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CRL4s: the CUL4-RING E3 ubiquitin ligases

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Abstract

The evolutionarily conserved cullin family proteins can assemble as many as 400 distinct E3 ubiquitin ligase complexes that regulate diverse cellular pathways. CUL4, one of three founding cullins conserved from yeast to humans, uses a large β -propeller protein, DDB1, as a linker to interact with a subset of WD40 proteins that serve as substrate receptors, forming as many as 90 E3 complexes in mammals. Many CRL4 complexes are involved in chromatin regulation and are frequently hijacked by different viruses.

Cullins: the scaffold

The ubiquitin pathway regulates a wide range of diverse cellular processes by covalently attaching ubiquitin to specific substrate proteins either to alter their functions non-proteolytically or more commonly to promote their degradation by the 26S proteasome [1]. Ubiquitylation begins with the ATP dependent activation of ubiquitin by the E1 enzyme, and is followed by the subsequent transfer of ubiquitin to an E2 ubiquitin conjugating enzyme, and finally an E3 ubiquitin ligase is responsible for recognizing a specific substrate and promoting ubiquitin ligation. Over a thousand distinct E3 ligases, belonging to two major families, the HECT family and the RING family, are present in higher eukaryotes. Human cells contain as many as 28 HECT proteins and most, if not all, are believed to function as E3 ligases. More than 450 RING proteins are expressed in human cells and E3 ligase activity has been experimentally demonstrated for many of them.

Although not containing a RING domain themselves, members of the evolutionarily conserved cullin family can bind with a small RING protein, either ROC1 or ROC2 (for RING of Cullins; also known as Rbx and Hrt1), to constitute a large family of cullin-RING E3 ligases (CRLs). There are three cullins in yeast, six in *Caenorhabditis elegans* and *Drosophila melanogaster* (CUL1-6), up to nine in *Arabidopsis thaliana*, and six in humans (CUL1, 2, 3, 4A, 4B and 5). In addition, three other proteins, CUL7 and CUL9 (also known as PARC) in mammals and APC2 (anaphase promoting complex subunit 2) in all eukaryotes, contain significant sequence homology to cullins over a ~180 residue region and bind ROC or a homologous small RING protein, APC11. Unlike other RING E3 ligases, cullins do not bind substrates directly, but rather rely on substrate recruiting receptors that are typically joined to the cullin complex by a linker protein (Fig. 1). Remarkably, each cullin can associate with a different family of substrate receptors, leading to the assembly of as many as 400 distinct CRLs. A recent estimate suggests that 20% of all proteins subject to proteasomal degradation are targeted by CRLs [2].

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The founding cullin gene, *Cul1* (also called *Cdc53*), was first identified in budding yeast where its mutation blocks the G1-to-S transition [3] and leads to an accumulation of G1 cyclin [4], and was separately identified in *C. elegans* where loss of *Cul1* function caused hyperplasia of multiple tissues [5]. These diverse or even seemingly irreconcilable phenotypes were subsequently explained by the discovery that CUL1 can assemble into distinct E3 complexes and thereby ubiquitylate different substrates. CUL1 binds with a small protein, SKP1 (S-phase kinase-associated protein 1), which in turn binds with a conserved protein motif, the F-box, which is present in many different proteins [6]. Through additional protein–protein interaction modules, individual F-box proteins can then recruit different substrates to the CUL1–ROC1–E2 catalytic core [7]. In this modular SKP1–CUL1–F-box (SCF) assembly, CUL1 indeed acts as a scaffold, SKP1 as a linker and F-box proteins as substrate receptors (Fig. 1).

To bring specific substrates to CUL2- and CUL5-based ligases, a heterodimeric linker complex containing elongins B and C binds to an analogous N-terminal domain in CUL2 and CUL5 and to two similar protein motifs, the VHL-box and SOCS box. VHL (von Hippel-Lindau) and SOCS (suppressor of cytokine signaling) proteins, via their additional protein–protein interaction modules, target various substrates differentially to the CUL2–ROC1–E2 or CUL5–ROC2–E2 catalytic cores [8]. Omitting a linker, CUL3 utilizes its N-terminal domain to bind to a conserved 100-residue protein motif known as a BTB domain, which then target substrates to the CUL3–ROC1–E2 catalytic core [9] (Figure 1).

In this article, we will discuss the genetic function, molecular assembly and substrates of CUL4–RING ligases (CRL4s). We will compare CUL4's pleiotropic functions across several species, and review the current model for CUL4 assembly with its linker protein DDB1 (for damaged DNA binding) and its family of WD40 containing substrate receptors. Finally, we will highlight recent studies expanding CRL4^{CDT2} substrates on chromatin, providing new details on the structure of CRL4s, and analyzing new *Cul4A* mouse models. Additionally, we will discuss outstanding questions remaining in the field.

