

# COP9 Signalosome: A Multifunctional Regulator of SCF and Other Cullin-Based Ubiquitin Ligases

## Review

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**COP9 Signalosome (CSN) is a fascinating protein complex whose biochemical and physiological functions are only beginning to be understood. It is conserved throughout eukaryotes and is critical to the proper development of all multicellular organisms in which its function has been explored. Recent work suggests that CSN plays a key role in sustaining the activity of SCF and other cullin-based ubiquitin ligases, which may account for its essential roles in development. Here, we summarize what is known about CSN, and discuss hypotheses for how CSN promotes the activity of SCF ubiquitin ligases.**

The COP9 signalosome (CSN) is a multifunctional protein complex comprised of eight subunits, Csn1–Csn8 (Wei and Deng, 1999). All or most of these subunits are encoded in the genomes of humans, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the mustard weed *Arabidopsis thaliana*, the fission yeast *Schizosaccharomyces pombe*, and the budding yeast *Saccharomyces cerevisiae*. Although little was known about the biochemical function of CSN until recently, a striking 1:1 correspondence between the subunits of CSN and those of the lid subcomplex of the 19S regulatory particle of the 26S proteasome has encouraged proposals that CSN participates in ubiquitin-dependent proteolysis (Wei and Deng, 1999). Subunits of CSN also bear homology to several subunits of eukaryotic initiation factor 3 (eIF3), but the overall similarity is less than that observed with the lid. In this article, we review recent advances in our understanding of the biochemical and genetic functions of CSN. We propose that the multiple biochemical functions of CSN are focused upon the dynamic regulation of a cluster of ubiquitin ligases that share a cullin/RING-H2 core catalytic module. We suggest that the bewildering array of phenotypes observed in CSN mutants and functions ascribed to individual CSN subunits are testimony to the pervasive role of cullin-based ligases in cell physiology and organismal development.

### Biological Functions of CSN

#### **CSN Regulates Photomorphogenesis in Plants**

Mutations in individual CSN components are manifest as defects in signal transduction, transcription, cell cycle progression, and development. Overall, our best understanding of the physiological role of CSN derives from genetic studies of its role in photomorphogenesis in the plant *A. thaliana* (Osterlund et al., 1999). Photomorpho-

genesis refers to the broad spectrum of physiological and developmental changes that occur when a seedling is exposed to light. This developmental program is controlled in part by proteins that direct the synthesis of transcripts expressed only in illuminated plants. One key regulator is the DNA binding protein HY5. In the light, HY5 accumulates and induces the transcription of a suite of genes. In contrast, plants grown in the dark have trace amounts of HY5, and its target genes are quiescent. The negative regulation of HY5 in the dark requires *COP1*. In wild-type cells, HY5 is degraded rapidly by the proteasome pathway in the dark but is stable in the light, whereas in *cop1* mutants HY5 is constitutively stable (Osterlund et al., 1999). *COP1* bears RING and WD-40 domains, both of which are found in ubiquitin ligases. Thus, it was suggested that in darkness *COP1* binds and ubiquitinates HY5, thereby targeting it for degradation by the proteasome (Osterlund et al., 2000). However, the exact mechanism by which *COP1* promotes HY5 degradation has yet to be unraveled.

Insight into how light “short circuits” the *COP1*-dependent turnover of HY5 was provided by molecular analysis of the *COP1* protein (Osterlund et al., 1999). In the dark, *COP1* resides in the nucleus, where it presumably metes out a sentence of rapid degradation upon HY5. In the light however, *COP1* localizes to the cytoplasm, and presumably cannot gain access to nuclear HY5. How does *COP1* and its intracellular peregrinations relate to CSN? Deployment of *COP1* to the nucleus in the dark requires CSN: in cells lacking any one of the eight subunits of CSN, *COP1* takes up permanent residence in the cytoplasm regardless of whether the sun shines or not. Consequently, HY5 evades capture and accumulates to high levels in these mutants even in darkness, thereby accounting for their constitutive photomorphogenic phenotype. Despite considerable progress in the molecular characterization of photomorphogenesis, the biochemical mechanism by which CSN controls the localization of *COP1* remains unknown.

#### **Other Developmental Roles of CSN:**

#### **Patterning, Differentiation, Gametogenesis, and Organization of the Embryonic Cytoskeleton**

In addition to its role in the regulation of the nucleocytoplasmic distribution of plant *COP1*, CSN has also been linked to numerous processes in other organisms. In *Drosophila*, mutations in *Csn4* and *Csn5* subunits result in lethality during larval development and defects in oogenesis. More specifically, loss of *Csn5* results in activation of a DNA double-strand-break-dependent meiotic checkpoint that blocks developmental patterning of oocytes (Doronkin et al., 2002; Oron et al., 2002), and failure of photoreceptor neurons to differentiate (Suh et al., 2002). Although the precise mechanism underlying these phenotypes has yet to emerge, failure to degrade SCF substrates including the cell cycle regulator Cyclin E may play a contributory role (Doronkin et al., 2003). In *C. elegans*, *Csn5* was recovered in a two-hybrid screen for proteins that interact with the GLH RNA helicases (Smith et al., 2002). RNAi repression of *Csn5* results in sterile worms, a phenotype that recapitulates

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RNAi-mediated reduction of two GLH subunits. More recently, RNAi of Csn5 and other CSN subunits was shown to block downregulation of the Mei-1 subunit of the microtubule-severing protein katanin, resulting in a range of abnormalities that presumably arise from unscheduled disruption of microtubules, including defects in nuclear positioning, anaphase, and cytokinesis (Pintard et al., 2003).

