

CONTROL OF CULLIN-RING UBIQUITIN LIGASE ACTIVITY BY Nedd8

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Abstract: The Cullin-RING ubiquitin ligase (CRL) family, which may number as many as 350 different enzymes, has an enormous impact on cellular regulation. CRL enzymes regulate cell biology by conjugating ubiquitin onto target proteins that are involved in a multitude of processes. In most cases this leads to degradation of the target, but in some cases CRL-dependent ubiquitination acts as a switch to activate or repress target function. The ubiquitin ligase activity of CRLs is controlled by cycles of attachment and removal of the ubiquitin-like protein Nedd8. Conjugation of Nedd8 onto the cullin subunit of CRLs promotes assembly of an intact CRL complex and switches on ubiquitin ligase activity. Conversely, removal of Nedd8 switches off ubiquitin ligase activity and initiates CRL disassembly. Continuous maintenance of CRL function in vivo requires the activities of both the Nedd8-conjugating and deconjugating enzymes, pointing to a critical role of complex dynamics in CRL function. Here, we review how the Nedd8 cycle controls CRL activity and how perturbations of this cycle can lead to disease.

THE UBIQUITIN-PROTEASOME SYSTEM

Conjugation of ubiquitin to cellular proteins plays a key role in regulating many cellular and organismal processes.¹ Ubiquitin is covalently attached to target proteins via an isopeptide bond that links the C-terminus of ubiquitin to a lysine residue of the acceptor substrate.² Additional ubiquitins can be conjugated to any of the seven lysine residues of ubiquitin to form a polyubiquitin chain on the substrate. Assembly of a chain of ≥ 4 ubiquitins linked via the lysine-48 residue of the substrate-proximal ubiquitin (referred to as a Lys48-linked chain) marks cellular proteins for degradation by the 26S proteasome.³⁻⁴ Emerging evidence suggests that other linkages, including Lys11, can

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also target substrates for degradation by the proteasome.⁵ In contrast, monoubiquitination serves as a non-proteolytic signal in intracellular trafficking, DNA repair and signal transduction pathways.⁶

Ubiquitination of proteins is achieved through an enzymatic cascade that comprises ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes.^{2,7} E1 activates ubiquitin for transfer by adenylating its C-terminus and then forming a thioester linkage between the activated C-terminus of ubiquitin and an active site cysteine of E1. The activated ubiquitin is passed in a thioester linkage to the active site cysteine of an E2 enzyme. Substrate ubiquitination occurs when an E3 binds to both a molecule of substrate and an E2 thioesterified with ubiquitin (E2~Ub), bringing them into proximity with each other such that the ubiquitin can be transferred from the E2~Ub to the substrate. This transfer can either occur directly (i.e., with no intermediary) or via a covalent E3~ubiquitin thioester intermediate. The pairing of E2~Ub and substrates by E3s is the key determinant of substrate specificity in ubiquitination reactions. There are two major classes of E3s in eukaryotes, defined by the presence of either a HECT (Homologous to E6-AP C-terminus) domain or a RING (Really Interesting New Gene) fold.⁸ HECT-domain E3s contain a conserved cysteine that accepts ubiquitin from E2~Ub and then passes it on to substrate, whereas RING ligases promote the direct transfer of ubiquitin from E2~Ub to the substrate.

CULLIN-RING UBIQUITIN LIGASES

General Principles of CRL Organization

RING ligases are conserved from yeast to human and human cells potentially express more than 650 different types of these enzymes.⁹ The RING E3s that are perhaps the best understood of the members of the cullin-RING ligase (CRL) superfamily.⁹⁻¹² CRLs are modular multisubunit complexes that all contain a common core comprising a cullin subunit and a zinc-binding RING domain subunit. The cullin subunit folds into an extended structure that forms the backbone of CRLs. The C-terminal region of the cullin subunit forms a globular domain that wraps itself around the RING protein, which in turn recruits the E2~Ub to form the enzymatic core (Fig. 1). The N-terminal region of the cullin subunit, which resides at the opposite end of the elongated cullin structure, recruits substrate receptors via adapter proteins. SCF, the prototype of the CRLs, consists of the cullin Cul1, the RING domain protein Rbx1/Roc1/Hrt1, the adapter protein Skp1 and a substrate receptor protein such as Skp2 or β -TrCP. The substrate receptor proteins for SCF complexes share an F-box motif that links them to Skp1. The human genome encodes 69 F-box proteins¹³ and thus human cells potentially assemble 69 distinct SCF complexes, each with different substrate specificity. Other CRLs are assembled using other cullin and adapter/substrate receptor subunits. All told, there are nine different proteins that contain a region homologous to the C-terminal domain of Cul1 that binds a RING subunit. The complexes they nucleate are specified in Figure 2. Interestingly, eight of these proteins (Cul1, Cul2, Cul3, Cul4a, Cul4b, Cul5, Cul7 and PARC), share one of only two different RING proteins (Rbx1/Roc1/Hrt1 and Rbx2/Roc2/Hrt2). Only Apc2, which is the most atypical member of the family, assembles with the dedicated RING protein Apc11.¹⁴

