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Distinct and overlapping functions of the cullin E3 ligase scaffolding proteins CUL4A and CUL4B

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Abstract

The cullin 4 subfamily of genes includes *CUL4A* and *CUL4B*, which share a mostly identical amino acid sequence aside from the elongated N-terminal region in *CUL4B*. Both act as scaffolding proteins for modular cullin RING ligase 4 (CRL4) complexes which promote the ubiquitination of a variety of substrates. CRL4 function is vital to cells as loss of both genes or their shared substrate adaptor protein DDB1 halts proliferation and eventually leads to cell death. Due to their high structural similarity, *CUL4A* and *CUL4B* share a substantial overlap in function. However, in some cases, differences in subcellular localization, spatiotemporal expression patterns and stress-inducibility preclude functional compensation. In this review, we highlight the most essential functions of the *CUL4* genes in: DNA repair and replication, chromatin-remodeling, cell cycle regulation, embryogenesis, hematopoiesis and spermatogenesis. *CUL4* genes are also clinically relevant as dysregulation can contribute to the onset of cancer and CRL4 complexes are often hijacked by certain viruses to promote viral replication and survival. Also, mutations in *CUL4B* have been implicated in a subset of patients suffering from syndromic X-linked intellectual disability (AKA mental retardation). Interestingly, the antitumor effects of immunomodulatory drugs are caused by their binding to the CRL4^{CRBN} complex and re-directing the E3 ligase towards the Ikaros transcription factors IKZF1 and IKZF3. Because of their influence over key cellular functions and relevance to human disease, CRL4s are considered promising targets for therapeutic intervention.

Keywords

Ubiquitin ligase; CUL4A; CUL4B; CRL4; Cullin

1. Introduction: the ubiquitination cycle and E3 ligase function

Members of the cullin family serve as scaffolding proteins for modular, multi-subunit E3 ubiquitin ligase complexes. Ubiquitin is expressed at very high levels in the cell (in HEK293 cells, the concentration is approximately 85 μ M) and is dynamically attached and removed from proteins in a cyclical manner (Kaiser et al., 2011). The ubiquitination process relies on three different enzymes that are required for the covalent attachment of a ubiquitin molecule

to a substrate protein. First, E1 enzymes activate ubiquitin molecules via ATP-mediated adenylation followed by formation of an E1-ubiquitin thioester bond. Next, the activated ubiquitin molecule is transferred to an E2 conjugating enzyme. For RING (Really Interesting New Gene) E3 ubiquitin ligase complexes, the E2 is responsible for attachment of the ubiquitin to the substrate protein — as opposed to HECT domain E3 ligases, which act as an intermediate and possess catalytic activity. The primary function of RING E3 ligases is to regulate this transfer. Cullin RING ligase (CRL) complexes are composed of many different proteins – CRL4s alone have dozens of potential substrate receptors – but share a similar overall architecture (Lee and Zhou, 2007; Zimmerman et al., 2010).

Although substrate proteins can be ubiquitinated at multiple sites, ubiquitins are often added to other ubiquitin molecules — forming chains on the substrate. The qualities and significance of these chains depend on the specific lysine residue to which each ubiquitin molecule is conjugated. This process also happens dynamically as deubiquitinating enzymes can trim or process ubiquitins from chains. Polyubiquitination of substrate proteins typically serves as a tag for degradation by the proteasome, which is a large, multimeric protein complex that: deubiquitinates proteins (so that ubiquitin molecules can be “recycled”), linearizes substrates and processes them into smaller peptides via hydrolysis. However, conjugation of single ubiquitin molecules or K63 polyubiquitin chains does not induce proteasomal degradation but often serves to recruit other co-factors. The ubiquitin-proteasome system is thought to regulate the turnover of most proteins in the cell and E3 ligases, which dictate substrate specificity, actually outnumber kinases. Due to the importance of E3 ligases in virtually all aspects of cell biology, many of these proteins are being extensively investigated.

In this review, we focus on CRL4 complexes and how they regulate numerous key functions in the cell including: DNA repair and replication, chromatin-remodeling, cell cycle progression, embryogenesis (specifically, extra-embryonic development), sperm and blood cell differentiation, tumorigenesis, viral replication, intellectual disability and more.

2. Structures of the CRL4A and CRL4B ubiquitin ligase complexes

Despite sharing 82% identity, CUL4B differs from CUL4A in that the former possesses an elongated N-terminus (CUL4B is 149 amino acids longer than CUL4A). A nuclear localization signal (NLS) is present in the unique N-terminal extension of CUL4B, which is believed to mediate the nuclear localization of CUL4B (Zou et al., 2009). In contrast, CUL4A (which lacks an NLS) is predominantly found in the cytoplasm with a subpopulation of CUL4A protein residing in the nucleus where it induces the degradation of multiple nuclear proteins (Higa et al., 2003; Liu et al., 2009; Zhang et al., 2003). CUL4A expression oscillates during the cell cycle and shows high expression during S phase, which coincides with the downregulation of many CRL4 substrates that regulate cell cycle progression (Miranda-Carboni et al., 2008).

