Building and remodelling Cullin–RING E3 ubiquitin ligases

‘Ubiquitylation: mechanism and functions’ review series

John R. Lydeard¹, Brenda A. Schulman² & J. Wade Harper^{1*}

¹Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, and ²Departments of Structural Biology and Tumour Cell Biology, Howard Hughes Medical Institute, St Jude Children’s Research Hospital, Tennessee, USA

Cullin–RING E3 ubiquitin ligases (CRLs) control a plethora of biological pathways through targeted ubiquitylation of signalling proteins. These modular assemblies use substrate receptor modules to recruit specific targets. Recent efforts have focused on understanding the mechanisms that control the activity state of CRLs through dynamic alterations in CRL architecture. Central to these processes are cycles of cullin neddylation and deneddylation, as well as exchange of substrate receptor modules to re-sculpt the CRL landscape, thereby responding to the cellular requirements to turn over distinct proteins in different contexts. This review is focused on how CRLs are dynamically controlled with an emphasis on how cullin neddylation cycles are integrated with receptor exchange.

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See the Glossary for abbreviations used in this article.

Introduction

The proteome is constantly remodelled to suit the needs of the cell, through both synthesis and turnover. Protein turnover is controlled through two main systems: the ubiquitin–proteasome system (UPS) and lysosomal degradation system, including autophagy. Although selective autophagy can provide a means by which to control the abundance of particular organelles and even single proteins, it is best understood as a means to control bulk turnover of cellular components [1]. Conversely, the UPS is a particularly versatile and highly regulated system [2] that allows selective turnover of individual regulatory proteins, even when they are incorporated into higher-order multiprotein assemblies. On the one hand, the entire population of a given protein targeted by the UPS might be subject

to rapid tagging with ubiquitin and degradation by the proteasome. On the other hand, the UPS might control the degradation of only a small pool of a particular target protein, for example the population of a protein that has been phosphorylated by an upstream pathway. Thus, the UPS functions in space and time to sculpt the proteome and to control the abundance of active or inactive forms of signalling complexes and cellular machines. As illustrated in this Review, the UPS machinery that controls turnover of a wide swathe of the proteome is itself dynamically regulated through many mechanisms.

The tagging of proteins with particular types of ubiquitin chains serves to mark them for proteasomal degradation. This process occurs through an E1 (ubiquitin-activating enzyme)–E2 (ubiquitin-conjugating enzyme)–E3 (ubiquitin ligase) cascade [3–7]. E3 plays a central role in substrate targeting by binding directly to the substrate and presenting target lysine residues to receive ubiquitin from the E2, in the case of RING E3s, or from a ubiquitin-charged cysteine residue in HECT and RBR E3s [6,8–10]. Cullin–RING E3 ubiquitin ligases (CRLs), which were first discovered almost two decades ago [11–13], are a superfamily of RING E3s responsible for as much as 20% of ubiquitin-dependent protein turnover in cells [14]. Yet, many facets of their biology and mechanism remain poorly understood. CRLs, which have five main subclasses that were first identified in *Caenorhabditis elegans* (Sidebar A, Fig 1) [15], are modular assemblies built on a cullin scaffold [12,13,16]. They contain a carboxy-terminal globular domain (CTD) with an embedded RING finger protein (RBX1 or RBX2) that serves as the site for E2 binding and ubiquitin transfer activity [17,18], and an amino-terminal helical domain, which binds to distinct sets of substrate receptors (SRs) that specifically recruit a target protein destined for modification with ubiquitin [17,19,20]. The SR modules for CUL1, CUL2/5, CUL3 and CUL7 are structurally related, whereas those for CUL4A/B are divergent and contain motifs dissimilar to other CRLs [20–24]. As described below, many of the regulatory features of CRLs are thought to apply across CRL subfamilies regardless of the identity of the cullin and the specific SR module involved. Thus, for simplicity, we refer here to SR modules as general entities based on conserved features across CRL families.

The impact of CRLs on biology is evidenced by the large number of SR proteins identified, including ~200 in mammals (Fig 1) and even more in plants and worms [21,25,26]. The vast majority of these

¹Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

²Departments of Structural Biology and Tumour Cell Biology, Howard Hughes Medical Institute, St Jude Children’s Research Hospital, Memphis, Tennessee 38105, USA

*Corresponding author. Tel: (617) 432-6590; Fax: (617) 432-6591;

E-mail: wade_harper@hms.harvard.edu

receptors have not been studied in detail, but CRLs have been linked to many biological processes (Sidebar B) [19,27]. This complexity is undoubtedly reflected in the targets that CRLs ubiquitylate. The development of global approaches for matching CRLs with their substrates might potentially accelerate substrate identification, but elucidation of complex regulatory circuits that control target ubiquitylation will typically require focused studies [28–32]. Moreover, substantial effort has gone into the development of small-molecule inhibitors of the pathway, including SRs CDC4/FBXW7 and SKP2, the E2-conjugating enzyme CDC34 and the neddylation system (Sidebar C; [33]).

Given the vast assortment of CRL SRs, an important question is how their assembly into the repertoire of active CRL^{SR} complexes is regulated. Distinct SRs might be important for particular cellular or developmental events and, therefore, the timing of their appearance and activation might be critical for cellular or organismal homeostasis. Moreover, particular pairs of CRL^{SR} complexes might function in an antagonistic manner and their coexistence in active forms could be detrimental to the cell. Recent research has revealed that a complex regulatory framework controls CRL assembly and activation [34–39]. In this Review, we describe emerging themes in CRL regulation and highlight studies that have advanced understanding of how CRLs become activated and how CRL^{SR} identity is controlled in cells.