#### **CSN in Budding and Fission Yeasts**

Both budding and fission yeasts contain multisubunit CSN complexes. Although subunits of the fission yeast CSN are readily detected by homology searches (Mundt et al., 1999), the budding yeast complex is quite divergent and was discovered only very recently (Wee et al., 2002; Maytal-Kivity et al., 2003). It is interesting to note that although loss of CSN in all multicellular organisms examined to date results in drastic developmental defects that culminate in death, deletion of CSN subunits in fission or budding yeast is not lethal. Possible explanations for this discrepancy are discussed later on. However, the fission yeast Csn1 and Csn2 subunits are required for proper replication of DNA and for normal resistance to DNA damaging agents (Mundt et al., 1999). Interestingly, *suc22+*, which encodes the small subunit of ribonucleotide reductase (RNR), was identified as a multicopy suppressor of this phenotype (Liu et al., 2003). RNR catalyzes the production of deoxyribonucleotides for DNA synthesis. In normal cells, Spd1 negatively regulates Suc22 in some manner. Upon DNA damage or during S phase, the repressive effect of Spd1 is counteracted by its rapid degradation, which presumably frees RNR to produce dNTPs. However, in *csn1Δ* and *csn2Δ* mutants, Spd1 is not degraded and accumulates to high levels that repress Suc22 function, possibly by preventing redistribution of Suc22 from the nucleus to the cytoplasm. The mechanism by which Csn1 and Csn2 act is not known, but Spd1 turnover also requires Cul4, and Cul4 coimmunoprecipitates with Csn1, suggesting that Csn1-Csn2 directly activate Cul4.

#### **Miscellaneous Functions Linked to Individual CSN Subunits**

In addition to the myriad phenotypes seen in CSN mutants, CSN subunits have also been linked through protein-protein interactions to a broad range of cellular processes (Schwechheimer and Deng, 2001). Notably, Csn5/Jab1 has been uncovered numerous times in 2-hybrid screens, but it should be borne in mind that Csn5 binds Gal4 and thus may turn up as a false positive in two-hybrid assays (Nordgard et al., 2001). Csn5/Jab1 originally surfaced in a two-hybrid screen for proteins that bind the activation domain of c-Jun (Claret et al., 1996). Cotransfection of *JAB1* cDNA with reporter plasmids that contain an AP-1 promoter element responsive to c-Jun activity revealed that overexpression of Csn5/Jab1 boosts the transactivation capacity of c-Jun. Csn5/Jab1 was later uncovered in a screen for proteins that bind the CDK inhibitor p27 (Tomoda et al., 1999). Upon coexpression with p27 in transient transfection experiments, Csn5/Jab1 and a subset of other CSN subunits (Tomoda et al., 2002) promote export of p27 from the nucleus to the cytoplasm and its subsequent degradation by the proteasome. Csn5/Jab1 also took the bait in a screen for proteins that bind the cytokine known as macrophage migration inhibitory factor (MIF) (Klee-

mann et al., 2000). This factor is thought to play a key role in governing inflammation by counterbalancing the effects of glucocorticoids. Kleeman et al. argued that MIF exerts its biological functions by attenuating Csn5/Jab1 activity (Kleemann et al., 2000). Taken together these results and others implicate Csn5/Jab1 in gene transcription/oncogenesis, cell cycle control, immune response, and DNA metabolism. It remains to be seen whether these various functions involve the intact CSN or the pool of Csn5 that exists in low molecular weight complexes, and whether they require the isopeptidase active site of Csn5 (see below).

#### **The COP9 Signalosome Is a Nedd8 Isopeptidase CSN Binds SCF and Cleaves Nedd8-Cul1 Conjugates**

Recently, several reports have begun to shed light on the biochemical functions of the CSN. A key clue to unraveling the biochemistry of CSN was the observation that CSN copurifies with the Cul1 subunit of SCF ubiquitin ligase isolated from animal cells (Lyapina et al., 2001). This interaction was subsequently reproduced in *Arabidopsis* and CSN was shown to contribute to auxin perception, which is dependent upon SCF<sup>TIR1</sup> (Schwechheimer et al., 2001). This latter result provided important support for the idea that CSN is a novel positive regulator of SCF ubiquitin ligases. SCF is a multisubunit complex composed of 4 polypeptides: Cul1, Hrt1 (also known as Roc1 or Rbx1), Skp1, and one member of a large family of substrate binding proteins known as the F-box proteins (FBPs). Together, this complex of proteins belongs to a large class of enzymes known as E3 ubiquitin ligases (Deshaies 1999). These enzymes promote covalent attachment of ubiquitin to substrate proteins, which in turn are recognized and degraded by the 26S proteasome.