CUL4 genetics: pleiotropic effects with repeated connections to chromatin

CUL4 presents as a single gene in *Schizosaccharomyces pombe*, *C. elegans*, *Drosophila*, and *Arabidopsis* whereas mammalian cells express two closely related paralogues, *CUL4A* and *CUL4B*. Two closely related *CUL4* genes are also present in zebrafish and frog, but not in sea urchin, suggesting that the *CUL4* gene duplication is probably unique to vertebrates. Characterizations of null mutation or reduced expression of *CUL4* in these organisms have revealed a wide range of cellular and organismal defects, including many associated with deregulated chromatin.

S. pombe Cul4 mutations cause multiple chromatin defects

Cul4 (also known as *pcu4*) deletion in fission yeast results in severely growth retarded, but viable cells that are extremely elongated with decondensed chromosomes [10]. The defect in chromosome condensation can be attributed in part to a role for Cul4 in maintaining the heterochromatin formation through its interaction not with Ddb1, but instead with Rik1, a protein that is distantly related to mammalian DDB1. Using Rik1 as linker protein, Cul4 binds the Clr4 histone H3K9 methyltransferase and is required for Clr4 localization to heterochromatin [11–13].

C. elegans cul-4 mutations cause CDT1 accumulation and DNA re-replication

Inactivation of *cul-4* in *C. elegans* by RNA interference (RNAi) results in a developmental arrest at the L2 larval stage and causes massive DNA re-replication. In *cul-4* RNAi animals, there is a sustained accumulation of the DNA replication-licensing factor, CDT-1 (chromatin

licensing and DNA replication factor 1), after S-phase completion whereas wild-type cells express undetectable CDT-1. Additionally, the nuclear export of the other replication licensing factor, Cdc6, is inhibited indirectly due to a failure to ubiquitylate the p21 homolog CKI-1 and contributes to the dramatic re-replication phenotype. Removal of one genomic copy of *cdt-1* suppresses the *cul-4* re-replication phenotype. These results demonstrate a function of CUL-4 in maintaining genome stability in part by facilitating CDT-1 degradation during the cell cycle and thus preventing aberrant re-initiation of DNA replication [14].

Arabidopsis CUL4 mutations impair development

Arabidopsis CUL4 is expressed abundantly and broadly in almost all tissues examined, including inflorescences, stems, roots, siliques, both light- and dark-grown seedlings and leaves. *Arabidopsis* expresses two different *CUL4* isoforms from a single gene, with one (CUL4-L) containing an additional 50 amino acid sequence at the N-terminus than the other (CUL4-S). Reduced *CUL4* expression in *Arabidopsis* by transgene-mediated co-suppression or RNAi results in pleiotropic *cop*-like (constitutive photomorphogenesis) phenotypes and widespread developmental defects in lateral roots, abnormal vascular tissue, and stomatal development [15,16], providing genetic evidence supporting the notion that CUL4 can assemble multiple distinct CRL4 complexes and regulate many different substrates.

Mammalian CUL4A and CUL4B are not entirely redundant

In mammals, genetic analysis of *CUL4* function is complicated by the existence of two closely related genes, *CUL4A* and *CUL4B*, which encode proteins sharing 80% identity with CUL4B containing an extended N-terminus (Figure 2A). A *Cul4A* mutant mouse created by deleting exon 1 (*Cul4A*^{Δ1/Δ1}, Figure 2B) was reported to cause early embryonic lethality and wide spread defects in many organs and animal death in adult mice when conditionally deleted [17]. This phenotype led to the early impression that the two *CUL4* genes in mammals are functionally distinct. Recently, a different *Cul4A* mutant mouse strain targeting exons 17-19 (*Cul4A*^{Δ17-19/Δ17-19}) which encode the ROC binding region and Nedd8 modification site unexpectedly showed no apparent phenotype [18]. This significant discrepancy was explained by the inadvertent disruption in the *Cul4A*^{Δ1/Δ1} mutant strain of a very close neighboring gene, *Pcid2*, which encodes an uncharacterized protein with homology to essential proteasome subunits [18]. Supporting this explanation, deletion of exons 4-8 (*Cul4A*^{Δ4-8/Δ4-8}) which encode for a portion of the DDB1 binding domain resulted in only a mild decrease in the proliferation in mouse embryonic fibroblasts (MEFs) and viable mice [[19], Pradip Raychaudhuri, personal communication].