Overview of CRL regulatory machinery

CRL regulation can be viewed in its simplest form as a means by which to toggle individual CRL^{SR} complexes between on (active) and off (inactive). When on, the CRL^{SR} complex can engage substrates and catalyse ubiquitin transfer, whereas in the off state the CRL is inactive as an E3 ligase. The regulatory architecture of the CRL system, however, is much more complex and has many mechanisms for establishing the activity status of dozens of CRLs simultaneously and dynamically in cells. For example, there might be several distinct off states for individual CRLs. Here, we define the basal state of a CRL complex as being composed of a cullin, a RING finger protein (RBX1 or RBX2) and an SR module. As expanded on below, however, the available data indicate that the CRL is unlikely to be in this basal state for a substantial amount of time and, in principle, will probably populate a wide range of architectures during its lifetime. Relevant events that modify the CRL architecture include: activation through the process of neddylation; association of the active neddylated form with the COP9 signalosome (CSN) complex, a multi-subunit deneddylase that can sterically block substrate access, catalyse cullin deneddylation, and remain bound to the CRL; loss of SR through proteolytic degradation; and SR exchange via a CAND1-driven mechanism [21,34–38,40–43]. A confounding factor to understanding this regulation is that in cells these different events are generally not synchronized for CRLs *en masse*, or for individual CRL^{SR} complexes. Thus, CRL^{SR} complexes *in vivo* are an admixture of distinct regulatory architectures that are difficult to deconvolute and quantify [36]. In addition, the balance between particular CRL assemblies might be dictated by the abundance of specific substrates that can effectively displace CRL^{SR} complexes from their CSN-bound assemblies, as described below [35,36]. Finally, there is likely to be competition among individual CRLs for the main regulatory elements (CSN, CAND1 and NEDD8-pathway proteins), as these components seem to be limiting relative to the total abundance of cullins in cells, and different cells will probably have not only a different array and total abundance of SRs but also potentially different levels of regulatory factors [36]. Thus, elucidating the dynamics of CRL

Glossary

BC	elongin BC
BTB	broad complex, tramtrack, 'bric-a-brac'
CAND1	cullin-associated and neddylation-dissociated 1
CRL	cullin RING ligase
CTD	carboxy-terminal globular domain
CSN	COP9 signalosome
CSN1	COP9 signalosome subunit 1; also known as GPS1 and COPS1
Cul	cullin
DCAF	DDB1 CUL4 associated factor
DCN1	defective in cullin neddylation 1
DDB1	DNA damage-binding protein 1
ERAD	endoplasmic-reticulum-associated degradation
GEFs	guanine nucleotide exchange factor
GVM	glomvenous malformation
HECT	homologous to the E6-AP carboxyl terminus
NAE	NEDD8-activating enzyme
NatC	N-acetyl transferase
NEDD8	neural precursor cell expressed developmentally downregulated protein 8 system
PIP box	PCNA interaction protein motif
PONY	potentiating neddylation
RBR	RING-between-RING
Rbx	RING box
RING	really interesting new gene
SKP1	S-phase kinase-associated protein 1
SCF	SKP1–CUL1–F-box protein
SR	substrate receptor
SREF	substrate receptor exchange factor
SOCS	suppressors of cytokine signalling
UBC	ubiquitin-conjugating
UPS	ubiquitin-proteasome system
VHL	von Hippel–Lindau

architecture *in vivo* presents major technical and intellectual challenges. That the current models of this regulation have been built primarily on the basis of structural and biochemical studies and that some implications of the existing models have not been fully tested *in vivo* is, therefore, unsurprising.

Mechanism of cullin neddylation

Nedd8 is a ubiquitin-like protein (58% identical to ubiquitin) that is covalently linked to a conserved lysine residue in a C-terminal winged-helix motif in cullins (Lys720 in CUL1) through an E1 NEDD8-activating enzyme (NAE)–E2 NEDD8-conjugating enzyme cascade, wherein UBC12 and its close orthologue UBE2F are dedicated E2s [43–47]. Early studies demonstrated that cullin neddylation is required for efficient ubiquitylation and/or turnover of CRL substrates *in vivo*, which has been borne out through the development of MLN4924, a small-molecule inhibitor of NAE [14,48–50]. Inhibition of CRL activity by MLN4924 results in accumulation of a host of CRL targets [14,43,51,52].

Structural and biochemical studies revealed a complex mechanism underlying cullin neddylation that involves dual E3 activity and co-translational modification of neddylation E2s to strongly activate the transfer of NEDD8 to the cullin [39,44,53,54]. Early studies indicated that UBC12 can neddylate CUL1 *in vitro* in a manner that requires RBX1 [55–57]. Genetic and biochemical data obtained initially in budding yeast and *C. elegans*, however, revealed that a novel conserved protein, DCN1, is necessary for maximum neddylation of yeast CUL1 orthologue Cdc53 *in vitro* and *in vivo* [58].

Sidebar A | Cullin–RING E3 ubiquitin ligase architecture

Cullin–RING E3 ubiquitin ligases (CRLs) are modular complexes that form an elongated horseshoe-like structure. In humans, one of six cullin proteins—CUL1, CUL2, CUL3, CUL4A/CUL4B, CUL5 and CUL7—form the central CRL scaffold. At the catalytic core, the cullin carboxy-terminus is bound to the amino-terminus of a RING finger protein—RBX1 or RBX2. RBX2 uniquely associates with CUL5, whereas RBX1 binds to the other cullins. The C-terminal RING domain of RBX1/2 engages an E2-conjugating enzyme to mediate ubiquitin transfer. Cullin N-termini bind to a collection of distinct substrate-receptor modules to recruit different targets. There are three classes of module. (i) The substrate-receptor module for CUL3-based E3s are single proteins that contain a broad complex, tramtrack, bric-a-brac (BTB) fold that interacts with the N-terminus of CUL3 and an additional protein-interaction domain that binds to substrates. (ii) The substrate receptors (SRs) for CUL1, CUL2, CUL5 and CUL7 use one of two BTB-fold proteins (SKP1 or ELOC) to interact with the N-terminus of their respective cullin, and SKP1 and ELOC contain additional sequence elements that associate with specific classes of substrate-binding receptor proteins—F-box proteins for SKP1 or BC/SOCS-box proteins for ELOC (for example, the F-box motif is a 40-residue structure that interacts with SKP1 to form the SR module for CUL1-based E3s, which are commonly referred to as SCF for Skp1–Cul1–F-box ligases). (iii) The substrate modules for CUL4A/B are composed of DDB1 and members of the DCAF family of SRs. DDB1 is unrelated to SKP1 and ELOC, but also associates with the N-terminus of the cullin. For each receptor family, between 20 and 100 specific receptor proteins have been identified [21,22]. In addition, PARC/CUL9 has been shown to bind to RBX1 and to be neddylated, but it does not associate with SKP1 or F-box proteins [101], and its molecular functions and adaptors remain to be identified.

