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Deconjugation of Nedd8 from Cul1 Is Directly Regulated by Skp1-F-box and Substrate, and the COP9 Signalosome Inhibits Deneddylated SCF by a Noncatalytic Mechanism*[S]

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Background: A detailed description of the kinetics of deneddylation of cullin by CSN has been lacking.
Results: Selected factors and SCF subunits are able to inhibit deneddylation to varying degrees. CSN interferes with SCF-mediated ubiquitination through a noncatalytic mechanism.
Conclusion: Deneddylation of Cul1 by CSN is regulated by F-box protein, substrate, and other factors.
Significance: Our work reported here could facilitate the development of directed therapies.

COP9 signalosome (CSN) mediates deconjugation of the ubiquitin-like protein Nedd8 from the cullin subunits of SCF and other cullin-RING ubiquitin ligases (CRLs). This process is essential to maintain the proper activity of CRLs in cells. Here, we report a detailed kinetic characterization of CSN-mediated deconjugation of Nedd8 from SCF. CSN is an efficient enzyme, with a k_{cat} of $\sim$1 s^{-1} and K_m for neddylated Cul1-Rbx1 of $\sim$200 nM, yielding a k_{cat}/K_m near the anticipated diffusion-controlled limit. Assembly with an F-box-Skp1 complex markedly inhibited deneddylation, although the magnitude varied considerably, with Fbw7-Skp1 inhibiting by $\sim$5-fold but Skp2-Cks1-Skp1 by only $\sim$15%. Deneddylation of both SCF^{Fbw7} and $SCF^{Skp2\text{-}Cks1}$ was further inhibited $\sim$2.5-fold by the addition of substrate. Combined, the inhibition by Fbw7-Skp1 plus its substrate cyclin E was greater than 10-fold. Unexpectedly, our results also uncover significant product inhibition by deconjugated Cul1, which results from the ability of Cul1 to bind tightly to CSN. Reciprocally, CSN inhibits the ubiquitin ligase activity of deneddylated Cul1. We propose a model in which assembled CRL complexes engaged with substrate are normally refractory to deneddylation. Upon consumption of substrate and subsequent deneddylation, CSN can remain stably bound to the CRL and hold it in low state of reduced activity.

Cullin-RING ubiquitin ligases (CRLs)[4] are heteromeric enzymes comprising cullin, RING domain, and substrate receptor subunits (1, 2). The cullin subunit serves as the backbone of the enzyme, displaying on one end a substrate receptor complex that recruits substrates for ubiquitylation and on the other end a RING domain subunit (Rbx1/Roc1/Hrt1) that recruits a ubiquitin-conjugating enzyme that transfers ubiquitin to substrate.

CRLs comprise a family of up to 240 enzymes that exert a profound effect on eukaryotic cells and organisms. CRLs, in aggregate, appear to account for $\sim$20% of total protein degradation by the ubiquitin-proteasome system (3) and have been implicated in myriad processes that underlie normal development and physiology (4). CRLs and the pathways they control are also prominent targets of mutation in human diseases. For example, the most common genetic defect observed in colon cancer is of the APC pathway, which mediates degradation of β-catenin via the CRL known as $SCF^{\beta\text{-}TrCP}$ (5). Likewise, the CRL subunit Fbw7/Cdc4 is a prominent human tumor suppressor gene (6). Given their central roles in controlling numerous cellular processes, there is considerable impetus to understand how CRLs work and how they are regulated. Although much of the regulation in CRL pathways is focused on the substrates, the CRLs themselves are well documented to be regulated by conjugation of the cullin subunit with the ubiquitin-like protein, Nedd8 (7, 8).

The COP9 signalosome is an 8-subunit complex that was originally discovered based on its role in controlling light-regulated development in *Arabidopsis thaliana* (9). Insight into the molecular basis of CSN action began to emerge with the finding that in human cells, CSN forms a stable complex with a particular subfamily of CRLs known as SCF ubiquitin ligases (10). A similar interaction was also detected in *Arabidopsis* (11). Importantly, CSN was shown to control Nedd8 modification of the Cul1 subunit of SCF (10). Whereas $\sim$10–20% of Cul1 is typically modified with Nedd8 in wild type *Schizosaccharomyces pombe*, in CSN-deficient cells the entire population of Cul1 is neddylated. The reason for this is that CSN-deficient cells lack an enzymatic activity that deconjugates Nedd8 from Cul1 (*i.e.*, deneddylates). Subsequently, it was shown that this enzymatic activity resides within the Csn5 subunit of CSN and is specified by a novel metalloenzyme motif referred to as the

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[4] The abbreviations used are: CRL, cullin-RING ubiquitin ligase; CSN, COP9 signalosome; SCF, Skp1-Cul1-F-Box.

JAMM domain (12). It is now widely appreciated that CSN mediates deneddylation of all cullins and appears to be the only enzyme capable of doing so with good efficiency (7, 8).

In vitro, CSN inhibits CRL activity (13). This can be readily understood, because conjugation of Nedd8 to Cul1 stimulates the activity of SCF (14–17). However, multiple lines of genetic evidence indicate that CSN is required to sustain CRL activity in cells (7). This apparent paradox is resolved by the observation that CSN-mediated inactivation of CRLs counteracts autocatalytic breakdown of substrate receptor subunits (18–20). Thus, the principal physiological function of CSN appears to be to sustain optimal levels of CRL activity.

Several lines of evidence point toward CSN playing an important role in human cancer and potentially being a novel molecular target for cancer therapy. The catalytic subunit of CSN, Csn5, is overproduced in many human cancers, and its overproduction often correlates with poor survival (21). Interestingly, the coding region for Csn5 is co-amplified along with that for c-Myc in some aggressive human breast cancers (22). Simultaneous overexpression of the two proteins synergistically activates c-Myc target genes, implicating Csn5 as a positive regulator of c-Myc. Moreover, expression of a catalytically inactive mutant of Csn5 greatly attenuates growth of a c-Myc-driven tumor in mice (23). Likewise, knockdown of Csn5 suppresses growth of hepatocellular carcinoma in mice (24). Finally, four subunits of CSN were shown to be required for the growth of human colon cancer cells that express GTPase mutant K-Ras, but not of isogenic cells in which the oncogenic *KRAS* allele was deleted (25). Taken together, these results suggest that CSN promotes cancer and nominate Csn5 as a target for cancer therapy.

To successfully target CSN in cancer will require a better understanding of how CSN works in cells, which in turn will require a better quantitative understanding of the biochemical properties of CSN. Although it has been known for 10 years that CSN catalyzes the removal of Nedd8 from Cul1, the quantitative parameters for this reaction have yet to be described, because of the complexity of both the enzyme and its substrate. Here, we establish enzyme and substrate preparations and quantitative biochemical assays that allowed us to measure the steady-state kinetic parameters for substrate deneddylation by CSN. Our most important finding is that different F-box proteins can directly inhibit deneddylation of their Cul1 partner to variable extents, and this inhibition is potentiated upon binding of substrate. We also find that CSN has unusually high affinity for its reaction product, unmodified Cul1-Rbx1. Finally, we document that CSN can also inhibit the ubiquitin ligase activity of purified, unmodified SCF, pointing to a new and unexpected role of CSN as a stoichiometric inhibitor of the activity of deneddylated CRLs.