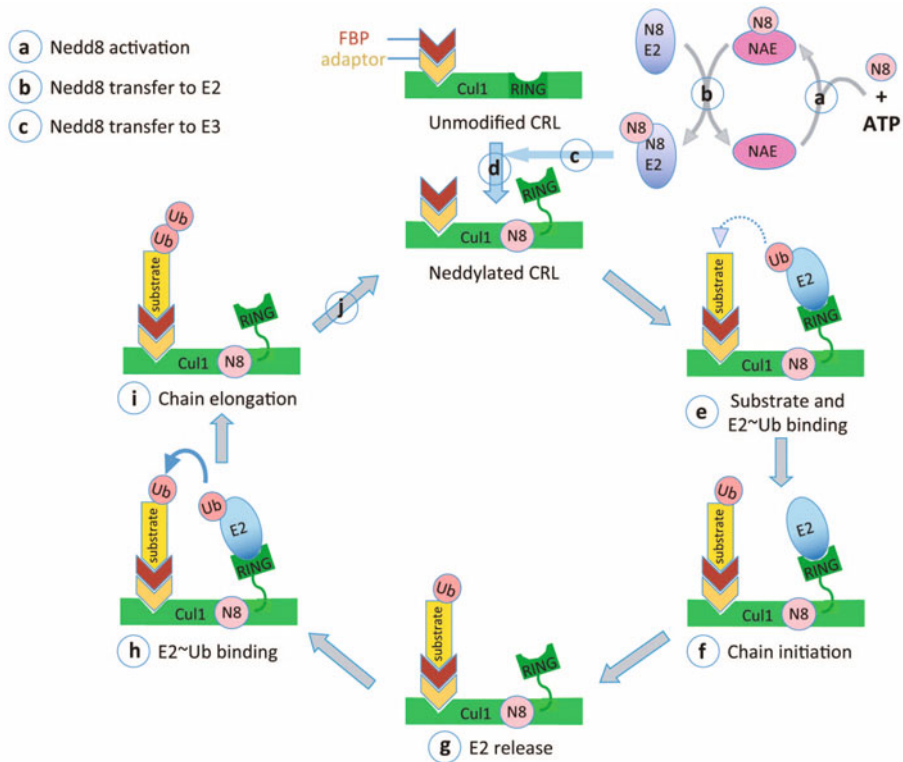


Figure 1. Reaction cycle for substrate ubiquitination by an activated cullin-RING ligase (CRL). a) Nedd8 (N8) is first activated when it reacts with Nedd8-activating enzyme (NAE) in the presence of ATP. b) Activated Nedd8 is then transferred from NAE to the Nedd8 conjugating enzyme (N8 E2). c) Nedd8 is subsequently transferred from Nedd8 E2 to CRL. Nedd8 is covalently attached to the cullin subunit via an isopeptide bond on a conserved lysine residue. d) Conjugation of Nedd8 causes the RING subunit to spring out from the cullin resulting in increased CRL activity. e) Members of the SCF subfamily of CRLs recruit E2~Ub via the RING domain and substrates via the F-box protein (FBP). The FBP is linked to the cullin subunit (Cu1) by the adaptor, Skp1. Ubiquitin (Ub) is transferred from E2~Ub to the lysine residue of the bound substrate to initiate chain formation (f). g) After ubiquitin is discharged to the substrate, the apoE2 dissociates from the CRL. h) Another E2~Ub is recruited to the RING for subsequent ubiquitin transfer. i) Ubiquitin is transferred from E2 to the lysine residue of ubiquitin conjugated to substrate resulting in chain elongation. Dissociation of discharged E2 clears the way for subsequent recruitment of E2~Ub to build a longer ubiquitin chain. j) At some point, the ubiquitinated product dissociates. The length of the ubiquitin chain acquired by the substrate is a function of the rates of E2 cycling and product dissociation. If the rate of E2~Ub binding, ubiquitin transfer and E2 dissociation are fast compared to product dissociation, long chains will be generated. Conversely, if the rate of E2 cycling is slow, product will dissociate before long chains can be acquired.

Recruitment of E2 Enzymes by CRLs

The E2~Ub that is recruited by SCF to ubiquitinate substrate has been the subject of some debate. Yeast SCF complexes specifically employ Cdc34 as the E2,¹⁵⁻¹⁷ whereas human SCF utilizes either Cdc34 or UbcH5c *in vitro*.¹⁸ The identity of the E2 used by human SCF *in vivo* remains uncertain and few studies address this issue.¹⁹⁻²⁰ UbcH5 and Cdc34 have markedly different biochemical properties. Cdc34 is relatively poor at

