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Review

Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization

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Abstract. Cullin-RING E3 ubiquitin-Ligases (CRLs) are the most prominent class of ubiquitin-ligases. By controlling the stability of a cohort of key regulators, CRLs impinge on many cellular and biological processes such as immunity, development, transcription, cell signalling and cell cycle progression. CRLs are multi-subunit complexes composed of a catalytic site and a substrate recognition module nucleated around a cullin scaffold protein. Most eukaryotic genomes encode at least five distinct cullins, and each of these cullins recruits a specific substrate-recognition module such that CRL complexes are modular. Despite their considerable diversity, CRLs are regulated by similar mechanisms. In particular, recent observations indicate that conformational variability induced by CRL dimerization and by conjugation of the ubiquitin-like protein NEDD8 on the cullin subunit stimulates substrate polyubiquitination. In this review, we discuss the composition of CRL complexes and the various molecular mechanisms controlling their activity.

Keywords: ubiquitin-dependent proteolysis, neddylation, deneddylation, COP9 Signalosome, cullin-associated and neddylation-dissociated (CAND1).

Introduction

Protein degradation is a rapid and specific mechanism ideally suited to regulate irreversible processes such as cell cycle transitions. A major pathway controlling protein degradation is the ubiquitin-proteasome system, which targets substrate proteins for rapid proteolysis upon conjugation to ubiquitin, a small conserved protein of 76 residues. Substrate proteins are covalently linked to ubiquitin after a series of trans-thioesterification reactions catalyzed by an enzymatic cascade [1]. The C-terminal glycine residue of ubiquitin is first thio-esterified to an E1 (ubiquitin activating) enzyme in an ATP-dependent manner, transferred to an E2 (ubiquitin conjugating) enzyme also as a thioester linkage and finally, through the action of an E3 (ubiquitin-ligase), transferred to a lysine residue on the substrate as an isopeptide linkage. Reiteration of the catalytic cycle assembles a polyubiquitin chain, which targets the substrate to the 26S proteasome [2].

Central to the ubiquitin-proteolytic system are E3-ligases, which must specifically interact with both the ubiquitin-loaded E2-enzyme and selectively recruit substrates. Two main classes of E3 are evident at the primary sequence level: those bearing a HECT (Homologous to E6-AP C-Terminus)-type catalytic domain and a prominent class containing a RING (Really Interesting New Gene) domain. In contrast to

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HECT-type E3 enzymes, which contain an active cysteine residue and form an obligate thioester bond with ubiquitin prior to transfer to the substrate, RING-type E3s serve as a docking site for the ubiquitin-activated E2, which provides catalytic activity. The RING domain was first linked to the ubiquitin-proteolytic system through its discovery in subunits of two cell cycle-regulated E3-ligases: the APC/C (Anaphase Promoting Complex/Cyclosome) [3, 4] and the SCF (Skp1 – Cullin- F-box) [5–10]. The APC/C is a large multiprotein complex that controls entry and exit from mitosis by triggering the degradation of a cohort of substrates including securin and the mitotic cyclins mainly via two different degrons, called the KEN box and the D-box. APC/C recognizes these degrons using two evolutionarily conserved subunits containing WD40 propeller domains called CDC20/Fzy and CDH1/Hct1/Fzr [11, 12]. The SCF was originally discovered concomitantly in budding yeast and *C. elegans* through genetic studies on cell division [13–18]. Subsequent biochemical characterization of this complex revealed that the SCF core complex is composed of a cullin scaffold subunit (Cdc53 in budding yeast/CUL1 in mammals), the small adaptor SKP1, and the RING-H2 finger protein RBX1 (also called HRT1 or ROC1) [7, 18, 19]. SKP1 links the N-terminal part of CUL1/Cdc53 to a myriad of substrate-recognition subunits termed the F-box, which share a small motif of approximately 40 residues required for SKP1 binding [20–23]. Through its C-terminal part, CUL1/Cdc53 binds RBX1, which acts as a docking site for the ubiquitin-activated E2 enzyme (Fig. 1). In the last decade, SCF complexes have been implicated in a cohort of processes and a recent review summarizes the critical roles played by these complexes in various cellular and biological processes [24].

The SCF complex is the archetype and the founding member of a larger family of cullin-based ubiquitin-ligases nucleated around different cullin subunits (CUL1 to CUL7). Each cullin recruits a distinct substrate recognition module such that bioinformatic analyses predict the existence of more than 500 distinct cullin-based E3-ligases. In this review, we summarize what has been learned about CRL complexes over the past ten years. In particular, we present the general composition and architecture of the various CRL subfamilies and the molecular mechanisms regulating their activity.

Cullin-RING E3-ligases are modular

Cullins are evolutionarily conserved from yeast to mammals; sequence homology spans the entire protein but is highest at the C-terminus, characterized by the cullin homology domain that spans approximately 200 residues. The human genome encodes at least seven cullins including CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7 [13, 17, 25] and two proteins, PARC (Parkin-like cytoplasmic protein) [26] and APC2 (subunit of the APC/C complex) [4], which also contain a cullin homology domain, but are different from the typical cullins and are thus not considered in this review. Most eukaryotic genomes encode homologues of all cullins except budding and fission yeast, which only contains homologues of CUL1, CUL3 and CUL4, termed Cdc53/Pcu1, CUL3/Pcu3 and Rtt101/Pcu4, respectively. Each cullin nucleates multi-subunit ubiquitin-ligases containing the same catalytic core, which is composed of the cullin-RBX1 heterodimer bound to the ubiquitin-loaded E2 enzyme, except for CUL5, which also binds RBX2 (Fig. 1). The cullin-RING architecture is conserved in the APC/C in which the catalytic core resides in the APC2/APC11 heterodimer [9, 27]. Even though all cullin-based E3-ligases contain the same catalytic core, they use different substrate-recognition modules. In most cases these modules use a BTB (Bric-à-Brac, Tramtrack and Broad complex)-fold adaptor to interact with the amino-terminal domains of cullin, which is only conserved between cullin orthologues, and thus provides specificity for the interaction with the various substrate-recognition modules [28].

Cullin-based E3-ligases are thus modular and, except for the CUL1-based SCF complexes, the nomenclature of the SCF-like complexes is not yet settled such that various acronyms have been used to define these complexes [29]. In this review, we will use the acronym CRL for Cullin-RING E3-Ligases followed by the number of the cullin, with the identity of the substrate-recognition subunit mentioned in superscript. For example, we will use the acronym $CRL4^{CDT2}$ to define the CUL4-based E3-ligase that uses CDT2 as a substrate-recognition subunit.