EXPERIMENTAL PROCEDURES

Purification of CSN—293F23V5 cells (gift from Z. Q. Pan, Mount Sinai School of Medicine, New York) stably expressing FLAG-Csn2 and Csn3-V5 were adapted to grow under suspension conditions in FreeStyle 293 expression medium (Invitrogen). When an approximate cell density of 5×10^5 cells/ml was reached in a culture volume of 500 ml, the cells were pelleted, washed once in cold PBS, pelleted again, and flash frozen in liquid N_2. CSN was affinity-purified using anti-FLAG M2 resin (Sigma) as previously described (26), except binding occurred over a 2-h period, 1 mM EDTA was used, EGTA was omitted, and Complete Mini EDTA-free Protease Inhibitor Mixture (Roche Applied Science) was used instead of individually adding different inhibitors. Following affinity purification, CSN was concentrated and immediately loaded onto a Superdex 200 gel filtration column equilibrated with 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 10% glycerol (storage buffer). Fractions containing CSN were pooled, concentrated with an Amicon Ultra-4 30-kDa molecular mass cut-off (Millipore), and aliquoted for storage at −80 °C.

Purification of Nedd8—The artificial gene His8-PKA-Nedd8 was designed to aid in the purification of *in vitro* neddylated Cul1 and to be radiolabeled with ^{32}P for quantitative enzymatic analysis. The gene encoding the amino acid sequence MHHHHHHHHRRGSLMLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQMNDEKTAADYKILGGSVLHLVLALRGG was purchased from DNA2.0 and put into their expression plasmid pJexpress414. Expression was in BL21 (DE3) bacterial cells under ampicillin selection. 1-liter cultures were grown at 37 °C until an A_{600} of ~1.0 was reached. The temperature was dropped to 16 °C before the addition of isopropyl β-D-1-thiogalactopyranoside. Induction was carried out at 16 °C overnight. Bacterial pellets were lysed by sonication in a buffer containing 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM dithiothreitol, and Complete Mini EDTA-free protease inhibitor mixture (Roche Applied Science). The lysates were clarified by centrifugation and incubated with 1.5 ml of nickel beads (Qiagen) per pellet from a 1-liter culture. Binding to resin was allowed to occur for 2 h at 4 °C. The resin was washed two times with 50 column volumes of lysis buffer, followed by elution with 25 mM Tris, pH 7.5, 100 mM NaCl, and 200 mM imidazole. This mixture was then loaded onto a Superdex 75 gel filtration column equilibrated in storage buffer composed of 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 10% glycerol. Fractions with His8-PKA-Nedd8 were concentrated, and the purified protein was frozen at −80 °C.

Expression and Purification of Recombinant Proteins—All proteins as outlined in supplemental Table S3 were expressed in either *Escherichia coli* or Hi5 insect cells and purified using standard procedures. Ubxd7 was a gift from W. den Besten. Dcn1 was a gift from B. Schulman (St. Jude, Memphis, TN). CSN expressed and purified from *E. coli* was a gift from N. Zheng (University of Washington, Seattle, WA). Phosphorylated Cyclin E-Cdk2 was a gift from B. Larimore (Clurman laboratory, University of Washington, Seattle, WA).

In Vitro Neddylation Reaction—Cul1-Rbx1 was neddylated with His8-PKA-Nedd8 (HPN8) using conditions previously described (14), with the exception that His8-PKA-Nedd8 was used at a concentration of 40 μM. Cul1-Rbx1 conjugated with HPN8 was purified by standard column chromatography techniques, using first S-Sepharose, followed by binding to nickel affinity matrix and then Superdex 200 gel filtration. For the experiments shown in supplemental Fig. S1 (*C* and *D*), neddylation reactions were terminated by the addition of 5 μM

MLN4924 and then diluted directly into a deneddylation reaction.

In Vitro Deneddylation Reaction—Deneddylation reactions in Figs. 2*E* and 3 were carried out with 0.8 nM CSN in a 1× deneddylation buffer containing 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 5% glycerol, and 1 ng/μl ovalbumin. Deneddylation reactions in Fig. 2*A* and supplemental Figs. S1, *C* and *D*, and S3) were carried out with 1 nM CSN in deneddylation buffer containing 25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 1% glycerol, 1 ng/μl ovalbumin. Deneddylation reactions in Fig. 2 (*B–D*) were carried out with 1 nM CSN in deneddylation buffer containing 25 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1% glycerol, 15 ng/μl ovalbumin 25 mM trehalose. See figure legends for the time of reaction and the concentrations of the reaction components. All of the reactions were performed at room temperature (23–25 °C). Deneddylation reactions were quenched with reducing SDS-PAGE buffer and separated by SDS-PAGE on 16% gels. The gels were dried and exposed to a phosphor screen for analysis. Quantification was performed with ImageQuant (GE Healthcare) and plotted using GraphPad Prism. All of the values reported are the averages of at least two independent experiments. Rates of deneddylation were calculated by dividing the signal for free ^{32}P-labeled HPN8 by the summed signals for ^{32}P-labeled HPN8-Cul1 and free HPN8 to obtain the percentage of deneddylation. The amount of free Nedd8 formed was then calculated as: (% deneddylation) * (concentration of input HPN8-Cul1-Rbx1)/100. For the experiments shown in Fig. 2 (*A–D*), initial rates were calculated excluding the first 10 s, because of the apparent biphasic nature of some of the reactions, which we ascribe to a small fraction of Cul1 that did not assemble with F-box-Skp1 or substrate and was therefore deneddylated more rapidly.

Ubiquitylation Reaction—All of the ubiquitylation reactions were carried out as previously described (15) in a reaction buffer composed of 25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM dithiothreitol. All of the reactions were performed at room temperature (23 °C). See figure legends for the time of reaction and the concentrations of the reaction components. Ubiquitylation reactions were quenched with reducing SDS-PAGE buffer and separated on 16% Tris-glycine SDS-PAGE gels. The gels were dried and exposed to a phosphor screen for analysis. Quantification was performed with ImageQuant (GE Healthcare) and plotted using GraphPad Prism. All of the values reported are the averages of at least two independent experiments, except supplemental Fig. S4.

Cell Culture, Immunoprecipitation, and Western Blot—HEK293T cells were obtained from ATCC and grown under standard cell culture conditions in DMEM supplemented with 10% FBS. For transfection, Lipofectamine2000 was used according to the manufacturer's instructions (Invitrogen). A description of the plasmids used in this study for transfection and immunoprecipitation is provided in supplemental Table S4. Twenty-four hours post-transfection, the cells were rinsed in cold phosphate-buffered saline and lysed in 400 μM cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.1% Nonidet P-40, Complete Mini EDTA-free protease inhibitor mixture (Roche Applied Science)). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4 °C, followed by incubation with either anti-HA (Covance), anti-M2 FLAG (Sigma), or anti-Myc (Covance) and 30 μl of Tris-Acryl protein A (Pierce) at 4 °C for 2 h with rotation. Resins were washed four times with lysis buffer and resuspended in SDS sample buffer. The proteins bound to resin were resolved by SDS-PAGE on a 7, 10 or 12.5% gel. Immunodetection was performed with antisera to Cul1 and Skp1 (Invitrogen), Cand1 (Calbiochem), Cks1 (Invitrogen), Csn5 (Santa Cruz), Myc (Covance), and Rbx1/Roc1 (BIOSOURCE).