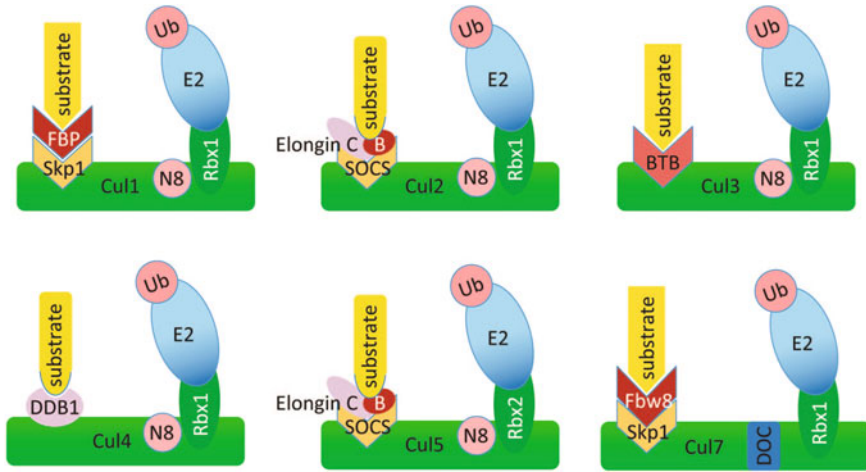


Figure 2. Modular assembly of cullin RING ligases (CRLs). The C-terminal domain of the cullin subunit associates with a RING domain protein (Rbx1 or Rbx2) to form the catalytic core that recruits E2-ubiquitin thioester. The N-terminal domain of cullin interacts with substrate receptor either directly or via an adaptor protein that recruits substrates. All cullins are known to be modulated by the conjugation of Nedd8.

transferring ubiquitin to unmodified substrate, but extends ubiquitin chains on modified substrate very rapidly—up to 100-times faster than it initiates them.^{18,21} Another feature of Cdc34 is that it is very specific for forming Lys-48 linkages and this specificity is intrinsic to Cdc34. UbcH5, on the other hand, is adept at transferring ubiquitin to naïve substrate, but unlike Cdc34 shows no preference for elongating the attached ubiquitin into a chain.¹⁸ Indeed, once a substrate bears a single ubiquitin modification, both the K_M and k_{cat} for subsequent ubiquitin transfers by UbcH5 decrease, suggesting that UbcH5 binds more tightly to the modified substrate, but in a configuration that has relatively low ubiquitin transfer activity.¹⁸ A second difference between UbcH5 and Cdc34 is that the former exhibits no preference for Lys-48 linkages and forms polyubiquitin chains linked through multiple lysine residues.²² It is unclear how this lack of chain linkage specificity would influence the metabolism of ubiquitin chains assembled by UbcH5.

It has been suggested for the yeast APC (Anaphase Promoting Complex) that one E2 (Ubc4) is used to initiate chains whereas a second E2 (Ubc1) elongates the chain.²³ A similar model could explain the conflicting observations that have been made with SCF. However, it remains unclear how chain-initiating and chain-elongating E2s would not interfere with each other's actions and SCF would know to recruit the proper E2 depending on the stage of substrate modification. Given this concern, other possibilities should be entertained. For example it is possible that UbcH5 is not a physiological E2 for SCF substrates. Two lines of argument support this proposal. First, high concentrations of UbcH5 are required to saturate SCF ($K_M \sim 1\text{-}2 \mu\text{M}$) and the requisite effective concentrations may not be achievable in vivo due to titration of UbcH5 by numerous other RING domain proteins.²⁴ Meanwhile, Cdc34 has a far lower K_M for SCF (100-200 nM)²⁵ and could easily saturate SCF in vivo, based on estimates we have made of Cdc34 concentration in yeast cells.²⁶ Second, because UbcH5 is not fast at building chains, most substrates that are modified by UbcH5-SCF do not acquire a chain of ≥ 4 ubiquitins before they dissociate from SCF. By contrast, whereas Cdc34 is

inefficient at initiating ubiquitination, the majority of substrates that are modified by Cdc34 go on to acquire a chain of ≥ 4 ubiquitins prior to dissociating from SCF.¹⁸ It should be borne in mind that not only are the ubiquitin chains generated by UbcH5 short, but most likely they comprise a mixture of different ubiquitin-ubiquitin linkages of uncertain potency in sustaining turnover by the proteasome *in vivo*.

Recent kinetic studies have helped to shed light on how Cdc34 can be so adept at building ubiquitin chains on substrate rapidly. First, it appears that Cdc34~Ub has a noncovalent binding site for the ubiquitin that accepts the thioester.²⁷ Although this binding site has low affinity ($K_M \sim 500 \mu\text{M}$ for yeast Cdc34), it should be easily saturated in the context of an SCF complex bound simultaneously to ubiquitinated substrate and Cdc34~Ub, due to the proximity between these two molecules. A second feature of Cdc34 that enables rapid chain assembly is the exceptionally rapid dynamics of the Cdc34–SCF complex. Although this complex forms with extremely high affinity ($K_D \sim 20\text{--}100 \text{ nM}$ depending upon the modification state of Cdc34 and Cull1),¹⁸ it is remarkably unstable. Discharged Cdc34 dissociates from SCF with an off-rate of $\sim 30 \text{ sec}^{-1}$,²⁵ which leads to a predicted on-rate of $\sim 4 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$.²⁵ This exceeds by 2–3 orders of magnitude the predicted on-rate for protein–protein interaction based on random diffusion. Exceptionally fast binding of Cdc34~Ub and SCF is mediated by the acidic tail of Cdc34, which engages in an electrostatic interaction with a basic “canyon” on the underside of Cull1. The extremely rapid dynamics of Cdc34–SCF interaction enables chain assembly to occur at rates approaching 4–5 ubiquitin transfers per second.²¹