Despite their high degree of sequence homology, wide expression and apparent compensation of *Cul4A* loss by *Cul4B* in the mouse, the two *CUL4* genes are not entirely functionally redundant. Deletion of exons 17-19 resulted in an increase in stability of several CRL4 substrates, including DDB2, xeroderma pigmentosum complementation group c (XPC) and p21, and enhanced DDB2-dependent global genomic repair activity of UV-damaged DNA in MEFs, and led to resistance to UV-induced skin cancer in mice [18]. These findings, which are consistent with the enhanced resistance to UV-induced carcinogenesis in DDB2 transgenic mice [20], point to a role for CUL4A in restricting, rather than promoting, the activity of DNA damage responsive proteins by promoting DDB2 degradation, a function that is apparently not fully compensated by CUL4B. Although a *Cul4B* mutant mouse has not been reported, several familial mutations in *CUL4B* have been identified in association with X-linked mental retardation (XLMR) in humans [21–23]. Supporting a distinct genetic function of *CUL4B* gene, the N-terminus of CUL4B, but not CUL4A, has been shown to uniquely binds to the dioxin receptor (AhR), assembling a CUL4B specific ubiquitin ligase complex that targets estrogen receptor α (ER- α) for degradation [24]. DDB1, the linker that is commonly used by both CUL4A and CUL4B for interacting with different substrate receptors, was identified, along

with CUL4B in the AhR-immouncomplex, but its function in promoting CUL4B-mediated substrate degradation remains unclear as it was not apparently required for substrate recruitment.

***S. cerevisiae* CUL8 (RTT101) functions like CUL4**

Notably, *S. cerevisiae*, which contains CUL1 and CUL3 orthologues as judged by both sequence conservation and binding with SKP1 and BTB proteins, respectively, does not contain a CUL4 ortholog. Instead, budding yeast express a unique cullin, Cul8 (also known as Rtt101), that, although not sharing primary sequence homology with CUL4, appears to assemble CRL4-like complexes and performs similar functions as CUL4 in other organisms. The *cul8Δ* cells are viable, but have a slower growth rate, are delayed in anaphase progression, are sensitive to fork arrest induced by DNA alkylation and accumulate DNA damage, suggesting a function for Cul8 in maintaining genomic integrity [25,26]. Cul8 interacts with Mms1, a protein that was initially identified by its function in protecting against the DNA-alkylating agent methyl methanesulfonate (MMS) as well as DNA damage during replication. Mms1 protein shares low, but significant primary sequence similarity (18% with human DDB1) with DDB1, which is absent in budding yeast, and could fold into a DDB1-like β-propeller structure. Cul8 binds Mms1 through an N-terminal sequence analogous to the CUL4 DDB1-binding domain and at the same time Mms1 can recruit additional proteins such as Crt10 and Mms20 that might function as substrates or substrate receptors [27].

DDB1: the linker

A direct DDB1–CUL4 association was first noted in a DDB2 immunocomplex [28]. The significance of this binding was not immediately clear as nearly all studies on DDB1 at that time focused on its role in DNA repair and no study had linked DDB1 to protein ubiquitylation. Later, genetic analyses of *DDB1* mutants in *S. pombe*, *C. elegans*, *Arabidopsis*, *Drosophila* and mouse (Box 1), revealed broad functions for DDB1 beyond DNA repair. Three subsequent studies firmly established a functional role of DDB1 in CUL4-mediated ubiquitylation thereby leading to the eventual discovery of DDB1-mediated assembly of multiple CUL4-based E3 complexes.

Text Box 1

Cellular and organismal phenotypes of DDB1

S. pombe

Deletion of *cul4* in *S. pombe* (also known as *pcu4*) resulted in severe growth retardation, but viable cells that are extremely elongated with decondensed chromosomes [10]. *ddb1Δ* fission yeast cells are viable, but are hypersensitive to various of DNA damage agents, are delayed in DNA replication progression, accumulate DNA damage, and show elongated phenotypes, abnormal nuclei and retarded growth [75,88]. Many of these phenotypes are similar to those observed in *cul4Δ* cells [10]. Notably, *S. pombe* contains an additional protein, Rik1 that shares 21% identity with DDB1 and also assembles E3 ligases with Cul4.

Arabidopsis

Unlike other organisms, *Arabidopsis* contains two *DDB1* genes, *AtDDB1A* and *AtDDB1B*, complicating the assessment of the functional dependency of CUL4 on DDB1. *AtDDB1B* disruption is lethal whereas the only effect of homozygous loss of *AtDDB1A* is decreased UV tolerance for a short period of time immediately following UV irradiation [89,90]. The inability of *AtDDB1A* to complement *AtDDB1B* is puzzling given that *AtDDB1A* is 91% identical to *AtDDB1B* over the entire 1088 residues, also binds to AtCUL4

[16], and is widely and concurrently expressed in many tissues; however *AtDDB1B* is often expressed at higher levels [91]. This puzzle lacks a clear solution.