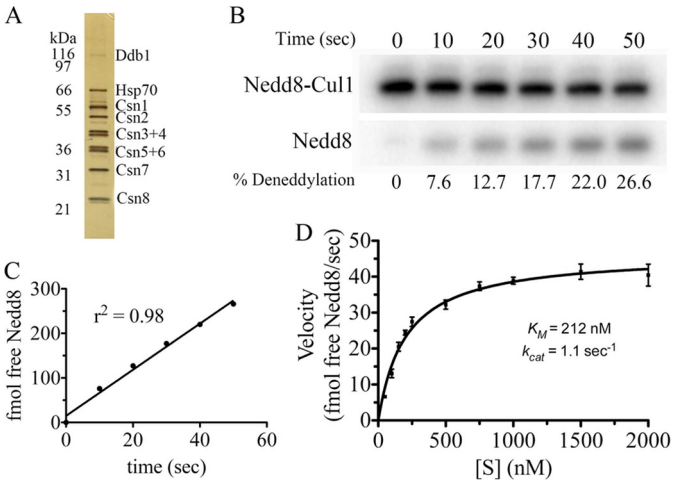

FIGURE 1. **Characterization of *in vitro* deneddylation assay components and enzymatic properties of human CSN.** *A*, purified CSN from HEK293 cells was fractionated by SDS-PAGE and analyzed by silver staining to check for purity and stoichiometry of enzyme subunits. *B*, purified Cul1-Rbx1 (50 nM) conjugated with ^{32}P-labeled HPN8 was incubated with 1 nM CSN in a total reaction volume of 50 μl. At the indicated time points, the aliquots were quenched and evaluated by SDS-PAGE followed by autoradiography. *C*, PhosphorImager quantification of *B*. *D*, the initial rate of deneddylation by 0.8 nM CSN at different concentrations of substrate is plotted. K_m and k_{cat} were estimated by fitting the curve to the Michaelis-Menten equation.

RESULTS

We set out to characterize in depth the deneddylating activity of CSN by developing an *in vitro* assay in which the conversion of neddylated Cul1 to Cul1 could be quantitatively measured. CSN was purified from HEK293 cells that stably express the Csn2 subunit modified with an N-terminal FLAG tag (27). CSN recovered from an anti-FLAG affinity column was further enriched by gel filtration prior to being used for the experiments described here. All eight subunits were found to be present in the purified material, in apparently stoichiometric amounts (Fig. 1*A*). Mass spectrometry analysis of purified CSN identified two co-fractionating proteins (Ddb1 and Hsp70) that could not be totally removed by conventional methods such as high salt or treatment with ATP and Mg^{2+} (data not shown).

To develop a quantitative, multiturnover assay for deneddylation, we first sought to generate a labeled form of Nedd8. To this end, we fused a sequence encoding eight histidines followed by a protein kinase A phosphorylation site to the N terminus of Nedd8 to generate His-PKA-Nedd8 (HPN8). When Cul1 was neddylated with HPN8 (HPN8-Cul1) and subsequently purified by gel filtration and nickel-nitrilotriacetic acid chromatography, the resulting CSN substrate was 98% neddylated as determined by SDS-PAGE (supplemental Fig. S1, *A* and *B*). After radioisotope labeling with [^{32}P]ATP and cAMP-de-