Substrate Recruitment to CRLs

Unlike the nature of the E2 used by SCF, the matter of how substrate is recruited is better understood. Substrate recruitment by ubiquitin ligases has been reviewed recently²⁸ and substrate recruitment by SCF ubiquitin ligases in particular has also been discussed in depth,^{10,12} so we will not go into detail here. Substrates recruited to SCF for ubiquitination are usually covalently modified by phosphorylation, although other covalent modifications, including glycosylation and ribosylation, have been reported to serve as signals for recruitment. The structures of several phosphorylation-based degrons bound to their cognate substrate receptor subunit of SCF have been solved and in each case the covalent modification makes defined molecular contacts that enable its specific recognition.^{29–32}

MECHANISM AND REGULATION OF CRLs

Whereas substrate recruitment to SCF is now understood in molecular detail for some complexes, the actual ubiquitination reaction has resisted detailed description. The ubiquitination reaction catalyzed by E2~Ub–SCF can be subdivided into two steps by both kinetic and mutational analysis: transfer of the first ubiquitin to substrate (chain initiation) and polymerization of ubiquitin chains by formation of ubiquitin-ubiquitin linkages (chain elongation).^{18,21,27} Perplexingly, the original structural studies of SCF sub-complexes suggested that there should exist a $\sim 50 \text{ \AA}$ gap between bound substrate and the active site cysteine of E2 within an E2~Ub–SCF–substrate ternary complex.^{29–33} For SCF to facilitate chain initiation, the substrate lysine that is to be modified must come in close proximity with the thioester bond that joins ubiquitin to E2. Thus, publication of the structural studies served to highlight how little we know about how SCF works.

CRLs Are Activated by Nedd8 Conjugation

We now appreciate that (at least part of) the answer to the conundrum of how SCF and other CRLs can ubiquitinate substrate across a ~ 50 Å gap lies in the fact that these enzymes are activated by a reversible covalent modification of the cullin subunit with the ubiquitin-like protein, Nedd8.³⁴ Covalent modification of cullins with Nedd8, which is often referred to as neddylation, is essential in all eukaryotes tested to date,^{35–36} with the exception of budding yeast.^{37–38} The conjugation of Nedd8 requires a ubiquitin-like enzyme cascade involving the Nedd8-activating enzyme AppBp1-Uba3, one of two Nedd8-conjugating enzymes (Ubc12/UBE2M and UBE2F),^{39,40} the RING protein Rbx1 or Rbx2 and the activator Dcn1,⁴¹ resulting in neddylation of Cul1 at the highly conserved lysine 720³⁴ (Fig. 3). A lysine is found in the equivalent position in Cul2, Cul3, Cul4A, Cul4B, Cul5, Cul7 and PARC and

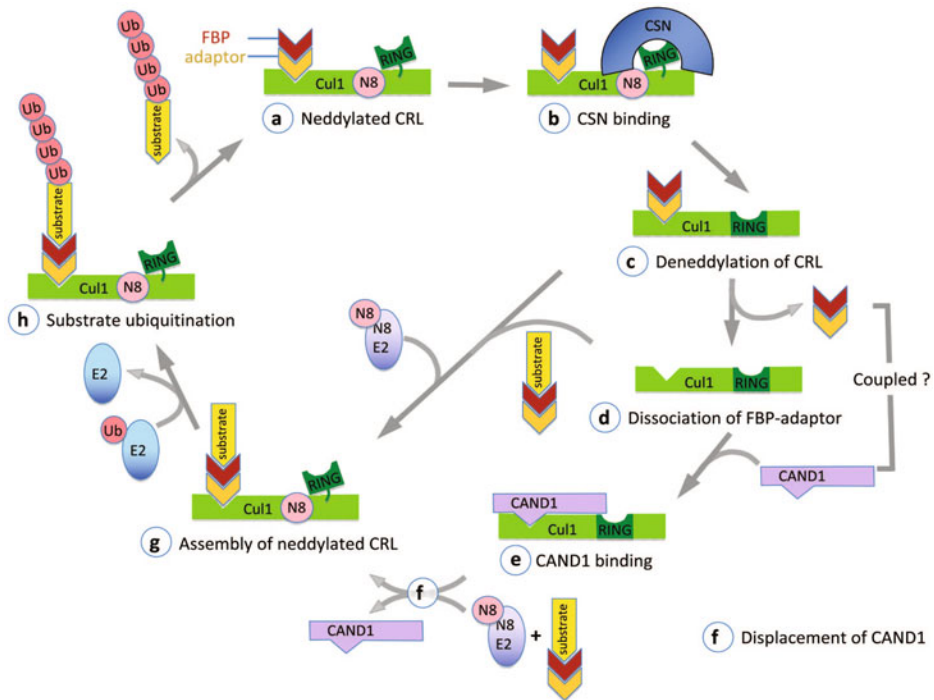


Figure 3. Neddylation and deneddylation cycle in substrate ubiquitination and CRL regulation. a) SCF in neddylated state possesses higher ligase activity for substrate ubiquitination. b) COP9-Signalosome (CSN) binds CRLs and recognizes neddylated cullin. The complementary binding surfaces on CRL and CSN are not known and thus the nature of the interaction depicted here is speculative. c) CSN deneddylates cullin to yield unmodified CRL with low ubiquitination activity. This deneddylated complex might be immediately reneddylated (shunt to (g)), or the F-box–Skp1 complex might dissociate from Cul1 (d) before reneddylation and binding of a new F-box–Skp1 occurs (shunt from step d to step g). Alternatively, Cul1–Rbx1 might become sequestered in a complex with CAND1 (e). Dissociation of F-box–Skp1 may or may not be coupled to binding of CAND1. The fluxes *in vivo* through the various sub-pathways linking steps c–g are not known. e) In the presence of neddylation machinery and substrate receptor–adaptor module, CAND1 is displaced from cullin. f) CAND1 displacement results in the assembly of active neddylated CRL complex. g) Neddylated CRL in the presence of E2–Ub builds ubiquitin chains on substrate, yielding ubiquitinated substrates.