For simplicity, therefore, we refer to Cdc53 as yeast CUL1 throughout. This activity required the potentiating neddylation (PONY) domain of DCN1, which can interact with both the cullin and UBC12 [54,59]. The lack of co-precipitation of RBX1 and DCN1 implied that DCN1 promotes neddylation independently of RBX1, which was inconsistent with previous work [55,56]. Subsequent structural and mechanistic analysis clarified this picture, demonstrating that the RBX1 RING domain can adopt multiple conformations, including a conformation that would enable the active-site cysteine of bound NEDD8–UBC12 to face the recipient lysine in the cullin [60,61]. The conformational flexibility of the RING domain in conferring neddylation was further supported by crosslinking and mutational data, which demonstrated that previously observed conformations of the RING domain did not support neddylation [60]. In addition, an N-terminal helix extension in UBC12 physically associates with the PONY domain of DCN1 (Fig 2A), which enhances the ability of the RBX1–RING-bound UBC12 to approach the neddylation site [53]. Thus, maximum NEDD8 transfer occurs when DCN1 and RBX1 are functioning together [53].

Budding yeast contains a single *DCN1* gene, whereas the human genome encodes five DCN1-related proteins. Why this gene family has expanded and whether individual DCN proteins function in specific CRL activation pathways remains unclear. Indeed, initial attempts to demonstrate specific roles for any DCN protein in the activation of cullin neddylation in human cells were disappointingly negative, which possibly reflects functional redundancy [62,63]. In addition, when bacterially produced and purified components were used, human DCN1 did not effectively stimulate cullin neddylation by recombinant UBC12 purified from bacteria [39]. The resolution of this paradox came from the discovery that UBC12

Fig 1 | Architecture of human cullin–RING E3 ubiquitin ligase system. The number of human SRs for each CRL complex is indicated on the left. CUL4A and CUL4B are represented as a single CRL. The CRL regulatory apparatus is composed of the neddylation system, the deneddylation system and the SR-exchange factor. UBC12 is the neddylation E2 for RBX1-based CRL complexes, whereas UBE2F is the E2 for CRL5–RBX2. DCN1 is a co-E3 for both RBX1 and RBX2. The CSN deneddylates CRLs. CAND1 is a CRL exchange factor that interacts with both the amino- and carboxy-terminal regions of the cullin–RING complex. CSN subunits are indicated, an asterisk indicates that CSN5 is the catalytic subunit. CRL, cullin–RING E3 ubiquitin ligase; CSN, COP9 signalosome; N, NEDD8; S, substrate; SR, substrate receptor; U, ubiquitin.

is acetylated at its N-terminus *in vivo*, and this modification is critical for optimum activation of UBC12–DCN1-dependent neddylation of both the yeast and mammalian cullins [39]. The N-terminal helix extension in UBC12 is positioned to facilitate the engagement of a hydrophobic pocket in DCN1 by the N-terminal acetyl-methionine moiety (Fig 2B). In essence, acetyl-methionine acts as

a unique amino-acid structure that is highly complementary to the binding site in DCN1, and increases the binding constant of UBC12 for DCN1 by more than two orders of magnitude [39]. N-terminal acetylation is required for human DCN1-dependent stimulation of cullin neddylation *in vitro*, whereas yeast UBC12 acetylation increases the rate of neddylation afforded by yeast DCN1 [39]. This finding is consistent with the fact that deletion of the main N-acetyl transferase for DCN1 in budding yeast NatC led to a partial decrease in cullin neddylation *in vivo* [39]. Previous work has demonstrated that mammalian CUL5 primarily uses RBX2 as its cognate RING finger, and this CUL5–RBX2 module requires a second neddylation E2, UBE2F, for activation [18,44]. Like UBC12, UBE2F is also acetylated at the N-terminus, which promotes NEDD8 transfer to CUL5 [64]. Similarly, UBC12 N-terminal acetylation is important for stimulating neddylation of CUL1 and other cullins that use RBX1 as the RING finger protein [64]. Nevertheless, the role of the five DCN orthologues in mammals remains largely unknown. The PONY domains of the individual family members can activate several cullin–RBX pairs to varying degrees, but the impact on *in vivo* neddylation has been difficult to assess with RNA interference, probably because of at least partial redundancy among the various DCN family members [63,64].

Consequences of cullin neddylation for CRL activity

How neddylation regulates CRL activity depends on context. Crystallographic studies of several cullin–RING assemblies (CUL1–RBX1, CUL1–RBX1–SKP1–F-box, CUL1–RBX1–CAND1, CUL5^{CTD}–RBX1 and CUL4A–RBX1–DDB1) in the unneddyated forms indicated a rigid structure [17,61,65,66]. Early modelling studies (Fig 2C) placed the catalytic cysteine of the RBX1-bound E2 approximately 50 Å from the substrate [17], which raised the question of how the ubiquitin was actually transferred to the substrate and to the elongating ubiquitin chain. Subsequent analysis of neddyated CUL5^{CTD}–RBX1 [61] revealed a striking reorganization of the cullin CTD, which places the RING domain of RBX1 in an orientation distinct from previously observed structures. Crosslinking studies indicated that, upon neddylation, RBX1 is no longer packed against the cullin in the same way (Fig 2C,D) [61,67,68]. Thus, one can think of the un-neddyated state as off or inactive and the neddyated state as on or active. An important unanswered question concerns the extent to which the mobility of the RING domain is important for both the initial ubiquitin transfer step and for processive polyubiquitination, although it is clear that neddylation activates these events (Sidebar D) [61,67,68]. The answer will probably require crystallographic and kinetic analyses of neddyated CRLs bound to charged E2s and substrate. Methods developed recently to examine the structure of a HECT E3-activated ubiquitin–substrate complex that mimics the ubiquitin transfer step could provide a route towards addressing this question [69].

Although the activation of the intrinsic activity of a CRL is one mode of regulation by neddylation, it is not the only one. Indeed, CRL neddylation can block association with the CAND1 exchange factor and enhance association with the CSN complex, as described in detail below.