Drosophila

Ddb1 is allelic to a previously defined locus termed *piccolo* (*pic*) [66]. Semi-lethal *piccolo* mutants were originally characterized based on shared irregularities in bristle, wing, and body segment growth [92]. *Ddb1* mutants growth arrest at the second instar stage, thus failing to develop completely [66].

Mouse

In the mouse, *Ddb1* deletion results in early embryo lethality before E12.5 [93]. Conditional deletion of *Ddb1* in the central nervous system (CNS) leads to neuronal and lens degeneration, brain hemorrhages, and neonatal death with strong selective elimination of nearly all proliferating neuronal progenitor cells and lens epithelial cells by apoptosis [93]. Accumulation of DNA damage and selective killing of proliferating cells were also observed in epidermal cells in which *Ddb1* was conditionally deleted [94].

DDB2 and *CSA*, encode two related WD40 proteins which control two different pathways of nucleotide excision repair, global genome repair (GGR) and transcription-coupled repair (TCR), and when mutated they lead to two distinct hereditary diseases, xeroderma pigmentosum and Cockayne syndrome, respectively. In a search for the molecular basis underlying the function of *DDB2* and *CSA* in DNA repair, it was surprising to find that they assemble into similar complexes that contain *DDB1*, *CUL4A*, *ROC1* and all 8 subunits of *COP9* signlaosome [29]. Separately, an investigation of human *DET1*, the homolog to *Arabidopsis DET1a*, a negative regulator of plant light responses, identified an E3 complex that contains *CUL4A*, *ROC1*, *COP1* and *DDB1* and promotes ubiquitylation and degradation of the proto-oncogenic transcription factor c-Jun [30]. Furthermore, analyses of endogenous *ROC1* and *CUL4A* complexes identified *DDB1* as a near-stoichiometric *CUL4A* binding partner. In this study, *in vivo* and *in vitro* ubiquitylation assays established *DDB1* as an essential component for the *CDT1* ubiquitylation by the *CUL4A*– *ROC1*– E2 catalytic core [31]. Together, these studies provided the basis for the subsequent discovery that *DDB1* links multiple WD40 proteins to the *CUL4*– *ROC1*– E2 catalytic core.

When compared with both *SKP1* (160 residues), *elongin B* (161 residues) and *elongin C* (112 residues), a notable feature of *DDB1* (1140 residues) as the linker for *CUL4* is its large size. This difference could reflect the need of *DDB1* to interact with more divergent substrate receptors (WD40 proteins) than other linkers. It also raises the possibility that *DDB1* might interact with additional groups of substrate receptors or substrates. The presence of additional *DDB1*-related proteins potentially expands the versatility of *CUL4* in the assembly of E3 complexes. Computational analyses suggested that two mRNA processing factors, *SAP130* (spliceosome-associated protein 130) and *CPSF160* (cleavage and polyadenylation specificity factor 160), share significant primary sequence homology with *DDB1* (16% and 20%, respectively), contain multiple WD40-like repeats (21 in *SAP130* and 18 in *CPSF160*) and could fold into three β -propeller domains [32]. Very little is known about *CPSF160*. *SAP130*, by contrast, has been identified in two related GCN5 histone acetyltransferase-containing transcription complexes, the STAGA and TFTC complexes [33,34]. Recently, a direct connection of *SAP130* with the cullin family was made by the demonstration of its binding with CSN and, surprisingly, with multiple cullins (*CUL1*, 2 and 4A) [35]. *SAP130* binds to both C-terminal and N-terminal sequences in *CUL1*, similar to another cullin regulator, *CAND1* (cullin-associated and neddylation-dissociated 1), and can form a complex with the linkers and substrate receptors of both *CUL1* (*SKP1*–*SKP2*) and *CUL2* (*elongin B* and *VHL*). This finding, together with the presence of *DDB1* in the *SAP130*-containing STAGA and TFTC complexes [34], suggests that *SAP130*, unlike *DDB1*, is not a *CUL4*-specific linker.

Fission yeast Rik1, initially identified by its function in maintaining gene silencing and heterochromatin formation, shares 21% identity with DDB1 and was co-purified with Cul4, Roc1, as well as Clr4 histone H3K9 methyltransferase and Lid2 H3K4 demethylase [11,12,36,37]. Fission yeast Ddb1 was not found in Rik1-containing complexes, as expected if Rik1 replaces Ddb1 as a linker for Cul4 in these complexes. A WD40 protein, Clr8 (also called Dos1 or Raf1), co-purifies with the Rik1–Cul4 complex, is required for heterochromatin formation, and appears to function as a structural component in a manner analogous to a substrate receptor. Thus far, Rik1 is the only protein besides DDB1 that is known to function as a linker for CUL4.