all of these cullins are conjugated with Nedd8.⁴²⁻⁴⁵ The cullin homology domain-containing Apc2 subunit of the Anaphase-Promoting Complex does not contain the equivalent lysine and does not appear to undergo modification with Nedd8.

Conjugation of cullins with Nedd8 has a range of effects on cullin function and assembly state. A substantial fraction of Cul1 in eukaryotic cells is sequestered into an inactive complex with CAND1.⁴⁶⁻⁴⁷ A recent exception was noted in *S. pombe*⁴⁸ where only a minor fraction of Cul1 is sequestered. CAND1 binds Cul1 in an extended manner and thereby disrupts both the association of Cul1 with Skp1 and conjugation of Nedd8 to lysine-720.⁴⁹ Nedd8 conjugation coupled with F-box-Skp1 binding displaces CAND1 from Cul1, enabling the assembly of intact and functional SCF complexes.⁴⁹⁻⁵⁰ However, the Nedd8 conjugation pathway remains essential even in the absence of CAND1 suggesting that Nedd8 regulates CRLs by other mechanisms that are critical for life.⁵¹ Early studies reported that Nedd8 modification of Cul1 stimulates ubiquitination of the substrates p27^{Kip1} and I κ B α by SCF^{Skp2} and SCF ^{β -TrCP} respectively.⁵²⁻⁵⁶ and it was suggested that neddylation activates SCF by stabilizing its association with Ubc4, a close relative of UbcH5.⁵⁷ Subsequent NMR studies revealed a potential Nedd8 binding site on Ubc4,⁵⁸ suggesting a mechanism for how Nedd8 conjugation could stabilize E2 recruitment. However, the same site on UbcH5 binds ubiquitin and promotes ubiquitination by the E3 BRCA1, which is not modified by Nedd8.⁵⁹ Moreover, Cdc34 lacks the equivalent binding site and it was not shown by any direct measurement that Nedd8 conjugation improves the affinity for Ubc4 in a manner that depends on its putative noncovalent Nedd8 binding site. These observations raised the question of whether enhanced recruitment of E2~Ub is the primary mechanism by which neddylation activates SCF.

Nedd8 Conjugation Causes a Major Conformational Change in Cul5

The major mechanism by which Nedd8 conjugation stimulates ubiquitin transfer within the E2–E3–substrate complex was revealed by a confluence of X-ray crystallographic and biochemical studies. In a herculean effort, the Schulman laboratory solved the structure of the C-terminal domain (CTD) of Cul5 bound to Rbx1 in the unmodified and Nedd8-conjugated states.⁶⁰ Nedd8 conjugation induces a massive conformational change in the complex. The H29 helix of Cul5^{CTD} rotates ~45° relative to the α/β portion of the Cul5^{CTD}. This results in a marked repositioning of the 4-helix bundle (4HB), winged-helix B (WHB) and α/β subdomains within Cul5^{CTD}. In the unmodified state, the WHB cradles the RING domain of Rbx1. Upon reorientation of the 4HB, WHB and α/β domains, the WHB and RING are levered apart, freeing the E2-binding RING domain of Rbx1 to spring forth from the surface of Cul5. Rbx1 remains tightly bound to Cul5 via a long β -strand (S1) that forms an extended β -sheet with the S1, S2 and S3 strands in Cul5^{CTD}. This β -sheet is connected to the RING domain via a flexible linker that is found in two different conformations in the crystal of Nedd8-conjugated Cul5^{CTD}–Rbx1. Taken together, these data suggest a model wherein Nedd8 conjugation releases the Rbx1 RING domain, which catapults from the surface of Cul5^{CTD} like a jack-in-the-box. Although the RING domain remains tethered to Cul5^{CTD}, the linker is flexible, which allows the RING domain to sample three-dimensional space in the void that separates E2 from substrate in the unmodified SCF complex. Modeling suggests that the RING domain with its bound E2 could potentially come very close to substrate bound to the F-box subunit of SCF.