Composition of CRL complexes

In the canonical SCF complexes, the BTB-fold adaptor SKP1 connects the unique N-terminal part of CUL1 to F-box proteins, which recognize their substrates through various substrate interaction motifs, such as WD40 motifs or Leucine Rich Repeats (LRR) (Fig. 1). CUL7 also uses SKP1 but, intriguingly, so far only one F-box protein has been shown to bind CUL7 [25]. In CRL2 and CRL5 complexes, the BTB-fold adaptor ElonginC connects CUL2 and CUL5 to BC-box proteins, which in turn bind their substrates primarily through LRR or ankyrin repeats (Fig. 1). Despite the fact that both CUL2 and CUL5 use the same adaptor, they engage different classes of substrate-recognition subunits, termed VHL-box and

SOCS-box proteins respectively, which bind their cognate cullin through specific regions thus providing cullin specificity [30]. The ubiquitin-like protein ElonginB is also part of CRL2 and CRL5.

In CRL3 complexes, a single polypeptide containing a BTB domain and a substrate-binding interface, which often consists of Kelch repeats, merges the function of SKP1/F-box or ElonginC/BC-box heterodimers and bridges the N-terminal part of CUL3 to substrate [28] (Fig. 1).

Finally, heterodimers composed of the large DDB1 protein, which does not harbour a BTB-fold but three seven-bladed ß-propellers, called BPA, BPB and BPC and a member of the DCAF family (Ddb1-CUL4-associated factor), function as substrate-recognition module in CRL4 complexes (Fig. 1). Most of the DCAF proteins contain WD40 repeats and share a short motif termed WDxR that is required for DDB1 binding [31, 32]. However, residues outside the WD40 repeats may also secure the interaction between DCAF proteins and DDB1/CUL4. CUL4A and CUL4B use an overlapping set of DCAF proteins and exhibit partially redundant functions, but they may also associate with distinct substrate-adaptors for specific cellular functions. The topology of the CRL4 complex is also conserved in fission yeast [33, 34] and in budding yeast [35].

Substrate-recognition subunits represent large families of proteins such that the potential number of CRLs is staggering with more than 500 hundred members. One key question is whether all these proteins indeed function as bona fide CRL subunits. The answer to this question is unknown as there is currently no simple rule to predict whether F-Box, BC-box, BTB or DCAF proteins assemble into CRL complexes. At least, those that contain an additional protein-protein interaction motif are more likely to be part of CRL complexes. For example, the budding yeast F-box proteins Ctf13 and Rcy1, which do not incorporate into SCF complexes, lack such domains [36, 37].

Are there additional CRL subunits?

Several accessory factors have been found to associate with core CRL subunits including SGT1, CKS1, alphaB-crystallin and DDA1 (DDB1 and DET1 Associated factor). Sgt1 activity is required to sustain SCF activity. In particular, Sgt1 may promote assembly of Skp1-based complexes along with its co-chaperone Hsp90 [38, 39]. CKS1 and alphaB-crystallin function as specificity factors and promote the binding of phosphorylated P27 and cyclin D1 to SCF^{SKP2} and SCF^{FBX4} complexes respectively [40, 41]. Finally, DDA1, which binds DDB1 through its BPC [32], interacts physically and functionally with multiple CRL4s [42], but its precise function in CRL4 regulation remains to be elucidated.

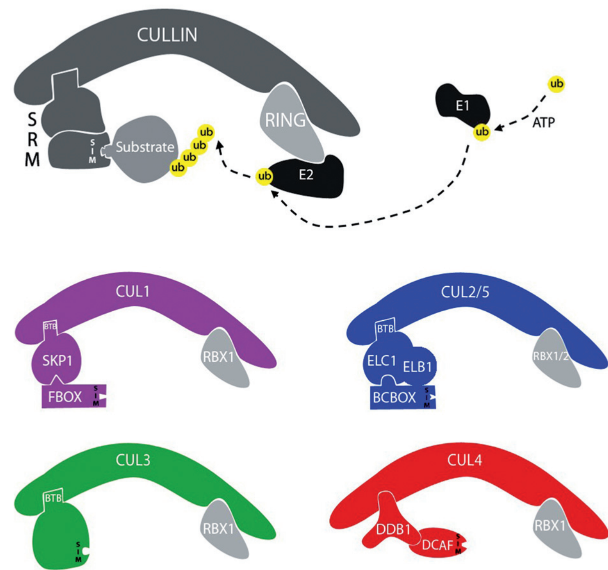

Figure 1. Composition of cullin-RING E3-ligases (CRLs). CRLs are nucleated around a cullin scaffold protein, which recruits the substrate-recognition module through its N-termini and the catalytic site through its C-terminal part. The basic composition of E3-ligases containing CUL1 (purple), CUL2/5 (blue), CUL3 (green) and CUL4 (red) is now well established. In the SCF and CRL2/5 complexes, SKP1 and ElonginC, bridge the interaction between the cullin and the F-box and BC-box proteins, respectively. In CRL3 complexes, a single polypeptide fulfils the function of SKP1/ElonginC and F-box/BC-box dimers and bridges CUL3 to substrates. Finally, in CRL4 complexes, DDB1 engages the N-terminal part of CUL4 and recruits DCAF proteins. The substrate-recognition subunits of these various CRL complexes bind their substrates through various substrate-interaction motifs (SIM) such as WD40, Kelch or leucine-rich repeats.

Cullin-RING E3-ligases have an extended architecture

Various CRL complexes including the SCF^{SKP2} [43] and the DDB1-CUL4A-RBX1-SV5-V complexes [31, 44] have been crystallized such that the 3D-architecture of CRL complexes is now emerging. In these complexes, CUL1 and CUL4A adopt an elongated arc-shaped structure with a helical amino-terminal domain composed of three repeats of five-helix bundles (cullin repeats 1–3) and a globular C-terminal domain.

