

Structural Basis for a Reciprocal Regulation between SCF and CSN

Radoslav I. Enchev,^{1,2} Daniel C. Scott,^{3,4} Paula C.A. da Fonseca,² Anne Schreiber,^{2,5} Julie K. Monda,³ Brenda A. Schulman,^{3,4,*} Matthias Peter,^{1,*} and Edward P. Morris^{2,*}

¹ETH-Zurich, Institute of Biochemistry, Department of Biology, Schafmattstr. 18, CH-8093 Zurich, Switzerland

²Division of Structural Biology, The Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, UK

³Department of Structural Biology

⁴Howard Hughes Medical Institute

St. Jude Children's Research Hospital, Memphis, TN 38105, USA

⁵Present address: ETH-Zurich, Institute of Biochemistry, Department of Biology, Schafmattstr. 18, CH-8093 Zurich, Switzerland

*Correspondence: brenda.schulman@stjude.org (B.A.S.), matthias.peter@bc.biol.ethz.ch (M.P.), edward.morris@icr.ac.uk (E.P.M.)

<http://dx.doi.org/10.1016/j.celrep.2012.08.019>

SUMMARY

Skp1-Cul1-Fbox (SCF) E3 ligases are activated by ligation to the ubiquitin-like protein Nedd8, which is reversed by the deneddylating Cop9 signalosome (CSN). However, CSN also promotes SCF substrate turnover through unknown mechanisms. Through biochemical and electron microscopy analyses, we determined molecular models of CSN complexes with SCF^{Skp2/Cks1} and SCF^{Fbw7} and found that CSN occludes both SCF functional sites—the catalytic Rbx1-Cul1 C-terminal domain and the substrate receptor. Indeed, CSN binding prevents SCF interactions with E2 enzymes and a ubiquitination substrate, and it inhibits SCF-catalyzed ubiquitin chain formation independent of deneddylation. Importantly, CSN prevents neddylation of the bound cullin, unless binding of a ubiquitination substrate triggers SCF dissociation and neddylation. Taken together, the results provide a model for how reciprocal regulation sensitizes CSN to the SCF assembly state and inhibits a catalytically competent SCF until a ubiquitination substrate drives its own degradation by displacing CSN, thereby promoting cullin neddylation and substrate ubiquitination.

INTRODUCTION

Cullin-RING ligases (CRLs) constitute the largest family of E3 ubiquitin ligases (Deshaies and Joazeiro, 2009). The modular CRL architecture explains their pervasive but highly specific functions. As exemplified by the archetypical Skp1-Cullin1-F-box (SCF) complexes, CRLs are nucleated by one of seven structurally elongated cullin protein scaffolds. The conserved cullin C-terminal domain assembles the CRL catalytic core by binding a RING-finger protein, typically Rbx1, which in turn promotes ubiquitin transfer from an associated E2 enzyme. The distal N-terminal end of the cullin binds a substrate receptor

(SR) module, which recruits the ubiquitination target. The modular and variable SRs allow recognition of numerous substrates by the same CRL catalytic core.

Human SCF complexes use one of more than 60 SRs, which bind Skp1 through their F-box domain. Skp1 bridges the interaction of Cul1 and the SR, which in turn recruits specific ubiquitination substrates via a distinctive degron motif (Duda et al., 2011). As examples, Fbw7's WD-40 domain recognizes the phosphorylated form of the cell-cycle regulator CyclinE (Hao et al., 2007), while Skp2 uses a leucine-rich repeat domain together with the coreceptor Cks1 to recruit a phosphorylated form of the cyclin-dependent kinase (Cdk) inhibitor p27 (Hao et al., 2005).

CRL catalytic activity is also controlled by covalent attachment of the ubiquitin-like protein Nedd8 to the cullin's winged-helix B (WHB) domain. Similar to ubiquitination, neddylation is mediated by an enzymatic cascade, including the Nedd8-conjugating E2 enzyme Ubc12, which is activated by the RING domain of Rbx1 with stimulation by Dcn1 (Kurz et al., 2005; Scott et al., 2011). Non-neddylated cullin-Rbx1 complexes can bind an inhibitor, CAND1, which prevents neddylation and competes with SR association (Goldenberg et al., 2004). Neddylation favors a conformational rearrangement of the cullin C-terminal domain and Rbx1, which prevents CAND1 binding and enhances CRL-mediated ubiquitination activity (Duda et al., 2008; Saha and Deshaies, 2008; Yamoah et al., 2008).

Cullins are deneddylated by the Cop9 signalosome (CSN). The CSN comprises eight different subunits, Csn1 through Csn8, named in order of descending molecular weight. Together, they unleash the zinc-metalloprotease activity of Csn5 by an unknown mechanism (Sharon et al., 2009). The active site of Csn5 is located within its N-terminal MPN domain (Cope et al., 2002; Lyapina et al., 2001) and most probably functions similarly to the thermolysin-like mechanism described for the homologous deubiquitinating enzyme AMSH-LP (Sato et al., 2008). Csn6 also comprises an MPN domain, which, however, lacks the conserved zinc-coordinating residues and is thus thought to serve a scaffolding function. The remaining six subunits are characterized by PCI domains, comprising a C-terminal winged helix preceded by a bundle of bihelical repeats (Dessau et al., 2008; Scheel and Hofmann, 2005). The PCI domains of Csn1,

Csn2, Csn3, and Csn4 are preceded by long N-terminal extensions, predicted to contain further helical repeats (Enchev et al., 2010; Pick et al., 2009). There is a striking similarity between the CSN subunit composition and that of the lid subcomplex of the 26S proteasome, which also comprises two MPN- and six PCI-domain-containing subunits with 1:1 sequence correspondence (Enchev et al., 2010; Pick et al., 2009).

CAND1 has been implicated in exchange of SRs upon CSN-mediated SCF deneddylation (Schmidt et al., 2009). Thus, SCF regulation has been thought of as a cycle of assembly with SRs, neddylation, substrate ubiquitination, CSN-mediated deneddylation, and CAND1-stimulated disassembly/reassembly and/or neddylation (Bosu and Kipreos, 2008; Cope and DeShaies, 2003). However, in cells, only a small subset of non-neddylated cullins is found in complex with CAND1 (Bennett et al., 2010), and it remains unclear how the neddylation/deneddylation cycle is coordinated with substrate availability. Moreover, cellular CRLs exist in a wide range of assembly states, and upon inhibition of deneddylation, both neddylated and non-neddylated CRLs stably associate with CSN, with or without SR modules (Bennett et al., 2010; Olma et al., 2009). How the different CRL assembly and activation states influence CSN and vice-versa remains poorly understood. Specific signals and SR binding have been suggested as regulators of CSN-CRL4 complex formation (Fischer et al., 2011; Groisman et al., 2003), but neither the structural basis nor the functional significance of forming stable complexes between CSN and the products of its enzymatic reaction is understood. Intriguingly, CRL4 enzymes, which may not require neddylation for activity, can be inhibited through CSN by an unknown mechanism (Fischer et al., 2011). Similarly, a recent study reported that CSN can inhibit SCF activity through a yet unknown, noncatalytic mechanism and that the SCF assembly state influences the CSN deneddylation activity (Emberley et al., 2012). Collectively, the available data imply that CSN and CAND1 regulate CRL function on multiple levels, the relationships and mechanisms of which remain incompletely characterized.

To understand the functional implications of the CSN interactions with CRLs, we determined structures of several CSN and CSN-SCF complexes through electron microscopy. We applied a hybrid structural approach to obtain pseudoatomic molecular models, which were further validated biochemically. Surprisingly, we found that CSN can occlude both SCF functional sites—for E2 enzymes and for ubiquitination substrates—which are located at opposite ends of the SCF complex. Consequently, CSN-SCF complex formation results in inhibited activation of Ubc12 by non-neddylated SCF and of Cdc34 by neddylated SCF and competes with binding to CAND1 and ubiquitination substrate. Our findings thus define the structural and biochemical basis underlying noncatalytic regulation of SCFs by CSN and imply that ubiquitination substrates can trigger SCF activation.

RESULTS

Structural Electron Microscopy Analysis of CSN Complexes

To gain structural and functional insights into the binding of CSN to fully assembled SCFs, we reconstituted various CSN-SCF

complexes in vitro. In addition to wild-type CSN, we also produced a recombinant CSN complex harboring a Csn5 subunit with an H138A point mutation in its active site, which interferes with zinc chelation, is deneddylation defective (Cope et al., 2002), and thus stably associates with neddylated SCFs.

Purified complexes of CSN^{Csn5H138A} with neddylated SCF^{Skp2/Cks1} (referred to as CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1}) or SCF^{Fbw7} (referred to as CSN^{Csn5H138A}-SCF~N8^{Fbw7}) (Figure 1A) were subjected to negative-stain electron microscopy and single-particle analysis. We determined the structure of the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} complex by ab initio angular reconstitution and iterative rounds of refinement (Figures 1B and S1A). To differentiate between the CSN and SCF components within the map we analyzed electron microscopy images of negatively stained apo CSN complexes. We calculated the structure of apo CSN, using an initial reference derived from the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} structure (Figures 1C and S1B). Comparison between the two maps showed that the apo CSN structure well matched a large portion of the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} map, allowing the segmentation of the latter into its CSN and SCF components (Figure 1D), which was confirmed by difference analysis (Figure S3A). The CSN density is characterized by a well-resolved mesh of discrete patches of elongated densities, consistent with our previous data (Enchev et al., 2010). The remaining region, which therefore corresponds to SCF~N8^{Skp2/Cks1}, forms a separate elongated curved density, connected to CSN through both of its ends.

Next, we investigated whether this CSN-binding mode is structurally conserved among the SCF family through analysis of CSN^{Csn5H138A}-SCF~N8^{Fbw7}. The CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} structure was used as an initial reference for the analysis of CSN^{Csn5H138A}-SCF~N8^{Fbw7} (Figure S1C). Indeed, the resulting structure (Figure 1E) is overall very similar to CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1}. Difference analysis with the apo CSN map indicated that SCF~N8^{Fbw7} also employs both of its ends to bind CSN and adopts a comparable position (Figure 1F and Figure S3A).