DWD/DCAF/CDW proteins: the substrate receptors

Following the discoveries that CUL1, CUL2 and CUL3 each interacts with multiple substrate receptors and that CUL4 performs pleiotropic functions, it was anticipated that CUL4 very likely would also interact with a protein motif present in multiple proteins. Elucidating the CUL4-interacting motif, however, proved to be challenging and was made possible by the discovery of DDB1 as a major functional partner of CUL4. Taking different approaches -- proteomic, bioinformatic and structural analyses -- four independent studies collectively identified and experimentally demonstrated 50 different DDB1 binding WD40 proteins, referred to variously as DWD (DDB1-binding WD40), DCAF (DDB1–CUL4 associated factors) or CDW (CUL4–DDB1-associated WDR) proteins [38–41] and reviewed in [42]. It was also clear that only a subset of, but not all WD40 proteins, interact with DDB1 and CUL4. DDB1-binding proteins contain a common motif, variably defined as the “double DxR box” with two DxR motifs located at the end of two consecutive WD40 repeats [40], the “DWD box”, a 16-residue stretch that correspond to the second half of a WD40 repeat and an Arg residue at position 16 following the WD dipeptide [38], the “DXXXXR/KXWDXR/K” motif as the subdomain of WD40 repeats [39], or simply as the “WDXR” motif to emphasize the Arg residue following the WD dipeptide [41].

Three common features of DDB1-binding WD40 proteins emerge from these analyses. (1) Some DWD/DCAF/CDW proteins contain one, most contain two and a few contain three such motifs. (2) The Arg residue following the WD dipeptide is critically important and point mutation of this single residue in some DWD/DCAF/CDW proteins is sufficient to disrupt its binding with DDB1. Notably, point mutations of this Arg residue, Arg273, in the DWD box of DDB2 is found in several human patients with xeroderma pigmentosum group E (XP-E) and disrupts DDB2–DDB1 binding [43]. Unexpectedly, when the co-crystal structure of human DDB1 and zebrafish DDB2 revealed that the corresponding Arg residue in zebrafish DDB2 (Arg309) did not contact DDB1 [44], leaving the exact contribution of this Arg to DDB1 binding unclear. (3) Additional residues besides the Arg are required for binding with DDB1. For example, a 6-amino acid sequence in the DWD/DCAF/CDW protein WDR21 which lies outside the WD40 repeats is important for binding with DDB1 [45]. This region was identified by sequence homology in some, but not all, DWD/DCAF/CDW proteins. Although the exact residues that contact DDB1 are not yet precisely defined, the current definition of the DWD/DCAF/CDW motif is highly predictive of and likely structurally important for DDB1 binding proteins.

How many DWD proteins are there? The human genome encodes about 320 unique WD40 proteins. Database searches using the DWD box predicted that as many as 90 DWD proteins, or a third of total WD40 proteins, could bind to DDB1 [38]. Similar searches predicted 33 DWD proteins in fission yeast, 36 in *C. elegans*, 75 in *Drosophila*, 78 in rice and 85 in *Arabidopsis* [38,46]. When 11 predicted *Arabidopsis* DWD proteins were tested for their direct interaction with DDB1 by yeast two-hybrid assay, all were found to be positive [46], suggesting that most of these predicted DWD proteins indeed bind to DDB1. Notably, 11 human proteins contain both an F-box and WD-40 repeats (FBXW proteins), including 8 with a predicted DWD

box. The experimental demonstration that FBW5, as well as two additional FBXW proteins, APG16L and KATNB1 [38], can bind with DDB1 and CUL4 suggests that an individual substrate receptor (a FBXW protein) might target a substrate to two different E3 ligases, potentially further expanding the reach of CRLs.

CRL4 substrates

At present, nearly two dozen proteins are reported to be degraded by a CRL4 (Table 1), including several that are not ubiquitinated by CRL4 during normal cell growth, but are diverted to CRL4 by a virus (Box 2). Evidence is solid for some and incomplete or correlative for others, and among the CRL4 substrates, CDT1 currently is the best characterized. Several interesting features unique to CRL4 emerged from these studies.

