR
15219970_At1g22920_Ath   78-179   TIEIMGLMQGK-TEGD----TIIVMDAFAL-29-GRLENVVGWYHSHPGYGCWLSGIDVSTQMLNQQYQEPFLAVVIDPTR
6319985_Rri1p_Sce       105-216   NIEIMGILMGF-TLKD----NIVVMDCFNL-29-GAKLNVVGWFHSHPGYDCWLSNIDIQTQDLNQRFQDPYVAIVVDPLK
17483211_FLJ14981_Hsa   290-392   RSEVVGYLGGRWDVNS---QMLTVLRAFPC-22-LRGLSLVGWYHSHPHSPALPSLQDIDAQMD-9-NGFQPCLALLCSPY
7297828_CG4751_Dme      302-405   VREVCGYLGGTWDMNT---HTLSITKTYPC-22-QDQLLLVGWYHSHPKFQAEPTLRDCDAQLD-10LTYTPCVSLIISPY
15620889_KIAA1915_Hsa   493-594   MAEVIGLLGGRYSEVD---KVVEVCAAEPC-22-VRGFSVIGWYHSHPAFDPNPSLRDIDTQAK-6-RGGAKFIGMIVSPY
1168719_C6.1A_Hsa        31-140   KEEVMGLCIGELNDDT-23-RIVHIHSVII-32-GRPMRVVGWYHSHPHITVWPSHVDVRTQAMYQMMDQGFVGLIFSCFI
15231308_At3g06820_Ath   18-131   TEEIMGLLLGDIEYSK----NGESATAMIW-39-GRTTRVIGWYHSHPHITVLPSHVDVRTQAMYQLLDSGFIGLIFSCFS
7243127_KIAA1373_Hsa    292-385   GIETCGILCGKLTHNE------FTITHVIV-24-QHDLLTLGWIHTHPTQTAFLSSVDLHTHCSYQLMLPEAIAIVCSPKH
14043382_AMSH_Hsa       278-371   GVETCGILCGKLMRNE------FTITHVLI-24-QQGLITLGWIHTHPTQTAFLSSVDLHTHCSYQMMLPESVAIVCSPKF
7301945_Amsh_Dme        271-364   NIETCGVLAGH-LSQN-----QLYITHIIT-24-QMQLITLGWIHTHPTQTAFLSSVDLHTHCSYQIMMPEALAIVCAPKY
11499780_AF2198_Afu      20-101   PDEFIALLSGS-KDVMD----ELIFLPFVS-16-PIGMKVFGTVHSHPSPSCRPSEEDLSLFTR---FGKYHIIVCY-PYD
14590365_PH0451_Pho      22-104   PREVAGFLRMK-DGVFE---EVLIVPKGFF-12-PHDESIKGTFHSHPSPFPYPSEGDLMFFSKFG---GIHIIAAF-PYD
16554503_VNG1818a_Hsp    21-113   PREACGVFVGE-RDGDL----RRVTAVRRV-26-AVGREVVGFYHSHPVGPGRPSATDREHAQ------WPDRVYVVASLA
15789943_VNG0778C_Hsp    20-120   AREAAQDSHPD-EYLGL--DGYVVTDVLVI-17-PNDMRNVGSIHSHPNGVLAPSDADRSMF-GKG---QLHIILGH-PYG
7514470_aq_1691_Aae      21-114   PYETCGLLIGK-SEG-----GIRIAYEAFE-28-SKGMEIVGVYHSHPDHPDRPSQFDLQRA-------FPDLSYIIFSVQ
17230399_all2907_Nsp     24-106   ETEADNFTQEI-NKTNI---TSPTSSLKRR-16-DKSLNIIGIYHSHPDHPAIPSECDRLYA-------WAGYSYIIVSVQ
15805429_DR0402_Dra      22-114   PREVCGALGG--WVRGE---QVQAHALYPL-27-REGLDLVALYHSHPHGPAAPSASDRRLAA------YPVPYLIADPAA
11347692_PA0639_Psa      20-104   PRECCGLIVRG-VRQ------RRYVACRNA-18-EDQGEVLAIVHSHPDVPATPSMADRVSCELHG---LPWVILSW-PEG
15640211_VC0181_Vch      37-138   TPESAGVLIGE-RRGQ----HLVVCDISEP-29-AGTHLYLGEWHTHPEDRPFPSATDRHSWRRNIVSDESMLLLIVGRKD
17546377_RSc1658_Rso    140-231   FIEAARKAAPN-EHA-----AWVVWDSRTG-24-EDHESLVVDMHSHGALAAFFSEQDNRDDAGE----VKISCVVGDLAD
16131509_RadC_Eco       121-205   EREIFMVIFLD-SQH-----RVITHRRLFS-18-KINASALILAHNHPSGCAEPSKADKLITERI----IKSCQFMDLRVL
16079856_YsxA_Bsu       128-212   TQEHFVCLYLN-TKN-----QVIHKRTVFI-18-KRSAASFICVHNHPSGDPTPSREDIEVTRRL----FECGNLIGIELL
20090827_RadC_Mac       125-209   KKEKFITLYLD-TKN-----QILKEEVVSI-18-LESSASVIMVHNHPSGDPSPSREDIMVTEKL----VEGGKLLGIDIL
15801143_YkfG_Eco        55-139   EREEFRVLYLN-NQN-----QLIAGETXFT-18-YHNAAAVVLAHNHPSGEVTPSKADRLITERL----VQALGLVDIRVP

consensus/80%                     ..E.h.hh....................   ......l..hHsHPs..s..S..D.............h.hh.....
Secondary Structure               .HHHEEEEEE.........EEEEEEE..     ....EEEEEEE...........HHHHHHHHH.......EEEEEEE...
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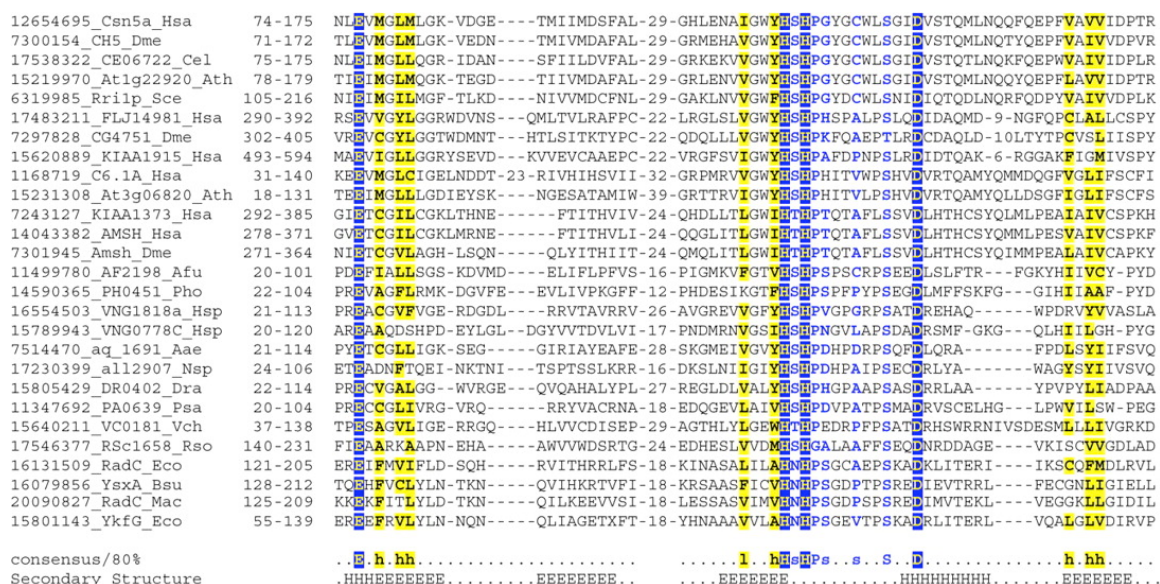
Figure 3-2

Figure 3-3: CSN dependent deneddylation is dependent upon divalent cations

(**A**) Deneddylation is inhibited by EDTA and 1,10-phenanthroline (o-PT). *csn5Δ* lysates were incubated either alone or with wild-type (Cul1-myc13) lysates in the presence or absence of 1mM o-PT or 20mM EDTA for 30 minutes at 30ºC. Lysates were resolved by SDS-PAGE and analyzed by western blot against Cul1.

(**B**) Purified pig spleen CSN (psCSN) deneddylating activity is sensitive to divalent cations and metal chelators. Prior to the assay, psCSN was incubated for 5 minutes with 0.5% methanol (lane 2), 1mM o-PT (lane 3 and 5), 1mM 1,7-phenanthroline (lane 4), 1mM o-PT plus 2mM $NiCl_2$ (lane 6), 1mM zinc acetate (lane 7), or 1mM $NiCl_2$ (lane 8). For lane 5, psCSN was treated with o-PT for 5 minutes prior to the addition of $NiCl_2$.