Molecular Models for CSN and CSN-SCF Complexes

Given the similarity of CSN and the 26S proteasome lid subcomplex (Pick et al., 2009), we considered recent electron microscopy studies defining molecular boundaries of the proteasome lid and proposing pseudoatomic models with constituent subunits for the fission and budding yeast, as well as for human lid subcomplexes (da Fonseca et al., 2012; Lander et al., 2012; Lasker et al., 2012). A comparison of the CSN region of the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} map and the human lid (Figure S2A) revealed substantial similarity. We thus assigned individual subunits within the CSN density to locations corresponding to their homologs in the lid. Atomic models for all human CSN subunits from I-TASSER (Roy et al., 2010) (Figure S2B) were docked into the CSN density. The resulting model (Figure 2) is characterized by a close match between the protein density and the atomic coordinates. There is little density unoccupied by the docked models, and there is no significant spatial overlap between the docked coordinates. Furthermore, the atomic models and the corresponding density segments of the

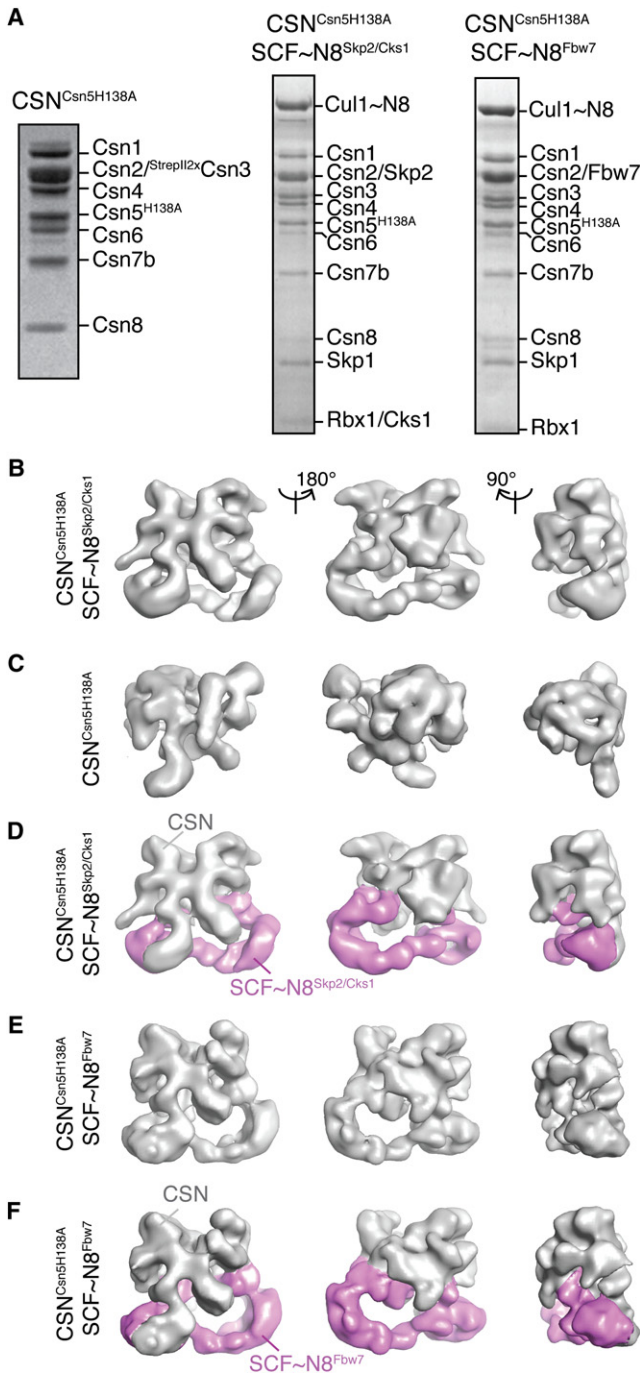


Figure 1. Reconstitution and Single-Particle Electron Microscopy Analysis of CSN-SCF Complexes

(A) Coomassie-stained SDS-PAGE of recombinant CSN^{Csn5H138A} and reconstituted CSN-SCF complexes after gel filtration.

(B–F) Surface views of electron microscopy density maps of (B) CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1}, (C) CSN^{Csn5H138A}, (D) CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} segmented into its SCF~N8^{Skp2/Cks1} (purple) and CSN (gray) subcomplexes. (E) CSN^{Csn5H138A}-SCF~N8^{Fbw7}, and (F) CSN^{Csn5H138A}-SCF~N8^{Fbw7} segmented into its CSN^{Csn5H138A}-SCF~N8^{Fbw7} (purple) and CSN (gray) subcomplexes.

See also Figures S1 and S3.

CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} map demonstrate strong correlation (Figure S2D).

In the model, the PCI subunits in CSN form an approximately coplanar surface (Figure 2, right-hand panel). Their major interaction interfaces are formed from the C-terminal winged-helix domains that form an arc (Figure 2, left-hand panel), from which the extended N-terminal domains radiate to form the characteristic ribbon-like densities in the map. In the model, the four longest subunits (Csn1, Csn2, Csn3, and Csn4) are docked into the central region of the arc, which is capped at each end by the shorter Csn7 and Csn8 subunits. The N-terminal peripheral density assigned to Csn4 in the model is less well recovered, possibly due to conformational heterogeneity in this region.

In contrast to the PCI subunits, the MPN subunits Csn5 and Csn6 are predicted to adopt globular conformations (Figure S2B). In the model, these two subunits form a protrusion on the side opposite the PCI subunits (Figure 2, center and right-hand panels), with Csn6 located over the center of the winged-helix arc and Csn5 offset in the direction of the Csn1 and Csn2 N-terminal domains and extending away from the plane of the PCI subunit cluster.

To test the assignments for Csn2 and Csn5, we produced CSN subcomplexes lacking the Csn5 subunit, CSN^{ΔCsn5}, or solely containing the PCI domain of Csn2, CSN^{Csn2ΔN1-269} (Figures S1D, S2C, S4F, and S4G). CSN^{ΔCsn5} formed a complex with SCF~N8^{Skp2/Cks1} (Figures S3D and 4C). We subjected these complexes to electron microscopy and single-particle analysis. We analyzed CSN^{ΔCsn5}-SCF~N8^{Skp2/Cks1}, using the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} structure as an initial reference (Figures S1E, S3B, and S3C). The major difference between the refined CSN^{ΔCsn5}-SCF~N8^{Skp2/Cks1} and CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} maps is that the former lacks density in the region assigned to Csn5 (Figure S3B), which matches well with the Csn5 atomic model. Similarly, we analyzed CSN^{Csn2ΔN1-269}, using the apo CSN model as a reference (Figure S1F). The resulting map closely resembles the apo CSN structure but lacks a peripheral, extended, and curled density (Figure S3B), which matches well with the location and dimensions of the segment in which we docked the predicted TPR-like fold of the PCI-associated module of Csn2.

Having identified the CSN region in our maps of the CSN-SCF complexes and modeled individual CSN subunits, we interpreted the remaining density in terms of the known atomic structures of SCF components. We generated models for the neddy-lated SCF complexes on the basis of the SCF^{Skp2(F-box domain)} structure (Zheng et al., 2002) but used a model for the neddy-lated conformation of the C-terminal domain of Cul1¹⁻⁶⁹⁰ (Duda et al., 2008) and the structures of the two respective SR assemblies, Skp1-Skp2/Cks1 (Hao et al., 2005; Schulman et al., 2000) and Skp1-Fbw7 (Hao et al., 2007). We docked these as rigid bodies into the corresponding densities of the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} and CSN^{Csn5H138A}-SCF~N8^{Fbw7} maps (Figure 3), with close agreement in both cases. The extreme C-terminal domain of Cul1 (Cul1⁶⁹¹⁻⁷⁷⁶), encompassing Helix29 and WHB, forms one structural entity of a size similar to Rbx1 and Nedd8, and all three are known to be flexibly oriented to each other (Calabrese et al., 2011; Duda et al., 2008; Duda et al., 2012; Fischer et al., 2011), thus precluding their

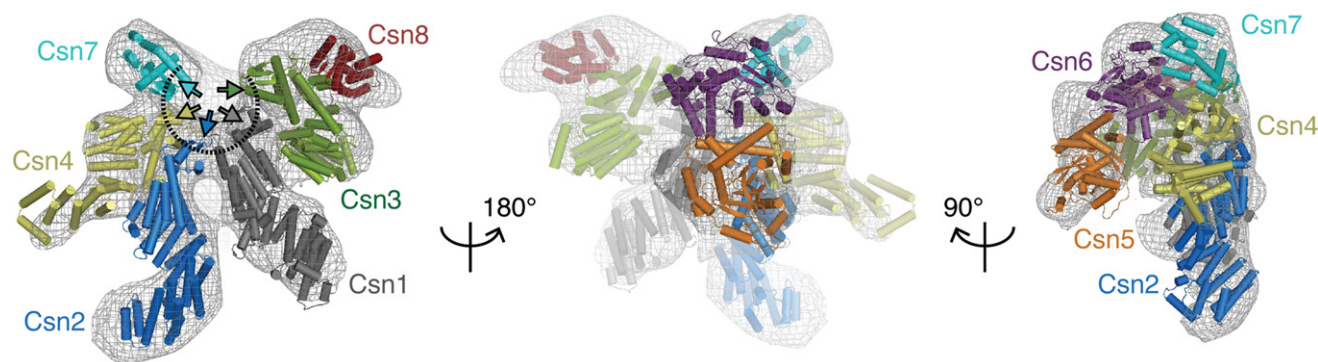


Figure 2. Molecular Model for CSN

The CSN segment from the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} map is shown as gray mesh. Left, PCI cluster side view. A dotted arc and color-coded arrows indicate the approximately coplanar positions of the winged-helix domains. MPN subunits are omitted for clarity. Center, opposite side, characterized by a protrusion formed by the two MPN domain subunits, Csn5 and Csn6. Right, view showing the edge of the coplanar PCI cluster. The protrusion formed by the Csn5 and Csn6 MPN subunits is left of the PCI cluster. Csn6 is more closely integrated with the PCI cluster, while Csn5 is angled away. See also Figure S2.

definitive docking in the map. Nevertheless, after the CSN and Cul1¹⁻⁶⁹¹/Skp1/Skp2/Cks1 segments are assigned, there is a portion of density left unaccounted for (Figure S3D), which matches well a neddylated WHB domain of Cul1 as well as Rbx1. Thus, a putative model is shown in Figures S3E and S3F, demonstrating that WHB~Nedd8 and Rbx1 can, in principle, be accommodated in the groove between Csn5 and Csn2 (see also Figure 6A).