Text Box 2

Viral Hijacking of DDB1– CUL4

Many viruses are known to exploit the ubiquitylation pathway to evade innate cellular antiviral mechanisms or otherwise benefit viral propagation [95]. CRL4s are targeted by several viruses including members of the paramyxovirus, herpesvirus, lentivirus, and hepadnavirus families. Although these diverse viruses commonly target DDB1, they appear to disrupt CRL4s in different ways. Viruses from the paramyxovirus family including simian virus 5, mumps virus and human parainfluenza virus type 2, use CUL4–DDB1 to degrade signal transducer and activator of transcription (STAT) proteins that respond to interferon signaling and initiate cellular antiviral responses [79,80]. The crystal structure of simian virus 5 V (SV5-V) protein in complex with DDB1 showed that despite almost no primary sequence homology with DDB2, SV5-V protein similarly inserts a helix between β -propellers BPA and BPC [96]. Hepatitis B virus X protein (HBx) also binds to DDB1; this binding is thought to facilitate viral replication [97,98], possibly by causing an extended S-phase [99]. Binding assays show that SV5-V and HBx bind DDB1 in a mutually exclusive manner [98]. Furthermore, structural analysis revealed that HBx, as well as SV5-V contains primary sequence homology to DDB2 helix h1 that inserts into a cavity formed by BPA and BPC in DDB1 [44]. Collectively, these structural analyses suggest that HBx and SV5-V viral proteins, although lacking WD40 repeats and sharing little primary sequence homology with each other, might form a similar structural motif and bind to a region in the BPA-BPC pocket of DDB1 in a manner similar to that of DDB2. The cellular protein(s) targeted by HBx to DDB1– CUL4 ligase remains unknown, raising the possibility that HBx facilitates viral propagation by interfering with the ubiquitylation of a host protein by, instead of targeting a novel host protein to, the DDB1– CUL4 ligase. This could also be the case for the murine gamma herpesvirus 68 M2 protein that binds to CUL4–DDB1 and impairs DNA damage response and inhibits apoptosis through unknown cellular mechanisms [100]. Most recently, several groups have established that HIV-1 Vpr and the related simian immunodeficiency virus protein Vpx bind to CRL4^{VprBP}. Though it remains unclear how Vpr–VprBP association promotes viral replication, the hijacked E3 ubiquitin ligase complex degrades uracil-DNA glycosylase UNG2, a substrate not targeted by CRL4^{VprBP} in the absence of Vpr [77]. Indeed, mechanisms for how CRL4 is exploited by so many divergent viruses remains to be shown in detail, but clearly this broadly expressed ubiquitin ligase is an attractive target.

PCNA is required for the ubiquitylation of multiple PIP box proteins by CRL4^{CDT2}

A unique feature of CRL4^{CDT2} mediated CDT1 ubiquitylation is the requirement of proliferating cell nuclear antigen (PCNA) [47–50], a cofactor of DNA polymerases that forms

a trimeric ring to encircle DNA and make a sliding clamp [51]. The function of PCNA in CRL4^{CDT2}-mediated ubiquitylation remains unclear, but is not limited to CDT1 alone. Recently, three additional CRL4^{CDT2} substrates, both human and *C. elegans* p21, a CDK inhibitor, [52–54], *C. elegans* polymerase η [55], and *Drosophila* E2f1 [56], have all been reported to be degraded in a PCNA-dependent manner. All four CRL4^{CDT2} substrates contain a conserved PCNA-interacting motif (PIP box) and bind PCNA directly, and this direct binding is required for their ubiquitylation. Although the CDT1–PCNA interaction is necessary to recruit the CRL4^{CDT2} E3 complex to chromatin [41], this effect has not been clearly demonstrated for the other three CDT2 substrates. Humans express 47 PIP box proteins, and most have chromatin-associated functions [51]. It will be interesting and important to determine whether and how many other PIP box proteins are also ubiquitylated by CRL4^{CDT2}. Further, no direct interaction between PCNA and CDT2 has been reported, leaving open the possibility that the function of PCNA could extend beyond CDT2 to other CRL4 ligases. It is tempting to speculate that PCNA provides the biochemical basis for the strong links between CUL4–DDB1 and chromatin biology [57].