In all CRL complexes, the substrate recognition modules bind to the first N-terminal cullin repeat (CR1). In particular, helices 2 and 5 (H2 and H5) of CUL1, CUL2/5, CUL3 and CUL4 are engaged in interaction with SKP1, ElonginC, BTB proteins and DDB1, respectively. These helices are well conserved between cullin orthologues and mutation of specific

conserved residues disrupts binding to the corresponding adaptor. For example, mutations of helices 2 and 5 of CUL3 abolish its binding to BTB proteins [28, 45]. Likewise, deletion of CUL5 H2 impairs its binding to ElonginC [46]. CUL4A also engages DDB1 through these two helices, but further embraces DDB1 through its unique N-terminal extension [31, 47]. This bipartite binding mode between CUL4A and DDB1 represents an interesting variation in cullin-adaptor interactions. Conversely, specific residues in the adaptor molecules provide strict specificity for their cognate cullin. Substrate-recognition subunits may also contribute to specificity. Indeed, the 3D structure of the SCF^{SKP2} complex revealed specific contacts between CUL1 and the F-box of SKP2, suggesting that the sequence of the F-box influences the assembly of SCF complexes [43]. Likewise, bioinformatic analyses and solution structure determination of the SOCS3-ElonginBC ternary complex revealed that VHL- and SOCS-box proteins share key residues essential for direct binding to CUL2 and CUL5 respectively [30, 46, 48, 49]. Based on these observations it is tempting to speculate that the sequences of the BTB and DCAF proteins may similarly influence CUL3 and CUL4 binding respectively.

The cullin C-terminal domain (CTD) contains a four-helix bundle, an α/ß domain and winged-helix B (WHB) subdomains, which acts to recruit RBX1 through intermolecular ß-sheet [43]. Indeed, RBX1 and CUL1 form an intermolecular ß-sheet consisting of a combination of ß strands from both proteins. The same hold true for the CUL4A/RBX1 and CUL5/RBX1 interactions [31, 50].

The 3D structure of several substrate-recognition modules bound to their substrates is known such as the SKP1/FBW7 heterodimer bound to the phosphodegron of cyclin E [51], SKP1/ß-TRCP bound to ß-catenin [52] or the budding yeast Skp1/Cdc4 heterodimer bound to the phosphodegron of cyclin E [53]. In all cases the linker region between the F-box and the WD40 propeller domain forms a stalk-like structure and acts to optimally position the substrate for ubiquitination by the SCF-bound activated E2. However, for isopeptide bond formation between the lysine side chain of the substrate and the ubiquitin carboxyl terminus, the substrate must directly contact the thioester linkage that joins ubiquitin to the E2. Intriguingly, the estimated distance between a CRL-bound substrate and the active-site cysteine of the E2 reaches approximately 50 Å [51–53]. Moreover, this distance and the catalytic geometry are expected to change during ubiquitin chain elongation. Hence, it is difficult to envision how rigid CRL complexes can promote assembly of long multiubiquitin chains on substrate within a 50 Å cleft. Importantly, recent results now indicate that neddylation (i.e. modification of the cullin subunit by the ubiquitin-like protein NEDD8) induces a drastic conformational change of the C-terminal part of the cullin that frees RBX1 and allows it to adopt various conformations to help bridge the 50 Å gap, thereby facilitating ubiquitin transfer. In the next section, we present the neddylation pathway and summarize these recent findings.

The ubiquitin-like protein NEDD8 closes the gap

In an enzymatic cascade similar to ubiquitination, the ubiquitin-like protein NEDD8 (Neural precursor cell Expressed Developmentally Down-regulated protein 8) is covalently attached to a conserved C-terminal lysine residue of all cullin family members, with the exception of APC2, the cullin-like subunit of the APC/C complex [54–56]. Sequence analysis and crystal structure determination of the SCF^{SKP2} complex have revealed that the neddylated lysine is embedded in a conserved region that spans less than 10 amino acids and is positioned at the rim of a cleft formed by conserved residues from the cullin and RBX1 [43, 56]. NEDD8 conjugation is catalyzed by an E1-like heterodimer of ULA1 and UBA3, which are homologous to the N- and C-terminal parts of the ubiquitin-activating enzyme, respectively. The heterodimeric E1 donates activated NEDD8 to a single dedicated E2 enzyme called UBC12 [57, 58]. Despite the high sequence (57% identical and 76% similar) and structural similarity between NEDD8 and ubiquitin [59], ULA1/UBA3 and UBC12 can only activate and transfer NEDD8 [55]. Acting at the end of the cascade, the evolutionarily conserved DCN1 (Defective in Cullin Neddylation) subunit – which does not form a thiolester bond with NEDD8 – stimulates the neddylation reaction *in vivo* and *in vitro* in conditions of limited amounts of UBC12 [60, 61]. DCN1 interacts with UBC12 and the cullins and thus fulfils all the criteria to function as a NEDD8 E3-ligase [60–62]. However, DCN1 may cooperate with RBX1 to promote cullin neddylation since RBX1 also binds cullins and UBC12 [63, 64], and has been shown to be essential for the neddylation reaction [65]. This prediction is further supported by the observation that DCN1 and RBX1 interact with each other [66]. DCN1 is conserved from yeast to humans and recent data indicate that SCCRO (Squamous Cell Carcinoma-Related Oncogene), the mammalian DCN1 orthologue, also promotes cullin neddylation, indicating that the function of DCN1 is evolutionarily conserved [62]. Importantly, SCCRO is amplified in numerous

human tumours and acts as an oncogene when overexpressed [67].

The importance of neddylation for cell cycle progression was first revealed more than 15 years ago through the analysis of the ts41 mutation in Chinese hamster ovary cells, which inactivate the NEDD8 conjugation pathway at non-permissive temperature [68, 69]. Later, it became evident that neddylation is critical for cell cycle progression and embryogenesis. In fact, the NEDD8 conjugating system is indispensable for viability in all species studied so far including mouse [70], *S. pombe* [71], *C. elegans* [72, 73], *A. thaliana* [63, 74], and *D. melanogaster* [75]. The only noticeable exception is budding yeast, in which neddylation is only essential when the function of the SCF is compromised [76].

In addition to cullins, several other proteins are regulated by NEDD8 conjugation, such as the tumour suppressors VHL, ribosomal proteins, p53 and MDM2 [54, 55]. However, in the context of this review, we only discuss the role of neddylation in the regulation of CRL complexes.