Analysis of a variety of mutants in the Rbx1 linker region as well as the Cul1–RING interface support the interpretation derived from the crystal structure.⁶⁰ Furthermore, small-angle X-ray scattering suggests that a similar conformational change occurs upon Nedd8 conjugation to Cul1. A particularly dramatic demonstration that this model is likely to be correct came from a cross-linking experiment.¹⁸ The idea was to ask whether conjugation of Nedd8 influences the formation of a crosslink between a β -catenin substrate peptide and the active site of E2 enzyme within a UbcH5–SCF ^{β -TrCP}– β -catenin complex. Whereas no cross-link was detected between β -catenin and UbcH5 when unmodified SCF was used, a strong cross-link was detected in the presence of Nedd8-conjugated SCF. The simplest explanation of this result is that Nedd8 conjugation induces a conformational change in SCF that brings the E2~Ub and the substrate into close approximation, as would need to occur during ubiquitin transfer. Detailed enzymological studies on Nedd8-conjugated SCF yielded additional data consistent with the idea that Nedd8 has a pervasive impact on the SCF complex. Nedd8 significantly enhances k_{cat} for ubiquitin transfer within the E2~Ub–SCF–substrate complex, particularly under single-turnover conditions. Binding analyses revealed that Nedd8 conjugation also stabilizes E2 recruitment (as measured by both K_M and K_D), but the effects on K_D are considerably smaller than the effects on k_{cat} .¹⁸ Besides the effect on E2 affinity, Nedd8 even appears to have a modest effect on transfer of ubiquitin to the low molecular weight nucleophile hydroxylamine. However, it seems likely that both of these latter effects may be indirect consequences of modest changes in the dynamic properties of the RING domain depending upon whether or not it is ensconced within the cullin CTD via interactions with the WHB subdomain. In addition to its effects on CRL activity, Nedd8 conjugation disrupts the binding site for CAND1, thereby enforcing its dissociation and promoting the assembly of an intact CRL. The effects of Nedd8 conjugation indeed appear to permeate nearly every aspect of CRL function and regulation.

DECONJUGATION OF Nedd8 BY THE COP9-SIGNALOSOME (CSN)

Nedd8-mediated activation of CRLs is part of a cycle wherein Nedd8 is being continuously conjugated to and deconjugated from cullins (Fig. 3). The recent availability of a chemical inhibitor of the conjugation process reveals that this cycle operates at a high rate.⁶¹ Thus, it is clear that to understand the impact of neddylation on CRLs, it is critical to understand the mechanism and regulation of Nedd8 deconjugation (deneddylation) as well as that of neddylation. Nedd8 conjugated to cullins is deneddylated by the COP9 signalosome complex (CSN).⁶² The CSN is comprised of eight subunits (Csn1–Csn8) and is highly conserved throughout the eukaryotic kingdom.^{63–64} Mutations in CSN components are manifest as defects in signal transduction,⁶⁵ transcription,⁶⁶ cell cycle progression⁶⁷ and development.^{68–69} Overall, our best understanding of the physiological role of CSN derives from genetic studies of its role in photomorphogenesis in *Arabidopsis thaliana*.⁷⁰ Photomorphogenesis refers to the broad spectrum of physiological and developmental changes that occur when a seedling is exposed to light. Genetic data suggest that CSN regulates photomorphogenesis by stimulating turnover of the transcriptional regulator Hy5 via the presumptive E3, COP1. COP1 is the substrate-binding subunit of a CRL complex that contains Cul4A, which provides a pleasingly simple molecular model for photomorphogenesis that ties together the genetic and biochemical data on CSN.⁷¹

COP9-Signalosome Defines a Novel Class of Metalloproteases

Insight into the mechanism by which CSN promotes cleavage of Nedd8 from Cull1 came from bioinformatic analyses of CSN subunits. Csn5 and a subset of other proteins that contain the JAB1/MPN/Mov34 domain were found to contain a highly conserved sub-motif, EX_nHS/THPX₇SX₂D.⁷² By analogy to zinc metalloproteases, we speculated that the His and Asp residues of this motif comprise a set of ligands that coordinate a catalytic zinc ion. Indeed, mutations in these conserved residues inactivate Csn5-dependent deneddylation of Cull1 in fission yeast and Nedd8 isopeptidase activity of purified pig CSN is sensitive to metal chelators. Based on these data we dubbed the conserved motif 'JAMM', for Jab1/Mpn domain Metalloenzyme. We and others went on to validate our predictions by solving the three-dimensional crystal structure of a JAMM domain protein from an archaeobacterium.⁷³⁻⁷⁴ Despite the insight into the active site of CSN, our overall understanding of the deneddylation reaction remains at a rudimentary level. Little is known about the molecular basis for substrate recognition or the dynamics of the process. The situation is exacerbated by the lack of structural data for the CSN complex or any of its individual subunits.

Consistent with its biochemical function as a Nedd8 isopeptidase, CSN behaves as an inhibitor of SCF *in vitro*. Deneddylation by CSN attenuates Cull1-RING-dependent ubiquitin chain synthesis⁷⁵ and also downregulates p27^{Kip1} ubiquitination by SCF^{Skp2} in a cell-free extract.⁷⁶ Nevertheless, multiple genetic studies indicate that CSN is required for proper CRL function *in vivo* in *A. thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *S. cerevisiae*, *S. pombe*, *Neurospora* and human tissue culture cells.^{48,77-83} It has been noted in these studies that cells lacking CSN function contain reduced levels of cullins and/or substrate receptor proteins due to increased turnover, possibly via an autoubiquitination mechanism. Likewise, although CAND1 can clearly inhibit active SCF ligases *in vitro*,⁴⁶⁻⁴⁷ inactivation of CAND1 by mutation leads to a dramatic loss of the *Arabidopsis* F-box protein Ufo1 and consequent reduction in function of SCF^{Ufo1} ubiquitin ligase activity *in vivo*.^{51,84} We and others have interpreted the contrasting negative roles of CSN and CAND1 *in vitro* versus their positive roles *in vivo* as evidence for an obligatory cycle of CRL assembly and disassembly, presumably involving reversible cycles of neddylation and deneddylation coupled with CAND1-dependent sequestration of cullin.^{63,85} If any part of this cycle is interrupted, CRL activity is downregulated.