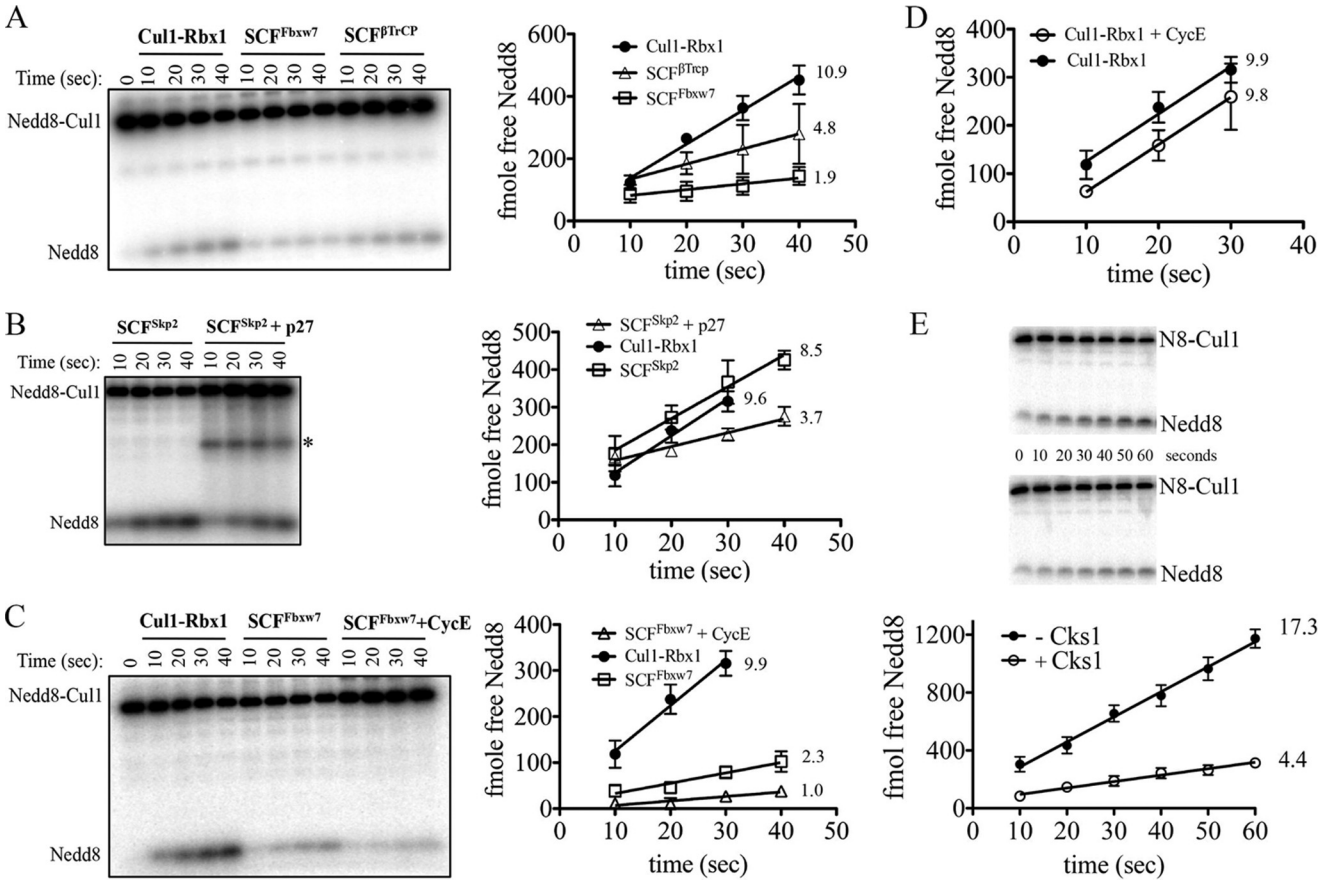

FIGURE 2. **F-box-Skp1 and substrate inhibit deneddylation by CSN.** *A*, purified Cul1-Rbx1 (500 nM) conjugated with ^{32}P-labeled HPN8 was preincubated for 10 min with 600 nM of Fbw7-Skp1 or β-TrCP-Skp1, followed by the addition of 1 nM CSN. At the indicated time points, aliquots were quenched and evaluated by SDS-PAGE (*left panel*) followed by PhosphorImager quantification (*right panel*). The rates (fmol of Nedd8 released from Cul1/s) are indicated to the right of each curve. The total reaction volume was 25 μl. *B*, same as *A*, except ^{32}P-labeled HPN8-Cul1-Rbx1 substrate at 50 nM was mock incubated or preincubated with 100 nM Skp1-Skp2-Cks1 in the presence or absence of 1 μM phospho-p27-cyclin E-Cdk2 (p27). Total reaction volume was 50 μl. The *asterisk* indicates [^{32}P] label incorporated into p27. *C*, same as *A*, except the ^{32}P-labeled HPN8-Cul1-Rbx1 substrate was at 50 nM, Fbw7-Skp1 was at 100 nM, and phospho-cyclin E-Cdk2 (CycE) was at 500 nM. *D*, same as *C*, except that Fbw7-Skp1 was omitted. *E*, ^{32}P-labeled HPN8-Cul1-Rbx1 substrate at 100 nM was preincubated 5 min with 300 nM Skp2-Skp1 plus or minus Cks1. Following assembly of SCF^{Skp2} complexes, the reactions were supplemented with ubiquitylation components (1 μM ubiquitin, 400 nM E1, 100 nM Cdc34, plus or minus 500 nM phospho-p27-cyclin E-Cdk2), incubated for 10 min, supplemented with ATP and Mg^{2+} to initiate ubiquitylation, and incubated a further 20 min prior to addition of CSN (0.8 nM). The total reaction volume was 50 μl. At the indicated time points, aliquots were quenched and evaluated by SDS-PAGE followed by phosphorimaging (*top panel*). Quantification of the phosphorimaging scans is shown in the *bottom panel*.

pendent protein kinase, >97% of the total signal from Nedd8 was attached to Cul1 as determined by phosphorimaging (Fig. 1*B*; time = 0). When CSN (1 nM) was added to HPN8-Cul1 (50 nM) and the reaction progress analyzed, deneddylation proceeded at a linear rate (Fig. 1, *B* and *C*).

Prior to performing kinetic analyses, we carried out a series of control experiments. First we confirmed that the phosphorylated His-PKA tag had little to no effect on the rate of deneddylation of Cul1 (supplemental Fig. S1*C*). Second, we compared rates of deneddylation by our FLAG-tagged CSN prepared from 293 cells with untagged CSN expressed in insect cells from recombinant baculoviruses (supplemental Fig. S1*D*) and untagged CSN expressed in *E. coli* (28) (supplemental Fig. S1*E*). The FLAG tag had no discernable effect on the activity of CSN isolated from eukaryotic cells. Meanwhile, the enzyme purified from *E. coli* was ~2-fold less active, possibly because of removal of terminal sequences from some subunits to facilitate expression.

To obtain enzymological metrics for CSN-mediated deneddylation, we evaluated reaction rate as a function of substrate concentration. CSN was held constant at 0.8 nM, and the initial rates of deneddylation at varying concentrations of HPN8-Cul1-Rbx1 were determined (supplemental Fig. S1*F*). The rates for each concentration of substrate were plotted and fitted to the Michaelis-Menten equation, which yielded a K_m of 212 nM and a k_{cat} of 1.1 s^{-1} (Fig. 1*D*) supplemental Table S1.

Within cells, the fraction of Cul1 that is modified by Nedd8 is higher for Cul1 bound to F-box proteins (16, 17, 29). We reasoned that this might arise from differential rates of deneddylation of Cul1, depending upon its assembly status. To test this possibility, we compared rates of deneddylation for HPN8-Cul1-Rbx1 *versus* three different HPN8-conjugated SCF holoenzyme complexes. Deneddylation of HPN8-SCF^{Fbw7} and HPN8-SCF$^{b\text{-}TrCP}$ was assayed at 500 nM. Strikingly, Fbw7-Skp1 had a major effect, reducing the rate of deneddylation by 5.8-fold (Fig. 2*A*). β-TrCP-Skp1 had a weaker effect, reducing the rate by 2.2-fold. However, the recombinant β-TrCP used for this experiment lacks the N-terminal 138 amino acids, which were removed to facilitate efficient expression (30). Interestingly, not all F-box proteins were inhibitory. Addition of Skp2-

Cks1-Skp1 reduced the rate of deneddylation by <20% (Fig. 2*B*). We do not understand the basis for this difference in behavior but note that endogenous Cul1 co-precipitated with transiently expressed F-box proteins was ≥50% neddylated in β-TrCP and Fbw7 immunoprecipitates but considerably less modified in Skp2 immunoprecipitates (supplemental Fig. S2, *A* and *B*, and data not shown).

In other work we have shown that a mutant of Skp1 (Skp1ΔΔ) used for crystallography (31) that lacks two acidic internal loops was able to bind Cul1-Rbx1 and assemble an active SCF complex but was unable to promote displacement of Cand1 from Cul1-Rbx1 (32).[5] Because Fbw7-Skp1 had the most potent effect on deneddylation, we tested the impact of the Skp1 loops in this context. Interestingly, the loop deletions reduced the inhibitory effect of Fbw7-Skp1 by ~2.5-fold (supplemental Fig. S3). However, the acidic loops of Skp1 were not sufficient to specify inhibition of deneddylation, because our Skp2-Cks1-Skp1 contained wild type Skp1.

Given the substantial effect of Fbw7-Skp1 on deneddylation, we next sought to test whether binding of substrate to Fbw7 might further influence deneddylation of the associated HPN8-Cul1. There is good reason to think this might be the case; Cul1 co-precipitated from cells with SCF substrates is essentially 100% neddylated (16), implying that substrate might either increase the rate of neddylation or decrease the rate of deneddylation above and beyond the effect of the F-box protein. Consistent with this possibility, the addition of the Skp2-Cks1 substrate $p27^{kip1}$ to $SCF^{Skp2\text{-}Cks1}$ complexes in fractionated cell lysate decreases Cul1 deneddylation (33). However, because this experiment was carried out with undefined protein fractions, a clear explanation for this phenomenon remains lacking.

To test the effect of substrate in a defined system, we compared the rate of deneddylation of HPN8-SCF^{Fbw7} in the presence and absence of full-length phospho-cyclin E bound to Cdk2. Cyclin E must be phosphorylated on at least two sites (T-380 and S-384) to serve as a substrate for SCF^{Fbw7} (34). The addition of phospho-cyclin E-Cdk2 further reduced the rate of deneddylation of HPN8-SCF^{Fbw7} by ~2.5-fold (Fig. 2*C*). The effect of substrate was specific, because phospho-cyclin E-Cdk2 had no effect on the rate of deneddylation of HPN8-Cul1 in the absence of Fbw7-Skp1 (Fig. 2*D*). Together, Fbw7 plus phospho-cyclin E reduced deneddylation by >10-fold. Note that this experiment was done with 50 nM substrate, in contrast to Fig. 2*A*, which was done with 500 nM. Thus, the inhibitory effect of Fbw7-Skp1 on deneddylation of HPN8-Cul1 was similar when CSN was either subsaturated or nearly saturated with substrate. To determine whether the effect of substrate applies to other SCF complexes, we tested the effect of phospho-p27-cyclin E-Cdk2 substrate on HPN8-$SCF^{Skp2\text{-}Cks1}$. The addition of substrate reduced the rate of deneddylation by ~2.3-fold (Fig. 2*B*), similar to what was seen in Fig. 2*C*. A similar magnitude of substrate-mediated inhibition was observed when SCF^{Skp2} complexes were assayed in the presence of phospho-p27-cyclin E-Cdk2 under conditions that were permissive (+Cks1) or not permissive (−Cks1) for substrate binding and ubiquitylation (Fig. 2*E*). Taken together, these results imply that substrate reduced the rate of deneddylation equivalently regardless of whether or not it was undergoing ubiquitylation.