NEDD8 conjugation stimulates the ubiquitin-ligase activity of CRL *in vitro* and *in vivo*, but how does NEDD8 act to activate CRL function? Using purified *in vitro* systems, several groups have shown that neddylated SCF complexes are more active than their non-neddylated counterparts towards different substrates such as Iκbα [77, 78] and p27 [79, 80]. Likewise, neddylated $CRL2^{VHL}$ is more active than the non-modified version and readily ubiquitinates HIF-1 alpha [81]. These observations indicate that neddylation activates CRL complexes, independently of the nature of the substrate. Indeed, *in vitro* neddylated CUL1/RBX1 complex, highly purified from bacteria, is significantly more active than its non-neddylated counterpart, clearly suggesting a direct role of NEDD8 in E3-ligase activation [82]. In an attempt to decipher the molecular mechanisms underlying this activation, Kawakami *et al.* showed that NEDD8 conjugation increases the affinity of the $SCF^{ß\text{-}TRCP}$ complex for ubiquitin-loaded E2 enzyme UBC4 [77]. Consistent with this observation, nuclear magnetic resonance analysis of the NEDD8/UBC4 complex, combined with mutational analysis, revealed that NEDD8 directly binds UBC4 [83]. Point mutations affecting the NEDD8 and UBC4 interaction, such as mutation of the solvent-exposed NEDD8 Ileu 44 residue, severely compromise SCF activity [83]. Together, these observations suggest that NEDD8 is acting as a landing pad for the ubiquitin-activated E2. However, other E2s such as CDC34 lack this putative NEDD8-binding site, which raises the question of whether recruitment of the ubiquitin-conjugated E2 is the primary molecular mechanism by which NEDD8 activates SCF. Furthermore, this model fails to explain how the 50 Å gap between the substrate and the activated E2 is filled.

To elucidate the exact role of neddylation in SCF activation, Saha and Deshaies undertook a detailed enzymatic study of the neddylated SCF complex versus its non-neddylated counterpart in a reconstituted system using purified components. They found that neddylation moderately stimulates the recruitment of the ubiquitin-activated E2, but has a more profound impact on ubiquitin transfer from SCF-bound E2 to the substrate lysine [84]. Substrate ubiquitination occurs in two steps and involves slow transfer of the first ubiquitin molecule to the lysine of substrate followed by rapid polymerization of ubiquitin chains [85]. Remarkably, NEDD8 not only stimulates transfer of the first ubiquitin molecule on the substrate but also greatly stimulates ubiquitin chain elongation. In fact, NEDD8 conjugation stimulated every evaluated parameter from E2 recruitment to substrate ubiquitination and chain elongation in a single substrate-E3 binding event. How could NEDD8 have such a drastic and global effect on the SCF? Using chemical cross-linkers, it was shown that ß-catenin can be cross-linked to ß-TRCP and UBC5 when engaged in neddylated $SCF^{ß\text{-}TRCP}$ interaction, but only to ß-TRCP when bound to a deneddylated version of the complex. Therefore, if the $SCF^{ß\text{-}TRCP}$ complex is not neddylated, ß-catenin is too far from UBC5 to be cross-linked, indicating that NEDD8 may induce a conformational change that helps to bridge the 50 Å gap. Consistent with this biochemical observation, the crystal structure determination of the neddylated C-terminal part of CUL5 bound to RBX1 (CUL5Ctd-RBX1), together with small-angle X-ray scattering analysis of neddylated CUL1Ctd-RBX1 relative to its non-neddylated counterpart, now indicate that neddylation induces a drastic conformational change of the WHB C-terminal part of the cullins, preventing its interaction with RBX1 [50]. Indeed, in the two CUL5/RBX1 heterodimers in the asymmetric unit, the RING domain of RBX1 no longer binds CUL5, but rather becomes flexibly linked to the cullin and free to adopt multiple conformations **(**Fig. 2**).** Therefore, upon cullin neddylation, RBX1 and its bound activated-E2 enzyme can be positioned in close proximity to the acceptor lysine of the substrate, thus stimulating the transfer of the first ubiquitin molecule. A flexible RBX1/E2-Ub heterodimer adopting various orientations can then accommodate a growing polyubiquitin chain [86].

Displacing the C-terminal inhibitory domain of the cullin is the critical basis for NEDD8-induced stimulation of CRL activity, since a single amino-acid substitution of the C-terminal part of CUL1 (Y761A),

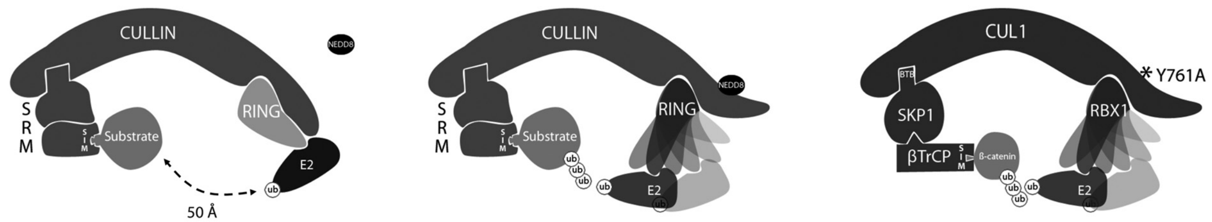

Figure 2. NEDD8 conjugation promotes CRL activation through conformational changes of the catalytic domain. NEDD8 (black circle) activates CRLs by promoting a drastic conformational change of the C-terminal part of the cullin that frees RBX1 ("open configuration") and allows it to adopt multiple orientations that bridges the 50 Å gap between the substrate and the activated E2 enzyme, thereby stimulating substrate ubiquitination (middle panel). A single amino-acid substitution of the C-terminal part of CUL1 (Y761A), which disrupts the CUL1/RBX1 interaction, bypasses the neddylation requirement and constitutively activates $SCF^{ß\text{-}TRCP}$ (right panel).

which disrupts the cullin/RBX1 interaction, bypasses the neddylation requirement and constitutively activates $SCF^{ß\text{-}TRCP}$ [87] **(**Fig. 2**).** Therefore, the observed increase in affinity of the activated E2 enzyme for the neddylated SCF complex is likely an indirect consequence of the dramatic conformational change of the C-terminal catalytic domain of the SCF, rather than the result of a direct interaction between NEDD8 and the activated E2. Moreover, the solvent exposed NEDD8 Ileu 44 residue, which was shown to prevent NEDD8 interaction with UBC4 in solution [83], is buried in the 3D structure of the neddylated CUL5-RBX1 heterodimer and contacts CUL5's WHB, explaining the effect of this mutation on SCF activity. Together these observations suggest that NEDD8 *per se* does not act as a landing pad for the activated E2, but rather facilitates the recruitment of the activated E2 enzyme by promoting RBX1 release.