In the on state, CRLs can be highly processive, giving rise to multiple catalytic cycles before substrate dissociation [70–72]. This feature has much to do with how a CRL-specific, ubiquitin-chain-forming E2 associates with the cullin–RING E3 scaffold. In budding yeast, Cdc34 is the dedicated E2 for ubiquitin chain

Sidebar B | Cullin–RING E3 ubiquitin ligase substrate recognition

Cullin–RING E3 ubiquitin ligases (CRLs) must target substrates for degradation in the appropriate cellular context. As an additional layer of regulation, CRLs often recognize substrates only after their post-translational modification. The requirements are unique to individual substrate receptors (SRs), but there are common themes that govern CRL substrate recognition. The archetypical one is the recognition of a short peptide degradation sequence in a phosphorylation-dependent manner (a phosphodegron). F-box proteins FBXW7, β -TrCP and SKP2 with its partner CKS1, all recognize distinct phosphodegrons in which a conserved serine or threonine is phosphorylated. For instance, SCF^{FBXW7} recognizes the phosphodegron pT-P-P-X-S motif (where X means any amino acid and p indicates phosphorylated residue) in substrates important for cell growth, including MYC, cyclin E and JUN. Other substrates, such as Notch, have a variation of this motif [102]. However, the recognition of phosphodegrons is not a requirement of SCF complexes, as FBXO2 and FBXO6 are involved in endoplasmic-reticulum-associated degradation by recognition of glycosylated proteins for degradation [103]. The CUL2–VHL tumour suppressor (CRL2^{VHL}) targets a range of substrates, including HIF1 α , under normoxic conditions by recognition of a specific hydroxylated-proline epitope [104,105]. Methyl-degrons have recently been identified for CRL4^{DCAF1} ligases [106]. Some SRs require dimerization or an additional receptor protein for efficient substrate recognition. SCF β -^{TrCP} and SCF^{FBXW7} dimerize to increase ubiquitylation activity by allowing multiple geometries to target various acceptor lysines [107,108]. CRL4^{CDT2} substrates, including CDT1, p21 and SET8, contain a PIP-degron and are degraded only when engaged with chromatin-bound PCNA [109]. SRs can also recognize DNA; for example, CRL4^{DDB2} detects ultraviolet-induced pyrimidine dimers in chromatin to facilitate nucleotide excision repair [110]. Some SRs require small molecules to act as glue and bridge interactions with substrates [111]. Additional CRL degron motifs will probably be identified for SRs in the coming years.

synthesis by SCF E3s [12,13,73]. Cdc34 exclusively promotes the synthesis of Lys 48-linked ubiquitin chains, because residues near its active site are complementary to the surface surrounding Lys 48 in ubiquitin [71], which is a structural feature conserved in human CDC34. This mechanism of chain-type specificity is integrated with features of the CRL that promote processive chain building. First, transfer of the first ubiquitin from the E2 to the lysine residue in the substrate is slower than subsequent ubiquitin-to-ubiquitin transfer rates [71,72]. This has been proposed to provide a proof-reading mechanism in which substrates that have suboptimum degrons—that is, only partly complementary degrons—dissociate from the CRL before attachment of the first ubiquitin. However, once the first ubiquitin is transferred, the rate of transfer of subsequent ubiquitins is increased, such that several ubiquitin-transfer cycles can occur before substrate dissociation. This speed ensures productive polyubiquitylation of optimum substrates and disfavors ubiquitylation of non-cognate targets, which would tend to have rapid off-rates [72]. Second, electrostatic complementarity between the acidic tail of the Cdc34 C-terminal and a basic canyon on the cullin protein renders the rate of encounter between charged E2 and the CRL faster than the diffusion limit. Because most Cdc34 is charged *in vivo* [74] and charged CDC34 has a higher affinity for neddyated CRLs than un-neddyated forms [68,70], this default state encourages processive target ubiquitylation.

Cdc34 is apparently the only E2 in yeast that functions with CRLs, whereas in mammals CDC34 and UBC5 have been implicated in ubiquitin-chain synthesis with CRLs. Humans have two and five CDC34 and UBC5 genes, respectively, which has

Sidebar C | Pharmaceutical inhibition of cullin–RING E3 ubiquitin ligase pathways

Mutation or dysregulation of cullin–RING E3 ubiquitin ligases (CRLs) can result in the development of cancer and other human diseases [19,24,27,112,113]. Targeting of the ubiquitin–proteasome system for pharmaceutical intervention through proteasome inhibition has been successful in the treatment of multiple myeloma and relapsed mantle-cell lymphoma [114,115]. Inhibition of CRL activation has become a promising way to treat cancer. The development of MLN4924, a first-in-class small-molecule inhibitor of NAE that prevents neddylation and, therefore, activation of CRLs could be a useful treatment of non-Hodgkin lymphoma or relapsed and/or refractory multiple myeloma [14,51,116]. CRLs are also hijacked by viruses, including adenovirus, paramyxovirus and HIV [117,118]. In this regard, the inhibition of the neddylation pathway also holds promise as a novel antiretroviral therapeutic to combat HIV [52].

CRLs can also be pharmaceutically inhibited at the ubiquitin transfer step. A remarkably specific small-molecule inhibitor allosterically inhibits CDC34 but not its paralogue CDC34B, which indicates that ubiquitin E2s are rational drug targets [119]. In addition to regulation of global CRLs, individual SRs might also be targeted, which enables therapeutic focus. The CRL4 receptor protein CRBN is the target of thalidomide [120], which is used (as well as its derivatives) to treat blood cancers [120,121]. CRL substrate receptors (SRs) CDC4/FBXW7 and SKP2 have also been targeted for small-molecule inhibition [122–124]. As more is understood about these pathways, we envision that the inhibition of CRLs and individual SRs is a promising and growing field of drug discovery [33].