Cullin deneddylation *in vivo* must occur in the presence of a substantial concentration of unmodified cullins as well as a large constellation of factors that bind cullins, any one of which might have an impact on the rate of deneddylation. To address this issue, we evaluated deneddylation of HPN8-Cul1-Rbx1 in the presence of different recombinant proteins purified from *E. coli* and added at a fixed concentration of 1 μM. Neddylated substrate was mixed with each potential regulator and allowed to interact for 5 min at room temperature before CSN was added, and the reaction progress was monitored. Interestingly, every single factor that was tested reduced the rate of deconjugation of HPN8 from substrate. The factors tested fell into two categories based on their ability to repress CSN deneddylase activity: moderate inhibitors (Ubc12, Dcn1, UbcH5C, and Nedd8), which repressed deneddylation by 2.2–4.8-fold, and strong inhibitors (Cul1, Ubxd7, Cdc34, and Cand1), which repressed deneddylation between 7.2- and 14.4-fold (Fig. 3*A*). Supplemental Table S2 contains initial rates of deneddylation in the presence of each factor tested. Based on these results, we pursued in more detail the inhibition of deneddylation by Cand1 and unmodified Cul1-Rbx1.

Cand1 was previously reported to interact exclusively with unmodified cullins, including Cul1-Rbx1 (35, 36). Consistent with this, the co-crystal structure of Cul1-Rbx1-Cand1 showed that the Nedd8 conjugation site of Cul1 is partially obscured by Cand1 (32). Moreover, the crystal structure of Nedd8 conjugated to the C-terminal domain of Cul5 showed how a Nedd8-induced conformational change blocks binding of the N-terminal domain of Cand1 (14). Therefore, we were surprised to find that Cand1 was a potent inhibitor of deneddylation (Fig. 3*A*). We investigated this property further in our *in vitro* deneddylation assay by keeping the concentrations of HPN8-Cul1-Rbx1 substrate (150 nM) and CSN enzyme (0.8 nM) constant and varying the concentration of Cand1. This experiment yielded an apparent inhibition constant of 160 nM (Fig. 3*B*). Two lines of evidence suggest that Cand1 inhibited deneddylation by binding substrate: the addition of Cand1 increased the K_m but did not affect the k_{cat} for deneddylation of HPN8-Cul1 (Fig. 3*C*), and Cand1 exhibited no effect on the initial rate of deneddylation of HPN8-$SCF^{Skp2\text{-}Cks1}$ (Fig. 3*D*). These data suggest that binding of the C-terminal domain of Cand1 to the N-terminal domain of Cul1 (which is blocked by Skp1-Skp2-Cks1) interferes with recruitment of CSN. Notably, in *Aspergillus*, Cand1 is naturally split into two polypeptides, and the polypeptide corresponding to the C-terminal portion of human Cand1 can bind Cul1 in the absence of the N-terminal portion (37).

CSN was also strongly inhibited by its reaction product, unmodified Cul1-Rbx1 (Fig. 3*A*). We determined the IC_{50} for Cul1 to be 260 nM when assayed at 50 nM substrate (Fig. 3*E*). This suggests that the Nedd8 modification must not confer a large amount of affinity for CSN, which is consistent with our observation that free Nedd8 was a weak inhibitor of deneddylation (Fig. 3*A*). The surprisingly strong apparent affinity of unmodified Cul1-Rbx1 product for CSN is consistent with our original discovery that CSN associates with a mutant Cul1 that

[5] N. Pierce and R. J. Deshaies, unpublished data.

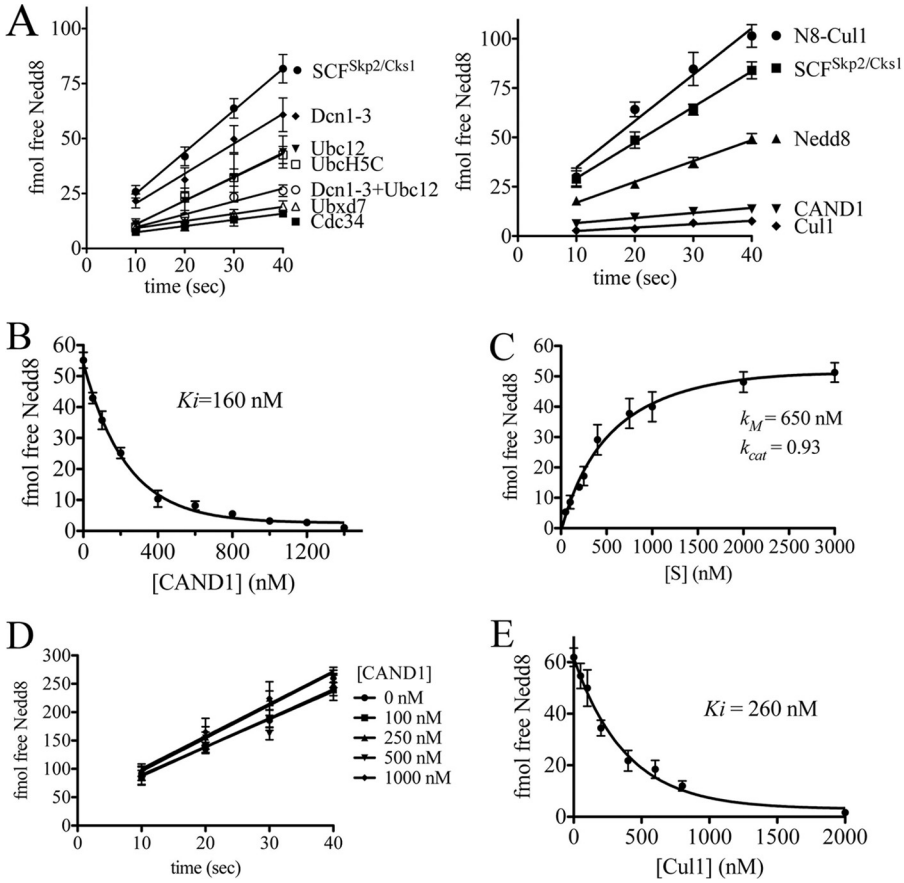

FIGURE 3. **Proteins that bind the C-terminal domain of Cul1 inhibit deneddylation.** *A*, ^{32}P-labeled HPN8-Cul1-Rbx1 (25 nM) was incubated for 10 min with 1 μM of the indicated factor prior to addition of 0.8 nM CSN. At the indicated time points, the aliquots were quenched and evaluated by SDS-PAGE followed by PhosphorImager quantification. The total reaction volume was 40 μl. *B*, same as *A*, except that substrate was 150 nM and was preincubated with the indicated final concentration of Cand1 prior to adding CSN. *C*, same as *B*, except that Cand1 (250 nM) and CSN (0.8 nM) were held constant, whereas the concentration of substrate was varied. The data were fitted to the Michaelis-Menten equation to estimate k_{cat} and K_m. *D*, ^{32}P-labeled HPN8-Cul1-Rbx1 (50 nM) was preincubated with 200 nM Skp1-Skp2-Cks1 for 10 min prior to addition of the indicated final concentrations of Cand1. Following a further 10 min of precincubation, CSN (0.8 nM) was added. At the indicated time points, the aliquots were quenched and evaluated by SDS-PAGE followed by PhosphorImager quantification. *E*, same as *B*, except that substrate was 50 nM and Cul1-Rbx1 was titrated.

lacks the extreme C terminus including the Nedd8 conjugation site (10) and raises the possibility that product dissociation might be rate-limiting for substrate deneddylation.