Further analysis of SCF-CSN interactions in fission yeast cells revealed that the degree of Nedd8 modification of the Cul1 subunit of SCF is perturbed in CSN-deficient mutants (Lyapina et al., 2001). Nedd8 (also known as Rub1) is highly related to ubiquitin, and like ubiquitin is covalently linked to other polypeptides by an isopeptide bond between the C terminus of Nedd8 and the  $\epsilon$ -amino group of a lysine residue within the target. Nedd8 is activated for attachment to substrates by a heterodimeric E1-like enzyme composed of Uba3 and Ula1 (also known as APP-BP1) subunits, and activated Nedd8 is then transferred to the Nedd8 conjugating enzyme Ubc12 (Lammer et al., 1998; Liakopoulos et al., 1998). Ubc12 directly transfers Nedd8 to its substrates, the only known ones being members of the cullin family of ubiquitin ligase subunits. All of the cullins appear to be modified on a single, conserved lysine residue. Although the Nedd8 attachment (i.e., neddylation) pathway is reminiscent of ubiquitination pathways, the attachment of Nedd8 does not appear to target proteins for degradation. Rather, neddylation of Cul1 enhances the ability of SCF to ubiquitinate proteins *in vitro* (Podust et al., 2000; Read et al., 2000; Wu et al., 2000). Normally, only a small fraction (~10%–40%) of *S. pombe* Cul1 is modified with Nedd8, but in CSN-deficient cells 100% of Cul1 is neddylated (Lyapina et al., 2001). Moreover, purified CSN can cleave Nedd8 from Cul1. Thus, both biochemical and genetic data indicate that CSN promotes cleavage of Nedd8 from Cul1.

### **A Novel Zinc Metalloprotease Motif in Csn5 Underlies Nedd8 Isopeptidase Activity of CSN**

Insight into the mechanism by which CSN promotes cleavage of Nedd8 conjugates came from bioinformatic analyses of CSN subunits, which uncovered a highly conserved submotif—EX<sub>n</sub>HS/THPX<sub>7</sub>SX<sub>2</sub>D—present in Csn5 and a subset of other proteins that contain the JAB1/MPN/Mov34 domain (Cope et al., 2002). By analogy to zinc metalloproteases, we speculated that the His and Asp residues of this motif comprise a set of ligands that coordinate a catalytic zinc ion. Indeed, mutations in these conserved residues inactivate Csn5-dependent deneddylation of Cul1 in fission yeast, and Nedd8 isopeptidase activity of purified pig CSN is sensitive to metal chelators. Based on these data we dubbed the conserved submotif “JAMM,” for JAB1/Mpn domain metalloenzyme. Strikingly, a *Drosophila csn5* cDNA bearing a JAMM mutation fails to restore viability and photoreceptor neuron differentiation to *csn5Δ* larvae, suggesting that the isopeptidase active site of Csn5 underlies at least two key developmental functions of CSN.

Bound zinc ions can play either a structural or catalytic role in proteins. Striking support for the catalytic role suggested for the JAMM motif of Csn5 is provided by the three-dimensional crystal structure of a JAMM protein from *Archaeoglobulus fulgidis* (X. Ambroggio, D. Rees, and R.J.D., unpublished data). As predicted by Cope et al. (2002), the conserved residues of the JAMM motif bind a zinc ion in a configuration reminiscent of that seen in well-characterized metalloproteases. Although the structural biology, genetic analysis, and chemical inhibition studies together argue convincingly that the JAMM motif of Csn5 comprises a Nedd8 isopeptidase active site, recombinant Csn5 subunit has not been shown to possess catalytic activity. It is possible that Csn5 must assemble with other subunits that either assist in substrate recognition (Lyapina et al., 2001; Yang et al., 2002) or relieve inhibitory constraints within the Csn5 polypeptide.

### **Other Biochemical Functions of CSN**

Besides its role as a deneddylating enzyme, several other functions of CSN and its component polypeptides have come to light recently. These new data support the view that CSN is a multifunctional regulator of cullin-based ubiquitin ligases.

#### **CSN Activates Deubiquitination by Ubp12**

Wolf and colleagues noted that the ability of CSN to inhibit ubiquitin ligase activity of Cul3 is more potent than its ability to promote Cul3 deneddylation, and in fact the deneddylation and Cul3 inhibitory activities of CSN can be uncoupled (Zhou et al., 2003). This observation led them to discover that CSN binds a conventional deubiquitinating enzyme of the cysteine protease family known as Ubp12. Groisman et al. (2003) also reported that CSN recruits a deubiquitinating activity that counteracts Cul4, but did not identify the enzyme. As is the case for Csn3–Csn7, deletion of the fission yeast gene that encodes Ubp12 has no effect on the growth rate of cells. However, the FBP Pop1 is modestly destabilized in both *csn5Δ* and *ubp12Δ* mutants. Based on these data, Zhou et al. (2003) proposed that Csn5 and Ubp12 work together to inactivate cullin complexes during cy-