Both SCF complexes adopt a peripheral position, approaching CSN through their substrate-recognition and E2-binding ends. At the E2-binding end, the Cul1 C-terminal domain and the region of the map modeled as the N-terminal TPR-like domain of Csn2 are connected by a continuous density, which appears to provide the major interaction between SCF and CSN in both complexes (Figure 3). At the substrate-recognition end of the CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} complex, the C-terminal α helices of Skp2 are located between the regions of the map modeled as the N-terminal domains of Csn3 and Csn1 (Figure 3A, middle panel, brown and purple arrows). Analogously, the WD40 repeat domain of Fbw7 is located in the corresponding region in the CSN^{Csn5H138A}-SCF~N8^{Fbw7} map (Figure 3B, purple arrows). On the other hand, for both CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} and CSN^{Csn5H138A}-SCF~N8^{Fbw7} complexes, the region into which the Cul1 N-terminal domain and the Skp1 subunit are docked is widely separated from CSN. Hence, it appears unlikely that there is any significant interaction between CSN and either the Cul1 N-terminal domain or the Skp1 subunit.

Determinants and Implications of CSN-SCF Interactions

To test and further explore the observed CSN-SCF interactions, we mixed various CSN and SCF complexes at 1:1 molar ratios and examined association by coelution over analytical size-exclusion chromatography (Figure 4). For comparison, data from the isolated CSN, SCF complexes, or different mutant complexes are shown in Figures S4A–S4E.

Our structural model predicts that CSN predominantly binds to the C-terminal portion of Cul1. Indeed, the coelution profiles

observed between CSN^{Csn5H138A} and a complex containing only the C-terminal domain of Cul1 and Rbx1 (Cul1^{CTD}/Rbx1), with and without neddylation, were comparable to the respective full-length Cul1/Rbx1 variants (Figure 4A). On the other hand, modulating the Cul1 C-terminal domain influenced CSN binding. In the presence of CSN^{Csn5H138A}, Cul1/Rbx1 migrated in two peaks, one corresponding to a complex with CSN and the other corresponding to free Cul1/Rbx1 (Figure 4A), while a greater proportion of the neddylated Cul1/Rbx1 (Cul1~N8/Rbx1) complex copurified with CSN^{Csn5H138A} (Figure 4A). Similarly, neddylated Cul1^{CTD}/Rbx1 complexes showed increased comigration with CSN^{Csn5H138A} compared to non-neddylated controls. Our structural model implies a potentially tenuous contact between CSN and the neddylated WHB domain of Cul1 (WHB~Nedd8, Figure S3C), and indeed, an intact WHB domain was required for binding of CSN and Cul1/Rbx1 complexes (Figure 4A). However, the WHB domain in isolation does not bind CSN sufficiently strongly to be detected by gel filtration (Figure S4B).

The structural analysis predicts that the Cul1 C-terminal domain approaches Csn2 to form a major interaction (Figure 3). Indeed, CSN^{Csn2 Δ N1-269}, lacking the N-terminal 269 residues of Csn2, as well as mutants with lesser truncations, failed to coelute with Cul1~N8/Rbx1 (data not shown) and displayed decreased deneddylation activity, whereas complexes lacking Csn5 generally behaved similarly to CSN^{Csn5H138A} (Figures 4, S2, and S4). Introduction of the Csn5^{H138A} active-site mutation to CSN^{Csn2 Δ N1-269} did not restore binding (Figure 4B).

In addition to the CSN-Cul1 C-terminal domain interface, we observed a more tenuous density connecting CSN and the SCF SRs (Figures 3A and 3B). The isolated SR modules Skp1/Skp2/Cks1 and Skp1/Fbw7 did not coelute with CSN, although the SRs appeared to enhance Cul1/Rbx1 association with CSN, since stoichiometric coelution of CSN and SCF^{Skp2/Cks1} or SCF^{Fbw7} over gel filtration did not require neddylation (Figures 4D and 4E). Moreover, the observed interaction of CSN-SCF^{Skp2/Cks1} was not substantially affected by deletion of either WHB or the Rbx1 RING domain (Figure 4E). However,

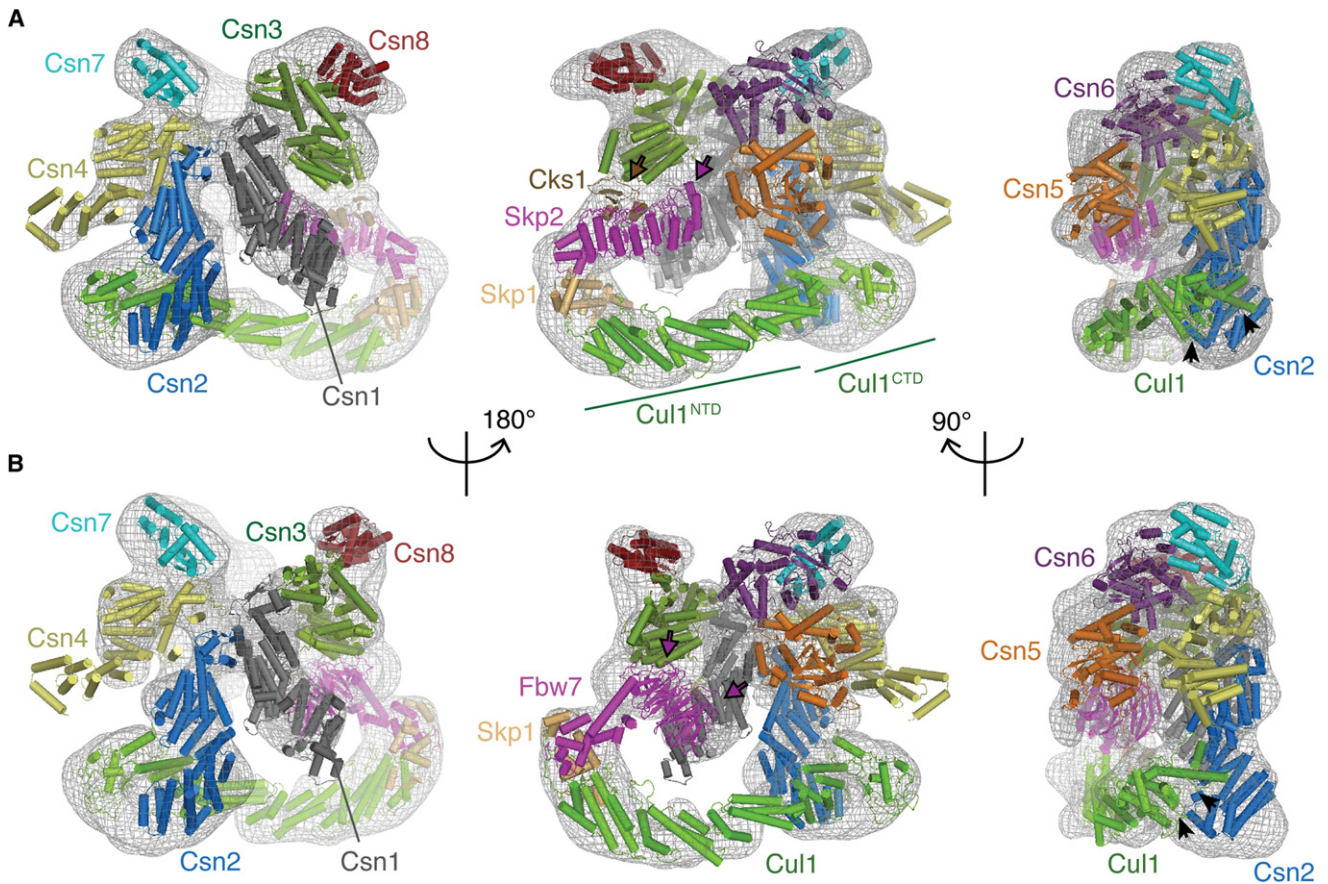


Figure 3. Molecular Models for CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1} and CSN^{Csn5H138A}-SCF~N8^{Fbw7}

(A) Molecular model for CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1}.

(B) Molecular model for CSN^{Csn5H138A}-SCF~N8^{Fbw7}.

Brown and purple (A) or purple (B) arrows in the central views indicate contact points between the SCF substrate receptors and CSN. Black arrows in the right-hand views indicate the basic canyon region of Cul1.

See also Figures S1 and S3.

SCF^{Skp2/Cks1} did not coelute with CSN^{Csn2ΔN1-104} (Figure S4I), confirming that the availability of SR is not sufficient to compensate for the requirement of the Csn2 N-terminal region. The CSN-SCF interaction was further explored by incubating preformed CSN-SCF^{Skp2/Cks1} complexes with CAND1 and analyzing the mix by gel filtration. Interestingly, we observed separation into CSN, CAND1-Cul1/Rbx1, and Skp1/Skp2/Cks1 complexes (Figure S4K).