(**C**) o-PT does not disrupt the CSN complex. psCSN was treated with 1mM o-PT and fractionated in the presence of 1mM o-PT by Superose 6 gel filtration. Fractions were analyzed by western blot with antibodies to Csn5 and Csn1.
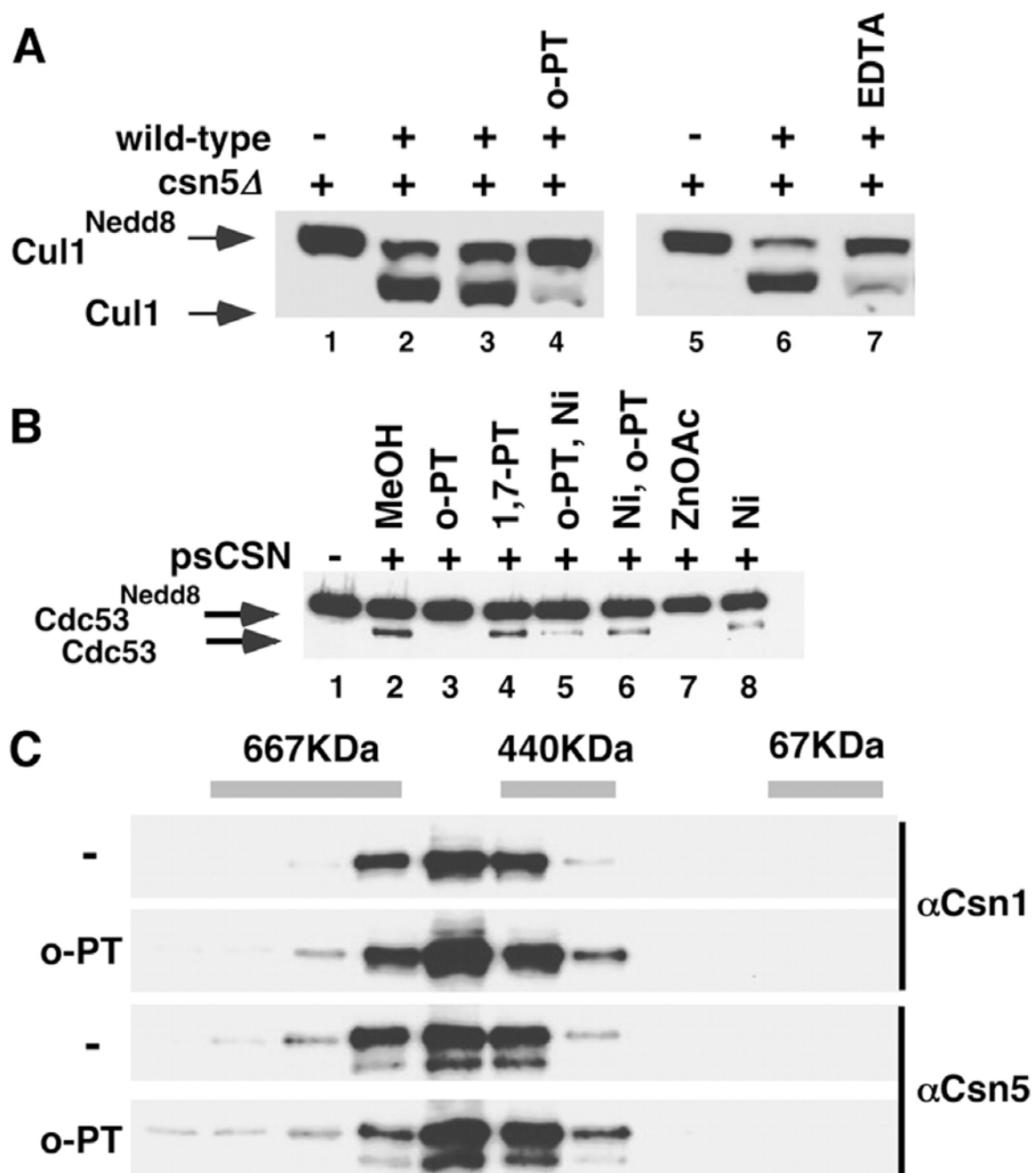
Figure 3-3

Figure 3-4: JAMM is essential for CSN dependent deneddylation

(**A**) Mutations in JAMM affect CSN dependent deneddylation *in vivo*. *S. pombe csn5Δ*

strains expressing either wild-type FLAG-tagged Csn5, or JAMM point mutants FLAG-

Csn5 (H118A), FLAG-Csn5 (H120A), or FLAG-Csn5 (D131N) were analyzed by

western blot with antibodies to FLAG (bottom) and Cul1 (top).

(**B**) Mutations in JAMM do not effect CSN assembly. Extracts from Csn2-myc13 *S.*

pombe expressing FLAG-tagged wild-type or JAMM mutant Csn5 were

immunoprecipitated with myc-antibodies and analyzed by western blot with antibodies to

FLAG.

(**C**) Mutations in JAMM do not affect CSN assembly. Extracts from Csn1-myc13 *S.*

pombe expressing FLAG-tagged wild-type or JAMM mutant Csn5 were

immunoprecipitated with myc-antibodies and analyzed by western blot with antibodies to

FLAG.

(**D**) Mutation of the JAMM motif in the *S. cerevisiae* Csn5 (Rri1) cannot rescue a *csn5Δ*

strain. SKP1^{myc9}*rri1Δ* cells expressing wild-type GST-tagged Rri1 or JAMM mutant Rri1

(H179A, H181A, or D192A) were analyzed by immunoprecipitation using anti-myc

followed by western blot with anti-Cul1 (Cdc53) antibodies (top panel). Expression of

ectopically expressed Rri1 was analyzed by western blot with antibodies to GST (bottom
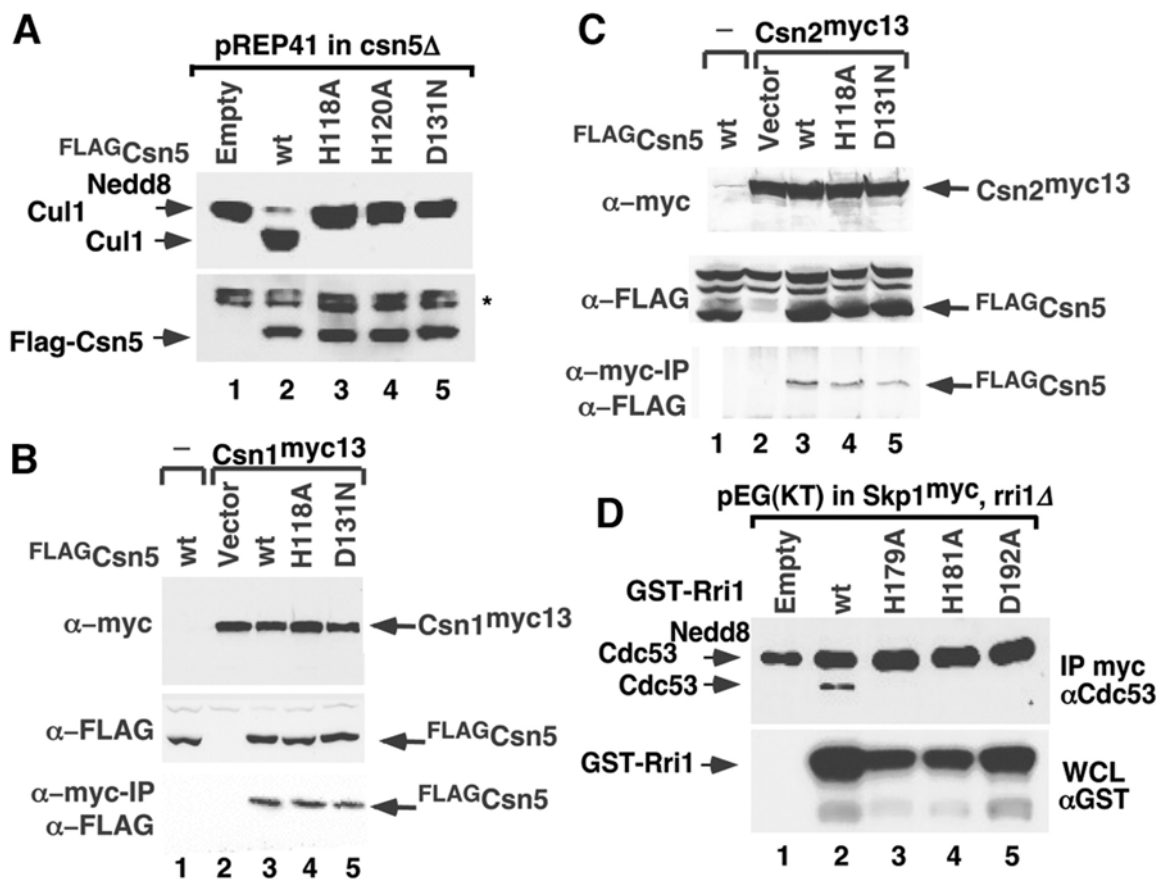
panel).