cles of operation. Specifically, they propose that once a substrate is degraded, Csn5 and CSN-associated Ubp12 counteract or prevent inappropriate ubiquitin ligase activity. Ubp12 is suggested to rescue from degradation spuriously ubiquitinated ligase components so that they can focus their activity on legitimate substrates. Spurious ubiquitination of SCF subunits could be a problem if indeed Cdc34 disgorges its ubiquitin cargo only following its dissociation from SCF (Deffenbaugh et al., 2003). Meanwhile, Csn5 is envisioned to extinguish cullin-associated ligase activity. This hypothesis does not explain why CSN-deficient cells—which should lack both the Csn5 and Ubp12 inhibitory functions—are healthy. One would naively predict that the failure to counteract inadvertent ubiquitination of bystander proteins (*ubp12Δ*) coupled with the failure to downregulate cullin activity by deneddylation (*csn5Δ*) would lead to an unhealthy situation in which subunits of SCF complexes are inappropriately degraded. Possible explanations for why CSN is not essential in yeasts are considered later in this review.

#### **CSN Subcomplexes May Regulate Cullin-Based Ligases**

Although we have concentrated so far on discussing the properties of CSN polypeptides in the context of the hetero-octomeric CSN, it is now becoming clear that this is an oversimplification. Multiple CSN polypeptides can be found in complexes of molecular mass lower than the intact 500 kDa CSN (Mundt et al., 2002; Oron et al., 2002). Although the functional significance of these assemblages has remained obscure, Kato and colleagues recently proposed that a subcomplex comprising Csn4–Csn8 participates in p27 regulation (Tomoda et al., 2002). However, the best evidence for functional specialization of individual CSN subunits is provided by recent work showing that Csn1 and Csn2—but not Csn3, Csn4, and Csn5—are required for proper regulation of RNR (Liu et al., 2003), as was summarized earlier in this review. These observations provide strong support for the idea that Csn1 and Csn2 can regulate cullin function in three distinct ways: they can promote deneddylation as part of the intact CSN complex, they can indirectly counteract spurious ubiquitination by recruiting Ubp12, and they can stimulate Cul4 function by a distinct mechanism that does not require the other subunits of CSN. It remains to be seen whether Csn1 and Csn2 carry out the latter activity as part of CSN or as a separate complex.

#### **Role of CSN as a Regulator of Protein Kinases and the Proteasome**

Besides its role in promoting cullin activity, CSN has been proposed to serve as a regulator of protein kinase function. This proposal emanates from the observation that CSN copurifies with lipid and protein kinase activities. The first of these was identified as inositol trisphosphate 5/6 kinase (Wilson et al., 2001; Sun et al., 2002), and more recently casein kinase 2 and protein kinase D have been reported to associate with CSN (Uhle et al., 2003). Binding to CSN reduces inositol trisphosphate 5/6 kinase activity toward a protein substrate by 4-fold. However, until loss-of-function studies examine the effect of CSN on these kinases or vice versa, it remains equally plausible that these protein kinases either regulate CSN or are regulated by CSN. A final role that has

been suggested for CSN as a substitute for the analogous lid subcomplex of the 26S proteasome. This idea is based on both the similarity between the lid and the CSN, and on physical association between proteasome and CSN components (Deshaies and Meyerowitz, 2000; Schwechheimer and Deng, 2001; Peng et al., 2003). However, there are no strong functional data to support this proposition and the discovery of distinct deneddylation and deubiquitination activities for the CSN and lid, respectively, cast doubt on this hypothesis.

#### What Is CSN Doing, and When Is It Doing It?

We are now beginning to get a good grasp on the biochemical activities of CSN, but it remains mysterious how these biochemical activities are connected to cell physiology. Questions abound. For example, what exactly happens to SCF when the various activities of CSN are extinguished, and why? Are all SCF and cullin complexes equally dependent upon CSN, and if not, which ligases are most critically dependent upon CSN activity? Does CSN act constitutively upon its ligase substrates, or can the activity of CSN be influenced by regulatory cues? In this final section of the review, we take up these questions by discussing hypotheses for CSN regulation and function.

#### Is CSN Regulated?

The available evidence indicates that CSN can bind, deneddylate, and modulate the activities of Cul1, Cul3, and Cul4-based ubiquitin ligases (Lyapina et al., 2001; Groisman et al., 2003; Pintard et al., 2003; Zhou et al., 2003). Given that the human cullins may comprise upwards of 100 or more different ligase complexes that contain distinct substrate binding domains (e.g., F-box, SOCS box, and BTB domain proteins), CSN could potentially influence the degradation of hundreds of different proteins. Indeed, CSN has already been physically and/or functionally linked to multiple SCF ubiquitin ligase complexes, including SCF<sup>Cdc4</sup> (Cope et al., 2002; Doronkin et al., 2003), SCF<sup>UFO</sup> (Wang et al., 2003), SCF<sup>COI1</sup> (Feng et al., 2003), and SCF<sup>TIR1</sup> (Schwechheimer et al., 2001). This raises a key issue: can CSN independently regulate two different ligases at the same time?