CSN Can Compete with Ubiquitination Substrate for SCF Binding

Given that CSN approaches SRs in SCF complexes, we examined the potential influence of a ubiquitination substrate. The SCF^{Skp2/Cks1} substrate p27 is phosphorylated prior to SCF binding by Cdks. CyclinA/Cdk2 binds Skp2 and Cks1 and thus forms a complex with SCF^{Skp2/Cks1} and phospho-p27 (p-p27). p27 interacts with both Cdk2 and Skp2/Cks1, but only binding to the latter requires its phosphorylation (Hao et al., 2005; Spruck et al., 2001). The atomic coordinates of CyclinA/Cdk2 (Russo et al., 1996) can be docked onto the SCF^{Skp2/Cks1} structure

without steric clashes with other protein densities of our map (Figure 5A). In contrast, modeling the phospho-p27 (p-p27) N- and C-terminal fragments (Hao et al., 2005; Russo et al., 1996) raised the possibility that p-p27 and CSN might bind SCF^{Skp2}-CyclinA/Cdk2/Cks1 in a mutually exclusive manner (Figure 5A, arrow). Likewise, the proximity of CSN to Fbw7 may preclude a full-length substrate protein or protein complex from coexisting with CSN on SCF^{Fbw7} (Figure S6F).

To test whether CSN and p-p27 compete for SCF-binding, we first performed analytical size-exclusion chromatography with equimolar CSN^{Csn5H138A} and SCF^{Skp2}-p-p27/CyclinA/Cdk2/Cks1 in both neddylated and non-neddylated states (Figure 5B). Indeed, CSN^{Csn5H138A} formed a stable complex with SCF~N8^{Skp2}-CyclinA/Cdk2/Cks1, and p-p27 was excluded from the CSN complexes containing Cul1 (Figures 5B and S5). In the absence of neddylation, SCF^{Skp2}-CyclinA/Cdk2/Cks1 segregated as mutually exclusive complexes with both CSN and p-p27 in a dynamic equilibrium, most likely due to the less stable complex of CSN with non-neddylated SCF^{Skp2}-CyclinA/Cdk2/Cks1 (Figure 5B).

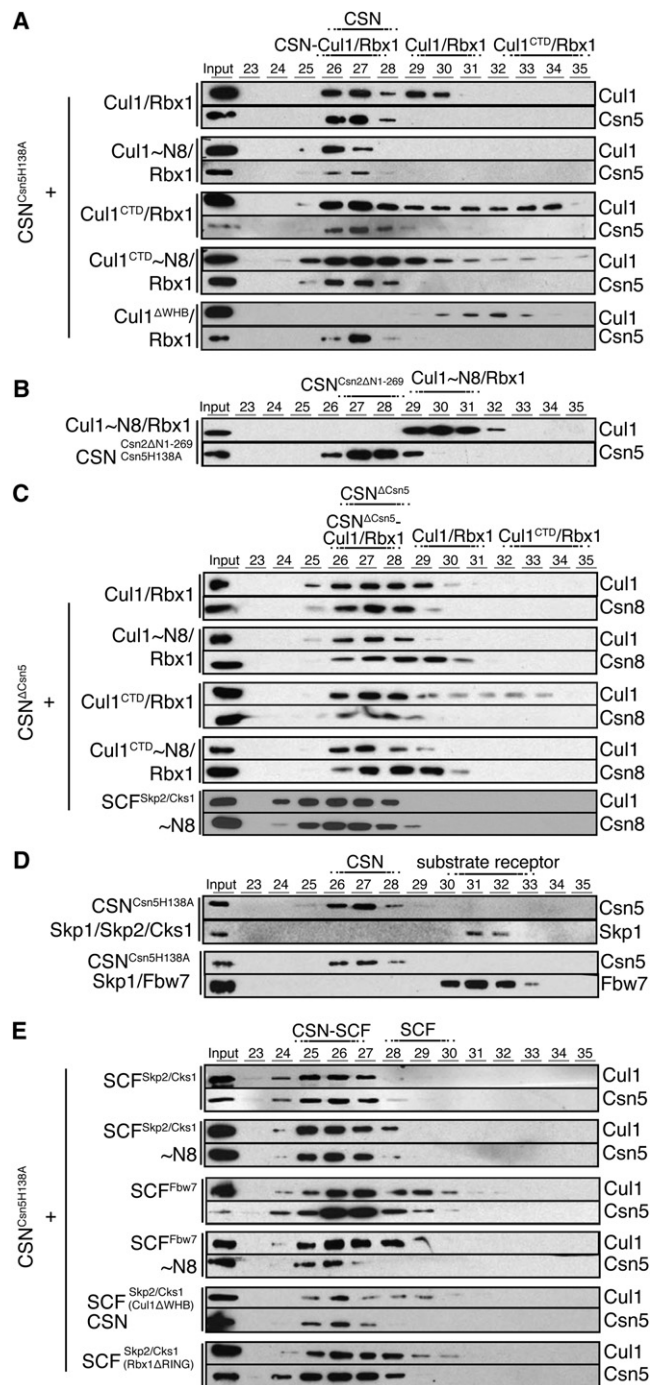


Figure 4. CSN-SCF Interactions Analyzed by Analytical Size-Exclusion Chromatography

Indicated SCF complexes were tested with equimolar amounts of (A, D, E) CSN harboring the Csn5^{H138A} active-site mutation, (B) CSN harboring an N-terminally truncated Csn2 and Csn5^{H138A}, or (C) a CSN complex lacking Csn5. Input and peak fractions (numbered) were blotted with the antibodies indicated to the right. Fractions in which particular complexes were eluted are indicated above each panel. Neddylated complexes are labeled with ~N8. See also Figure S4.

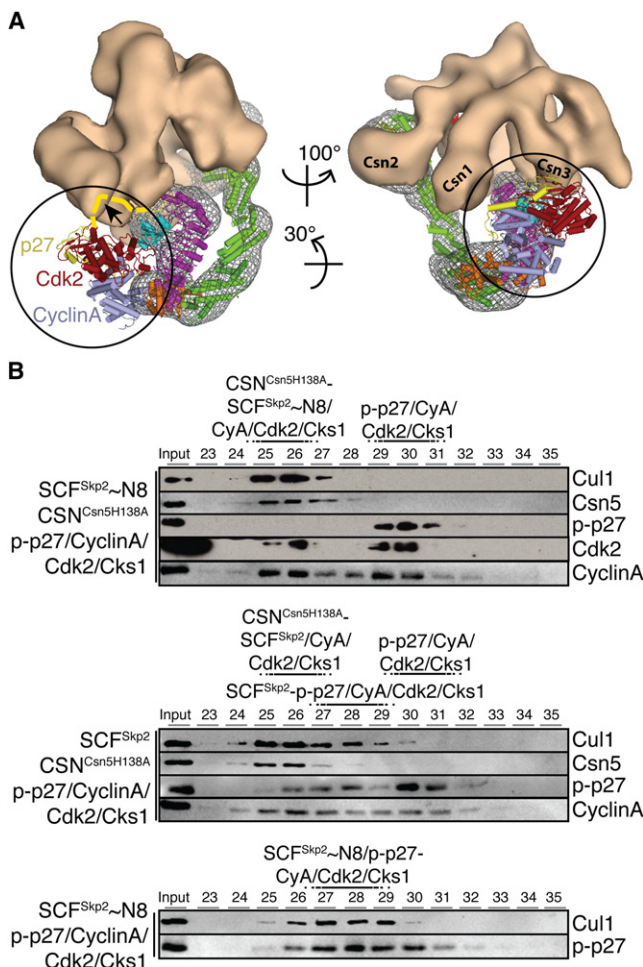


Figure 5. CSN-SCF Interactions in the Presence of Ubiquitination Substrate

(A) Views of the CSN^{Csn5H138A}-SCF^{Skp2}/Cks1~N8 map are shown with CSN as an orange surface and SCF^{Skp2}/Cks1~N8 as a gray mesh and the atomic coordinates as in Figure 3A. Docked Cdk2 (red), CyclinA (blue), and N- and C-terminal segments of p-p27 (yellow) are indicated by circles. A potential steric clash of p-p27 with the CSN density is indicated by a dashed yellow curve and an arrow on the left.

(B) Neddylated (~N8) or non-neddylated SCF^{Skp2} complexes were incubated with CyclinA/Cdk2/Cks1 and p-p27 in the presence or absence of equimolar amounts of deneddylation-defective CSN^{Csn5H138A} and were analyzed by size-exclusion chromatography.

See also Figure S5.

CSN-Mediated Deneddylation Depends on the SCF Assembly State

To explore the functional effects of different SCF assembly states, we examined CSN deneddylation activity toward different neddylated Cul1/Rbx1 complexes. Because endogenous and recombinant CSN displayed similar deneddylation activities (Figure S6A), all assays were performed with recombinant CSN. Consistent with the finding that the N-terminal domain of Cul1 is not necessary for CSN binding, deneddylation was comparable for neddylated full-length Cul1/Rbx1 (Cul1^{fl}/Rbx1), Cul1^{CTD}/Rbx1, and split-and-coexpressed Cul1/Rbx1 (referred

to here as Cul1^{SCE}/Rbx1) as substrates in our assays (Figure S6A). The latter is obtained by coexpression in bacteria of the Cul1 N-terminal domain (referred to as Cul1^{NTD}), the Cul1 C-terminal domain (referred to as Cul1^{CTD}), and Rbx1 as a total of three separate polypeptides, which assemble into a Cul1^{SCE}/Rbx1 complex whose structural and biochemical properties resemble those of full-length Cul1/Rbx1 (Duda et al., 2008; Goldenberg et al., 2004; Saha and Deshaies, 2008; Zheng et al., 2002).

Interestingly, the deneddylation activity toward Cul1^{SCE}~N8/Rbx1 was decreased upon addition of stoichiometric amounts of Skp1/Skp2/Cks1 or Skp1/Fbw7 (Figure S6B). Adding CyclinA/Cdk2 strengthened the inhibitory effect of Skp1/Skp2/Cks1, an effect maintained upon further addition of in vitro phosphorylated or unmodified p27 (Figure S6B). These results, which are consistent with a recent biochemical study (Emberley et al., 2012), suggest that Cul1/Rbx1 binding to Skp1/F-box modules, or their complexes with partner proteins, attenuates the deneddylation activity of CSN, possibly through product inhibition. We interpret the lack of deneddylation activity in the presence of substrate as being due to the lack of association between substrate-bound SCFs and CSN (Figure 4B).