made molecular analysis of these pathways *in vivo* more complicated than in yeast. Early studies indicated that UBC12 and/or CDC34 could promote conjugation of ubiquitin onto the CRL substrates I κ B α or p27 *in vitro*, and that overexpression of catalytically inactive UBC12 or CDC34 could inhibit TNF α -induced I κ B α degradation *in vivo*, presumably through a dominant-negative mechanism [50,75–77]. Studies that used RNA interference, which depleted the isoforms for each E2, indicated that UBC12 and CDC34 proteins are both required for I κ B α turnover *in vivo* [78]. The requirement for two E2s was rationalized by the finding that, *in vitro*, CDC34 poorly ubiquitylates I κ B α , but addition of UBC12 substantially increases the ability of CDC34 to promote chain elongation on I κ B α [78]. UBC12 promoted monoubiquitylation of I κ B α , and it was proposed that this form of I κ B α is a substrate for CDC34 in a two-step mechanism [78]. Other studies, however, indicated that CDC34A or CDC34B is sufficient to elongate highly processive ubiquitin chains, not only on I κ B α but also on cyclin E, which is a substrate of SCF^{FBXW7} [35,72,79,80]. UBC12 orthologues lack the C-terminal acidic tail characteristic of CDC34 proteins, and would, therefore, not be expected to use a mechanism analogous to that used by CDC34 to control the dynamics of the CRL encounter. This difference possibly explains why UBC12 primarily monoubiquitylates I κ B α *in vitro*. Nevertheless, examples of CRL-dependent monoubiquitylation of target proteins are emerging and UBC12 could potentially function in these pathways, leaving Lys 48 chain production to the dedicated CRL E2, CDC34 [81]. Moreover, the basic canyon found in CUL1 is conserved in all mammalian cullins [70], which indicates that chain assembly through CDC34 in mammals might use a similar kinetic mechanism to that proposed for CUL1. Further experimental studies are required to understand the complexity of E2 utilization for various classes of CRL in mammals.

Fig 2 | Structure of selected cullin-binding components of neddylation machinery and the effect of cullin neddylation on SCF structure. (A) DCN1 (violet) co-recruits UBC12's N-terminal helix (cyan) and the WHB subdomain at the carboxy-terminal from a cullin (CUL1, green) [39]. (B) Close-up view of panel A, with DCN1 shown in surface view coloured by electrostatic potential (red, negative; blue, positive), which highlights the hydrophobic pocket that binds to UBC12's N-acetyl methionine. (C) Model for an unneddylated but fully assembled CRL in complex with an E2–ubiquitin intermediate, based on superposition of structures of CUL1–RBX1–SKP1–SKP2–CKS1–p27 phosphopeptide and RING–UBCH5–E2 [17,125–127]. The p27 phosphopeptide is shown in spheres. The substrate is distal from the E2–ubiquitin active site. (D) Model of a neddylated CRL showing the potential for RBX1 RING domain rotation, which is based on superimposing common features of CUL1–RBX1 with NEDD8–CUL5^{CTD}–RBX1 [61]. NEDD8 is shown in yellow covalently linked to the repositioned portion of CUL1. The location of the RBX1 RING domain found in some unneddylated CRL structures is shown in blue, with alternative positions found in neddylated structures shown in sky and cyan. CRL, cullin–RING E3 ubiquitin ligase.

CRL inhibition by the CSN deneddylase

One of the most perplexing aspects of the CRL system is that CRLs must undergo cycles of neddylation and deneddylation. This action is now beginning to be understood in the context of CRL–receptor exchange, as described in detail below, and is inextricably linked to the CSN deneddylase. Although the deneddylase activity of the CSN was discovered more than a decade ago [34,36,42,82–84], only recently has the complexity of its roles in CRL homeostasis emerged. The CSN is composed of eight subunits, most of which contain PCI domains (Figs 1,3A). CSN5/COPS5 is the catalytic subunit and contains a zinc-metalloenzyme active site [83,85]. Data indicate that the CSN is structurally related to the 19S regulatory particle of the proteasome, which also contains PCI domains [86–89]. As a deneddylase, it seems feasible that the primary function of the CSN would be to transiently associate with CRLs and to remove NEDD8. However, studies have revealed a more complex picture.

First, 10–20% of SCF complexes are tightly associated with CSN complexes at steady-state in human cells, and the extent of CSN–SCF association is only reduced by around twofold in the absence of CUL1 neddylation [36]. Thus, the CSN has an affinity for SCFs independent of their neddylation state *in vivo* [36]. This affinity has been shown *in vitro* with CSN complexes devoid of CSN5/COPS5 activity [38,40,41]. Indeed, electron microscopy images of SCF^{SKP2/CKS1} bound to CSN indicate the primary involvement of CSN2/COPS2 in engaging the cullin–RBX1/2 module, whereas other CSN subunits approach the variable SR arm (Fig 3A,B) [41]. The finding that CSN physically approaches and could interact with the SR arm is surprising given the structural diversity of these modules (Fig 1), but could also partly explain why the fraction of particular CRLs found in association with CSN can differ. For example, the extent of CSN association with CUL4B approaches 40%, but is < 5% for CUL2 and CUL3 [36]. The neddylated cullin CTD–RING module is buried by the CSN in a manner that blocks the access of CDC34, thereby suppressing ubiquitin ligase activity (Fig 3A) [41].

Second, and surprisingly, data from several studies, including the initial identification of the CSN as a CRL deneddylase [83], indicate that CSN subunits can associate avidly with neddylated CRLs [36,83,90]. Quantitative studies indicate that as much as 50% of cullins associated with CSN complexes retain neddylation, as determined with tagged CSN5 or CSN6 subunits in lysates generated in the presence of CSN inhibitor *o*-phenanthroline to block post-lysis deneddylation [36,40]. These findings indicate that either association of the CSN with a neddylated CRL itself is not sufficient for isopeptide bond hydrolysis or that an unknown CSN inhibitory factor exists. Nonetheless, the data indicate a second signal could dictate the timing of NEDD8 removal from the cullin [36]. One caveat is that these studies have generally relied on epitope-tagged CSN subunits that might be capable of interacting with CRLs but lack enzymatic activity due to incomplete assembly with the CSN5 catalytic subunit.