At their N-terminal ends, both CUL4 proteins bind to DDB1, which was first identified as the larger subunit of the heterodimeric *UV-damaged DNA binding* (UV-DDB) protein complex. DDB1 functions as a substrate adaptor protein by forming a connection between

the CUL4 scaffold and multiple substrate receptors known as DCAFs (DDB1–CUL4-associated factors) (Harper and Tan, 2012; Higa and Zhang, 2007; Lee and Zhou, 2007; Zhang et al., 2008; Zimmerman et al., 2010). The highly promiscuous binding capabilities of DDB1 enable CUL4 complexes to interact with a large network of proteins and regulate a variety of different processes in the cell. Sophisticated crystallography work has shown that CUL4A and CUL4B complexes have very similar structures (Angers et al., 2006; Fischer et al., 2011; Scrima et al., 2008). The N-terminal end of CUL4B is known to contain an NLS and its isolated structure has been resolved by Vollmar et al. (unpublished). This peptide has been reported to participate in substrate recruitment (Ohtake et al., 2007).

The cullin homology domain binds to RING proteins (Rbx1/ROC1), which then recruit active, ubiquitin-conjugated enzymes (Fig. 1). Therefore, the C-terminal ends of cullins serve as the catalytic centers of CRL complexes. Cullins also undergo post-translation modifications that affect their function as ligase proteins. NEDD8, a ubiquitin-like molecule which also has an E1–E2–E3 enzymatic cascade, is conjugated to Lys619 and Lys873 on human CUL4A and CUL4B, respectively (Pan et al., 2004). Cullin neddylation is thought to promote CRL activity (Saha and Deshaies, 2008). Structural studies on the Rbx1-paired C-terminal domains of CUL1 and CUL5 show higher flexibility and range of motion in the E2-recruiting protein following cullin neddylation (Duda et al., 2008). As a result, the ubiquitin-conjugated E2 comes in closer contact with the substrate protein at the opposite end of the CRL complex. Some CRLs are able to form dimers and one group has shown that CRL4^{DCAF1} can form dimers in vitro via DCAF1–DCAF1 interactions, which enhance CRL4 function (Ahn et al., 2011).

Selective recruitment of certain subunits and NEDD8 attachment are not the only regulators of CRL activity. The COP9 signalosome (CSN) is a multimeric protein complex that negatively regulates CRL function by de-neddylating cullins — the CSN5 subunit of the signalosome possesses isopeptidase activity (Cope et al., 2002). The CSN also has high affinity for deneddylated SCF and inhibits its ubiquitin ligase activity (Emberley et al., 2012). CRL dependence on neddylation is further exploited by the inhibitor protein CAND1, which obstructs the C-terminus of cullins and prevents NEDD8 conjugation (Goldenberg et al., 2004). CAND1 also blocks interactions between cullins and their respective substrate adaptors/receptors at the N-terminus (Duda et al., 2011). The non-receptor tyrosine kinase c-Abl also appears to antagonize CAND1 inhibition of CRL4A. Interestingly, this function is carried relies on the tyrosine kinase domain yet is independent of c-Abl catalytic activity (Chen et al., 2006).

3. Gene expression patterns in normal mammalian tissue

In the first report characterizing human *CUL4A* cDNA, Northern blot analysis showed that *CUL4A* transcript is very highly expressed in the testis, heart and skeletal muscle with notably high expression in the thymus, prostate, ovaries, small intestine and pancreas (Chen et al., 1998). Further array-based analyses (using a larger amount of tissue subsets) have confirmed high expression in the pancreas, testes and in T cells but, most notably, *CUL4A* transcript is expressed at consistently high levels in multiple types of blood cells ranging from CD34⁺ progenitors and CD33⁺ myeloid cells to mature lymphocytes (Su et al., 2004).

In mice, *Cul4a* transcript is expressed more highly in progenitor cells than mature leukocytes (Su et al., 2004). Organism-wide tissue analysis of CUL4A protein expression (via Western blotting) in 5 month old mice shows the highest protein expression in: testes, brain, liver and heart (Kopanjan et al., 2011).

CUL4B transcript is also highly expressed in human leukocytes, but to a lesser degree than *CUL4A* (Su et al., 2004). Relative to median expression levels, *CUL4B* mRNA is expressed at least 2-fold higher in pituitary cells, adipocytes and smooth muscle cells. In mice, *Cul4b* transcript is very highly expressed in placental cells and embryonic stem cell lines as well as the testes (Su et al., 2004). According to human tissue analysis conducted as part of the Human Protein Atlas project, CUL4B is most highly expressed in pancreatic tissue, endocrine glands, the cerebellum, the lower GI tract, bone marrow and the testes (there is no information in this database on placental tissue or specific blood cell lineages) (Uhlen et al., 2015). CUL4B is highly expressed in extra-embryonic tissues during early mouse development and is required in those tissues for the production of viable offspring (Chen et al., 2012; Jiang et al., 2012; Liu et al., 2012).