On one extreme, CSN can be envisioned to act constitutively and without prejudice on the entire pool of cullins. Indeed, ablation of CSN deneddylating activity results in the entire pool of Cul1 becoming neddylated (Lyapina et al., 2001). On the other extreme, the functional interaction of CSN with individual ligases may be precisely controlled by intracellular or extracellular signals. Three observations support (but do not prove) the idea that the action of CSN is regulated in some manner. First, Groisman et al. (2003) showed that two distinct Cul4 complexes associate differentially with CSN upon UV irradiation of cells, and consequently the Cul4 subunit is differentially deneddylated. Second, sunlight blocks the ability of CSN to promote the COP1-dependent repression of HY5 (Osterlund et al., 1999). Third, neddylated Cul1 can be coimmunoprecipitated with CSN (Lyapina et al., 2001), suggesting that deneddylation within the enzyme-substrate complex can somehow be inhibited.

The examples cited above raise a second important question: if indeed the action of CSN upon cullin ligases

is regulated, is CSN itself controlled, or is deneddylation (and/or its other functions) regulated at the level of substrate? In support of substrate-level control, there is little evidence that global cullin neddylation state changes in response to intra- or extra-cellular signaling save for an increase in Nedd8-modified Cul1 in plants exposed to sunlight (Wang et al., 2003). There are multiple ways that the action of CSN upon a specific SCF complex might be controlled. For example, the presence of substrate bound to SCF could conceivably inhibit recruitment of CSN or promote recruitment of Ubp12, which could explain why only neddylated Cul1 molecules coprecipitate with the SCF  $\beta$ -TrCP substrate I $\kappa$ B $\alpha$  (Read et al., 2000). Alternatively, a regulated covalent modification or protein-protein interaction might govern access of CSN to different ligase complexes—the same mechanism by which a single SCF complex can differentially control the turnover of diverse substrates. In the case of photomorphogenesis, sunlight activates binding of cryptochrome to COP1 (Wang et al., 2001), which could conceivably occlude COP1 from interacting with CSN. Regardless of the exact mechanism(s) at play, it is clear that CSN could simultaneously modulate the activity of many different ubiquitin ligases in response to many different signals. Clearly, this is a topic that demands further study.

#### CSN Promotes Activity of Cullin-Based Ligases

A comparison of biochemical and genetic data leads to some confusion about whether CSN is a positive or negative regulator of cullin-based ubiquitin ligases. In vitro, CSN promotes both deneddylation of cullins (Lyapina et al., 2001) and opposes their ubiquitin incorporation activity by virtue of Ubp12 (Zhou et al., 2003). Since neddylation of Cul1 stimulates its ubiquitin ligase activity by promoting recruitment of E2 enzyme in a reconstituted system (Kawakami et al., 2001), CSN should behave as a negative regulator of SCF, and indeed has been shown to act as such in vitro in either physiological (Yang et al., 2002) or substrate-independent (Lyapina et al., 2001; Groisman et al., 2003; Zhou et al., 2003) ubiquitin ligase assays.

By contrast, multiple genetic studies support the opposing view. If CSN antagonizes cullin function, then reduction of CSN function should enhance cullin activity or suppress loss-of-function mutations in cullin-based ligases. Exactly the opposite is seen, however. Reduction or loss-of-function mutations in CSN subunits either mimic or exacerbate the effects of mutations that compromise the SCF<sup>Cdc4</sup> (Cope et al., 2002; Doronkin et al., 2003), SCF<sup>UFO</sup> (Wang et al., 2003), SCF<sup>COI1</sup> (Feng et al., 2003), DDB2.com, CSA.com (Groisman et al., 2003) and SCF<sup>TIR1</sup> (Schwechheimer et al., 2001) complexes, as well as the Cul3- and Cul4-based complexes that promote Mei-1/katanin (Pintard et al., 2003) and Spd1 (Liu et al., 2003) turnover, respectively. Although the genetic studies definitively establish CSN as a positive regulator of cullin function, most have not determined which activity of CSN is responsible. However, point mutations within the JAMM motif of Csn5 exacerbate temperature sensitive alleles of SCF<sup>Cdc4</sup> subunits in budding yeast (Cope et al., 2002), suggesting that the CSN's Nedd8 isopeptidase acts positively on SCF.

### **Does CSN Promote Cycles of SCF Assembly?**

The divergent genetic and biochemical results indicate that ubiquitin ligase activity of SCF is not strictly dependent upon CSN, but that CSN is required to sustain SCF activity over time in the context of a cell. Why might this be? We propose that CSN controls cycles of SCF assembly/disassembly that occur *in vivo*, but do not come into play in standard *in vitro* reconstitution systems. For the sake of simplicity we focus our discussion on SCF and the deneddylase activity of CSN, but it should be kept in mind that other activities of CSN contribute to the regulation of SCF and the other cullin-based ligases (Liu et al., 2003; Zhou et al., 2003).

In theory, multiple aspects of SCF function might be cyclical and stimulated by CSN, including: recruitment of ubiquitin-charged E2 or repetitive charging of SCF-bound E2, recruitment of a naïve substrate molecule upon dissociation of a ubiquitinated substrate, or formation of active SCF complexes and their disassembly into cullin/Hrt1 and F-box/Skp1 subcomplexes. The first two possibilities seem unlikely based on *in vitro* data: SCF<sup>Cdc4</sup> can rapidly promote attachment of up to 20 ubiquitins on a single lysine of Sic1 (Petroski and Deshaies, 2003) and SCF <sup>$\beta$ -TrCP</sup> can rapidly ubiquitinate a 100-fold molar excess of phosphorylated  $\beta$ -catenin peptide (Wu et al., 2003) in the absence of CSN, arguing that cycles of neddylation/deneddylation are not required to sustain multiple rounds of recruitment of charged E2 or substrate (for a different viewpoint, see Pintard et al., 2003). However, it remains possible that either of these processes is antagonized by a factor that is absent from the *in vitro* systems and that is counteracted by CSN *in vivo*.