Third, the association of CSN with potentially active (neddylated and receptor loaded) CRLs renders these complexes functionally inactive and, in essence, removes this population of CRLs from the active cellular pool [40,41]. Biochemical studies, however, indicate that the association of CSN with CRLs can be reversed by the presence of substrates [40,41]. Incubation of preformed CSN–CRL complexes with substrates results in a loss of CRL binding and engagement of the substrate by the CRL complex [38,40,41]. Competition between substrates and the CSN, therefore, provides

Sidebar D | In need of answers

- (i) What is the SKP1–CUL1–F-box protein (SCF) substrate landscape? Which motifs are recognized by individual substrate receptors (SRs)?
- (ii) How are cullins built *in vivo*? How are cullin–RING E3 ubiquitin ligases (CRLs) activated and how is individual CRL–SR complex formation spatially and temporally controlled in cells?
- (iii) Does CAND1 act as an exchange factor on all SRs or is it a unique mechanism for a subset of modules? How does CAND1 perform the SR exchange for individual cullins, particularly with structurally divergent CUL4–DDB1?
- (iv) What are the kinetics of cullin–COP9 signalosome (CSN) binding and deneddylation of SCF complexes?
- (v) Why has DCN1 diverged in humans? Can all DCN1 family members act with UBC12/UBE2F to neddylate cullins *in vivo*?
- (vi) To what extent is cullin neddylation and RBX1/2 RING mobility critical for the initial ubiquitylation and subsequent polyubiquitylation of SCF substrates?
- (vii) Does CSN have an inhibitory role independently of its ability to deneddylate cullins?

a potential mechanism for controlling the abundance and architecture of CRLs *in vivo* (Fig 4A). When substrates are abundant for a particular CRL^{SR}, these substrates can successfully compete with CSN for engagement. Current models posit that as the concentration of a substrate for a specific SR dissipates in space and time, CSN increasingly engages the CRL^{SR}, which leads to removal of the CRL^{SR} from the active CRL pool and potentially to its deneddylation (Fig 4A). Dissociation of the deneddylated CRL^{SR} from the CSN would then provide a substrate for CAND1-dependent receptor exchange, as described below, thereby recycling the cullin–RING scaffold to fit the needs of the cell (Fig 4A). This substrate competition model has been developed to a large degree on data from structural and *in vitro* studies and has not been exhaustively tested *in vivo* [38,40,41]. Indeed, testing this model would require a means by which to remove all of the substrates of a particular SR followed by a quantitative assessment of the extent of association of the CRL^{SR} with CSN. Because several substrates would be required, investigation would be experimentally challenging. An alternative approach might be to use SRs containing point mutations in the degron-binding site. The expectation is that this receptor would mimic a situation in which substrates are depleted, giving rise to a quantitative shift in CSN assembly relative to the wild-type SR. Moreover, the absence of synchrony *in vivo* makes analysis of individual CRL^{SR} complexes, their substrates, and the extent of neddylation and CSN engagement challenging.

Building CRL complexes through CAND1

A model is emerging of CRL regulation by assembly of SR modules with the cognate cullin–RING assembly, which then seems to be coupled to rapid DCN1-dependent and RBX1/2-dependent neddylation, at least when substrates for the particular CRL^{SR} are abundant (Fig 4A). Of course, this is an oversimplification, as multiple SRs for a particular cullin are present at any given time and the abundance of different SRs changes due to synthesis and degradation. How does the cell know precisely what array of SR modules to engage with a cullin at any given time? Presumably, this is dictated in part by the array of substrates that are present, which will be coupled with the developmental or functional state of the cell. But there might be situations in which rapid changes in the constellation of

Fig 3 | Structures of the CSN–SCF and CAND1–CUL1–RBX1 complexes. **(A)** Two views of the structure obtained by electron microscopy of catalytically inactive CSN bound to SKP1 (dark blue)–SKP2 (purple)–CKS1 (pink)–NEDD8 (yellow)–CUL1 (green)–RBX1, with density for these proteins shown as grey mesh and for CSN in red. Because NEDD8, the CUL1 WHB domain to which it is linked, and the RBX1 RING domain are similar sizes, only their approximate locations are indicated [41]. **(B)** Crystal structure of CUL1 (green)–RBX1 (blue)–CAND1 (red). The NEDD8 acceptor lysine (Lys 720) on CUL1 is shown in spheres [66].

active CRL^{SRs} need to occur. For example, certain receptor proteins such as S-phase F-box protein SKP2 accumulate during specific phases of the cell cycle and need to be efficiently incorporated into CRL complexes to maintain timely cell-cycle progression [19]. Data indicate that this remodelling is promoted by the CAND1 protein in a process that depends on both the array of SR modules available and the neddylation state of the available cognate cullin [34,35,37].

CAND1 has had an enigmatic past. This heat-repeat protein was first suggested to function as an inhibitor of CRLs by binding specifically to the cullin–RING assembly in a manner that was blocked by cullin neddylation [91,92]. This model was strengthened by structural analysis of CAND1–CUL1–RBX1, which revealed that the cullin scaffold is draped by CAND1 on both the N-terminal SKP1 binding site and C-terminal junction with RBX1 to block the NEDD8-acceptor lysine, which resulted in a cullin–RBX1–CAND1 complex that is incompetent for NEDD8 or ubiquitin ligation (Fig 3B) [66]. Genetic studies, however, indicated that CAND1 is a positive regulator of CRL function, at least in specific pathways linked with particular SR modules [82,93–97]. Moreover, addition of SKP1/SKP2—the SR module responsible for ubiquitination of p27—to preformed CAND1–CUL1–RBX1 complexes led to dissociation of CAND1 from CUL1–RBX1 [98], which suggests reversibility in CAND1 function. This apparent paradox has been resolved by the finding that CAND1 can promote SR exchange *in vitro* and its removal from cells can alter the steady-state distribution of receptors associated with CUL1 [34,35,37].

An important insight came from a kinetic analysis of SCF assembly with a series of fluorescence resonance energy transfer probes linked with different components of the CRL [35]. The SR module (composed of SKP1 and F-box protein FBXW7) binds extremely tightly to unneddylated or neddylated CUL1–RBX1, with a dissociation rate of $\sim 10^{-6}$ and an association rate of $\sim 10^6$, which gives a K_D value in the picomolar range. Assuming other SRs for SCF complexes have similar binding constants, SR exchange seemed unlikely to occur on a biologically relevant timescale with previously assembled SCF complexes in the absence of a catalyst. Contrary to prediction, addition of CAND1 to previously assembled but unneddylated SCF^{FBXW7} complexes increased the rate of SR-module dissociation by several orders of magnitude (to 1.3 s^{-1}), but this effect was almost completely abrogated if the SCF complex was neddylated, which is consistent with the ability of NEDD8 to block the access of CAND1 to the cullin scaffold [35]. One potential caveat is that some F-box residues also interact with CUL1 [17], and therefore, although the bulk of the interactions of SR with CUL1 occur through SKP1, small differences in F-box structure could alter the intrinsic association between particular SR modules and CUL1. Studies in *Schizosaccharomyces pombe* also indicate that yCand1 favours F-box proteins with specific motifs [42].