COP9-Signalosome as a Regulator of Human Disease

CSN is emerging as a potential player in human disease with several different connections having been made, particularly in cancer. Early studies noted a correlation in cancer cells between elevated expression of Csn5 and reduced levels of the SCF substrate and cyclin-dependent kinase inhibitor p27^{Kip1},⁸⁶⁻⁸⁸. Csn5 overexpression promotes p27^{Kip1} turnover, but it remains unclear how this relates to CSN function. Subsequently, it was shown that overexpression of Csn5 and Myc in human breast cancer cells induces a regulon of 512 genes linked to the wound healing response in untransformed cells.⁸⁹ An activated wound healing response in cancer cells is a powerful predictor of metastasis and death in multiple primary human tumors.⁹⁰ Recent studies from the same lab reveal that proliferation of breast cancer cells *in vitro* requires Csn1, Csn5 and Csn6.⁹¹ Moreover, defective proliferation of Csn5-depleted cells is not rescued by expression of a JAMM domain mutant. These data suggest that the isopeptidase activity of Csn5 promotes an

increase in Myc transcriptional activity, which in turn activates the wound response regulon.⁹¹ It is tempting to speculate that this effect is mediated by deneddylation and downregulation of SCF^{FBW7}, which recognizes N-terminal Myc Box I and antagonizes Myc transcriptional function by enhancing its proteasomal turnover.⁹²⁻⁹³ Another case in which CSN has been implicated to act in an oncogenic capacity is osteosarcoma. Analysis of an amplicon located at 17p11.2 suggests that overexpression of the COPS3 (Csn3) gene can be a causative factor in osteosarcoma.⁹⁴ More recently, colorectal cancer cells expressing oncogenic K-Ras but not isogenic cells deleted for the K-Ras oncogene were shown to depend on several CSN subunits for their survival.⁹⁵ Interestingly, genes in the neddylation pathway are also required for survival of cells expressing oncogenic K-Ras. Together, the studies on the wound response and K-Ras implicate CSN as an excellent candidate target for treatment of a subset of breast cancers as well as colorectal cancers powered by a mutant K-Ras.

Another link between CSN and cancer was suggested by investigations on the nucleotide excision repair proteins Csa and Ddb2.⁹⁶ Loss of Csa is a cause of Cockayne's syndrome whereas loss of Ddb2 is found in a subset of patients with the cancer-prone syndrome Xeroderma pigmentosum. These proteins serve as the putative substrate recognition subunits of CRL complexes comprising Ddb1, Cul4A and Rbx1. In the absence of UV irradiation, the assembled CRL4^{DDB2} ubiquitin ligase (Ddb2–Ddb1–Cul4A–Rbx1) exists as a soluble complex in the nucleus and is bound to CSN. When the nucleotide excision repair pathway is activated by UV damage to DNA, CRL4^{DDB2} dissociates from CSN and binds tightly to the damaged chromatin. Chromatin-bound CRL4^{DDB2} becomes neddylated and is now competent to ubiquitinate its substrates. By contrast, in the absence of UV irradiation, the CRL4^{CSA} (Csa–Ddb1–Cul4A–Rbx1) is not complexed with CSN. When UV irradiation damages chromatin, CRL4^{CSA} binds to RNA polymerase IIo that is stalled at DNA lesions and recruits CSN, presumably resulting in inactivation of CRL4^{CSA} ubiquitin ligase E3 activity.

CONCLUSION AND FUTURE PERSPECTIVES

Other Functions for the Nedd8 Regulatory System

A number of Nedd8-conjugated proteins other than the cullins have been described in the literature, including Mdm2,⁹⁷ p53,⁹⁷ VHL⁹⁸ and ribosomal proteins.⁹⁹ The physiological ramifications of these modifications (which are typically found only on a very small fraction of target molecules) remain to be fully explored. As has been pointed out by Rabut and Peter, none of the Nedd8-modified proteins (besides the cullins) discovered to date satisfy fully a set of criteria that they proposed for the validation of physiologic targets of the Nedd8 conjugation pathway.¹⁰⁰ A matter of particular concern is that the ubiquitin conjugation system has demonstrated the capacity to conjugate Nedd8 *in vitro* and so it is critical to establish by functional ablation of the Nedd8 conjugation pathway that the neddylation of a given protein is indeed specific. Currently, the noncullin Nedd8-modified proteins for which there exists the most convincing evidence are p53 and VHL. Clearly, more work on alternative targets of Nedd8 is urgently needed.