Figure 3-4

Figure 3-5: Functional interaction between SCF and CSN

(**A**) Deletion of Rri1 in S. cerevisiae enhances the temperature-sensitive phenotypes of mutant SCF strains. Strains indicated were grown in complete media (YP) with dextrose (D) at 25 °C to mid-log phase, and serial dilutions were spotted onto YPD plates. Cells were grown at 25 °C or 33 °C for 2 to 3 days.

(**B**) Genetic interactions between Rri1 and SCF are dependent upon the JAMM motif. Indicated strains were constructed to express either wild-type or JAMM mutant Rri1 under the Galactose promoter. Cells were grown in YPD at 25°C to mid-log phase, and serial dilutions were spotted onto YPD or YP Galactose plates. Cells were grown at 25 °C or 33 °C (*cdc34-2*, *rri1Δ*) or 34 °C (*skp1-12*, *rri1Δ*).

(**C**) Deletion of Rri1 in *skp1-12* strains stabilizes the SCF substrate Sic1. skp1-12 and skp1-12, rri1Δ strains were transformed with a plasmid containing GAL-inducible HA-tagged Sic1. Strains were grown in YP-Galactose to induce expression of HA-Sic1 following arrest in mitosis with nocodazole. Cells were transferred to YPD to silence GAL expression and time points were taken as indicated. Cells were lysed and analyzed by western blot using an antibody against the HA epitope.
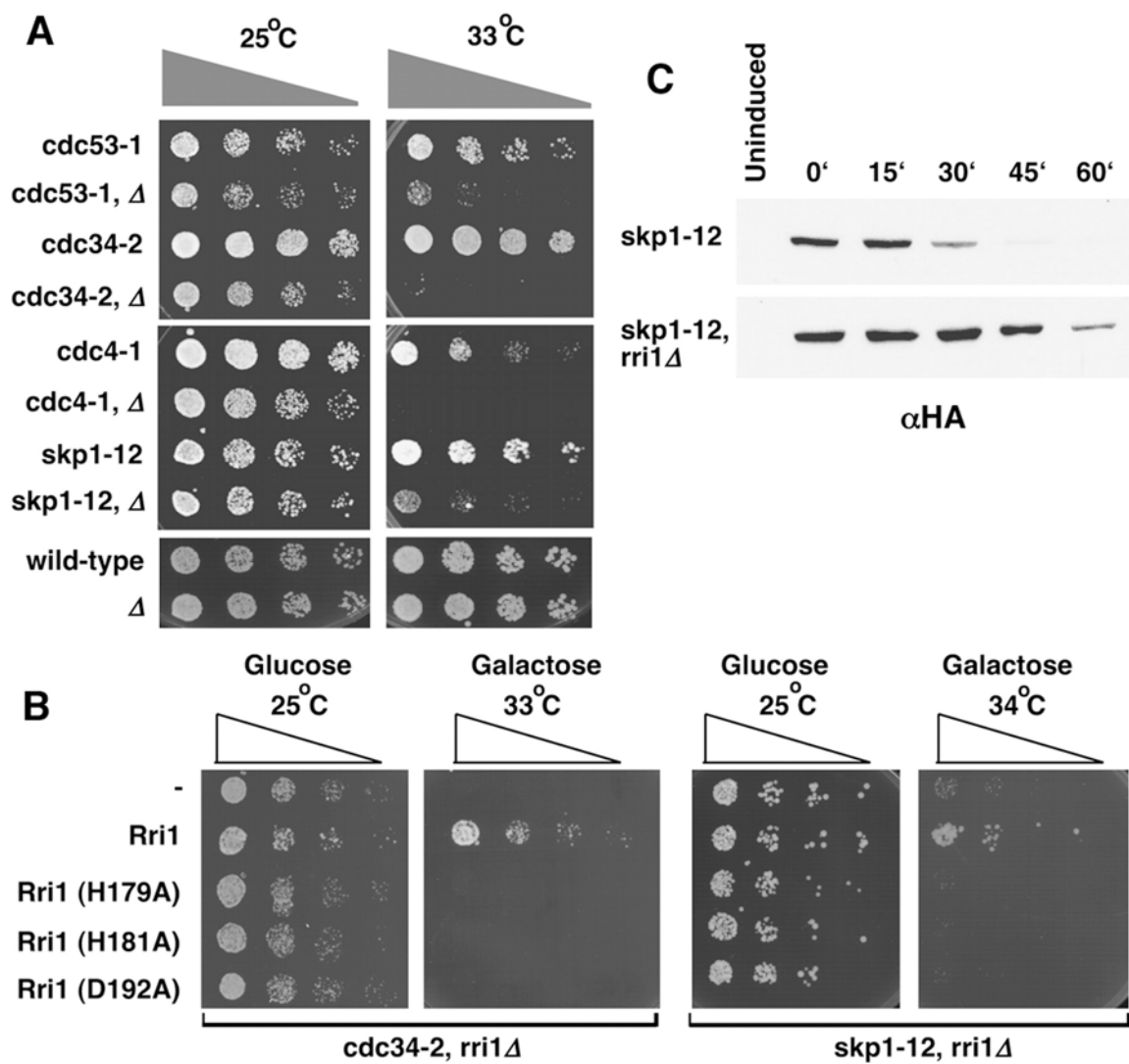
Figure 3-5

Figure 3-6: JAMM is essential for proper development in *Drosophila*

(A - H) Developing eye discs of second-third instar wild-type (wt), CSN5-null (Δ/Δ),

CSN5-null/hs-Csn5 (Δ/Δ; hs-wt), and CSN5-null, hs-Csn5 (D148N) (Δ/Δ; hs-D148N)

were stained for the R cell markers Elav and Chaoptin.

(I) Expression of wild-type Csn5 and Csn5 (D148N). Whole cell extracts were made

from second to third instar larvae and analyzed by western blot against Csn5. Lane 1:

wild-type. Lane 2: CSN5-null. Lane 3: CSN5-null, hs-CSN5. Lane 4: CSN5-null, hs-

CSN5 (D148N).

(J) Lethality of CSN5-null can be recovered by expression of hs-CSN5 but not hs-CSN5

(D148N). Percentage recovery of the genotypes indicated resulting from the cross of

CSN5-null/CSN5+ and Df/+; CSN5-transgene (wild-type and D148N). Percentages

reflect approximately 80 progeny scored. (CSN5N, CSN5-delete; +, wild-type; Df,

deficiency spanning CSN5 locus; hs, heat-shock).
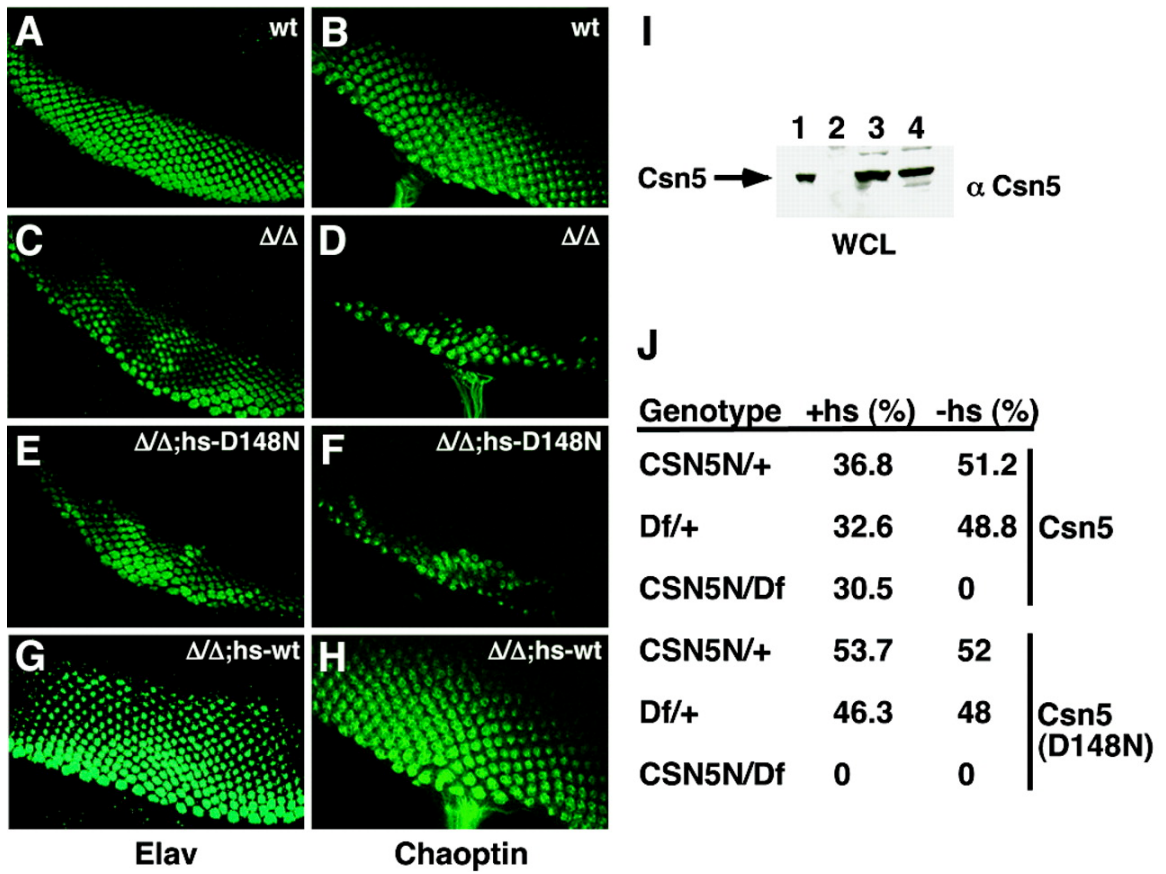
Genotype	+hs (%)	-hs (%)	
CSN5N/+	36.8	51.2	
Df/+	32.6	48.8	Csn5
CSN5N/Df	30.5	0	
CSN5N/+	53.7	52	
Df/+	46.3	48	Csn5 (D148N)
CSN5N/Df	0	0	