Why is the budding yeast SCF complex largely independent of cullin neddylation? Based on these structural data one would predict that *S. cerevisiae* Cdc53 has unique features rendering it more flexible, thereby mimicking the effect of neddylation. Sequence analysis reveals that Cdc53 has a unique glycine-rich insertion preceding the WHB domain, which may impact on flexibility [50].

To summarize these important observations, cullin neddylation relieves the autoinhibitory activity of the extreme C-terminal domain of the cullin, and acts allosterically as a molecular switch to control the activity of cullin-RING E3-ligases. Taken together these observations are consistent with a model whereby NEDD8 conjugation on the cullin allows for greater structural flexibility to bridge the 50 Å gap and to bring the thioester bond between the E2 and ubiquitin close to the acceptor lysines of the substrate and ubiquitinated substrate.

CRL dimerization: it takes two to tango

SCF and CRL substrates are heterogeneous in size, conformation, nature and numbers of degrons. Furthermore, they are often ubiquitinated on multiple lysine residues such that the optimal distances between the acceptor lysine of the substrate and the E2-Ub thioester bond can vary considerably. The mechanistic requirements for CRL-mediated ubiquitination are thus extremely complex. By inducing conformation variability of CRL complexes, NEDD8 conjugation contributes to the polyubiquitination of these heterogeneous substrates. Besides neddylation, recent observations indicate that SCF and CRL dimerization is another mechanism by which these complexes acquire conformational variability that helps to accommodate various substrates.

CRLs dimerize through their substrate-recognition subunits

A wealth of evidence indicates that a large number of CRLs dimerize through their substrate-recognition subunits. For example, multiple F-box proteins dimerize, including FBW7 [51, 88] and its homologues SEL-10 in *C. elegans* [89], Cdc4 in budding yeast [90] and Pop1/2 in fission yeast [91, 92]. Similarly, FBX4 [93], ß-transducin repeat containing protein 1/2 (ß-TRCP1/2) [94], and Met30 also dimerize [90]. F-box proteins dimerize through a small domain, called the D-domain, which is located N-terminal to the F-box motif. The D domain spans approximately 40 residues and folds in three alpha-helices that self associate to form a parallel dimer [95]. In contrast to its hydrophobic nature, the dimer interface is poorly conserved at the amino acid level and only critical hydrophobic residues (leucine or isoleucine) are conserved spatially.

Likewise, BTB proteins dimerize or heterodimerize, such as MEL-26 [96], RhoBTB2 [97], KLHL9/13 [98], PLZF [99], ZBTB7 [100] and KEAP1 [97, 101]. In contrast to ELC1 and SKP1, which only share the BTB-fold, all these proteins contain an amino-termi-

nal extension, which constitutes a dimerization interface. This interface does not overlap with the predicted surface by which BTB proteins interact with CUL3 [28, 102]. As for the D-domain, the dimerization interface of BTB proteins is overall hydrophobic, but is poorly conserved at the amino acid level [102]. Whether other CRL complexes dimerize remains to be seen. It has been reported that VHL (Von Hippel-Lindau tumour suppressor)-mediated dimerization of the CRL2^{VHL} complex is critical for its activity [103], but in another study the authors failed to detect dimeric CRL2 complexes [104]. CRL4 may also dimerize [104], but the dimerization unit remains to be found. Further work is now required to investigate the dimerization properties of CRLs and, in particular, to determine whether CRLs dimerize exclusively through their substrate-recognition subunits or through other mechanisms. Indeed, it has been reported that CRL3 dimerization can be mediated by CUL3 neddylation [105].

CRL dimerization is essential *in vivo*

The functional importance of CRL dimerization is underscored by the observation that substrate-recognition subunits mutated in their substrate-binding interface but retaining their dimerization properties, act in a dominant-negative fashion *in vivo*. For example, the product of the *mel-26(sb45)* allele fulfils these criteria and acts in a dominant manner *in vivo* [96]. Likewise, overexpression of Cdc4 versions that are defective in substrate binding interferes with SCF^{Cdc4} function in budding yeast. Importantly, this dominant-negative effect can be alleviated by introduction of a D-domain mutation into the same allele [90, 95]. Moreover, alleles encoding monomeric Cdc4 fail to complement a *cdc4*Δ mutation and artificial dimerization of these versions partially restore SCF^{Cdc4} function *in vitro* and *in vivo* indicating the essential role of SCF^{Cdc4} dimerization *in vivo* [95]. Similarly, dimeric SCF^{FBW7} is more efficient in cyclin E ubiquitination than the monomeric version [51, 88], and SCF^{FBW4} and CRL3^{KEAP1} dimerization is also essential for cyclin D1 and NRF2 degradation respectively [93, 101]. Along the same lines, it is worth noting that Fbw7 has been isolated as a haploinsufficient tumour suppressor, and several cancer-associated mutations in Fbw7 may thus act in a dominant-negative fashion [106].

CRL dimerization induces conformational variability

As discussed previously in this review, CRLs do not have catalytic activity *per se* and only function by increasing the concentration of reactants and optimally positioning them to favour the ubiquitination reaction. Therefore, dimerization may regulate SCF/CRL activity by increasing the local concentration of substrate and ubiquitin-activated E2, by facilitating substrate recognition or by optimally orienting the acceptor lysines of the substrate and the ubiquitin-loaded E2 enzymes. The importance of SCF dimerization has been extensively studied in the case of the SCF^{Cdc4} complex in budding yeast. This complex controls entry into S-phase by triggering the degradation of the CDK inhibitor Sic1. Sic1 recognition and ubiquitination is triggered upon its phosphorylation in late G1 phase of the cell cycle by G1 cyclin/CDK activity on multiple sites [51, 107, 108]. Since Sic1 contains multiple degrons, one would predict that dimerization is required for efficient Cdc4-Sic1 interaction. For example, both Sic1-binding sites of the SCF^{Cdc4} dimer might simultaneously engage two Sic1 degrons and thereby increase the affinity of the interaction. However, monomeric and dimeric forms of Cdc4 have the same affinity for phospho-Sic1 [95]. Therefore in this context, SCF dimerization does not affect Cdc4 affinity for Sic1, but rather influences substrate positioning and lysine acceptor site utilization, suggesting that the configuration of the SCF^{Cdc4} dimer is critical. Indeed, small-angle X-ray scattering analysis of the dimeric Skp1/Cdc4 complex and modelling of the entire SCF^{Cdc4} complex revealed that the two Sic1-binding sites of Cdc4 and the two E2-binding sites lie in the same plane in a suprafacial orientation [95]. Such a configuration juxtaposes two ubiquitin-activated E2 enzymes in close proximity to the substrate, and provides different distances between the substrate and the activated E2, thereby increasing the reaction kinetics. In particular, such a configuration appears critical for efficient chain elongation.