CSN-SCF Binding Interferes with Cdc34 Activity

CSN has been shown to impede CRL4 autoubiquitination independently of deneddylation (Fischer et al., 2011). To gain structural insights into whether and how CSN might affect SCF-mediated ubiquitination, we docked the E2 Cdc34 onto the Rbx1 RING domain on the basis of available structures of other E2-RING E3 complexes (Zheng et al., 2000). Although the orientation of Rbx1's RING domain relative to the cullin subunit is known to be flexible (Calabrese et al., 2011; Duda et al., 2008; Fischer et al., 2011), Rbx1 can be positioned as a rigid body together with neddylated Cul1^{CTD} (Duda et al., 2008) with a reasonable fit in the electron microscopy map (Figure S3F). Strikingly, with Rbx1's RING domain in this orientation, or in any other previously reported conformation, steric hindrance would prevent the simultaneous interaction of Cul1/Rbx1 with CSN and Cdc34 (Figure 6A). Moreover, part of the CSN density appears in close proximity to the region most likely corresponding to the basic canyon of Cul1, which recruits the specialized acidic tail of Cdc34 with high affinity (Kleiger et al., 2009) (Figure 3, right-hand panels, black arrows). Structural modeling thus predicts that CSN binding prevents a catalytic interaction of Cul1/Rbx1 and Cdc34. As evident from analytical size exclusion (Figures 5B and S5), CSN and p-p27 binding to neddylated SCF^{Skp2/Cks1} is mutually exclusive. Thus, under the equilibrium conditions of the ubiquitination assays, CSN^{Csn5H138A} or CSN^{ΔCsn5} is expected to strongly reduce the occupancy time of both p-p27 and Cdc34. Combined with the low efficiency of Cdc34 as a priming E2, addition of these CSN constructs resulted in a decreased length of the polyubiquitin chains assembled by SCF^{Skp2/Cks1}/Cdc34 on in vitro phosphorylated full-length p27 (p-p27; Figure 6B, left, and Figures S6C and S6D). CSN^{Csn2ΔN1-269} only partially prevented p-p27 ubiquitination in this lower-molecular-weight region, most likely due to deneddylation by the relatively high and stoichiometric CSN:SCF concentrations used in this assay (Figure S6C).

In cells, CSN's binding to neddylated SCFs would most likely result in very rapid deneddylation (see Figure S6B). To test whether CSN binding can exert an inhibitory effect on non-neddylated SCF, we used a non-neddylatable Cul1 construct with an Arg-to-Lys mutation of its neddylation site (Cul1^{K720R}/Rbx1), which shows residual ubiquitination activity at long time points (Duda et al., 2008; Saha and Deshaies, 2008). This effect was strongly attenuated by stoichiometric addition of CSN^{Csn5H138A} (Figure S6D). The decreased polyubiquitin chain length observed upon addition of CSN^{Csn5H138A} was not due to an obvious contamination with a deubiquitinating enzyme (Figure S6E). Moreover, CSN^{Csn5H138A} counteracts SCF~N8^{Fbw7} activation of processive Cdc34-mediated ubiquitination of a short Cyclin E phosphopeptide (Figure 6B, right). Notably, because of its small size, this Cyclin E peptide may circumvent the steric clash within the SCF^{Fbw7}-CSN complex, as shown by analytical size-exclusion chromatography (Figures S6F and S6G).

Because the data suggest that CSN interferes with ubiquitin-transfer activity of Cdc34 independent of substrate-SR interactions, we performed pulse-chase assays to monitor SCF-mediated activation of Cdc34's intrinsic ubiquitin-transfer activity. In brief, in the pulse reaction, we generated a thioester-linked Cdc34 conjugate with a ³²P-labeled lysine-less (K0) version of ubiquitin. Use of lysine-less ubiquitin prevents polyubiquitin chains from forming during the pulse. In the chase, we added unlabeled wild-type ubiquitin and monitored ligation of radiolabeled K0 ubiquitin by the appearance of diubiquitin chains. Consistent with previous studies, Cdc34-mediated diubiquitin synthesis was stimulated by all complexes containing neddylated Cul1/Rbx1 (Figures 6C and S7A). In addition to neddylated Cul1/Rbx1 itself, this includes neddylated Cul1^{CTD}/Rbx1, SCF^{Skp2/Cks1}, and SCF^{Fbw7} (Saha and Deshaies, 2008). Paralleling the effects on substrate ubiquitination, addition of CSN^{Csn5H138A} and CSN^{ΔCsn5}, but not CSN^{Csn2ΔN1-269}, markedly decreased diubiquitin formation (Figure 6C and Figure S7A).

Finally, we used Glmn to probe the accessibility of the RING domain of Rbx1 in the presence or absence of CSN. Glmn can stably interact with the Rbx1 RING domain (Duda et al., 2012), and as with Cdc34, docking its structure onto Rbx1 suggested a steric clash with CSN (Figure S7B). Indeed, addition of Glmn resulted in inhibition of deneddylation, probably due to CSN's inability to bind the Cul1~N8/Rbx1-Glmn complex. Consistently, an Arg547-to-Ala mutant Glmn impaired for Rbx1 binding did not inhibit CSN deneddylation activity (Figure 6D).

CSN and Ubiquitination Substrate Binding Differentially Regulate SCF Neddylation

The close structural integration of CSN with the Cul1 C-terminal domain-Rbx1 region raises the possibility that CSN binding might also affect neddylation. Indeed, addition of CSN^{Csn5H138A} moderately decreased neddylation of Cul1^{SCE}/Rbx1 and substantially reduced neddylation of Cul1^{SCE}/Rbx1 complexed with Skp1/Skp2/Cks1 (Figure 6E). Addition of Skp1/Skp2/Cks1 as well as CyclinA/Cdk2 in the absence of CSN^{Csn5H138A}, on the other hand, had no major inhibitory effect on Cul1 neddylation (data not shown). Importantly, however, addition of CyclinA/Cdk2 in complex with in vitro phosphorylated p27

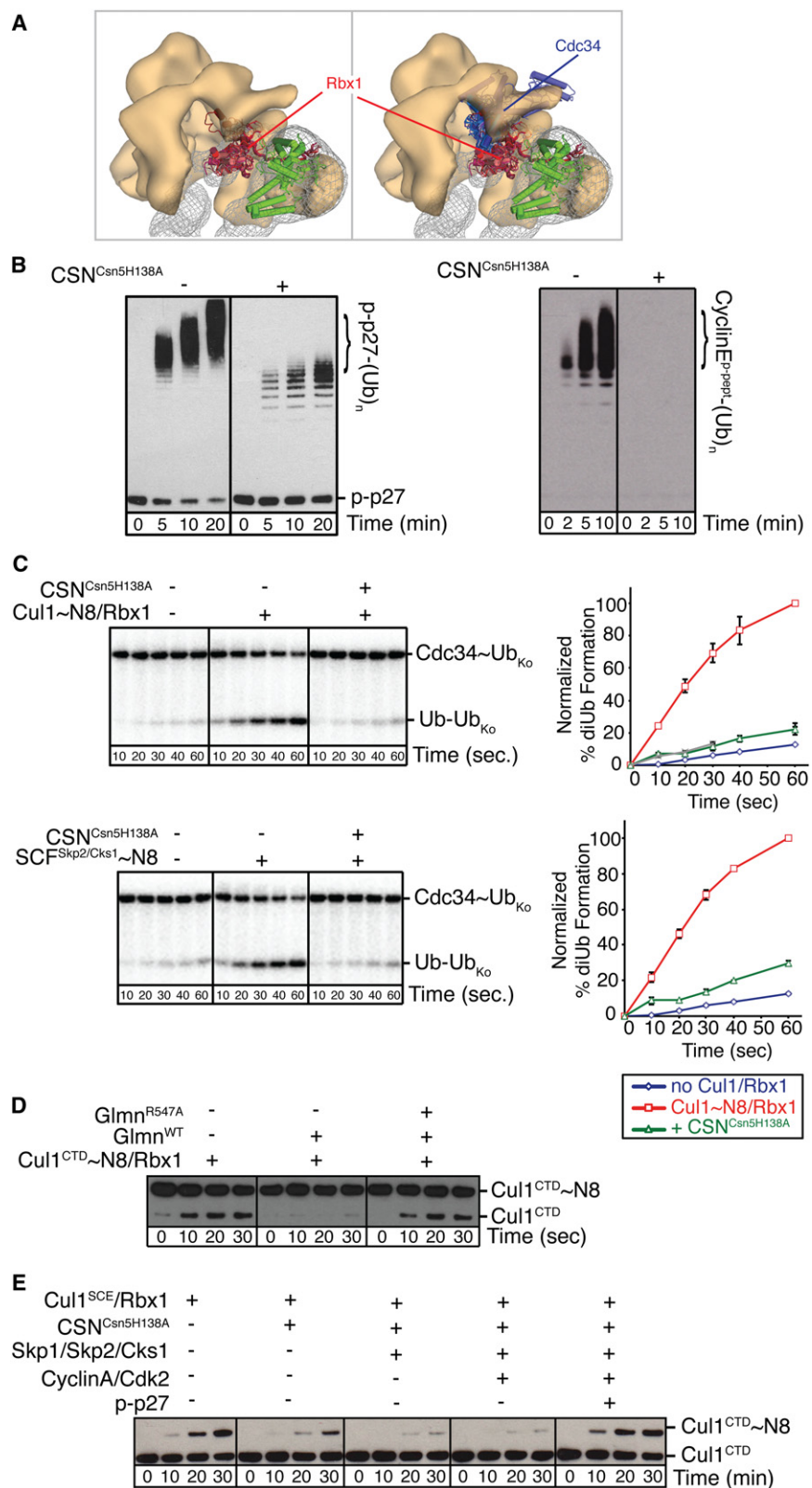


Figure 6. CSN-SCF Binding Regulates Ubiquitination and Neddylolation

(A) Zoomed-in surface view of CSN^{Csn5H138A}-SCF~N8^{Skp2/Cks1}, color-coded as Figure 5A, showing an overlay of all reported Rbx1 conformations without (left) or with (right) the docked Cdc34 model. Cul1⁴¹¹⁻⁶⁹⁰ (C-terminal domain without Helix29 and WHB) is shown in green, Rbx1 and Cdc34 orientations are shown as hues of red and blue.