Figure 3-6

Tables

Table 3-1: Identification of proteins present in psCSN by mass spectrometry

	Description	#peptides	% coverage	Yeast homolog
CSN	Gps1/CSN1	11	18.70%	none
	CSN2	16	34.10%	none
	CSN3	11	25.30%	none
	CSN4	19	48.10%	none
	CSN5	16	45.50%	Rri1
	CSN6	14	42.80%	none
	CSN7a	12	46.90%	none
	CSN7b	2	19.70%	none
	CSN8	3	20.10%	none
Proteasome	Rpn7	4	12.30%	Rpn7
	Rpn6	4	14.20%	Rpn6
	Rpn8	4	14.30%	Rpn8
	Rpn9	3	10.60%	Rpn9
	Rpn3	3	7.10%	Rpn3
	Rpt3	2	12.20%	Rpt3
	Rpn8	2	6.80%	Rpn8
	Rpn2	2	3.10%	Rpn2
	20S alpha type 6	2	8.90%	Scl1
	Rpn11	2	7.10%	Rpn11
	Rpn5	2	6.10%	Rpn5

	Description	#peptides	% coverage	Yeast homolog
HSP	HSP90 alpha	10	15%	HSC82/HSP82
	BiP protein [Homo sapiens]	7	15%	Kar2/SSA1/SSA2
	HSP60	2	6.60%	HSP60
	HSP 71	4	9.60%	SSA1-4/KAR2/SSB2
PP2A	PP2A subunit B (PR55 delta)	2	5.20%	
	PP2A subunit A (PR65 alpha)	8	21.60%	TPD3
	PP2A subunit B (PR55 alpha)	3	9.20%	CDC55
	PP2A subunit B (PR61 epsilon)	1	3.20%	RTS1
MCM	MCM6	11	18%	MCM6/CDC47
	MCM2	10	16.60%	MCM2/MCM3
	MCM4/cdc21 homolog	6	8%	CDC54/MCM6/CDC46
Cytoskeleton	alpha-actinin	15	22.50%	SAC6/CMD1(Low)
	Kinesin-like protein	8	4.90%	KIP3/CIN8/KAR3/KIP1
	Kinesin-heavy chain	5	7.20%	KIP1/KIP3/CIN8
	tubulin alpha-1 chain	2	7.50%	TUB1/TUB3/TUB2/TUB4
	myosin, light polypeptide 6, alkali, smooth muscle and non-	2	19.20%	CMD1/MLC1/CDC31
Misc.	ubiquitin activating enzyme E1-like	2	4.30%	Uba1
	CULLIN HOMOLOG 1	2	4.10%	Cdc53
	endoplasmin precursor	6	10.70%	HSC82/HSP82

Description	#peptides	% coverage	Yeast homolog
Retinoblastoma-binding protein 4	8	15.60%	HAT2/MSI1
G1/S transition, RB7, HAT	9	20%	HAT2/MSI2
Skb1 (protein methyltransferase)	19	24.20%	HSL7
HDA1/Rpd3	6	17.80%	Rpd3/Hos2/Hos1
HDA2	6	17.40%	Rpd3/Hos2
Talin	5	3.60%	
goodpasture antigen-binding	4	11.90%	none (UBP9 similarity)
metastasis associated gene 1	3	4.80%	none (YGL131c
phosphodiesterase 2A, cGMP-stimulated	3	6.60%	None (Pde2 similarity)
UV-damaged DNA binding factor	3	5.60%	none (Rse1 similarity)
valosin-containing protein	3	3.70%	CDC48 (homology to AFG2/YLL034
KIAA1421 protein/putative	3	2.40%	Vps13
110 KDA CELL MEMBRANE GLYCOPROTEIN	2	9.10%	(similarity to rpn13)
TRIPEPTIDYL-PEPTIDASE II	3	4.20%	none
coatomer protein complex, subunit	2	4.30%	SEC27
translation elongation factor	2	7.80%	TEF2/TEF1
hepatoma-derived growth factor	2	7.10%	none
unknown/putative cop1 homolog	2	2.80%	None
(IMP DEHYDROGENASE 2)	2	6.60%	IMD4/3/2/1
muscle specific gene; hypothetical	2	15.60%	similar to VMA13/UBP1
hProtein Disulfide Isomerase	2	21.70%	PDI1

Description	#peptides	% coverage	Yeast homolog
unknown (similar to usp)	2	2.90%	YPL249C
unknown (similar to Tom1 and Nedd4)	2	1.60%	Similar to Tom1/RSP5
dendritic cell protein-similar to CSN7b	2	9.40%	similarity to rpn9
unknown	2	7.30%	none
sperm surface protein	2	3.70%	none
titin, cardiac muscle	2	0.10%	
unknown (RING, Zinc motifs)	1	1.40%	none

Table 3-2: *S. cerevisiae* **yeast strains used in this study**

Yeast Strains:

<u>*S. cerevisiae*</u>:

<u>Stain</u>	<u>Genotype</u>
RDY 385	MAT a, *cdc4-1*, his7, ura1
RDY 597	MAT a, *cdc34-2*, his3Δ, *ura3-52*, GAL+
RDY 690	MAT a, ura3, trp1, ade2, *cdc53-1*
RDY1377	MAT a, *can1-100*, *ade2-1*, leu2-3, -112, *trp1-1*, *ura3-1, Skp1-12*
RDY 1716	MAT a, ubc12::KanMX, *his3Δ1, leu2Δ 0, met15 Δ 0, ura3Δ0*
RDY 1721	MAT a, *his3Δ1, leu2Δ 0, met15Δ 0, ura3Δ0*
RDY 1735	MAT a, rri1::KanMX, *his3Δ1, leu2Δ0, met15Δ0, ura3 Δ 0*
RDY 1832	MAT α, RRI1::KanMX, *can1-100, leu2-3, -112, his3-11, trp1-1, ura3-1, ade2-1*
RDY 1835	MAT a, RRI1::KanMX, *can1-100, leu2-3, -112, his3-11,*

trp1-1, *ura3-1*, *ade2-1*, pep::TRP1, bar1::LEU2,

skp1::HIS3, SKP1::Myc9

RDY 1914   MAT a, rri1::KanMX, ura3, trp1, ade2, *cdc53-1*

RDY 1915   MAT a, rri1::KanMX, *cdc34-2*, his3Δ, ura3-52, GAL+

RDY 1916   MAT a, rri1::KanMX, *cdc4-1*, his7, ura1

RDY 1917   MAT a, rri1::KanMX, *can1-100*, *ade2-1*, *leu2-3, -112,*

trp1-1, *ura3-1*, *skp1-12*

RDY 2096   MAT a, pci8::KanMX, his3Δ1, leu2Δ0, met15Δ0,

ura3Δ0

RDY 2097   MAT a, YOL117W::KanMX, his3Δ0, leu2Δ0,

met15Δ0, ura3Δ0

Table 3-3: *S. pombe* **yeast strains used in this study**

RDY 1712   csn5Δ

RDY 1568   Pcu1^{myc13}

RDY 2092   csn5Δ, Csn1^{myc13}

RDY 2093   csn5Δ, Csn2^{myc13}

Table 3-4: Plasmids used in this study

Plasmid	RDB Number
pREP41	1549
pREP41-FLAG-Csn5	1469
pREP41-FLAG-Csn5 (H118A)	1487
pREP41-FLAG-Csn5 (H120A)	1488
pREP41-FLAG-Csn5 (D131N)	1489
pEG(KT)	1572
pEG(KT)-Rri1	1471
pEG(KT)-Rri1 (H179A)	1478
pEG(KT)-Rri1 (H181A)	1491
pEG(KT)-Rri1 (D192A)	1494

Chapter 4: Role of Deneddylation In The Stabilization of F-box Proteins

Gregory A. Cope and Raymond J. Deshaies.