CRL4 ubiquitylation of histones does not lead to degradation

Several studies have observed CRL4^{DDB2}-dependent ubiquitylation of core histones and repair factor XPC during DNA repair [58,59] as well as ubiquitylation of DDB2 by DDB1–CUL4 [60–63]. The consequences of these ubiquitylation events to the substrates and to the DNA repair process remains unclear. One model posits that via ubiquitylating histones and presumably loosening up chromatin [59], DDB2 is ubiquitylated and degraded, thereby facilitating the access of other repair factor, such as XPC, to the DNA lesion. XPC ubiquitylation, by contrast, enhances its DNA binding affinity, but does not promote its degradation [64]. The view that DDB2 ubiquitylation promotes the binding of other factors to the lesion is challenged by evidence that loss of DDB2 ubiquitylation enhances rather than inhibits DNA repair activity as was seen in the absence of *c-Abl* [65] and more recently in cells lacking *Cul4A* [18]. Perhaps, DDB2 ubiquitylation functions to restrict repair activity to the lesion instead of spreading to undamaged DNA. More intriguingly, it remains unclear how one E3 ligase (DDB1–CUL4–ROC1) apparently causes monoubiquitylation of some substrates (H2A, H3 and H4) and polyubiquitylation of others that leads to degradation in one case (DDB2), but not in another (XPC). It will be important to determine whether different ubiquitin lysine residues are linked to different substrate and if so, whether ROC1 can itself switch between different E2s or if additional factors control the ROC1–E2 binding to conjugate different ubiquitin lysines to different substrates.

Not all substrates of CRL4 ligases are associated with chromatin

Not all CRL4 substrates identified to date are associated with chromatin, as illustrated by the ubiquitylation of cytoplasmically localized TSC2 (tuberous sclerosis 2) by CRL4A^{FBW5} [66], membrane associated protein Merlin by CRL4A^{VprBP} [66,67], and ER- α *in vitro* by CRL4B^{Ahr} [24]. Another issue related to CRL4 substrate that should be mentioned is that a DWD/DCAF/CDW protein might itself be a substrate, as has been suggested for DDB2 [64]. In the case of SCF E3 ligases, several F-box proteins, including Cdc4 and Grr1 [68], are degraded by the CUL1–ROC1–E2 core after the substrate they recruited is degraded, presumably to ensure the release and sustain the supply of the CUL1–ROC1 core for the assembly with other F-box proteins into a new SCF E3 complex.

Future outlook

Given the number of demonstrated and predicted DWD/DCAF/CDW proteins and the number of proteins interacting with CUL4 and/or DDB1, there is good reason to anticipate that a potentially large number of CRL4 substrates will be identified in the near future. We will also

likely know the answers to three challenging issues related to CRL4. What is the biochemical function of PCNA in promoting CRL4^{CDT2} mediated substrate ubiquitylation? How does a single DDB1– CUL4– ROC1 E3 complex differentially cause monoubiquitylation as well as non-proteolytic and proteolytic polyubiquitylation? What are the biochemical mechanisms by which CRL4s contribute to gene silencing, heterochromatin formation and chromosome condensation? Answers to these issues will also have broad implications beyond CRL4 biology and should help to advance the fields of ubiquitin and chromatin biology.

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Glossary

- RING finger** First identified in the really interesting new gene (RING), this cysteine and histidine rich domain coordinates two zinc atoms in a cross brace manner. Rather than directly binding ubiquitin, RING fingers promote the transfer of ubiquitin directly from the E2 to the substrate by allosterically activating the E2
- HECT domain** A domain that is homologous to E6-AP carboxyl terminus and contains an evolutionally conserved cysteine residue that forms a thioester linkage with ubiquitin
- NEDD8** Initially identified as a neural precursor cell-expressed and developmentally down-regulated human gene, encoding for an 81 amino acid protein that shares 60% identity with ubiquitin, evolutionary conserved in all eukaryotes and can covalently bind cullins to activate CRL activity
- DDB** Damaged DNA Binding protein complex, first identified as a heterodimeric complex involved in DNA repair, consists of the 127 kDa subunit DDB1 and a 48 kDa subunit DDB2. DDB1 is now recognized as the linker protein in CRL4 complexes and has additional roles beyond DNA repair
- WD40** A domain defined at the primary sequence level by a Gly–His dipeptide and a Trp–Asp (WD) dipeptide separated by 20 – 30 residues, and is typically ~40 residues in length. WD40 repeats are commonly found in many proteins of diverse function, and are typically thought to form β-propeller structures
- BTB** First identified in the *Drosophila* proteins Bric-a-brac, Tramtrack, and Broad-complex, the BTB domain is found in approximately 200 human proteins. BTB proteins are thought to function as the substrate receptor for CRL3 complexes. Also known as poxvirus and zinc finger (POZ) proteins
- F-box** This domain is approximately 40 amino acids and was first identified in Cyclin F. It subsequently was shown to be required for binding CRL1 linker Skp1. There are 78 F-box proteins identified in humans that are thought to function as substrate receptors for CRL1 complexes
- Elongin** The Elongin complex, consisting of A, B, and C subunits was named for its ability to stimulate transcription elongation by RNA polymerase II. The Elongin B and C heterodimer has subsequently been shown to function as a linker for CRL2 and CRL5 complexes