(B) In vitro ubiquitination of p-p27 (left) CyclinE^{phosphopeptide} (right), assayed in the absence (-) or presence (+) of catalytically inactive CSN^{Csn5H138A}. Unmodified and polyubiquitinated substrates [- (Ub)_n] were detected by immunoblotting with p-p27 antibodies and biotin antibodies, respectively.

(C) Pulse-chase [³²P]-ubiquitin (Ub) transfer from Cdc34 to lysine-less ubiquitin (Ub_{Ko}) in the presence (+) or absence (-) of CSN^{Csn5H138A} and neddylated Cul1/Rbx1 (upper panel) or neddylated SCF^{Skp2/Cks1} (lower panel). Formation of di-Ub was assayed by autoradiography (left-hand panels) and quantified by plotting the percentage of di-Ub formation as a function of time (right-hand panels).

(D) Deneddylation of Cul1^{CTD}~N8/Rbx1 by CSN was assayed as described in Figure S6B in the presence (+) or absence (-) of wild-type (WT) or Rbx1-interaction-defective R547A mutant Glmn.

(E) Neddylolation (~N8) of Cul1^{SCE}/Rbx1 was measured by immunoblotting with Cul1 antibodies, in the presence (+) or absence (-) of CSN^{Csn5H138A}, Skp1/Skp2/Cks1, and CyclinA/Cdk2 and p-p27.

See also Figures S6 and S7.

relieves the CSN^{Csn5H138A}-mediated inhibition of neddylation (Figure 6E). These experiments support the notion that CSN inhibits neddylation of bound SCF complexes by blocking access to Ubc12, implying that CSN couples SCF neddylation with substrate availability.

DISCUSSION

Recent studies showing that over 30% of cellular CRLs exist in stable complexes with CSN demonstrate that CRLs and CSN do not interact only as short-lived catalytic intermediates (Olma et al., 2009; Bennett et al., 2010). Here, we report molecular models of CSN in complex with two fully assembled SCFs, which provide a structural framework for understanding stable CSN-SCF species and, combined with biochemical assays, reveal unexpected noncatalytic modes of SCF regulation by CSN.

Molecular Architecture of CSN

The proposed molecular model for CSN provides a glimpse into the overall three-dimensional arrangement of the eight CSN subunits. We have experimentally validated the global locations of Csn2 and Csn5 and docked homology models into the corresponding segments (Figure S2D). Although the docking statistics for the remaining subunits appear to be of similarly high quality, we note that the models are only predictions and that their exact conformation and/or orientation cannot be determined at the resolution of the present study. Nevertheless, our model is supported by the strong similarity between the density distribution of the CSN complex described here and that of its homolog, the lid of the 26S proteasome, whose subunit arrangement has been established at higher resolution (Figure 2A) (da Fonseca et al., 2012; Lander et al., 2012; Lasker et al., 2012). Furthermore, our model is supported by most available subunit-interaction data. For example, the subunit proximities match well with the tandem mass spectrometry analysis of recombinantly produced and reconstituted CSN (Sharon et al., 2009) as well as additional subunit contacts, including Csn1-Csn4 and Csn2-Csn4 (Serino et al., 2003; Tsuge et al., 2001). In our model, interaction between Csn1 and Csn2 involves their C-terminal winged-helix domains in agreement with the experimental observation that their PCI domains are sufficient for incorporation into the complex, whereas their N-terminal portions do not copurify with CSN (Tsuge et al., 2001; Yang et al., 2002). In fact, a splice variant of Csn2, known as Alien, is missing the winged-helix domain and functions as a CSN-independent corepressor of the thyroid hormone receptor (Tenbaum et al., 2003). Indeed, our model is consistent with the major interactions among the elongated PCI-domain-containing subunits Csn1, Csn2, Csn3, and Csn4 being through their winged-helix domains (Figure 2). Interestingly, although the overall molecular architectures of CSN and the proteasome lid are strikingly similar, these two complexes appear to recognize their substrates by different structural elements. While CSN requires the N terminus of Csn2 to position the neddylated cullin C-terminal domain in proximity to Csn5 (Figure 3), the proteasome paralog of Csn2, Rpn6, is a scaffold, linking the lid to the 20S core. Recognition of polyubiquitinated substrates relies on nonlid subunits such as Rpn10 and Rpn13.

Molecular Determinants of the CSN-SCF Interaction

No structural information for a CSN-CRL interaction was previously available. Extrapolating from X-ray crystallographic data for other CRL-binding partners, one might have predicted either a relatively narrow interface, as observed between Cul1/Rbx1 and E2 enzymes and/or Dcn1, or a very broad interface engulfing most of the cullin, as for Cand1 (Duda et al., 2011). However, our structural and biochemical analysis revealed that CSN localizes to both SCF functional sites. In our model, this two-pronged interaction between CSN and SCF involves primarily the Csn2/Csn5 region and the cullin C-terminal domain, as well as the Csn1/Csn3 region and the SCF SR (Figure 3).

Interestingly, our data revealed that Csn5 is dispensable for assembly of the remaining seven subunits, consistent with its peripheral location in the complex (Figure 2) and the fact that such a Csn5-free CSN subcomplex can still bind SCF but not deneddylate it (Figures 4C and S4G). Furthermore, cullin neddylation stabilized the CSN-SCF interaction, even in the absence of Csn5 (Figure 4), suggesting that a Csn5-Nedd8 contact is not essential for the interaction. It seems likely that Csn2, and perhaps other CSN subunits, preferentially binds to the neddylated conformation of the cullin C-terminal domain and/or Rbx1. Notably, although Csn5 harbors a catalytic JAMM motif, the subunit has to be assembled in the CSN complex for deneddylation to be observed (Sharon et al., 2009). Our molecular model thus suggests an unexpected explanation: Csn5 itself cannot recognize neddylated SCF substrates but apparently requires Csn2 and the rest of the complex to position the Nedd8 moiety attached to the CRL correctly in its active site. Moreover, this result implies that in contrast to Csn2, depletion of Csn5 or addition of chemical Csn5 inhibitors may not affect the formation rate or stability of CSN-CRL complexes in cells. Importantly, in our *in vitro* assays, loss of Csn5 had no effect on the noncatalytic modes of CSN-mediated SCF inhibition. We therefore caution against using Csn5 depletion or chemical inhibition as the sole means of inactivating CSN function.

We also identified CSN-density segments modeled as Csn1 and Csn3 as approaching the SCF SRs, consistent with binding of a beta-barrel SR and Csn1 (Tsuge et al., 2001). Indeed, we observed small structural differences in the densities assigned to the N termini of Csn1 and Csn3 between the apo CSN and the CSN-SCF complexes (Figure S3A), which may indicate conformational rearrangements associated with SCF binding and are reminiscent of the conformational changes reported for binding of the lid to the proteasome (Lander et al., 2012). However, this interaction appears less substantial than the Csn2-dependent Cul1 C-terminal domain interaction (Figure 3), and when examined by analytical size-exclusion chromatography (Figure 4B) and pull-down assays (not shown), CSN does not appreciably associate with SRs in the absence of Cul1/Rbx1. Nevertheless, the presence of SRs increases Cul1/Rbx1 association with CSN (Figure 4). Intriguingly, CSN can preferentially form complexes with CRL subsets, determined partly by the identity of the SRs (Olma et al., 2009). Additional studies are needed in order to shed light on the structural basis for such preferences and their functional implications.

CSN Regulates SCF Activity by Multiple Mechanisms

The functional significance of the prevalent CSN-CRL complexes found in cells has remained poorly understood. The structural models presented here suggest a surprisingly complex, multilayered mechanism of CSN-mediated inhibition of SCF activity. Apart from deneddylation, our data suggest that CSN binding exerts a double-pronged attack on both SCF functional sites by sterically hindering productive interactions of SCF with other factors. Indeed, we demonstrate that CSN competes with the Rbx1 RING interactors Glmn (Duda et al., 2012; Tron et al., 2012), the ubiquitin E2 enzyme Cdc34, and the Nedd8-E2 Ubc12, as well as a ubiquitination substrate. Other RING interactors have also been shown to interfere with CSN-mediated deneddylation (Emberley et al., 2012). Consistently, we showed that CSN binding is sufficient to inhibit SCF-mediated ubiquitination, even when catalytically inactive CSN^{Csn5H138A} complexes are used. Importantly, wild-type CSN has been reported to inhibit CRL4 ubiquitination activity by an unknown, noncatalytic mechanism (Fischer et al., 2011), suggesting that CSN inhibition mediated by steric hindrance is conserved across the CRL family.

CSN and CAND1 Differentially Regulate CRL Assembly and Activity

Very little is known about the regulation of CSN activity. Unexpectedly, we observe that the assembly of Cul1/Rbx1 with SRs also downregulates the deneddylation activity of CSN, implying that SR-free cullins are better deneddylation substrates. This is consistent with findings that binding to SRs correlates with increased Cul1/Rbx1 neddylation in cells (Chew and Hagen, 2007; Kawakami et al., 2001). Together, our data provide the structural and mechanistic basis for a two-branched model of regulating SCF assembly and ubiquitination activity by CSN and CAND1, which may have evolved to regulate distinct Cul1/Rbx1 assemblies. CAND1 recognizes non-neddylated Cul1/Rbx1 complexes, and its interaction with Cul1/Rbx1 is in a dynamic equilibrium with the Cul1/Rbx1-Skp1/F-box interaction, thereby facilitating reassembly of non-neddylated cullins with SR modules (Bornstein et al., 2006; Schmidt et al., 2009; Siergiejuk et al., 2009) (Figure S4K; D.C.S. and B.A.S., unpublished data). We presume that there are no protein factors favoring directionality in the equilibrium. On the other hand, CSN regulates the ubiquitination activity of assembled SCFs through the multiple mechanisms detailed above. Importantly, in addition to deneddylation, the CSN-SCF interaction inhibits Rbx1-Ubc12-mediated Cul1 neddylation, thereby sequestering and protecting assembled SCF complexes in an inactive state.