To conclusively demonstrate that CSN can form a stable interaction with unmodified SCF, we mixed purified CSN with purified SCF$^{Skp2\text{-}Cks1}$ and fractionated the mixture on a Superdex 200 size exclusion column. SCF$^{Skp2\text{-}Cks1}$ that was not mixed with CSN was used for comparison purposes. In the presence of CSN, a fraction of the SCF$^{Skp2\text{-}Cks1}$ molecules was shifted to higher molecular weight fractions, corresponding to fractions that contained CSN (Fig. 4*A*).

To test whether unmodified full-length Cul1 (*i.e.*, the product of deneddylation) exhibits significant binding to CSN in cells, we transiently transfected HA epitope-tagged wild type and K720R Cul1 expression constructs into HEK293 cells. K720R lacks the site on Cul1 to which Nedd8 is conjugated. Twenty-four hours post-transfection, the cells were lysed, and the ^{HA}Cul1 was immunoprecipitated with HA antibody. The immunoprecipitates were then immunoblotted with antibodies to detect ^{HA}Cul1 as well as the endogenous Skp1, Rbx1, Csn5, and Cand1 proteins (Fig. 4*B*). Whereas wild type and K720R ^{HA}Cul1 were expressed at similar levels and bound similar amounts of Rbx1 and Skp1, the K720R mutant actually retrieved more Csn5 than wild type. By comparison, a recent proteomic study reported equivalent association of CSN with wild type and K720R-Cul1 in HEK293T cells (38). As independent confirmation of this result, we examined the interaction of endogenous Cul1 with transiently expressed FLAG-tagged Csn5. Cul1 co-immunoprecipitated with ^{FLAG}Csn5 was exclusively in the unconjugated form (Fig. 4*C*). Even if deconjugation occurred within the CSN-SCF complex *in vitro*, this result emphasizes the point that unlike traditional enzymes, CSN did not rapidly let go of its substrate upon deconjugating it. To more directly compare the association of CSN with neddylated and unmodified Cul1, we repeated this experiment with ^{FLAG}Csn5-ASA, which is mutated for two of the histidine residues that play a critical role in forming the active site that mediates deneddylation (12, 39). Although most of the endogenous Cul1 that co-immunoprecipitated with ^{FLAG}Csn5-ASA was modified with Nedd8, a substantial pool of unmodified Cul1 was recovered, confirming that unmodified cullin substrate can associate stably with CSN. Finally, to explore structure-activity relationships in greater depth, we also evaluated binding of endogenous CSN to ^{HA}Cul1 variants that could

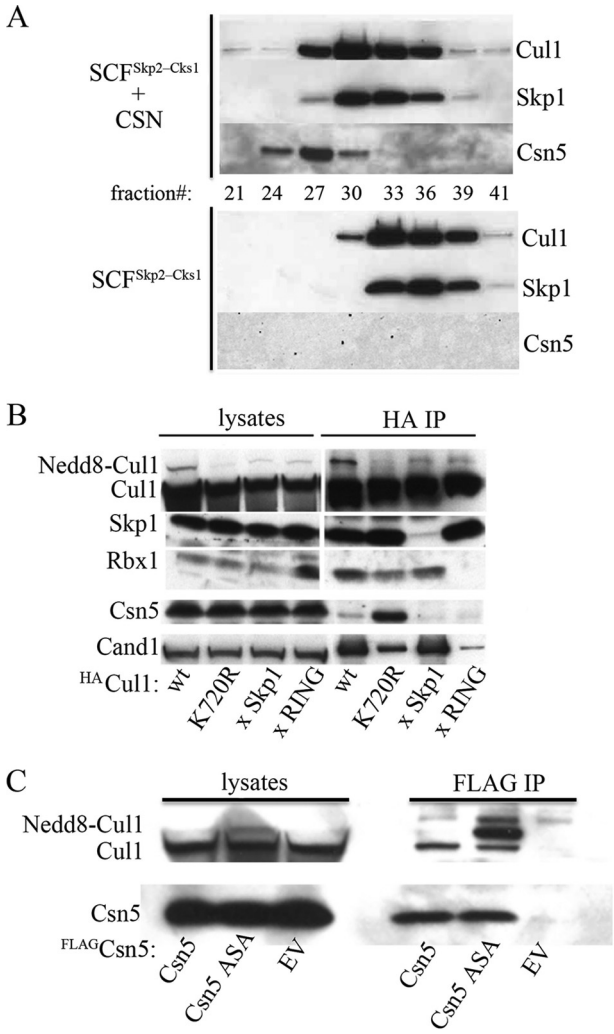

FIGURE 4. **CSN forms a stable complex with both neddylated and unmodified Cul1.** *A*, purified $SCF^{Skp2-Cks1}$ (600 nM) was incubated for 15 min in either the presence (*top panel*) or the absence (*bottom panel*) of 300 nM purified CSN. Complexes were passed through a Sephadex 200 size exclusion column, and every third fraction was separated by SDS-PAGE and Western blotted with antisera to the indicated proteins. *B*, the indicated HA-tagged Cul1 constructs were transfected into HEK293 cells. Twenty-four hours post-transfection, the lysates were generated, and ^{HA}Cul1 was immunoprecipitated with anti-HA antibody. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with antisera to the indicated proteins. Cul1 was detected with anti-HA. *xRING* and *xSkp1* refer to point mutants of Cul1 that were deficient in binding Rbx1 and Skp1, respectively. K720R has an arginine . substitution at the Nedd8 conjugation site (lysine 720). *C*, the indicated FLAG-tagged Csn5 constructs were transfected into HEK293 cells. Twenty-four hours post-transfection, the lysates were generated, and ^{FLAG}Csn5 was immunoprecipitated (*IP*) with anti-FLAG antibody. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with antisera to Csn5 and Cul1 as indicated. *EV* refers to empty vector. *ASA* refers to a double point mutation that inactivates the JAMM domain of Csn5.

not bind to Skp1 and Rbx1. These mutants were generated by using the x-ray crystal structure of SCF as a guide (40). Both of these mutants bound less Csn5 (Fig. 4*B*). The failure of the RING-deficient mutant to bind CSN is consistent with the original finding that Csn2 binds Rbx1 in a yeast two-hybrid assay (10). Taken together, our data suggest that CSN exhibits a complex mode of interaction with Cul1 that is not dependent on Nedd8 and involves both ends of the elongated CRL complex.

Stable binding to substrate and/or product is unusual for an enzyme and suggested to us that CSN might regulate CRLs by mechanisms other than deconjugation of Nedd8. This possibility is further supported by our observation that multiple factors that interact with Cul1, including the E2 enzymes UbcH5C and Cdc34, inhibited deneddylation of HPN8-Cul1-Rbx1. We therefore set out to test whether CSN can inhibit SCF activity, independently of its effects on Nedd8 conjugation. An ubiquitylation reaction was set up that contained unmodified $SCF^{\beta TrCP}$ plus radiolabeled β-catenin peptide, ubiquitin, E1, and either UbcH5C or Cdc34. The ubiquitylation reaction was initiated by the addition of ATP and Mg^{2+} followed by the addition or omission of 300 nM CSN (which is slightly less than the estimated *in vivo* concentration of 500 nM in 293 cells) (38). The addition of CSN resulted in a 3.4–3.8-fold reduction in the rate of substrate conversion to products, independent of the E2 that was employed (Fig. 5). Inclusion of CSN affected both the extent of substrate conversion as well as the pattern of reaction products that were produced, indicating that CSN affected both ubiquitin chain initiation and elongation.