Nevertheless, these experiments indicated that CAND1 has the capacity to accelerate dissociation of SR modules from SCF complexes. Indeed, addition of CAND1 to mixtures of previously assembled unneddylated SCF ^{β -TRCP} and SKP1–FBXW7 resulted in

Fig 4 | CAND1 as a substrate receptor exchange factor. **(A)** Pathways for activation, SR exchange and deneddylation of CRLs. This model is based on the proposed role for CAND1 as an SR exchange factor [35], the role of the CSN complex as both a catalytic and binding-dependent inhibitor of CRLs, the action of which can be reversed by substrate [36,40,41], and the stoichiometries of the various components *in vivo* [36]. The model initiates with empty cullin (step 1), formed, for example, by new synthesis. This empty cullin could be engaged by a newly synthesized SR1 module (step 2) or potentially integrated into a CAND1 complex for exchange (not shown for clarity). The basal CRL complex could either enter into a CAND1-dependent exchange cycle (step 3) or could become rapidly neddylated and engage substrates (steps 4 and 5). As substrate abundance is diminished, CRLs can associate with CSN, and become deneddylated (step 6), followed by dissociation of the CRL complex to re-enter the cycle. The CAND1-exchange pathway leads to dissociation of SR1 and association of SR2 (either a pre-existing or newly synthesized module) through a proposed intermediate in which CAND1 and SR2 associate simultaneously with the cullin complex (step 3). Exchange of SR1 for SR2 would change which substrates are targeted in the cell. **(B)** Structure depicting how the β-hairpin of CAND1 (red) bound to CUL1 (green) clashes with SKP1 (blue), based on superimposing CUL1–RBX1–CAND1 and CUL1–RBX1–SKP1–SKP2^{F-box} structures [17,66]. The arrow denotes the location of the β-hairpin in CAND1. Some residues of SKP1 that are absent in the crystal structure might further clash with CAND1. CRL, cullin–RING E3 ubiquitin ligase; SR, substrate receptor.

an exchange of the SR module, as monitored by the acquisition of ubiquitin transfer activity towards cyclin E, a specific substrate of the SCF^{FBXW7} complex [35]. Thus, CAND1 seems to behave as an SR exchange factor (SREF) akin to guanine nucleotide exchange factors (GEFs), which promote exchange of GTP for GDP in GTPases. To examine the ability of CAND1 to function as a SREF in cells, the association of pre-existing CUL1 with SR modules was quantified in the presence and absence of CAND1 [35]. The expectation is that if CAND1 is important for exchanging newly synthesized SRs for existing SRs on CUL1, then differences in the abundance of various SRs would be seen by quantitative proteomics. The abundance of approximately 50% of detectable SRs was altered in the absence of CAND1 (1.5-fold to 8.0-fold), but no effect on RBX1

levels was observed. Thus, CAND1 is important for shaping the steady-state and dynamic landscapes of SCF^{SR} complexes *in vivo*, but seems to have some specificity in terms of the SR modules that it can exchange [35]. This finding is consistent with genetic studies in *C. elegans* that indicated selective requirements for *cand-1* in the control of specific SCF complexes during development [93]. Importantly, data in both budding and fission yeast indicate that the CAND1 orthologues yCand1 and knd1, respectively, probably play analogous roles, as the abundance of SR modules associated with *cul1* in *S. pombe* are altered in the absence of *knd1*, and the assembly of the Skp1–Grr1 SR with CUL1 in budding yeast in response to nutritional signals requires yCand1 [34,37]. In response to transfer from a fermentable to a non-fermentable carbon source, yeast

CUL1 association with SKP1 is lost due to the disassembly of CRL complexes. Deletion of γ Cand1 leads to an inability of SKP1 to dissociate from CUL1, which indicates an essential role for γ Cand1 in the exchange of SR modules *in vivo* [34]. Furthermore, $\Delta\gamma$ Cand1 cells responding to glucose through induction of the F-box protein Grr1 fail to assemble new SCF^{Grr1} complexes, presumably because the pre-existing CUL1 scaffolds are still associated with SR modules [34]. Deletion of *knd1* in *S. pombe* led to increased (up to twofold) or decreased (up to threefold) levels of F-box proteins associated with CUL1, dependent on the identity of the F-box protein, as indicated by quantitative proteomics [37]. In a metabolic pulse-labelling experiment, a decrease of twofold to threefold was found in the amount of SKP1 and detectable F-box proteins associated with CUL1 in the absence of *knd1*, which indicates a role for *knd1* in allowing newly synthesized SKP1 to assemble with CUL1 [37]. This decrease correlated with an increase of roughly twofold in the half-life of the SCF substrate Ams2 in *S. pombe* [37].

These provocative studies raise numerous questions (Sidebar D). Although most of the studies of CAND1 function described thus far have used CUL1-based CRLs, CAND1 also associates with other CRLs. The steady-state abundance of CAND1 in association with various CRLs, however, differs widely [36]: substantial amounts of CUL1, CUL4B and CUL5 are associated with CAND1 but only small amounts of CUL2, CUL3, CUL4A and CUL7 are stably associated with it [36]. Whether these differences simply reflect the steady-state abundance of the fraction of these CRLs undergoing active exchange or whether some cullins rely much less on CAND1 to shape their architecture is not clear. Biochemical studies with alternative CRLs are required to address this question and to determine the contribution of CAND1 to global CRL architecture *in vivo*. A second major question concerns how CAND1 actually performs the exchange reaction. Potential insight into this question comes from the finding that SKP1 lacking the residues that are predicted to clash with a β -hairpin in the CAND1 structure (Fig 4B) fails to undergo CAND1-dependent exchange [35], and that CAND1 lacking the β -hairpin can stably associate with SKP1 and the F-box motif in the presence of CUL1 [66]. This, together with the finding that the rate of F-box displacement by CAND1 saturates of 1.3 s^{-1} , led to the hypothesis of the existence of a transition state composed of a meta-stable CAND1–SR–CUL1 complex that would potentially be anchored by CAND1–CUL1 C-terminal domain–RBX1 interactions (Fig 4A) [35]. Such a transition state complex would either expel CAND1 to maintain the SR–CUL1 complex or would expel the SR to generate a CAND1–CUL1 complex that is competent for reaction with other SR modules to generate a new SCF^{SR} [35] (Fig 4A). If this is the case, it raises a question as to how other CRL receptors are displaced. While SKP1, ELOC and BTB proteins have similar folds and, therefore, could be displaced through similar mechanisms, the functional counterpart of SKP1 in the CUL4 CRL system DDB1 has a distinct molecular architecture. Thus, further mechanistic and structural studies are necessary to understand the molecular basis of the exchange reaction.