SCF complexes presumably exist in a dynamic equilibrium *in vivo*, because multiple FBPs compete for access to a common catalytic core composed of Cul1 and Hrt1. The notion that proper regulation of this dynamic process is important to sustain SCF function has gained force with the recent characterization of the extremely interesting Tip120A/CAND1 protein. CAND1 was found by four groups as a Cul1-interacting protein (Liu et al., 2002; Zheng et al., 2002; Hwang et al., 2003; Min et al., 2003; Oshikawa et al., 2003). Tight binding of CAND1 requires interaction with both the N- and C-terminal domains of Cul1, and is antagonized by either Skp1 (which competes with CAND1 for binding to the N-terminal domain of Cul1) or neddylation of Cul1's C-terminal domain. Accordingly, excess CAND1 inhibits SCF ubiquitin ligase activity *in vitro* or *in vivo* by sequestering unmodified Cul1 away from Skp1-FBP complexes, whereas depletion of CAND1 increases assembly of Skp1 with Cul1.

Although it is evident from its biochemical properties that CAND1 may underlie a cycle of SCF assembly and disassembly, it is unclear what provokes the dissociation of CAND1 from Cul1-Hrt1 to initiate SCF assembly, or the dissociation of Skp1 from Cul1-Hrt1 to initiate disassembly. An attractive idea is that the Nedd8 conjugating enzyme Ubc12 and CSN Nedd8 isopeptidase play key roles in controlling the CAND1 cycle. A model that illustrates this view is shown in Figure 1. In the remainder of the review we will use this model as a framework for thinking about the substantial body of literature on CSN, Nedd8, and CAND1.

The model shown in Figure 1 predicts that inhibition of either Nedd8 attachment, Nedd8 isopeptidase, or

CAND1 activities should lead to a reduction of SCF activity (Figure 2). This has indeed been shown in all three cases (Table 1). However, two classes of observations are not so readily explained by this model in its simplest form. First, several lines of evidence suggest that inhibition of neddylation has more severe consequences than inhibition of CSN (see Table 1 for references). This is seen, for example, in fission yeast where the neddylation pathway is essential but CSN-dependent deneddylation is not. In addition, a hamster cell line that has a mutation in Nedd8 E1 enzyme is thermosensitive for growth, whereas a normal human diploid fibroblast depleted of Csn5 by siRNA grows normally. A possible explanation for the more pervasive and stringent requirement for neddylation enzymes arises from thinking through the implications of Figure 2. In the absence of neddylation, there should be no SCF activity because eventually most or all of the Cul1-Hrt1 would become sequestered into inactive complexes with CAND1. However, in the absence of CSN, Cul1-Hrt1 could still dynamically recycle due to ongoing FBP synthesis and turnover. Indeed, different SCF complexes may exhibit differential requirements for CSN depending upon various factors, including the rate of synthesis and intrinsic stability of their FBP subunit. Another possible explanation for why loss of CSN has less impact than loss of neddylation enzymes is that CSN is required only when the cell needs to rapidly re-equilibrate its pool of cullin-based ligases in response to a stimulus (see next section for more detail).

The second observation that bears consideration is that in budding yeast, neither CSN nor the enzymes for Nedd8 attachment are essential for growth (Table 1). The explanation for this perplexing result may be simple: the budding yeast genome does not encode a CAND1-like molecule, and thus there is no need for Nedd8 attachment enzymes to liberate a pool of Cul1-Hrt1 for assembly of SCF complexes. An additional possible contributing factor is that in budding yeast, FBPs are degraded extremely rapidly ( $t_{1/2}$   $\sim$  5–30 min) by a cullin-dependent “autoubiquitination” mechanism (Zhou and Howley 1998; Galan and Peter 1999). The budding yeast repertoire of SCF complexes may thus be controlled simply by adjusting the relative rates of synthesis and turnover of individual FBPs (Deshaies 1999). By contrast, FBP turnover in animal cells appears to be more complicated. First, some mammalian FBPs—though unstable—are degraded with slower kinetics ( $t_{1/2}$   $\sim$  120 min for  $\beta$ -TrCP) (Davis et al., 2002). Second, other FBPs (e.g., Tome-1 and Emi1) are targeted for degradation *in trans* by other ubiquitin ligases in a regulated manner, such that they are only degraded rapidly during specific phases of the cell cycle (Ayad et al., 2003; Margottin-Goguet et al., 2003). Taken together, these observations imply that in animal cells, FBP degradation may not be an adequate mechanism to ensure rapid re-equilibration of the entire pool of SCF ligases following a stimulus.