UbcH5 and Cdc34 catalyze SCF-dependent substrate ubiquitylation with K_m values that differ by approximately an order of magnitude (15). Their equivalent sensitivity to inhibition by CSN suggested that CSN might not compete with E2 for binding to unmodified SCF. Consistent with this, 300 nM CSN exerted a similar fold inhibition of ubiquitylation in reactions that contained either 1 or 10 μM Cdc34 (supplemental Fig. S4*A*). We also evaluated whether CSN might compete with substrate. Regardless of whether cyclin E peptide substrate was present at 0.1 or 1 μM, 300 nM CSN inhibited SCF^{Fbw7} to a similar degree (supplemental Fig. S4*B*).

DISCUSSION

CSN mediates deneddylation of all cullins *in vivo*. However, the quantitative kinetic parameters of CSN-mediated deneddylation have remained largely unknown, in part because of the complexity of the enzyme and its substrate. In this work, we developed reagents and methods that enabled us to measure quantitatively the deconjugation of radiolabeled Nedd8 from a purified Nedd8-Cul1-Rbx1 substrate. Here, we consider the implications of our quantitative measurements for the physiological function of CSN.

Affinity of CSN for Substrate and Product—Our studies reveal that CSN has high affinity (K_m = 212 nM) for Cul1 substrate. We do not know the K_D for the interaction, but given that k_{cat}/K_m is in the diffusion-limited range, it is possible that K_D is considerably lower than K_m. Contrary to what has been suggested elsewhere (42), we also show that deneddylated cullin binds tightly with CSN, although not quite as tightly as neddylated substrate. At least four lines of evidence support this claim: 1) CSN activity was significantly inhibited by unmodified Cul1-Rbx1, with an IC_{50} of 260 nM (Fig. 3*E*); 2) unmodified $SCF^{Skp2-Cks1}$ was shifted to higher molecular weight fractions on a Sephadex 200 size exclusion column in the presence of CSN (Fig. 4*A*); 3) Csn5 stably co-precipitated with a mutant of Cul1 that cannot be neddylated (Fig. 4*B*); and 4) deneddylated Cul1 co-precipitated with both wild type Csn5 and a mutant that lacks deneddylase activity (Fig. 4*C*). These data add

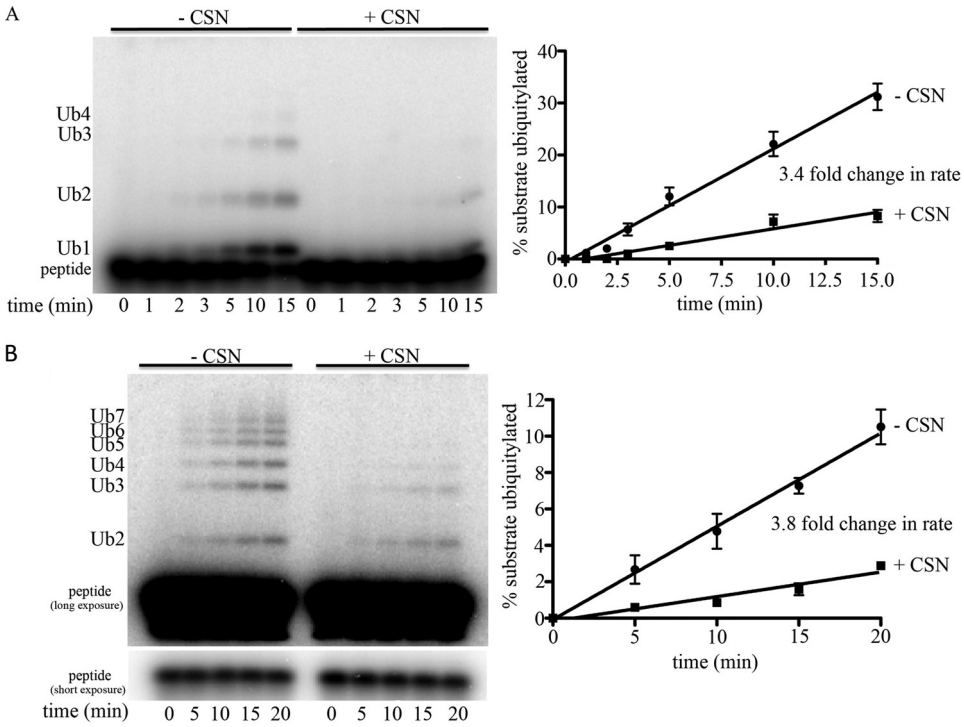

FIGURE 5. **CSN inhibits ubiquitylation by unmodified SCF.** *A*, $SCF^{\beta TrCP}$ (100 nM) and ubiquitylation components (1 μM ubiquitin, 400 nM E1, 100 nM UbcH5C, 600 nM ^{32}P-labeled-phospho-β catenin peptide) were incubated either in the presence or absence of 300 nM CSN for 10 min, after which ubiquitylation reactions were initiated by the addition of ATP and Mg^{2+}. Time points were harvested at the indicated times, fractionated by SDS-PAGE, and subjected to PhosphorImager quantification. *B*, same as *A*, except that 100 nM Cdc34 was used in place of UbcH5C.

to a considerable body of evidence that CSN binds tightly to its reaction product. Indeed, the role of CSN in CRL biology was first discovered based on its efficient co-immunoprecipitation with a C-terminal truncation mutant of Cul1 that lacks the lysine 720 to which Nedd8 is conjugated (10). In the recent quantitative proteomic study of Bennett *et al.* (38), they reported that even in the absence of Nedd8 conjugation (brought about by the addition of the Nedd8 conjugation inhibitor MLN4924 to cells), nearly 50% of Csn6 remains associated with cullins 1–5.

Given the surprisingly tight binding of unmodified Cul1 to CSN and the total relative concentrations of CSN (500 nM) and Cul1-Cul5 (~1250 nM) in cells (38), of which we estimate based on data shown in Ref. 39 that ~35% (~450 nM) is neddylated, it is plausible that the entire cellular pool of CSN is essentially saturated with neddylated and unmodified cullins. Indeed, based on the mole fraction of Csn6 that is bound to individual cullins, up to 60% of CSN remains associated with cullins following immunoprecipitation (38). It is a reasonable possibility that most of the remaining 40% of CSN was also bound to cullins in cells but dissociated during the preparation and washing of the immunoprecipitates. Thus, it is possible that in cells, dissociation of CSN from CRLs is rate-limiting for deneddylation.

Catalytic Rate of CSN—At saturating concentrations of Nedd8-modified Cul1 substrate, the maximal rate of CSN-mediated deneddylation is ~1 s^{-1}[11], Although reasonably fast, this k_{cat} is ~100-fold slower than the maximal rate of cleavage of the model substrate CbzGly-L-Phe by the zinc metalloprotease carboxypeptidase A (43). What is puzzling about the multiturnover rate of 1 s^{-1} is that it implies that the rate of product dissociation must be at least this fast. However, this is difficult to reconcile with the observations noted in the prior section that CSN binds to deneddylated CRLs with sufficient stability to survive gel filtration or immunoprecipitation. Resolution of this conundrum will require further experimentation.

A notable feature of the neddylation cycle is the rate at which it proceeds in cells. Application of the Nedd8 conjugation inhibitor MLN4924 to cells results in rapid loss of Nedd8 conjugates; although quantification was not reported, a conservative estimate is that 80% of neddylated cullins are consumed within 5 min (3). This represents a minimal rate for deneddylation, because earlier time points were not evaluated, and it takes a finite amount of time for the drug to penetrate cells and effect depletion of the pre-existing pool of Ubc12~Nedd8 thioesters. Regardless, this rate is easily achievable. At the estimated cellular concentrations of CSN and total Nedd8-conjugated cullins (500 and 450 nM, respectively) and the k_{cat}/K_m for deneddylation reported here (~5×10^6 M^{-1} s^{-1}), the pool of cullins could be extensively deneddylated within several seconds upon extinguishing Nedd8 conjugation activity.