- SOCS box** The suppressors of cytokine signalling (SOCS) box is a conserved motif found in CRL5 substrate receptors that includes the BC-box which binds to the Elongins B and C heterodimer and additional residues that specify binding with CRL5s
- VHL box** Named for homology to the Von Hippel-Lindau tumor suppressor, this domain contains the BC-box and additional residues that specify binding with CRL2s

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Figure 1. Assembly of Cullin–RING ubiquitin ligases (CRLs)

The cullin-ROC family of E3 ligases regulates the ubiquitylation of many substrates by assembling into multiple distinct E3 ligases. Each cullin uses a modular assembly to recruit different substrates to a common catalytic core by varying its substrate receptor. Cullin family members (green) from different organisms share similar mechanisms for assembling a multi-subunit complex to ubiquitylate specific substrate protein (light blue). An N-terminal domain interacts either directly with a protein motif (orange) present in substrate receptors (black) or via a linker (blue). Separately, a C-terminal domain binds with a small RING protein (ROC1 or ROC2, yellow) which recruits and allosterically activates an E2 enzyme (red) that transfers ubiquitin (Ub; color) to the substrate. Cullins are activated by the covalent conjugation with a ubiquitin-like modifier, NEDD8 (Nd8; purple). Human cells express an estimated 78 F-box proteins, 205 BTB proteins, about 90 DWD proteins and an estimated 50 BC-box proteins.

Figure 2. Schematic comparison of CUL4 proteins and mouse *Cul4A* mutants

A. Human cells contain two *CUL4* genes encoding CUL4A (NP_001008895) and two isoforms of CUL4B (NP_001073341 and NP_003579.3) proteins which differ by only 22 amino acids at the N-terminus.

B. Wild type mouse *Cul4A* gene and comparison of three targeted *Cul4A* mutants. Filled and open boxes denote coding and non-coding exons, respectively. Exons encoding the DDB1 and ROC 1 binding regions in CUL4A have been noted. Three mouse models targeting *Cul4A* have been generated to date, deleting exon 1, exons 4-8, and exons 17-19, respectively. Discrepancies in reported phenotypes might be due to unintentional disruption of a nearby neighboring gene, *Pcid2*.

Table 1

Substrates of CUL4-RING ligases

Substrate	Organism ^a	Receptor	Function	Reference
CDT1	<i>Hs, Xl, Dm, Ce, Sp</i>	CDT2 ^b	DNA replication	[14,31,41,47–50,69–72]
p21	<i>Hs</i>	CDT2 ^b	CDK inhibitor	[52–54]
E2F1	<i>Dm</i>	CDT2	Cell cycle	[56]
pol η	<i>Ce</i>	CDT2	DNA replication during damage	[55]
Spd1	<i>Sp</i>	Cdt2	DNA replication inhibitor	[73–76]
Merlin	<i>Hs</i>	VprBP ^c	tumor suppression	[67]
UNG2	<i>Hs</i>	VprBP-Vpr [?]	uracil DNA glycosylase	[77]
SMUG	<i>Hs</i>	VprBP-Vpr [?]	uracil DNA glycosylase	[77]
Histone H2A	<i>Hs</i>	DDB2	chromatin formation	[58]
Histone H3	<i>Hs</i>	DDB2	chromatin formation	[59]
Histone H4	<i>Hs</i>	DDB2	chromatin formation	[59]
XPC	<i>Hs</i>	DDB2	DNA damage repair	[64]
DDB2	<i>Hs</i>	DDB2 [?]	DNA damage repair	[61,64]
TSC2	<i>Hs, Dm</i>	FBW5	Tumor suppression	[66]
SKN-1	<i>Ce</i>	WDR23	Stress response and longevity	[78]
STAT1,2,3	<i>Hs</i>	SV5 ^d	Signal transducer and activator	[79–83]
ER-α ^e	<i>Hs</i>	AhR ^d	Estrogen receptor	[24]
c-Jun	<i>Hs</i>	?	Proto-oncogenic transcription factor	[30]
HOXA9	<i>Hs</i>	?	Development	[84]
CHK1	<i>Hs</i>	?	Cell cycle checkpoint	[85]
Dacapo	<i>Dm</i>	?	CDK inhibitor	[86]
Cyclin E	<i>Dm</i>	?	Cell cycle	[86]
β-catenin	<i>Hs, Dm</i>	?	Development	[87]

^a Abbreviations for the organisms: *Hs*, *Homo sapiens*; *Xl*, *Xenopus laevis*; *Dm*, *Drosophila melanogaster*; *Ce*, *Caenorhabditis elegans*; *Sp*, *Schizosaccharomyces pombe*

^b Also requires PCNA

^c Also known as DCAF1.

^d Not a WD40 protein

^e CUL4B-specific