Dimerization of SCF^{FBW7} is similarly required for efficient elimination of hypo-phosphorylated cyclin E, but it has not yet been elucidated whether dimerization acts to optimally position specific substrate lysines and an E2-active site, or to facilitate substrate binding or both. Further work is now required to determine whether CRL dimerization similarly affects ubiquitination of various substrates, but it is worth noting that the hypothetical CRL3^{BTB} complex also adopts a suprafacial configuration [102].

Regulation of CRL complexes by the Cop9 Signalosome and CAND1

CRL dimerization and cullin neddylation are critical events in the activation of CRL complexes. Hence these events must be tightly regulated. The Cop9 Signalosome hydrolyzes cullin-NEDD8 conjugates and together with CAND1 regulates CRL assembly

and activation. In this section we introduce the Cop9 Signalosome and CAND1 and discuss their roles in the CRL assembly cycle.

The Cop9 Signalosome hydrolyzes cullin-NEDD8 conjugate

The Cop9 Signalosome (CSN) is an evolutionarily conserved eight-subunit complex that was first identified in *Arabidopsis thaliana*, where it is required for the repression of photomorphogenic seedling development in the dark [109, 110]. A characteristic feature of the CSN subunits is the presence of two signature domains known as PCI (Proteasome, COP9, eIF3) and MPN (Mpr1-Pad1-N-terminal) domains, which are also present in two other large complexes, the eIF3 translation initiation factor complex and the proteasome lid. In particular, the proteasome lid and the CSN are both composed of the eight core subunits, six of the PCI class and two of the MPN class, and each subunit of the proteasome lid contains a paralogue in the CSN complex, such that there is a direct one-to-one correspondence between their subunits. In addition, both complexes may assemble into a similar ring-shaped structure with each paralogue located in the exact same position within the ring [111], suggesting a common origin for these complexes. The first link between the CSN and CRL deneddylation was established when the CSN was recovered in cullin immunoprecipitates and was found to deneddylate cullins [112, 113]. Subsequently, extensive protein sequence analyses identified a metalloprotease signature in the CSN5 subunit and site-directed mutagenesis confirmed that CSN5 is the enzyme deneddylating cullins [114]. Strikingly, a metalloprotease domain was also found in RPN11, the CSN5 paralogue of the proteasome lid, which was shown to process ubiquitinylated substrates in the 26S proteasome [115]. However, both recombinant CSN5 and RPN11 are catalytically inactive, suggesting an obligate role for complex assembly in enzyme activity. Consistent with these results, neddylated cullins accumulate upon inactivation of the CSN subunits.

What is the role of cullin deneddylation?

As previously discussed, neddylated CRLs are more active than their non-neddylated counterparts. Therefore, one would predict CRLs to be constitutively active in CSN mutants. Paradoxically, various genetic analyses in yeast [114, 116], plants [117], Drosophila [118] and *C. elegans* [119] revealed that reduction of CSN function attenuated rather than enhanced SCF and CRL function. To explain this apparent paradox, current models propose that CSN-mediated deneddylation of cullins is required to inactivate CRL complexes, and thereby prevent substrate recognition and CRL subunit instability by counteracting their auto-ubiquitination and subsequent degradation. Consistent with this model, the CSN prevents the autocatalytic instability of SKP2, cyclin F, FBW7, and FBX4 in human cell lines [120], FWD1 in *Neurospora crassa* [121], Btb3 and Pop1 in *S. pombe* [122, 123] and MEL-26 in *C. elegans* [124]. Core CRL subunits are also unstable when the CSN activity is compromised, such as the cullins CUL1 and CUL3 in Drosophila and in plants [125, 126], RBX1 in human cells [127], and CUL1 and SKP1 in *Neurospora crassa* [121]. A plausible mechanism for this function was provided with the discovery that the *S. pombe* deubiquitinating enzyme USP15 specifically interacts and cooperates with the CSN to prevent spurious ubiquitination of CRL subunits [123]. However, systematic proteomic analysis of the CSN complex in various systems including mammalian HEK 293T cells [42], *C. elegans* [124] and *Neurospora* [121] failed to detect any deubiquitination enzymes bound to the CSN. In addition, blocking cullin deneddylation by expressing a dominant-negative CSN5 mutant failed to rescue substrate-specific adaptor instability in mammalian cells [120], suggesting that the CSN deneddylase activity rather than its association with deubiquitination enzymes might be required to counteract degradation of substrate-recruitment factors. Despite preventing instability of CRL subunits, the CSN complex may fulfil several functions in CRL regulation. In particular, the CSN may trigger disassembly of CRL complexes and promote CRL subunit recycling (Fig. 4).

CRL regulation by CAND1

Another intriguing factor regulating the CRL assembly cycle is CAND1. CAND1 is an evolutionarily conserved HEAT-repeat containing protein of 120 kDa, which sequesters non-neddylated CUL1/RBX1 complexes, thereby preventing their assembly into SCF complexes [128–130]. Crystallographic analysis of the CUL1/RBX1/CAND1 ternary complex showed that CAND1 wraps around CUL1 in a head-to-tail fashion precluding SKP1 binding on one side, and neddylation on the other side by interacting with the cleft of the CUL1 CTD that bears the neddylated lysine residue [44]. However, CAND1 is unable to bind neddylated cullins since the C-terminally located CAND1 binding interface does not exist in neddylated CRLs, due to the NEDD8-induced reorientation of the cullin CTD [50]. By preventing both SCF assembly and cullin neddylation, CAND1 acts as a negative SCF regulator *in vitro*. Indeed, CAND1 has been shown to inhibit ubiquitination of SCF substrates such as IκBα or p27 *in vitro*. Paradoxically, SCF substrates accumulate in *cand1* mutants *in vivo*. For example in