### **CSN: A Key Player in Mediating the Dialog of Intercellular Communication?**

In thinking about models of CSN function, a striking observation that may bear greater attention is that many of the CSN-dependent processes that have come to light involve pathways that employ ubiquitin-dependent proteolysis to control the production or interpretation of an environmental signal. Both ubiquitin-dependent

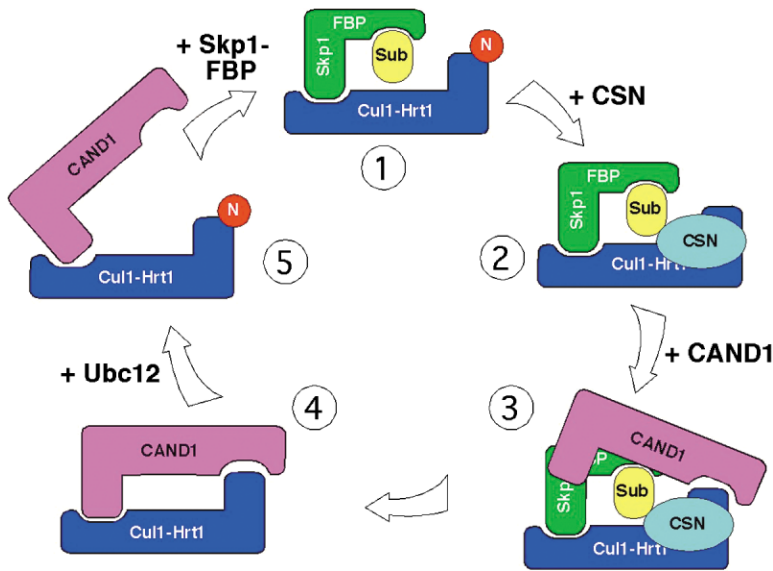


Figure 1. A Hypothetical Model for Nedd8-Driven Cycles of SCF Assembly and Disassembly

(1) Active SCF complex bound to substrate recruits CSN, which cleaves Nedd8 from Cul1 (2). This enables CAND1 to bind Cul1 (3) and eventually strip away Skp1-FBP, thereby sequestering Cul1 in an inactive state (4). Neddylation of Cul1 by Ubc12 weakens the grip of CAND1 on Cul1-Hrt1, and an incoming Skp1-FBP heterodimer delivers the coup de grace, displacing CAND1 from Cul1 to yield an active SCF complex (1). Although the model posits that the actions of CSN and Ubc12 initiate state transitions with CAND1 playing a subservient role, it is equally possible that the opposite is true, or that CSN-CAND1 and Ubc12-CAND1 act interdependently. Finally, it has been suggested that other factors might be required to enable Ubc12 to gain access to CAND1-bound Cul1. This model is similar in some respects but different in others to assembly models that have been discussed elsewhere (Liu et al., 2002; Hwang et al., 2003; Oshikawa et al., 2003).

proteolysis and CSN are required for correct perception of light versus darkness (Osterlund et al., 2000), auxin (Gray et al., 1999, 2001; Schwechheimer et al., 2001), jasmonate (Feng et al., 2003), and possibly pathogens (Azevedo et al., 2002). Moreover in animals, the best-described function of CSN involves cell-cell communication (Suh et al., 2002). *Drosophila* photoreceptor neurons R1–R6 project to the lamina, where they form synapses. Guidance of R1–R6 axons to their proper targets requires the presence of glial cells in the lamina. Remarkably, mutants that have reduced Csn5 activity in the R1–R6 photoreceptors exhibit a defect in glial cell migration, suggesting that CSN present in the growth cone is required to produce a signal that is then perceived as a guidance cue by migrating laminal glial cells. An essential function for CSN in the dialog that cells carry on with each other and with the environment would help to explain a striking conundrum: although mutants

of *Drosophila*, *Arabidopsis*, and mouse that lack CSN subunits die during development, severe attenuation of Csn5 expression in untransformed human diploid fibroblasts does not block cell growth (Table 1). Although CSN no doubt plays a role in cell autonomous processes such as DNA repair (Groisman et al., 2003), it may be that CSN's control over the repertoire of SCF complexes is more crucial in cells that must rapidly modify their physiological processes in response to variable signals received from other cells or the environment.

### Concluding Remarks

The past three years have witnessed an explosive advance in our understanding of CSN and its biochemical functions. Despite the impressive progress, a tremendous amount remains to be discovered about what CSN's activities are, how they work, how they might be regulated, and how these activities impinge on the