One potentially powerful approach to search for alternative Nedd8 conjugation targets is to evaluate neddylation in cells lacking deneddylation activity. Whereas blockade of CSN activity primarily induces the accumulation of Nedd8-modified cullins,^{62,101}

blockade of the enzyme Den1/NEDP1 that processes the precursor form of Nedd8 to yield mature Nedd8 causes the accumulation of numerous unknown Nedd8-modified proteins in yeast¹⁰² and *Drosophila* larvae.¹⁰¹ The *Drosophila* data were particularly intriguing because the increase of neddylated proteins in mutants lacking Den1/NEDP1 was shown to depend on the Nedd8 conjugation pathway and the pattern of accumulation differs greatly from that of mutants deficient in Csn5. These observations suggest that CSN may be dedicated for cullin regulation, but Den1/NEDP1 may control the deneddylation of a substantial pool of alternative Nedd8 targets. Several different proteomic searches for Nedd8 conjugates have been reported^{45,99,103-104} and it will now be of particular interest to repeat these analyses in cells deprived of Den1/NEDP1 activity.

The Nedd8 Pathway as a Target for Therapeutic Intervention

An important development in the past year was the report of a small molecule, MLN4924 (Millennium/The Takeda Oncology Company), which inhibits the activity of the Nedd8-activating enzyme (NAE) that primes Nedd8 for transfer to protein targets.⁶¹ MLN4924 effectively wipes out Nedd8 conjugation activity in vivo and this leads to extremely rapid (≤ 5 minutes) loss of neddylated cullins. This striking result suggests that cullins are being constantly neddylated and deneddylated at a blistering pace. In response to treatment with MLN4924, substrates for multiple CRLs begin to accumulate. Interestingly, different substrates show different dose-responses to MLN4924, suggesting that different CRLs may be differentially sensitive to depletion of NAE activity. The basis for such a differential response is not known. Based on its potency and specificity, MLN4924 promises to be of enormous value for basic research studies on the Nedd8 conjugation and deconjugation system. Apart from its utility as a research tool, MLN4924 shows excellent promise as a candidate therapeutic for treatment of cancer. MLN4924 showed marked activity in downregulating NAE activity in HCT-116 tumor cells xenografted into mice, resulting in deneddylation of cullins and accumulation of CRL substrates.⁶¹ Most impressively, MLN4924 elicited a strong reduction in the growth of the xenografted cancer cells.

Unresolved Questions

Although dramatic progress has been made in the past two years in understanding how Nedd8 conjugation regulates CRLs, much remains to be done. The most important unresolved question about the Nedd8 conjugation system is, how is the entire Nedd8 cycle controlled from the perspective of individual CRL complexes? What is perplexing is that cells express two different E2s and a handful of E3 enzymes to conjugate Nedd8 and two enzymes to deconjugate it (CSN and NEDP1/Den1), but meanwhile there are eight different cullin targets, each of which has the potential to assemble multiple distinct ubiquitin ligases. Based on the number of different putative cullin substrate receptors that are known, there may be as many as 350 different CRLs that are expressed in human cells and regulated by cycles of Nedd8 conjugation and deconjugation. It seems paradoxical that the regulation of such a large set of CRLs would be relinquished to such a small number of Nedd8-conjugating and deconjugating enzymes. Any signal that would serve to alter either Nedd8 conjugation or deconjugation activity could be expected to influence the activity of dozens to hundreds of CRLs, thereby influencing the turnover of hundreds to thousands of proteins, many of which are likely to function at cross-purposes.

It seems logical that there must exist some way to regulate the neddylation cycle in a more fine-tuned manner, such that the neddylation and deneddylation of individual CRL complexes can be controlled independently, enabling one flavor of CRL to be activated by neddylation at the same time that a distinct CRL complex is being decommissioned by CSN-mediated deneddylation. Indeed, this is precisely what happens to the CRL4^{DBB2} and CRL4^{CSA} complexes upon UV irradiation.⁹⁶ Such a mechanism could be based on substrate-mediated regulation of the Nedd8 cycle or could be controlled by a covalent modification (e.g., phosphorylation) that marks individual CRL complexes for neddylation or deneddylation.⁶³ The latter mechanism would echo CRLs themselves, which can ubiquitinate substrates on different schedules dictated by the protein kinases that mark the CRL substrates for ubiquitination.¹⁰⁵

Besides this major question about global versus complex-specific regulation of neddylation cycles, a number of more specific problems have so far resisted solution but seem primed for resolution. How does the putative Nedd8 E3, Dcn1, promote Nedd8 conjugation *in vivo*, even though it appears to have only very modest effects on this reaction *in vitro*? Is the Nedd8 conjugation reaction influenced by other polypeptides that engage the CRL (e.g., substrate, E2 enzyme)? What is the mechanism by which Nedd8 is cleaved from cullins by CSN (this includes the question of what is the molecular basis of substrate recognition)? Is CSN-mediated deconjugation regulated by factors that bind or modify CSN or that associate with the CRL substrate (e.g., Nedd8-conjugating factors, E2 enzymes and substrates)? Finally, will NAE prove to be a suitable target for cancer chemotherapy and might CSN and Den1/NEDP1 also be good candidates for pursuit? Clearly, much remains to be done to understand how the cycle of Nedd8 conjugation and deconjugation controls the repertoire of active CRLs and how modulation of this cycle might lead to new medicines to treat intractable diseases.

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