CRL Activation Requires Dissociation of CSN-CRL Complexes

Our work implies that the prevalent stably bound SCF-CSN complexes in cells (Bennett et al., 2010) are in a reciprocally inactivated state, which raises the question of how SCF-CSN complexes dissociate. The formation of CSN-CRL4s plays regulatory roles in the cellular response to DNA damage (Groisman et al., 2003)—a complex cellular process, meticulously regulated by post-translational modifications. It is thus conceivable that, for example, phosphorylation of the CSN-CRL-binding interface

could influence complex formation. Indeed, several kinases associate with CSN and have been shown to phosphorylate Csn2 and Csn7 (Sun et al., 2002; Uhle et al., 2003; Wilson et al., 2001). Moreover, Csn1, Csn3, and Csn8 have also been shown to be phosphorylation targets (Fang et al., 2008; Matsuoka et al., 2007). It will thus be important to investigate whether post-translational modifications could disrupt interaction interfaces between CSN and CRLs and thus play broad roles in negatively regulating their association.

Interestingly, our data suggest that CSN and the ubiquitination substrate p27 compete for SCF^{Skp2/Cks1}-binding (Figure 5). Similarly, addition of the SCF^{Fbw7} substrate phospho-CyclinE/Cdk2 downregulates deneddylation (Emberley et al., 2012). Moreover, CRL4 binding to chromatin-located substrates has been proposed to trigger to CSN-CRL4 dissociation (Fischer et al., 2011). Thus, the levels of ubiquitination substrates might also regulate CSN-CRL dissociation in vivo. Consistent with this notion, substrate-bound CRLs are fully neddylated in cells (Read et al., 2000). Here, we show that substrate promotes Cul1 neddylation even in the presence of catalytically inactive CSN. In cells, substrate-favored dissociation of CSN-SCF complexes could both allow neddylation and further promote polyubiquitination and proteasomal degradation of the substrate. CSN binding may prevent a futile neddylation/deneddylation cycle by coupling cullin neddylation to substrate availability. Unidirectionality may be further programmed by CSN's failure to deneddylate substrate-bound SCFs. Our data suggest that the molecular architecture of CSN is better suited to stably bind fully assembled SCFs rather than SR-free cullins. We therefore propose that CSN acts as a sensor for catalytically assembled SCFs and protects these complexes from disassembly, neddylation, and ubiquitin-mediated degradation until critical amounts of the cognate ubiquitination substrate have accumulated. In this model, CSN would not only work as an inhibitor of SCF activity but would also promote SCF function by maintaining its assembly in a state that ensures rapid and efficient substrate turnover.

EXPERIMENTAL PROCEDURES

Cloning, Expression, Purification, and Immunoblotting

Full description is provided in the Extended Experimental Procedures.

Analytical Size-Exclusion Chromatography

Protein concentrations were measured with a NanoDrop (Thermo Scientific) in 6 M guanidine. Equimolar (1 μ M) CSN and Cul1/Rbx1 variants were mixed on ice for 10 min in 15 mM HEPES, pH 7.8, 150 mM NaCl, 1% glycerol, 1 mM DTT. Skp1/Skp2/Cks1, p27/CyclinA/Cdk2 and/or CAND1 were added in slight excess (1.2 μ M). Nedd8, the WHB domain of Cul1 were added at 5 μ M. 200 μ l protein mixture was injected onto a Superose 6 10/300 size-exclusion column (GE Healthcare); the run was performed at 4°C, collecting 48 500 μ l fractions. Aliquots were analyzed by SDS PAGE on 4%–12% or 12% gels and immunoblotting.

Neddylation and Deneddylation Assays

Cul1/Rbx1 purified from insect cells (Enchev et al., 2010) and all other Cul1/Rbx1 constructs were neddylated by incubating 8 μ M Cul1^{fl}/Rbx1; 500 nM APPBP1-Uba3; 1 μ M Ubc12; 10 μ M Nedd8 at room temperature for 10 min in 50 mM Tris/HCl, pH 7.6, 100 mM NaCl, 2.5 mM MgCl₂, 150 μ M ATP. After addition of 10 mM DTT, products were purified over a Superdex 200 column (Duda et al., 2008). Neddylation assays involving CSN^{Csn5H138A}

addition contained 25 nM APPBP1/Uba3, 125 nM Ubc12, 125 nM Cul1/Rbx1, +/- 125 nM Skp1/Skp2/Cks1, +/- 1 μM or 3 μM CyclinA/Cdk2 or CyclinA/Cdk2/p-p27 in 25mM Tris pH 7.0, 50mM NaCl, 10mM MgCl₂, 1.5mM ATP, incubated on ice for 20 min. CSN^{Csn5H138A} was then added at 500 nM and incubated on ice for 5 min. Reactions were initiated by addition of 20 μM Nedd8. Aliquots were analyzed by SDS PAGE on 4%–12% gels and immunoblotting.

Deneddylation assays were performed in 50mM Tris, 50mM NaCl pH = 7.6 at room temperature using 2 nM CSN variants and 150 nM neddylation Cul1/Rbx1 variants and Skp1/F-box. 800nM CyclinA/Cdk2, CyclinA/Cdk2/p27, or CyclinA/Cdk2/p-p27 was added to ensure complex formation with Skp1/Skp2.

Ubiquitination Assays

Ubiquitination assays were performed at room temperature with 200 nM SCF variants and 100 nM Ube1, 500 nM Cdc34 and 50 μM ubiquitin. p27 was phosphorylated *in vitro* for 30 min at 30°C by mixing 4 μM CyclinA/Cdk2 and p27 in 40 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 1 mM ATP and 1 mM DTT. 200 nM phospho-p27/CyclinA/Cdk2 or 5 μM biotinylated CyclinE phosphopeptide were used as substrates. 500 nM (Figure 6B and S6C) or 200 nM (Figure S6D) CSN variants were added as indicated. Aliquots were analyzed by SDS PAGE on 4%–12% gels immunoblotting.

Diubiquitin Formation Assays

10 μM Cdc34 was loaded with 20 μM lysine-less [³²P]-ubiquitin ([³²P]-Ub^{R7}) with 100 nM ubiquitin E1 in 50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM MgCl₂, 1.5 mM ATP at room temperature for 25 min. The loading reaction was quenched by the addition of EDTA to 50 mM. Discharge reactions were performed in 25 mM Tris pH7.6, 100 mM NaCl, 50 mM EDTA. 250 nM ubiquitin, 500 nM Cul1~N8/Rbx1 +/- a 1:1 mix of Skp1/F-box were incubated on ice for 30 min to equilibrate SCF complex formation. The mixtures were placed at room temperature, and after a 5 min incubation, the indicated CSN variants were added to a final concentration of 1.25 μM followed by an additional 5 min incubation at room temperature. Discharge was initiated by the addition of Cdc34~[³²P]Ub^{R7} to a final concentration of 400 nM. Aliquots were analyzed on 4%–12% gels, dried, and exposed to a phosphorimager screen, scanned on a StormImager and quantified using ImageQuant TL v2003.02.

Electron Microscopy and Single-Particle Analysis

Electron microscopy data collection and analysis were similar to that described in (Enchev et al., 2010). Detailed description is given in the Extended Experimental Procedures.

ACCESSION NUMBERS

CSN^{Csn5H138A}_SCF~N8^{Skp2/Cks1} has been deposited in the EMDDataBank under accession code EMD-2173, CSN^{Csn5H138A}_SCF~N8^{Fbw7} under EMD-2174, CSN^{ΔCsn5}_SCF~N8^{Skp2/Cks1} under EMD-2175, CSN under EMD-2176, and CSN^{Csn2ΔN1-269} under EMD-2177.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.08.019>.

LICENSING INFORMATION

This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 Unported License (CC-BY-NC-ND); <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>.

ACKNOWLEDGMENTS

We thank D. Barford for providing access to equipment and laboratory space, F. Beuron for EM support, and R. Deshaies and A. Smith for critical reading.

R.I.E. is funded by an ETH/Marie Curie fellowship. E.P.M. is supported by the ICR; M.P. by the Swiss National Science Foundation, an ERC senior award, and the ETH-Zurich; and B.A.S. by ALSAC, HHMI, and NIH grant R01GM069530. B.A.S. is an Investigator of the Howard Hughes Medical Institute.