How are CRLs built *in vivo*?

The modular and dynamic nature of CRLs, coupled with the large number of SR modules and regulatory components, creates a complex CRL landscape in cells. Although our understanding of this complexity is limited, quantitative proteomic analysis of various CRL components in HCT116 and HeLa cells has revealed several

properties of the CRL landscape that are likely to apply in many other settings [36]. First, the CRL landscape is established by various factors, including the abundance of cullins, SR modules, and regulatory components, and rate constants for interchange, but they are not necessarily the same for every cell type. The abundance of cullins varies by around fivefold, but the abundance of CSN and CAND1 is substoichiometric relative to the total cullin concentration, such that only a small fraction of each CRL is assembled with the deneddylase or SREF at steady state [35,36]. Moreover, the association of these regulators with individual CRLs can vary widely. For example, CUL1, CUL4B and CUL5 dominate CAND1 assemblies at steady state in HCT116 cells, whereas a very small fraction of CUL3 and CUL4A are detected in complexes with CAND1, despite the fact that there seems to be a free pool of CAND1 [36].

Second, at steady state, a substantial fraction of an individual cullin exists in a neddylated form (for example > 50% of CUL1 in HCT116 cells), and near-instantaneous inhibition of the neddylation system by addition of MLN4924 results in rapid and complete deneddylation of the entire CUL1 pool (typically in less than 15 min) [14,36,99]. Thus, in the absence of a forward neddylation reaction, neddylated SCF complexes are able to rapidly cycle through an encounter with the CSN complex to become deneddylated, despite the fact that at steady state only ~20% of CUL1 is associated with CSN. This cycling is perplexing, given that ~50% of cullin associated at steady state with CSN is actually in the neddylated form [36]. These features suggest that CSN is likely to be in rapid equilibrium with SCF complexes, but kinetic studies akin to those performed with CAND1 [35] are needed to understand the dynamics of CSN–SCF encounters and how this relates to the rate of hydrolysis of the NEDD8–CUL1 isopeptide bond.

Third, the distribution of SR modules on individual cullins is likely to differ both between cell lineages and between states of the same cell type—for example, in different cell-cycle phases. In HCT116 cells, ~70% of CUL1 is associated with SKP1 (and presumably an F-box protein), but this pool of CRLs is probably undergoing considerable remodelling via CAND1 exchange [35–37]. In principle, this could reflect the presence of substrates for the vast majority of SCF^{SR}s formed at steady state, thereby blocking capture and deneddylation of idle SCF^{SR} complexes by the CSN. Alternatively, the relative abundance of cullin and CSN (~3:1) might limit the extent of sequestration.

Fourth, an abrupt loss of neddylation has little effect on the steady-state repertoire of SCF^{SR} assemblies, and has only a modest effect on the fraction of SCF associated with CAND1 or CSN (less than twofold) [36,99].

Fifth, although the CSN seems to be an important inhibitory partner of CRLs, an additional mode of negative regulation through binding of RBX1 to GLMN, a heat-repeat protein, has been revealed. GLMN binds to RBX1–CUL1 assemblies irrespective of their neddylation state, with binding constants in the double-digit nanomolar range. The activity of SCF^{SKP2} and SCF^{FBXW7} can be inhibited *in vitro* by the blocking of access to charged E2s by GLMN [79]. *In vivo*, however, GLMN seems to primarily affect the SCF^{FBXW7} pathway and regulates the abundance of the FBXW7 targets cyclin E and c-MYC [80]. Intriguingly, loss of GLMN, which is mutated in the vascular disorder glomuvenous malformation, leads to a pronounced loss in cellular levels of RBX1 and CUL1, as well as rapid turnover of FBXW7 [80,100]. GLMN is associated with other cullins through RBX1 and might mediate additional functions through alteration of

the abundance of these cullins [79]. GLMN can bind to a CAND1-CRL *in vitro* [79], but further work is needed to determine whether GLMN is integrated into the CAND1-dependent or CSN-dependent CRL regulation cycle.

Conclusions

The emerging picture is that many mechanisms probably contribute to CRL-network architecture (Fig 4A). Newly synthesized F-box proteins probably encounter SKP1 initially to form the SR module, which can either associate with newly synthesized or otherwise unoccupied CUL1 or newly produced SKP1-F-box modules might be exchanged on pre-existing SCF^{SR} complexes via CAND1. The fact that roughly one-half of the detectable F-box proteins associated with CUL1 are not affected by loss of CAND1 indicates that the synthetic route of CRL assembly is important, and CAND1 probably provides a major resculpting role when the constellation of F-box proteins or substrates is dramatically altered over a short time-scale, for example during changes in developmental or cell-cycle state [35]. An important open question is whether all SKP1-F-box modules are equally active in their ability to undergo exchange via CAND1, or whether the system has evolved to primarily exchange a subset of SR modules, and what structural features dictate use (Sidebar D). In the future, the development of molecular probes that allow individual assemblies of CRLs and their regulators to be dissected *in vivo* will be necessary to develop a more detailed understanding of how CRL assembly and function is controlled with temporal and spatial resolution.

Ubiquitylation: mechanism and functions

Other reviews in this series, which will be published in consecutive issues of EMBO reports, will cover:

Ubiquitin in the immune system, by Henning Walczak *et al*

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Ubiquitylation in stem cells, by Ioannis Aifantis *et al*

Understanding ubiquitylation one structure at a time, by Ron Hay *et al*

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CONFLICT OF INTEREST

J.W.H. is a consultant for Millenium Pharmaceuticals.

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