Summary

SCF ubiquitin ligases target numerous proteins for ubiquitin-dependent proteolysis, including p27 and Cyclin E. SCF is regulated by the ubiquitin-like protein Nedd8, which covalently modifies the Cul1 subunit. The removal of Nedd8 is catalyzed by a metalloprotease motif within Csn5. Here, we conditionally knock down Csn5 expression in HEK293 human cells. Analysis of SCF when Csn5 is suppressed indicates that the protein level of multiple F-box proteins is dramatically decreased. Molecular analysis indicates that this decrease is due to increased proteasome-dependent turnover of F-box proteins. We propose that loss of Csn5 results in a constitutively active SCF that targets F-box proteins for degradation.

Introduction

Proteins are marked for degradation to the 26S Proteasome via the covalent attachment of chains of the 76-amino acid protein ubiquitin (reviewed in Pickart 2001). This process involves three discreet steps. First, ubiquitin is activated by the ubiquitin-activating enzyme (E1) through the hydrolysis of ATP to AMP to yield a high energy thioester intermediate between the C-terminal glycine of ubiquitin and the catalytic cysteine of the

E1. Subsequently, ubiquitin is transferred onto the catalytic cysteine of one of many ubiquitin-conjugating enzymes that, in turn, release their cargo onto substrates with the help of ubiquitin ligase enzymes (E3).

One of the best-studied E3 ubiquitin ligase enzymes is the four-subunit complex SCF (reviewed in Petroski and Deshaies 2005). SCF consists of two activities: the first, contained within the Cul1 and RING-domain-containing Hrt1/Roc1/Rbx1 proteins, is the ability to activate the E2 to facilitate ubiquitin transfer from the E2 onto substrate; the second activity resides within the Skp1 and variable F-box proteins, which are thought to reel in substrates and feed them to the Cul1/Hrt1 sub-complex. The variability of the F-box proteins gives SCF the opportunity to access a wide array of substrates. In yeast, over 19 F-box proteins are known, in *A. thaliana* over 400, and in humans over 70 (Petroski and Deshaies 2005). This abundance of substrate adaptors gives SCF an enormous power over its minions.

SCF must be tightly controlled to prevent spurious ubiquitination. There are multiple ways in which this is accomplished. First, SCF is thought to be inactivated through the association of the protein CAND1 (Liu et al. 2002; Zheng et al. 2002). CAND1 binds to Cul1 and physically occludes Skp1 from binding and recruiting an F-box protein, keeping Cul1 and Hrt1 in an inactive form (Liu et al. 2002; Zheng et al. 2002). However, Cul1 can be covalently modified with the ubiquitin-like protein Nedd8, and this neddylation can displace CAND1 to allow for Skp1 to bind (Liu et al. 2002; Zheng et al. 2002). To shut down SCF, the Cop9 Signalosome (CSN) is thought to deneddylate Cul1, facilitating CAND1 binding and subsequent Skp1 release (reviewed in Cope and Deshaies 2003).

CSN is a highly conserved protein complex found from yeast to humans. CSN is composed of eight subunits, termed Csn1-Csn8 (Deng et al. 2000), and each of these subunits contains high homology to the 26S proteasome lid subcomplex and eukaryotic Initiation Factor 3 (eIF3)(reviewed in Cope and Deshaies 2003). CSN has been found to play enormously diverse roles in several different organisms (reviewed in Cope and Deshaies 2003). In *A. thaliana*, CSN components were identified in a screen for plants, which displayed a photomorphogenic defect (plants develop in the dark as they would in the light). In *D. melanogaster*, mutations in Csn4 and Csn5 result in pleitropic effects, including activation of meiotic checkpoints (Doronkin et al. 2002; Oron et al. 2002) and failure of photoreceptor neurons to differentiate (Suh et al. 2002). RNAi of Csn5 in *C. elegans* additionally results in pleitropic effects, including sterile worms and alterations in microtubules (Pintard et al. 2003a).

Although the molecular basis behind many of these phenotypes has yet to be elucidated, it is becoming evident that deneddylation of Cullins catalyzed by CSN is at least partially responsible. Transgenic flies and plants carrying mutations in the active site of JAMM fail to restore developmental phenotypes (Cope et al. 2002; Dohmann et al. 2005). Moreover, failure to deneddylate Cul3 in *C. elegans* leads to the accumulation of the microtubule severing protein Mei-1, causing microtubule defects (Pintard et al. 2003a; Pintard et al. 2003b).

The effects of the loss of deneddylation of Cullin proteins are still not understood. Failure to deneddylate causes a defect in Cullin-based ligase activity *in vivo* suggesting CSN acts positively on SCF (Schwechheimer et al. 2001; Cope et al. 2002; Doronkin et al. 2003; Feng et al. 2003; Groisman et al. 2003; Liu et al. 2003; Pintard et al. 2003b;

Wang et al. 2003); however *in vitro* data suggests a negative role for CSN regulation of

SCF (Lyapina et al. 2001; Yang et al. 2002; Groisman et al. 2003; Zhou et al. 2003).

What may account for this discrepancy? One hypothesis suggests that neddylation and

deneddylation affect the stability of substrate adaptor proteins *in vivo* (Cope and Deshaies

2003).

In an effort to investigate how loss of deneddylation can effect SCF activity, we

conditionally silenced the catalytic subunit of CSN in mammalian cells. Suppression of

Csn5 protein resulted in a significant decrease in the F-box proteins Skp2, Cyclin F,

Fbx7, Fbx4, and Fbw7. Moreover, mRNA transcripts of all but one of these F-box

proteins are unaltered when CSN5 is suppressed, suggesting the decrease in protein levels

is post-translation. Treatment with proteasome inhibitors significantly recovered the

protein levels. Finally, we found a dramatic increase in the protein and activity levels of

Cyclin E protein, a substrate of Fbw7, suggesting that loss of F-box proteins results in

substrate accumulation.

Results

Loss of CSN5 in HEK293 cells is not lethal

In an effort to analyze the effect of loss of deneddylation in human cells, we

utilized the inducible siRNA system developed by Clevers and colleagues (van de

Wetering et al. 2003) to conditionally down-regulate Csn5 protein levels. Treatment of

cells with doxycycline for eight days resulted in a drastic reduction in both Csn5 mRNA

and protein levels (Figures 1A and 1B). Despite this significant drop in Csn5 protein

levels, few morphological changes were observed and little change in cell cycle

distribution could be observed as monitored by FACS (data not shown). Analysis of other CSN components showed little changes in protein levels (Figure 1C). Given that loss of Csn5 results in accumulation of the neddylated form of Cullins, we analyzed Cullins 1-4 by western blot when we knocked down Csn5. Loss of Csn5 resulted in a marked enhancement of cullin neddylation status, most notably seen with Cul2 and Cul4 (Figure 1D).

Loss of deneddylation causes F-box protein instability

It has been proposed that loss of deneddylation could render F-box adaptor proteins unstable (Cope and Deshaies 2003). To test this hypothesis, we analyzed several F-box proteins when Csn5 is suppressed. Concomitant with loss of Csn5, we observed significant decreases in the protein levels of Skp2, Cyclin F, Fbw7, Fbx4, and Fbx7 (Figure 2A). To test whether this decrease in protein levels is post-translational, we performed RT-PCR on Skp2, Cyclin F, Fbw7, Fbx7, and Fbx4 mRNA transcripts. Despite the significant drop in protein levels, little change in mRNA levels could be observed with Skp2, Cyclin F, Fbw7, and Fbx7 suggesting the loss of protein occurs post-translationally (Figure 2B).

Loss of F-box proteins is proteasome dependent

We next examined whether the 26S proteasome is involved in the turnover of F-box proteins. Treatment of Csn5 knockdown cells with the proteasome inhibitor MG132 resulted in a marked stabilization of Skp2, Cyclin F, and Fbw7. In addition, similar results were obtained when we used the proteasome inhibitor LLnL. Therefore, loss of F-

box proteins when Csn5 protein levels are knocked down is dependent upon the 26S Proteasome.

Loss of Fbw7 results in a marked increase in substrate levels

Given the loss in protein levels of F-box proteins, we analyzed substrates of the F-box protein Fbw7. Induced down-regulation of Csn5 resulted in a marked increase in the protein levels of two Fbw7 substrates—c-myc and Cyclin E. Moreover, loss of Csn5 resulted in a loss of cell cycle regulation of Cyclin E protein abundance, suggesting a defect in Cyclin E turnover. Immunoprecipitation of Cyclin E complexes revealed an increase in Histone H1 kinase activity, suggesting that the increase in Cyclin E correlates with an increase in activity.

Overexpression of a non-degradable form of Cyclin E results in several growth defects, including chromosome instability (Spruck et al. 1999). In an effort to examine the effects on chromosome instability when Csn5 is knocked down, we analyzed cells induced for twenty-one days for chromosome abnormalities by FISH and kareotyping. Despite a significant increase in Cyclin E, we could not detect any chromosomal abnormalities (data not shown).