Inhibition of Deneddylation by F-box Proteins, Substrates, and Other Cul1-binding Factors—A key finding of this manuscript is that binding of F-box-Skp1 complexes to Cul1 can substantially reduce the rate of deneddylation. Fbw7-Skp1 slowed deneddylation by ~5-fold, and this effect was increased to >10-fold upon the addition of phospho-cyclin E-Cdk2 substrate. Thus, in the environment of the cell where CSN is essentially saturated with neddylated and unmodified cullins, fully

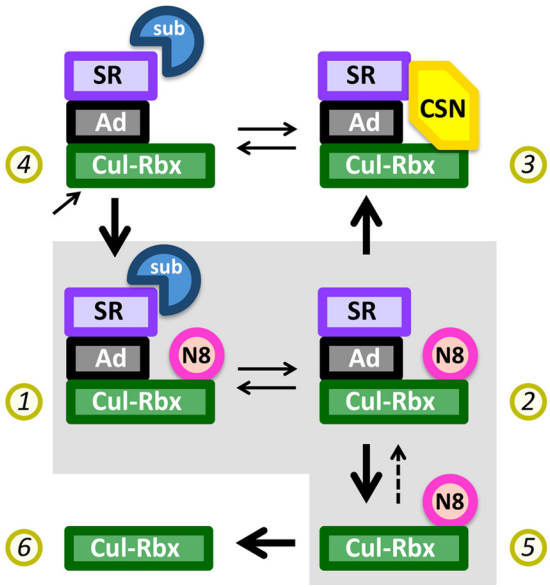

FIGURE 6. **Regulation of CRLs by reversible neddylation.** See text for details. Transitions marked by *single arrows* are vectorial. The intermediates at *steps 5* and *6* could re-form new CRL complexes by binding a different substrate receptor-adaptor module (*dashed* and *curved lines*, respectively).

assembled SCF complexes that are engaged with substrate are expected to be comparatively immune to the action of CSN. This has important implications for the regulation of SCF complex deactivation and disassembly. Accumulation of substrate for a particular CRL complex would be expected to lead to accumulation of the Nedd8-conjugated form of that complex (Fig. 6, *step 1*). Upon consumption of the substrate (Fig. 6, *step 2*), the CRL complex would either be subject to autoubiquitylation and degradation of the substrate receptor (Fig. 6, *step 5*) or recruit CSN and be deneddylated (Fig. 6, *step 3*). Reappearance of substrate would lead to displacement of CSN (Fig. 6, *step 4*) and re-formation of neddylated, active complex (Fig. 6, *step 1*). By this mechanism, CRL complexes for which substrate is present would be preferentially neddylated and activated. In the absence of CSN (Fig. 6, *gray zone*), this regulation would be undermined, and upon depletion of substrate for a particular CRL, the complex would remain conjugated to Nedd8 and active (Fig. 6, *step 2*), leading to autoubiquitylation and ultimately degradation of the substrate receptor and inactivation of the CRL (Fig. 6, *step 5*). We speculate that substrate adaptors that are most prone to become ubiquitinated in the absence of bound substrate will be the most sensitive to a loss of CSN function. Several important questions remain unanswered by this model. For example, how do F-box proteins and substrates inhibit deneddylation? Why does Fbw7-Skp1 but not Skp2-Cks1-Skp1 potently inhibit deneddylation? How does Cand1 fit into this cycle?

In addition to Fbw7-Skp1, which interacts with the N-terminal domain of Cul1, every factor that is known to contact Rbx1 and/or the C-terminal domain of Cul1 that was tested here (UbcH5c, Cdc34, Ubc12, Dcn1, Ubxd7, and Cand1) also inhibits deneddylation of Cul1 by CSN to some extent. We conclude that CSN is likely to make extensive contacts with Rbx1 and the C-terminal domain of Cul1. Our observation that Cand1 inhibits deneddylation is in direct conflict with a report to the contrary (44). We do not understand the reason for the discrepancy, but note that those authors measured deneddylation in a buffer that is completely lacking salt. We also draw attention to the inhibition of deneddylation by Ubxd7. Ubxd7 binds directly to the Nedd8 modification via an internal UIM domain (45, 46). Overexpression of Ubxd7 can cause accumulation of Nedd8-conjugated Cul2, suggesting that it can shield Cul2 from CSN *in vivo*.

CSN Inhibits CRL Activity by Catalytic and Noncatalytic Mechanisms—CSN initiates deactivation of a neddylated CRL complex by reversing the Nedd8 modification. The deconjugated CRL has severalfold less ubiquitin ligase activity (14–17). We suggest that in some instances, CSN remains stably bound to its product, and this reduces ubiquitin ligase activity even further. To investigate this possibility, we evaluated the effect of CSN on the basal activity of SCF that was not neddylated. Remarkably, 300 nM CSN (which is lower than its intracellular concentration) inhibited unmodified SCF by up to 3.5-fold. Equivalent inhibition of unmodified SCF occurred regardless of whether Cdc34 or UbcH5 was used as the E2 or whether Cdc34 was added at 0.1 (not shown), 1, or 10 μM. These observations suggest that the noncatalytic inhibitory activity of CSN was not competitive with respect to E2, even though E2 was able to inhibit deneddylation of HPN8-Cul1 by CSN. Likewise, CSN exhibited equivalent inhibition of SCF^{Fbw7} when assays were conducted with 0.1 or 1 μM cyclin E peptide substrate. We suggest that the E3 inhibition is a k_{cat} effect that arises because CSN restrains SCF in a low activity conformation. Regardless of the exact mechanism, noncatalytic regulation is likely to be physiologically relevant, because nearly 30% of Cul1 and >40% of Cul4B are stably bound to CSN in 293T cells (38). Indeed, genetic studies implicated fission yeast Csn1 and Csn2, but not Csn5, as being required for a specific function of CRL4 (47), and a recent publication reported that expression of catalytically inactive Csn5 partially restores proper CRL regulation in a Neurospora mutant that lacks Csn5 (48).

While this manuscript was being drafted, it was reported that CSN potently inhibits autoubiquitylation of substrate receptor subunits within $CRL4A^{DDB2}$ and $CRL4A^{CSA}$ complexes (49). Remarkably, this inhibition was relieved by the addition of a ligand for the substrate receptor. This suggests that binding of ligand/substrate to the CSN-CRL4A complex activates CRL4A by facilitating dissociation of CSN. Interestingly, $CRL4A^{CSA}$ efficiently polyubiquitinates its substrate CSB in the presence of CSN. Although this is in apparent conflict with our results, the kinetics of CSB ubiquitylation were not evaluated in the presence and absence of CSN, so it is possible that CSN retards ubiquitylation of CRL4A substrates much as it does for SCF as shown here.

Improvements in biochemical assay methods (15, 50–52) and mass spectrometry-based quantification (38, 53) have begun to give us a much clearer picture of the numerical parameters that govern the ubiquitin ligase activity of CRLs and the mechanisms that regulate this activity. Systematic pursuit of these approaches, coupled with structural biology and enzymology, promises to reveal a detailed picture of how these enzymes work, how their activities are controlled, and how these features relate to their physiological roles in cells. We

propose that this information will enable researchers to make good on the promise of basic science by developing novel medicines that target CRLs. Already, encouraging steps have been made in that direction (3, 41, 54, 55), and we can only hope, for the sake of patients, that there will be more to follow.

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