Received: August 15, 2012

Revised: August 24, 2012

Accepted: August 24, 2012

Published online: September 6, 2012

REFERENCES

- Bennett, E.J., Rush, J., Gygi, S.P., and Harper, J.W. (2010). Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* 143, 951–965.
- Bornstein, G., Ganoth, D., and Hershko, A. (2006). Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. *Proc. Natl. Acad. Sci. USA* 103, 11515–11520.
- Bosu, D.R., and Kipreos, E.T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell Div.* 3, 7.
- Calabrese, M.F., Scott, D.C., Duda, D.M., Grace, C.R., Kurinov, I., Kriwacki, R.W., and Schulman, B.A. (2011). A RING E3-substrate complex poised for ubiquitin-like protein transfer: structural insights into cullin-RING ligases. *Nat. Struct. Mol. Biol.* 18, 947–949.
- Chew, E.H., and Hagen, T. (2007). Substrate-mediated regulation of cullin neddylation. *J. Biol. Chem.* 282, 17032–17040.
- Cope, G.A., and Deshaies, R.J. (2003). COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. *Cell* 114, 663–671.
- Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V., and Deshaies, R.J. (2002). Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* 298, 608–611.
- da Fonseca, P.C., He, J., and Morris, E.P. (2012). Molecular model of the human 26S proteasome. *Mol. Cell* 46, 54–66.
- Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* 78, 399–434.
- Dessau, M., Halimi, Y., Erez, T., Chomsky-Hecht, O., Chamovitz, D.A., and Hirsch, J.A. (2008). The Arabidopsis COP9 signalosome subunit 7 is a model PCI domain protein with subdomains involved in COP9 signalosome assembly. *Plant Cell* 20, 2815–2834.
- Duda, D.M., Borg, L.A., Scott, D.C., Hunt, H.W., Hammel, M., and Schulman, B.A. (2008). Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. *Cell* 134, 995–1006.
- Duda, D.M., Scott, D.C., Calabrese, M.F., Zimmerman, E.S., Zheng, N., and Schulman, B.A. (2011). Structural regulation of cullin-RING ubiquitin ligase complexes. *Curr. Opin. Struct. Biol.* 21, 257–264.
- Duda, D.M., Olszewski, J.L., Tron, A.E., Hammel, M., Lambert, L.J., Waddell, M.B., Mittag, T., Decaprio, J.A., and Schulman, B.A. (2012). Structure of a Glomulin-RBX1-CUL1 Complex: Inhibition of a RING E3 Ligase through Masking of Its E2-Binding Surface. *Mol. Cell* 47, 371–382.
- Emberley, E.D., Mosadeghi, R., and Deshaies, R.J. (2012). Deconjugation of Nedd8 from Cul1 is directly regulated by Skp1-Fbox and substrate, and CSN inhibits deneddylation of SCF by a non-catalytic mechanism. *J. Biol. Chem.* Published online July 5, 2012. <http://dx.doi.org/10.1074/jbc.M112.352484>.
- Enchev, R.I., Schreiber, A., Beuron, F., and Morris, E.P. (2010). Structural insights into the COP9 signalosome and its common architecture with the 26S proteasome lid and eIF3. *Structure* 18, 518–527.
- Fang, L., Wang, X., Yamoah, K., Chen, P.L., Pan, Z.Q., and Huang, L. (2008). Characterization of the human COP9 signalosome complex using affinity purification and mass spectrometry. *J. Proteome Res.* 7, 4914–4925.
- Fischer, E.S., Scrima, A., Böhm, K., Matsumoto, S., Lingaraju, G.M., Faty, M., Yasuda, T., Cavadini, S., Wakasugi, M., Hanaoka, F., et al. (2011). The

- molecular basis of CRL4DDB2/CSA ubiquitin ligase architecture, targeting, and activation. *Cell* 147, 1024–1039.
- Goldenberg, S.J., Cascio, T.C., Shumway, S.D., Garbutt, K.C., Liu, J., Xiong, Y., and Zheng, N. (2004). Structure of the Cnd1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell* 119, 517–528.
- Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K., and Nakatani, Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* 113, 357–367.
- Hao, B., Zheng, N., Schulman, B.A., Wu, G., Miller, J.J., Pagano, M., and Pavletich, N.P. (2005). Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase. *Mol. Cell* 20, 9–19.
- Hao, B., Oehlmann, S., Sowa, M.E., Harper, J.W., and Pavletich, N.P. (2007). Structure of a Fbw7-Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. *Mol. Cell* 26, 131–143.
- Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., et al. (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J.* 20, 4003–4012.
- Kleiger, G., Saha, A., Lewis, S., Kuhlman, B., and Deshaies, R.J. (2009). Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates. *Cell* 139, 957–968.
- Kurz, T., Ozlü, N., Rudolf, F., O'Rourke, S.M., Luke, B., Hofmann, K., Hyman, A.A., Bowerman, B., and Peter, M. (2005). The conserved protein Dcn-1/Dcn1p is required for cullin neddylation in *C. elegans* and *S. cerevisiae*. *Nature* 435, 1257–1261.
- Lander, G.C., Estrin, E., Matyskiela, M.E., Bashore, C., Nogales, E., and Martin, A. (2012). Complete subunit architecture of the proteasome regulatory particle. *Nature* 482, 186–191.
- Lasker, K., Förster, F., Bohn, S., Walzthoeni, T., Villa, E., Unverdorben, P., Beck, F., Aebersold, R., Sali, A., and Baumeister, W. (2012). Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. *Proc. Natl. Acad. Sci. USA* 109, 1380–1387.
- Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A., and Deshaies, R.J. (2001). Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* 292, 1382–1385.
- Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., et al. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316, 1160–1166.
- Olma, M.H., Roy, M., Le Bihan, T., Sumara, I., Maerki, S., Larsen, B., Quadroni, M., Peter, M., Tyers, M., and Pintard, L. (2009). An interaction network of the mammalian COP9 signalosome identifies Dda1 as a core subunit of multiple Cul4-based E3 ligases. *J. Cell Sci.* 122, 1035–1044.
- Pick, E., Hofmann, K., and Glickman, M.H. (2009). PCI complexes: Beyond the proteasome, CSN, and eIF3 Troika. *Mol. Cell* 35, 260–264.
- Read, M.A., Brownell, J.E., Gladysheva, T.B., Hottelet, M., Parent, L.A., Coggin, M.B., Pierce, J.W., Podust, V.N., Luo, R.S., Chau, V., and Palombella, V.J. (2000). Nedd8 modification of cul-1 activates SCF(beta-TrCP)-dependent ubiquitination of I kappa Balpha. *Mol. Cell. Biol.* 20, 2326–2333.
- Roy, A., Kucukural, A., and Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5, 725–738.
- Russo, A.A., Jeffrey, P.D., Patten, A.K., Massagué, J., and Pavletich, N.P. (1996). Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature* 382, 325–331.
- Saha, A., and Deshaies, R.J. (2008). Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. *Mol. Cell* 32, 21–31.
- Sato, Y., Yoshikawa, A., Yamagata, A., Mimura, H., Yamashita, M., Ookata, K., Nureki, O., Iwai, K., Komada, M., and Fukai, S. (2008). Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature* 455, 358–362.
- Scheel, H., and Hofmann, K. (2005). Prediction of a common structural scaffold for proteasome lid, COP9-signalosome and eIF3 complexes. *BMC Bioinformatics* 6, 71.
- Schmidt, M.W., McQuary, P.R., Wee, S., Hofmann, K., and Wolf, D.A. (2009). F-box-directed CRL complex assembly and regulation by the CSN and CAND1. *Mol. Cell* 35, 586–597.
- Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M., and Pavletich, N.P. (2000). Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature* 408, 381–386.
- Scott, D.C., Monda, J.K., Bennett, E.J., Harper, J.W., and Schulman, B.A. (2011). N-terminal acetylation acts as an avidity enhancer within an interconnected multiprotein complex. *Science* 334, 674–678.
- Serino, G., Su, H., Peng, Z., Tsuge, T., Wei, N., Gu, H., and Deng, X.W. (2003). Characterization of the last subunit of the Arabidopsis COP9 signalosome: implications for the overall structure and origin of the complex. *Plant Cell* 15, 719–731.
- Sharon, M., Mao, H., Boeri Erba, E., Stephens, E., Zheng, N., and Robinson, C.V. (2009). Symmetrical modularity of the COP9 signalosome complex suggests its multifunctionality. *Structure* 17, 31–40.
- Siergiejuk, E., Scott, D.C., Schulman, B.A., Hofmann, K., Kurz, T., and Peter, M. (2009). Cullin neddylation and substrate-adaptors counteract SCF inhibition by the CAND1-like protein Lag2 in *Saccharomyces cerevisiae*. *EMBO J.* 28, 3845–3856.
- Spruck, C., Strohmaier, H., Watson, M., Smith, A.P., Ryan, A., Krek, T.W., and Reed, S.I. (2001). A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol. Cell* 7, 639–650.
- Sun, Y., Wilson, M.P., and Majerus, P.W. (2002). Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to CSN1. *J. Biol. Chem.* 277, 45759–45764.
- Tenbaum, S.P., Juenemann, S., Schlitt, T., Bernal, J., Renkawitz, R., Muñoz, A., and Baniahmad, A. (2003). Alien/CSN2 gene expression is regulated by thyroid hormone in rat brain. *Dev. Biol.* 254, 149–160.
- Tron, A.E., Arai, T., Duda, D.M., Kuwabara, H., Olszewski, J.L., Fujiwara, Y., Bahamon, B.N., Signoretti, S., Schulman, B.A., and DeCaprio, J.A. (2012). The glomovenous malformation protein Glomulin binds Rbx1 and regulates cullin RING ligase-mediated turnover of Fbw7. *Mol. Cell* 46, 67–78.
- Tsuge, T., Matsui, M., and Wei, N. (2001). The subunit 1 of the COP9 signalosome suppresses gene expression through its N-terminal domain and incorporates into the complex through the PCI domain. *J. Mol. Biol.* 305, 1–9.
- Uhle, S., Medalia, O., Waldron, R., Dumdey, R., Henklein, P., Bech-Otschir, D., Huang, X., Berse, M., Sperling, J., Schade, R., and Dubiel, W. (2003). Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. *EMBO J.* 22, 1302–1312.
- Wilson, M.P., Sun, Y., Cao, L., and Majerus, P.W. (2001). Inositol 1,3,4-trisphosphate 5/6-kinase is a protein kinase that phosphorylates the transcription factors c-Jun and ATF-2. *J. Biol. Chem.* 276, 40998–41004.
- Yamoah, K., Oashi, T., Sarikas, A., Gazdoui, S., Osman, R., and Pan, Z.Q. (2008). Autoinhibitory regulation of SCF-mediated ubiquitination by human cullin 1's C-terminal tail. *Proc. Natl. Acad. Sci. USA* 105, 12230–12235.
- Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Di Xiao, Wang, X., Rodriguez-Suarez, R.J., Zhang, H., and Wei, N. (2002). The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via neddylation of SCF Cul1. *Curr. Biol.* 12, 667–672.
- Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* 102, 533–539.
- Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M., et al. (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* 416, 703–709.