Discussion

Despite considerable progress over the last few years, little is know regarding the molecular consequence of the loss of deneddylation. Here, we report that suppression of Csn5 in human cells results in a drastic decrease in F-box protein levels. This decrease is post-translational and proteasome dependent, suggesting that loss of deneddylation

increases the ubiquitin-dependent turnover of F-box proteins. In agreement, loss of Csn5 causes the accumulation of an F-box protein substrate, Cyclin E. Deletion of CSN components in several different organisms causes a defect in SCF activity *in vivo* (Schwechheimer et al. 2001; Cope et al. 2002; Doronkin et al. 2003; Feng et al. 2003; Groisman et al. 2003; Liu et al. 2003; Pintard et al. 2003b; Wang et al. 2003). Our data suggest this defect is due, at least in part, to the loss of specific F-box proteins.

In agreement with our findings, recent studies in *S. pombe* and *Neurospora* revealed that loss of CSN renders Cullin adaptor proteins unstable (He et al. 2005; Wee et al. 2005). Although the exact mechanism by which Cullin adaptor proteins are turned over is unknown, given the increase in SCF activity *in vitro* when Cul1 is hyperneddylated (Lyapina et al. 2001; Yang et al. 2002; Groisman et al. 2003; Zhou et al. 2003), it would not be surprising if this turnover is triggered by an autocatalytic mechanism. Indeed, Cul1 has been implicated in the turnover of Skp2 (Wirbelauer et al. 2000), and preliminary studies suggest that inhibiting SCF activity *in vivo* suppresses F-box protein turnover (data not shown).

Given the effects of depletion of Csn5 on F-box proteins, it is extremely surprising that we could not detect any measurable cell morphological phenotype. For example, overexpression of Cyclin E (Spruck et al. 1999) or deletion of Fbw7 (Rajagopalan et al. 2004) causes chromosomal instability; however, we were unable to detect any measurable defect in chromosome status when we depleted Csn5 (data not shown). Given the effect of depletion of Csn5 on so many different F-box proteins, it is possible that opposing pathways are effected in such a way as to prevent the onset of chromosome instability.

We have shown that CSN can stabilize F-box proteins, thus acting positively on SCF. CSN has been shown to participate in several different processes, including development, transcription, and cell cycle progression (reviewed in Cope and Deshaies 2003). We propose that loss of deneddylation and F-box protein abundance underlies these phenotypes. Key questions remain unaddressed however. For example, does the mechanism we have uncovered participate in the turnover of F-box proteins in an unperturbed state? Is CSN deneddylating activity regulated? How do the cycles of neddylation and deneddylation intertwine? Indeed, answers to these questions are extremely important in unlocking the secrets SCF ubiquitin ligases.

Materials and Methods

Cell Culture, Cell Lines and plasmids: HEK293 cell lines were obtained from ATCC. Cell Lines were grown in Minimum Essential Media (MEM) supplemented with 10% FBS and grown at 37°C with 5% CO_2. For G1 cell cycle arrest, cells were grown in MEM media without Serum for 24 hours. For G2 cell cycle arrest, cells were treated with 330nM of Nocodazole (Sigma). All transfections were preformed using the calcium phosphate method. Briefly, cells were grown to a density of 1.2-2.0 X 10^6 cells per 6cm plate. 5ug of DNA was mixed to a volume of 450uL and 50uL of 2.5M $CaCl_2$ was added. 500uL of BBS (0.05M BES, 0.28M NaCl, 0.0015M Na_2HPO_4, pH 7.0) was added and solution vortexed to mix. Cells were re-fed with fresh MEM supplemented with 2.5uL/mL 25-hydroxycholesterol (Sigma). Precipitate was applied to cells and incubated

for 4 hours prior to removal with PBS. Cells were re-fed with fresh MEM and incubated

24-48 hours. All plasmids used in this study are listed in Table 1.

Construction of Stable Cell Lines: HEK293 cells were transfected with pGC95 (control

scrambled siRNA) or pGC93. 24 hours after transfection, cells were re-fed with MEM

supplemented with 5ug/mL puromycin and selected for 48 hours. Post-selection, cells

were grown for 7-10 days and single colonies were selected.

mRNA Analysis: RNA was purified from cells using Qiagen RNeasy kits. After

purification, RNA was normalized by concentration used as a template for stratascript

reverse transcriptase following manufacturers protocols (stratagene). Reverse transcribed

RNA was then used for PCR towards Csn5, β-Actin, Cyclin F, Fbx4, Fbx7, Fbw7, and

Skp2.

Protein Analysis: Native lysates were made by resuspending cell pellets in an equal

volume of buffer A (25mM TRIS (7.5), 150mM NaCl, 0.3% Triton X-100, 1mM EDTA

and 1mM DTT supplemented with 1mM PMSF, 0.25ug/mL pepstatin, and 5ug/mL each

of leupeptin, aprotinin, and chymotrypsin). Lysates were cleared by centrifugation and

normalized using the Biorad Protein Assay (Biorad). Denatured lysates were made by

resuspending cell pellets in an equal volume of boiling SDS buffer (2% SDS, 50mM

TRIS (7.5) and 5mM DTT) and boiling for 5 minutes. Lysates were sonicated and cleared

by centrifugation.

Antibodies: The following antibodies were used in this study: Fbw7 (Orbigen PAB-10563), Cyclin F (Santa Cruz Biotechnology sc-952), Cyclin E (Santa Cruz Biotechnology sc-198), Csn5 (Santa Cruz Biotechnology sc-9074), β-tubulin (Santa Cruz Biotechnology sc-9104) Fbx7 (Zymed 51-8000), and c-myc (Santa Cruz Biotechnology sc-42). Skp2 was generously provided by W. Krek, and Fbx4 was generously provided by W. Boelens.

Histone H1 kinase assay: Native lysates were immunoprecipitated for 1 hour with antibodies toward Cyclin E. Immunoprecipitates were washed 3 times in native lysis buffer (buffer A). Beads were resuspended in 10uL of reaction mix (for 100uL: 3uL 5mg/mL histone H1, 1.5uL 4.5mM ATP, 1.5uL γ-^{32}P ATP, 94uL of kinase assay buffer (10mM TRIS (7.5), 10mM MgCl$_2$, 50mM NaCl, 2mM EDTA, 1mM DTT, and 0.02% Triton X-100)). Reactions were incubated for 20 minutes at 21ºC and analyzed by SDS-PAGE followed by autoradiography.

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Figures

Figure 4-1: Suppression of Csn5 in HEK 293 human cells

(A) Expression of Csn5-specific siRNA suppresses endogenous Csn5 mRNA levels. Cells induced with doxycycline for eight days were harvested and analyzed by RT-PCR using oligos specific for Csn5.

(B) Expression of Csn5-specific siRNA suppresses endogenous Csn5 protein levels. Cells induced with doxycycline for eight days were harvested and lysed. Lysates were analyzed by western blot using antibodies specific toward human Csn5.

(C) Silencing of Csn5 does not appreciably affect the levels of other CSN subunits. Cells induced with doxycycline for eight days were harvested, lysates were made, and lysates were analyzed by western blot with the antibodies indicated.