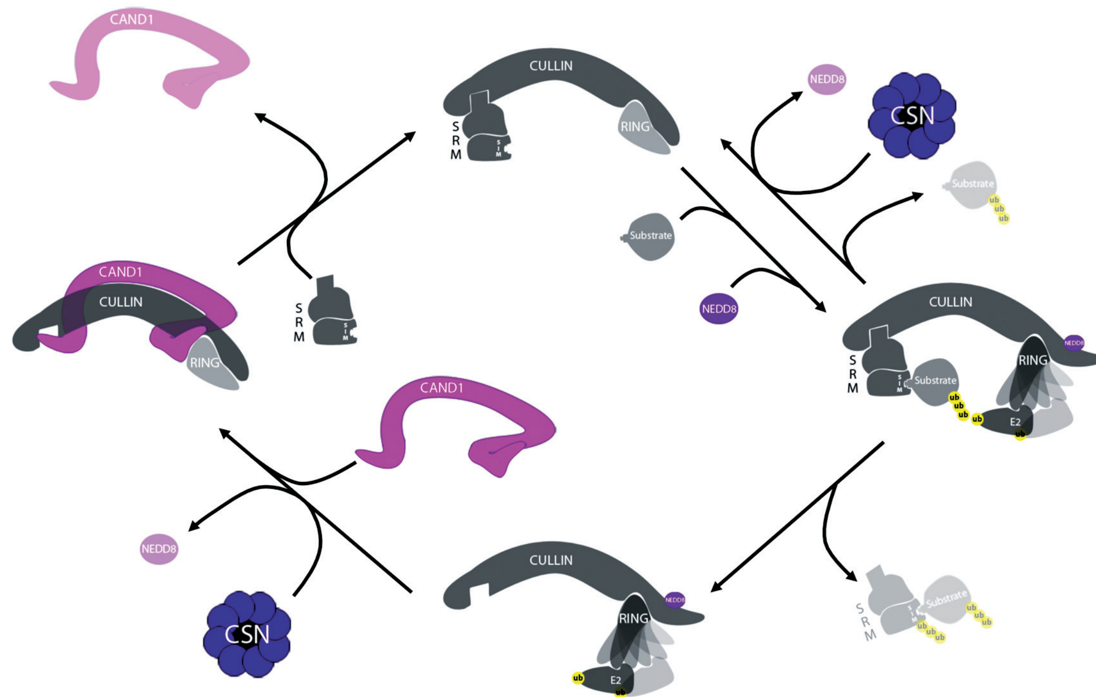

Figure 3. The CRL assembly cycle. Cullin/RBX1 (grey) heterodimers are sequestered by cullin-associated and neddylation-dissociated-1 CAND1 (pink), but upon binding of the substrate-recognition module (SRM), CAND1 dissociates and the CRL complex can be activated by neddylation of the cullin subunit. The Cop9 Signalosome (CSN, blue) hydrolyzes the cullin-NEDD8 conjugate and thereby inactivates CRL complexes.

plants, *loss-of-function* mutations in *cand1* confer multiple defects on SCF-regulated processes and results in the accumulation of Aux/IAA and RGA, the substrates of the SCF^{TIR1} and SCF^{SLY1} respectively [131, 132]. In human cells, CAND1 is similarly required for SCF^{SKP2} and $CRL3^{KEAP1}$ function [129, 133]. Zheng *et al.* reported that SKP2 protein levels decrease in *cand1* mutants, but that KEAP1 levels are not affected [129]. Likewise in plants, *cand1* inactivation does not affect the stability of the F-box proteins TIR1 [134], suggesting that CAND1 may not regulate SCF/CRL function by counteracting substrate adaptor instability. Instead, several lines of evidence indicate that CAND1 regulates SCF and CRL assembly and disassembly cycles **(**Fig. 3**)**. In particular, elegant genetic analysis in plants revealed that disrupting the CAND1/CUL1 interaction results in an increased abundance of assembled SCF^{TIR1} complex and, conversely, stabilization of the CUL1/CAND1 interaction diminishes the abundance of assembled SCF^{TIR1} [134]. Importantly, both increased and decreased CAND1/CUL1 compromise SCF^{TIR1} function, suggesting that CAND1-mediated cycling is critical for SCF function *in vivo* [134].

Is CAND1 an essential regulator of the SCF/CRL complexes?

Besides CUL1, CAND1 appears to bind all cullins, at least *in vitro.* This is consistent with the observation that CAND1 recognizes the overall elongated or arc-shaped structure that is adopted by all cullins [31, 43, 44]. However, *in vivo* the situation appears different. While CAND1 engages CUL1, CUL2, CUL3, CUL4A in human HeLa cells [130], only CUL1, CUL4A and CUL5 have been shown to bind CAND1 in human HEK293T cells [128]. Likewise, in *C. elegans*, CAND1 binds CUL2 [135], but does not appear to bind CUL3 [124]. Furthermore, *cand1* inactivation does not entirely recapitulate SCF/CRL inactivation. For example, *cand1* is neither essential in plants nor in *C. elegans,* suggesting that CAND1 contributes to the cycle of assembly and disassembly of CRLs only in particular physiological conditions. Specifically, CAND1 might play a critical role when several Skp1/F-box protein complexes compete at the same time for CUL1 binding. Along these lines, it is worth noting that CAND1 plays a significant role in plants, which contain more than 600 F-box proteins.

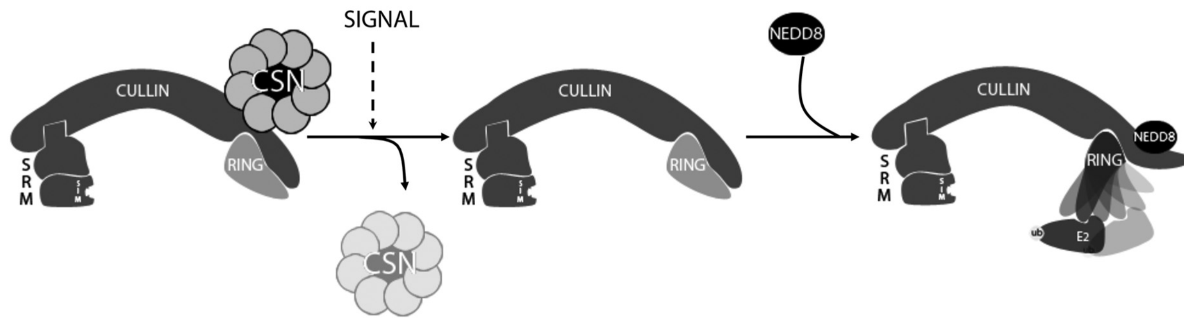

Figure 4. Signal-induced dissociation of pre-assembled CRL complexes from the Cop9 Signalosome. The Cop9 Signalosome (CSN) sequesters inactive pre-assembled CRL complexes (left panel) that are specifically activated by dissociation of the CSN and neddylation of the cullin upon a specific signal.