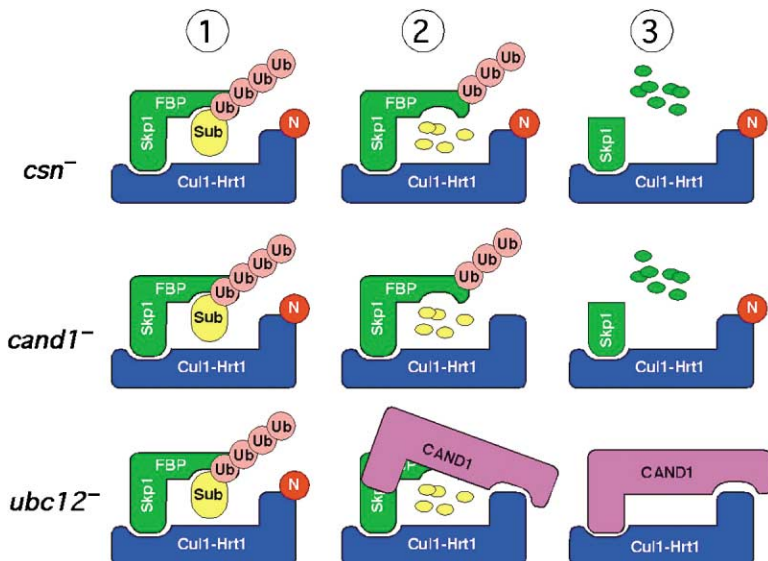


Figure 2. Predicted Consequences of Transiently Blocking Dynamic Cycles of SCF Assembly/Disassembly

CSN: Upon completion of substrate ubiquitination (1) and degradation (2), a cell depleted of CSN isopeptidase activity fails to cleave Nedd8 from Cul1, leaving Cul1 in a persistently active state and resulting in the eventual ubiquitination (2) and degradation (3) of the FBP. CAND1: Upon completion of substrate ubiquitination (1) and degradation (2), CSN cleaves Nedd8 from Cul1 (2). However, if CAND1 is inactivated, Skp1-FBP is not displaced from Cul1-Hrt1 and consequently the FBP is precociously ubiquitinated (2) and degraded (3). Ubc12: Upon completion of substrate ubiquitination (1) and degradation (2), CSN cleaves Nedd8 from Cul1 (2), enabling CAND1 to sequester Cul1-Hrt1 (2 and 3). However, upon inactivation of Ubc12, Cul1-Hrt1 complexes become irreversibly sequestered by CAND1.

Table 1. Properties of Mutants Deficient in Components of the Nedd8 cycle

Mutant or RNAi	Organism	Growth phenotype and impact on SCF/cullins	Ref.
<b>Nedd8 attachment</b>			
<i>uba3Δ, rub1Δ, ubc12Δ</i>	<i>S. cerevisiae</i>	viable, SCF active; synthetic lethal with <i>cdc34ts</i>	[1, 2]
<i>uba3Δ, ned8Δ, ubc12Δ</i>	<i>S. pombe</i>	inviable	[3]
<i>uba3Δ</i>	<i>M. musculus</i>	die in utero at periimplantation, SCF substrates accum.	[4]
<i>ts41</i> (in <i>ULA1</i> )	CHO cells	inviable; SCF and Cul2-VBC substrates accumulate	[5]
<i>ned8, ubc12, ula1</i> RNAi	<i>C. elegans</i>	embryonic arrest; larval epidermis differentiation defect	[6, 7]
<i>uba3ts (rfl1-1)</i>	<i>C. elegans</i>	inviable; substrate of Cul3 (Mei-1) accumulates	[7]
<i>nedd8</i>	<i>Drosophila</i>	inviable at 1st instar; SCF substrates accumulate	[8]
<i>axr1</i>	<i>Arabidopsis</i>	reduced auxin response; SCF <sup>TIR1</sup> substrate accumulates	[9]
<b>Nedd8 removal</b>			
<i>csn5Δ</i>	<i>S. cerevisiae</i>	viable; enhances <i>cdc34ts</i> phenotype	[10]
<i>csn5Δ</i>	<i>S. pombe</i>	viable, FBP partially destabilized	[11, 12]
<i>csn5</i> RNAi	<i>Arabidopsis</i>	inviable, SCF <sup>TIR1</sup> substrate accumulates	[13]
<i>csn5-null</i>	<i>Drosophila</i>	inviable at 3rd instar; SCF substrate accumulates	[14–16]
<i>csn4-null</i>	<i>Drosophila</i>	inviable at 3rd instar, albeit earlier than <i>csn5-null</i> .	[16]
<i>csn1-csn6</i> RNAi	<i>C. elegans</i>	inviable; substrate of Cul3 (Mei-1) accumulates	[17]
CSN5 siRNA	human cell line	viable; Cul4-dependent processes inhibited	[18]
CAND1 siRNA	human cell line	more Cul-Skp1 complex, less FBP (Skp2)	[19, 20]

1, Lammer et al., 1998; 2, Liakopoulos et al., 1998; 3, Osaka et al., 2000; 4, Tateishi et al., 2001; 5, Ohh et al., 2002; 6, Jones and Candido, 2000; 7, Kurz et al., 2002; 8, Ou et al., 2002; 9, Gray et al., 2001; 10, Cope et al., 2002; 11, Mundt et al., 2002; 12, Zhou et al., 2001; 13, Schwechheimer et al., 2001; 14, Suh et al., 2002; 15, Doronkin et al., 2003; 16, Oron et al., 2002; 17, Pintard et al., 2003; 18, Groisman et al., 2003; 19, Liu et al., 2002; 20, Zheng et al., 2002.

physiology of the organism. Key to answering these questions will be to examine in detail cells and organisms carrying point mutations that disable individual functions of CSN, and to use the resulting information to trace a path from molecular targets to organismal phenotype. With the tools at hand and the benefit of the insights gained over the past few years, the future holds great promise.

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