(D) Silencing of Csn5 results in altered Cullin neddylation. Cells were induced with doxycycline for eight days and harvested. Cells were lysed and analyzed by western blot using the antibodies indicated.
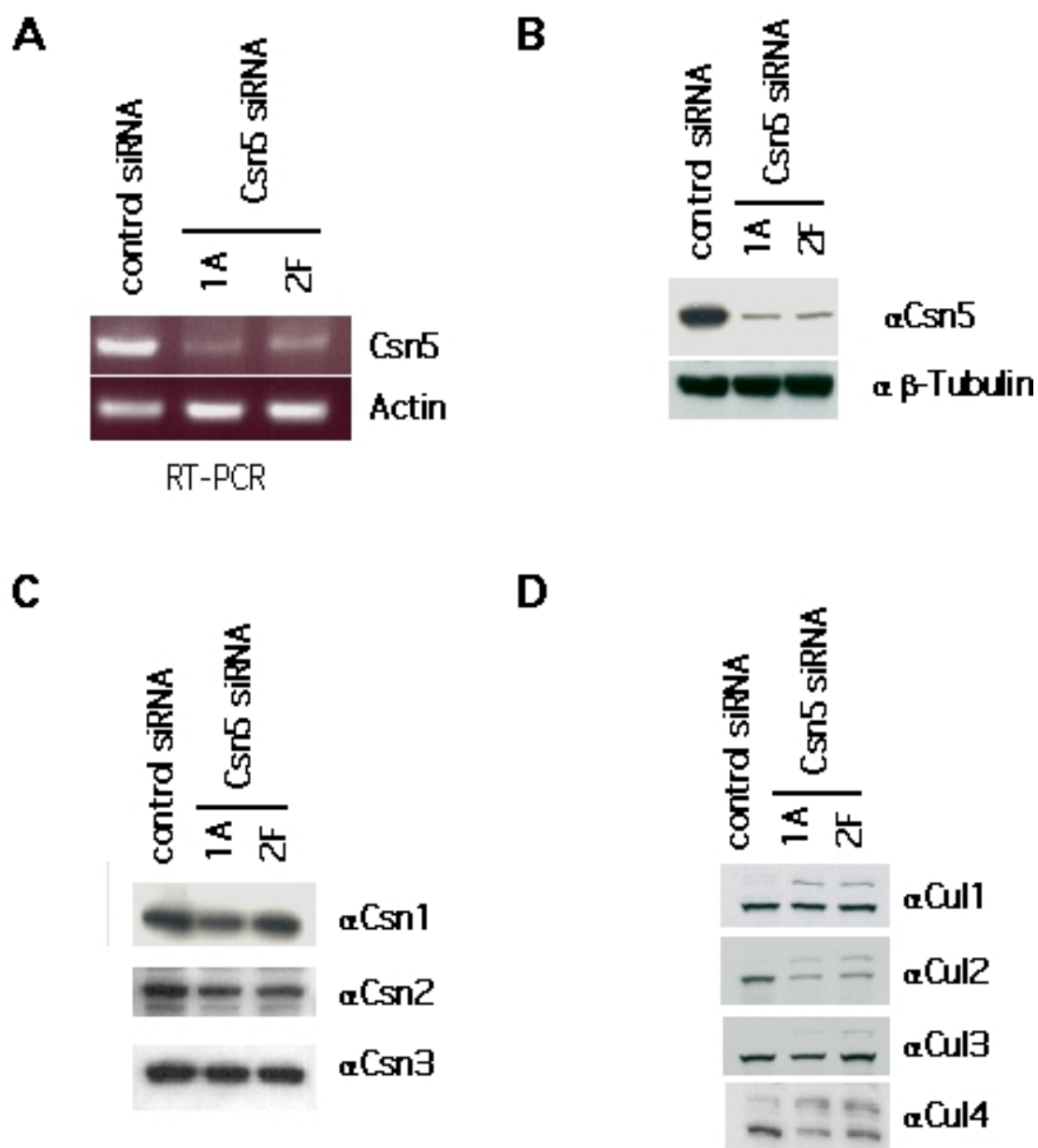
Figure 4-1

Figure 4-2: Loss of Csn5 results in a decrease in F-box protein levels

(A) Depletion of Csn5 results in a decrease in F-box protein levels. Cells were treated with doxycycline for eight days followed by lysis and analysis with antibodies toward the proteins indicated.

(B) Depletion of Csn5 does not effect all F-box protein mRNA levels. Cells were treated with doxycycline for eight days and analyzed by RT-PCT using oligo specific toward the mRNAs indicated.
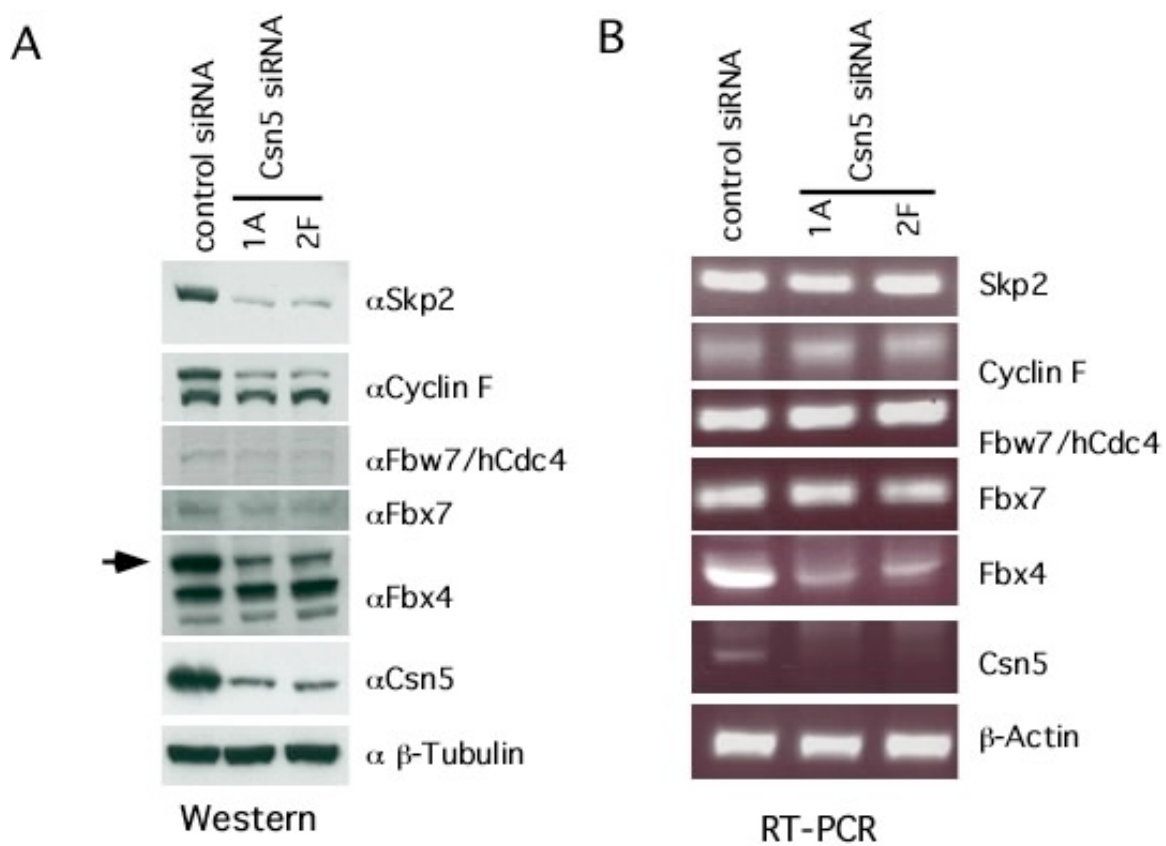
Figure 4-2

Figure 4-3: Loss of Csn5 results in proteasome dependent turnover of F-box proteins

(A) Treatment of doxycycline-treated cells with MG132 inhibits the turnover of F-box proteins. Cells were treated with doxycycline for eight days followed by treatment with MG132 (25uM) for eight hours. Cells were lysed in SDS and analyzed by western blot against the indicated proteins.
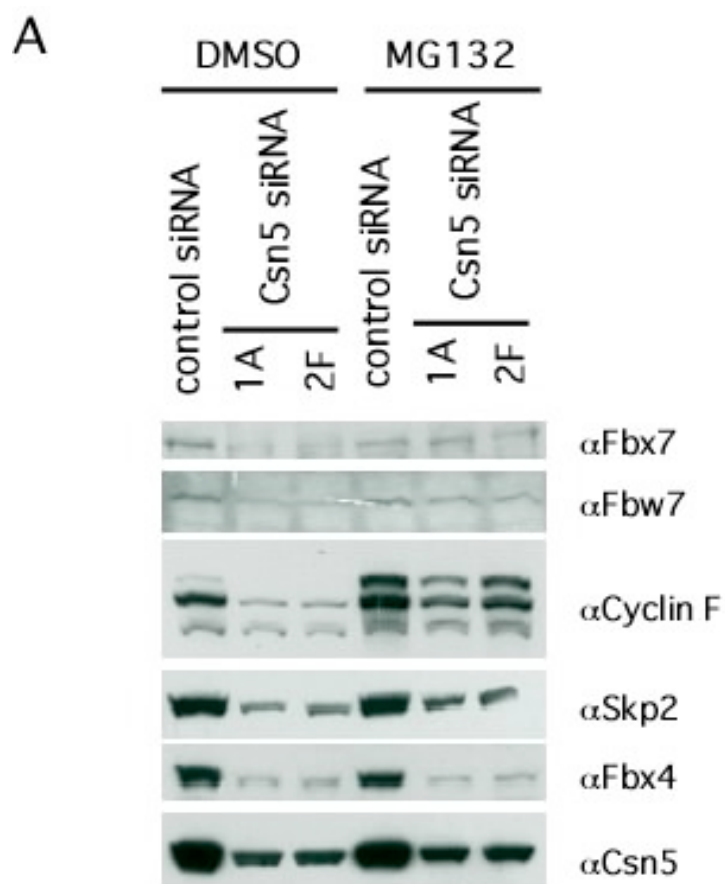
Figure 4-3

Figure 4-4: Loss of Csn5 results in an increase in Cyclin E, an F-box protein substrate

(A) Substrates of the Fbw7 SCF complex accumulate when Csn5 is depleted. Cells were induced with doxycycline for eight days followed by western blot analysis against the indicated proteins.

(B) Activity of the kinase Cyclin E/Cdk2 is increased when cells are depleted for Csn5. Cells were induced with doxycycline for eight days followed by lysis under native conditions. Lysates were immunoprecipitated with antibodies toward cyclin E and immuoprecipitates analyzed for kinase activity against histone H1.