How is CRL assembly and activation coordinated with substrate binding?

Substrate binding and cullin neddylation occur on different sites of the CRL complexes, which are separated by sometimes more than 100 Å. Intriguingly, substrate-bound CRLs are highly neddylated, clearly suggesting a connection between cullin neddylation and substrate binding [78]. An increasing number of experimental findings suggest that these events are indeed coordinated and subject to extensive regulation.

SCF/CRL assembly induced by substrate-recognition module/substrate complexes

Several recent reports now indicate that the availability of the adaptor bound to its substrate may dissociate CAND1 and thus promote CRL assembly and activation through cullin neddylation. For example, the availability of the F-box protein SKP2, along with its substrate p27, triggers assembly of the SCF^{SKP2} ubiquitin-ligase and its activation through neddylation of the CUL1 subunit *in vitro* [136]. In particular, SKP2 in complex with SKP1 dissociates CAND1 while the substrate p27 blocks CSN-mediated deneddylation of CUL1. Likewise, coexpression of VHL along with its substrate HIF-1 alpha results in a dramatic increased of neddylated CUL2 [137]. Moreover cullin mutants unable to bind substrate-recognition modules exhibit reduced neddylation [138], indicating that CRL assembly and activation is mediated, at least in part, by substrate-recognition modules bound to their substrates. However, this type of regulation might be CRL specific and other CRLs might be activated through slightly different mechanisms.

Signal-induced dissociation of pre-assembled CRL from the CSN

Specific cues may trigger dissociation of fully assembled but inactive CRL complexes from the CSN, and in turn, their activation through cullin neddylation. For example, inactive $CRL4^{DDB2}$ ubiquitin-ligase is tightly bound to the CSN, but rapidly activated upon UV irradiation by dissociation from the CSN and subsequent neddylation [139]. This type of regulation is ideally suited when cells have to respond rapidly to a specific signal or insult such as signalling molecules or DNA-damaging agents. Importantly, systematic proteomic analysis of the mammalian Cop9 Signalosome revealed that many CRLs are tightly bound to the CSN complex and might be released upon a specific signal or insult. Since most of these CSN-interacting CRLs appear to regulate DNA metabolism, it is tempting to speculate that the release of multiple CRLs from the CSN may orchestrate DNA replication and the DNA-damage response, and concomitantly initiate DNA repair while preventing DNA replication [42] **(**Fig. 4**).**

Coordination of substrate phosphorylation and CRL activation through dimerization

CRL dimerization is critical for CRL function, but whether signalling events regulate CRL dimerization is unknown. Very interestingly, a recent report revealed that dimerization of the $SCF^{FBX4/alphaB\text{-}crystallin}$ complex is temporally regulated during the cell cycle, through phosphorylation-induced dimerization of the F-box protein FBX4, and coordinated with substrate phosphorylation [93]. The $SCF^{FBX4/alphaB\text{-}crystallin}$ complex targets phospho-cyclin D1 for degradation during cell cycle progression. At the G1/S boundary, cyclin D1 is phosphorylated by GSK3ß and exported to the cytoplasm in a CRM1-dependent manner where it is recognized by the $SCF^{FBX4/alphaB\text{-}crystallin}$ and polyubiquitinated [140]. Importantly, besides phosphorylating cyclinD1, GSK3ß phosphorylates FBX4 on its serine 12

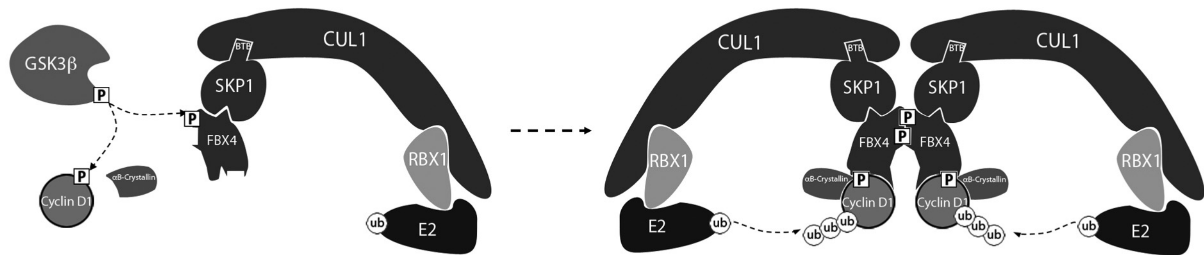

Figure 5. GSK3ß coordinates cyclin D1 phosphorylation and SCF$^{FBX4/alphaB\text{-}crystallin}$ dimerization at the G1-S transition. At the G1/S boundary, the kinase GSK3ß phosphorylates and targets cyclin D1 to its cognate E3-ligase SCF$^{FBX4/alphaB\text{-}crystallin}$. Concomitantly, GSK3ß activates the SCF$^{FBX4/alphaB\text{-}crystallin}$ through phosphorylation of the F-box protein FBX4. Upon phosphorylation, FBX4 promotes self-association and activation of the SCF$^{FBX4/alphaB\text{-}crystallin}$ complex and cyclin D1 ubiquitination.

residue and this phosphorylation event promotes dimerization of the SCF$^{FBX4/alphaB\text{-}crystallin}$ complex (Fig. 5) [93]. The importance of FBX4 dimerization for cyclin D1 degradation is mirrored by the observation that most of the cancer-associated mutations in FBX4 occur in the N-terminal region of FBX4 and interfere with its dimerization [93]. Other CRLs might be regulated similarly and future work is now required to further investigate this exciting aspect of CRL regulation.

Concluding remarks and perspective

During the last decade, tremendous progress has been made in identifying the composition and architecture of the various CRL complexes and the numerous regulatory mechanisms controlling their activity. Despite this progress, we are far from understanding the role and regulation of CRLs. For example, if bioinformatic predictions hold true, several hundreds or more distinct complexes compose the CRL family. So far, however, fewer than fifty substrates have been identified in humans [29]. Likewise, while neddylation and dimerization are emerging as key regulatory mechanisms activating CRL complexes, how these events are coordinated with substrate binding remains unclear. In particular, further investigation is needed to elucidate the regulation of the interaction between the Cop9 Signalosome and CRLs.

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