(C) Cell cycle regulation of Cyclin E is lost when cells are depleted for Csn5. Cells were induced with doxycycline for eight days followed by arrest at the indicated cell cycle stages. Cells were analyzed by western blot with the indicated antibodies.
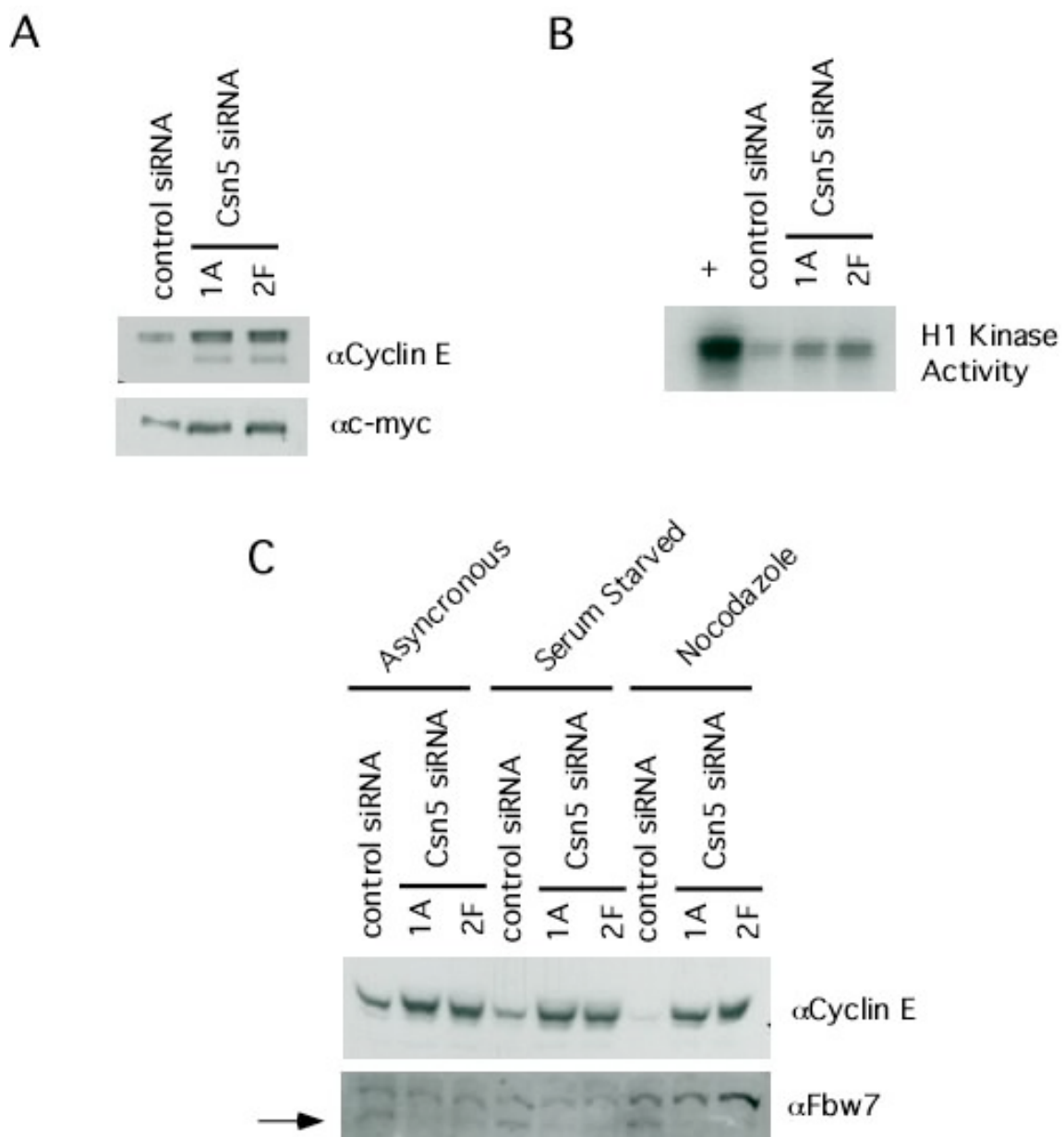
122

Figure 4-4

Tables

Table 1: Plasmids used in this study

Plasmid	Reference	Description
pGC95	This study	Tetracycline inducible scrambled siRNA
pGC93	This study	Tetracycline inducible siRNA to human Csn5.
RJD 1419	This study	CMV Flag-Csn5
RJD 1500	This study	CMV Flag-Csn5 (ASA)
RJD 1501	This study	CMV Flag-Csn5 (D151N)
RJD 942	(Lyapina et al. 1998)	HA-Cul1
RJD 1192	(Wu et al. 2000)	HA-Cul1 (1-498)

Chapter 5: Future Directions

Despite the enormous amount of progress that has been made on SCF and CSN in the last several years, there are still key questions that need to be addressed. These questions not only address the specific mechanism of Cul1 regulation, but also address more global questions regarding CSN and Nedd8.

SCF Regulation by Nedd8 and CSN

A key question that remains regarding SCF regulation concerns the dynamics of neddylation and deneddylation. Our work and the work of others suggest that SCF exists in two different states, an active and an inactive state (see Chapter 4). In the latter, SCF is sequestered by the regulator CAND1, which occludes Skp1 and the F-box protein from binding. A key question lies in how neddylation and CAND1 release are intertwined—how they both occur and how are they regulated. Does neddylation stimulate CAND1 release or vice versa? How are these processes tied into Skp1 and the F-box protein? Does F-box protein binding to cargo stimulate neddylation and/or CAND1 release? These questions are difficult to address given the heterogeneous nature of SCF *in vivo*, and a highly purified *in vitro* system will be essential in establishing solid conclusions.

In the alternative active state, once neddylation of Cul1 and binding of Skp1 occurs, SCF resides in a form able to ubiquitinate substrates and/or F-box proteins. Do SCF and CSN communicate to deneddylate Cul1 and revert to the inactive state? Is CSN activity regulated? Are other factors involved? Similar to the above scenario, given the

heterogeneous nature of SCF *in vivo*, a purified system would be essential to address these questions.

CSN and The Regulation of Neddylated Proteins

To date, eight neddylated proteins have been identified (Cul1-5, VHL, p53, and MDM2) (Hori et al. 1999; Stickle et al. 2004; Xirodimas et al. 2004). Moreover, deletion of CSN results in a stockpile of neddylated proteins in *S. pombe* (Lyapina et al. 2001), suggesting that Cullins are not the only neddylated proteins *in vivo*. One question that remains is how many other proteins are neddylated? Are these regulated by CSN? How does neddylation change their activity? Despite the potential complexity of the answer to this question, the route to the answer is ostensibly simple. Purification of an affinity tagged Nedd8 from CSN depleted/deleted cells will isolate any conjugates, and these conjugates can be identified by mass spectrometry.

CSN and The Molecular Basis For Its Genetic Phenotypes

Although we have established that CSN-dependent deneddylation is essential to rescue *Drosophila* Csn5-null, the molecular basis behind this phenotype is still unknown. This is just one example of the many organisms that display complex phenotypes when CSN is deleted: what is the molecular basis of these phenotypes? Recent data from multiple groups suggest the deneddylation of Cullins underlie the turnover of key proteins involved in the development of these organisms (reviewed in Cope and Deshaies 2003). However there are still questions that remain unaddressed. What SCF substrates

are involved in these processes? What substrate adaptors facilitate the degradation of these substrates?

Investigating the molecular basis of these phenotypes is key to advancing the field.

Other CSN Activities

Although we have established that deneddylation is a main activity of CSN, other activities have been identified. For example, CSN has been shown to associate with the de-ubiquitinating enzyme Ubp12 in *S. pombe* (Zhou et al. 2003). This enzyme counteracts spurious adaptor protein ubiquitination and is dependent upon CSN (Wee et al. 2005). Another example is that CSN is associated tightly with Inositol Triphosphate 5/6 Kinase (Wilson et al. 2001; Sun et al. 2002), Casein Kinase 2, and Protein Kinase D (Uhle et al. 2003). These kinases somehow regulate p53 through phosphorylation (Bech-Otschir et al. 2001), although the nature of CSN participation in this process is unknown. Another unknown function to CSN is exemplified in the work done by Carr and Colleagues in *S. pombe* (Liu et al. 2003). Deletion of CSN1 and CSN2 results in the accumulation of a Ribonucleotide Reductase inhibitor Spd1. Spd1 is ubiquitinated in a Cul4-dependent manner; however the turnover of Spd1 is not deneddylation dependent, as it only accumulates in *csn1Δ* and *csn2Δ*. What is this mysterious activity of CSN? Finally, are there other activities associated with CSN that we are unaware of? Does CSN act as an alternative lid to the protease, as recently proposed (Li and Deng 2003)? These are questions that will require a good deal of effort to address.

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