4. Cellular and developmental functions of CRL4 ligases

4.1. DNA damage repair and chromatin remodeling

The UV-DDB complex is a heterodimeric complex composed of DDB1 – the large subunit and substrate adaptor of CRL4s – and DDB2, which is the smaller, DNA-binding subunit. The gene that encodes DDB2 is named *XPE* which stands for xeroderma pigmentosum (XP) complementation group protein E. XP is a rare autosomal recessive disorder that is characterized by extreme sensitivity to sunlight —specifically, UV rays (see (DiGiovanna and Kraemer, 2012) review for more information on this disease). The most common types of UV-induced DNA lesions are cyclopurimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), which are intra-strand linkages (usually occurring at C-T or T-T dimers) that distort DNA helical structure and can be recognized with high specificity by DDB2 and XPC (Fischer et al., 2011). After recognition of these lesions, the process of nucleotide excision repair (NER) is initiated — resulting in excision of the damaged section, synthesis of replacement DNA and ligation (the NER pathways are reviewed in (Nospikel, 2009)). After UV-irradiation, the UV-DDB heterodimer binds lesions on chromatin then recruits the CRL4 complex. UV rays also stimulate the neddylation, and therefore activation, of CRL4A complexes which causes the rapid ubiquitination and degradation of CRL4A substrates — including DDB2 itself (Groisman et al., 2003; Matsuda et al., 2005; Nag et al., 2001). In *Cul4a*^{-/-} embryo-derived fibroblasts, UV irradiation does not induce DDB2 degradation, which suggests that CUL4A is the CUL4 protein primarily responsible for DDB2 ubiquitination at the site of UV lesions (Liu et al., 2009).

Although DDB2 is considered a substrate of the CRL4A complex, it can also function as a DCAF substrate receptor and promote the ubiquitination of other proteins in a CUL4A-DDB1-dependent manner. Another DNA repair protein, XPC, is also a rate-limiting factor in the damage recognition phase of NER and can also bind to CPD and 6-4PPs on chromatin. DDB2 directly binds UV-damaged DNA and recruits the CRL4A complex to the damage site (Li, 2006; Luijsterburg et al., 2007). CRL4A^{DDB2} binds to and ubiquitinates XPC,

resulting in efficient NER activity at internucleosomal sites, which exhibit faster reaction kinetics than those within denser chromatin (Fei et al., 2011; Sugasawa et al., 2005). In fact, XPC ubiquitination occurs preferentially at open, nuclease “hypersensitive” sites in chromatin. In cell-free systems, XPC ubiquitination is required for NER activity when UV-DDB is present (Sugasawa et al., 2005). Recent work suggests that XPC may even protect DDB2 from CRL4A-mediated ubiquitination and subsequent degradation — allowing DDB2 to recognize damage and initiate repair at multiple sites (Matsumoto et al., 2015). *Cul4a*^{-/-} mice express higher levels of DDB2 and XPC and demonstrate higher rates of NER activity (Liu et al., 2009). Despite these advances, our understanding of the dynamic between CRL4A, DDB2 and XPC during the initiation of NER continues to mature.

In addition to regulation of genome-wide NER factors DDB2 and XPC, the CRL4 complex also associates with Cockayne Syndrome (CS) protein A, which is involved in transcription-coupled-NER (TC-NER). Patients with CS exhibit sensitivity to light (albeit less severe than XP patients) but the disease is largely categorized by developmental defects (see (Rapin et al., 2006) for a review of CS). When DNA lesions occur on actively transcribed portions of the genome and induce RNA polymerase stalling, the damage is resolved by the alternate TC-NER pathway, which does not require recognition by DDB2 or XPC. Instead, the stalled polymerase acts as a damage sensor — recruiting CSA, CSB and XAB2 to the damage site and initiating the repair process. After this point, the global genome- (GG-) and TC-NER pathways are resolved by the same repair factors (Nospikel, 2009). Initial studies showed that the CRL4A complex is able to associate with CSA protein following UV irradiation and that purified CRL4A^{CSA} is catalytically active in vitro (Groisman et al., 2003). In addition to their roles in NER, DDB2 and CSA have structural similarities and interact with DDB1 via an N-terminal helix–loop–helix motif (Fischer et al., 2011). CSA is required for the proteasomal degradation of the TC-NER damage recognition protein CSB, and the purified CRL4A^{CSA} complex is able to ubiquitinate CSB in vitro (Fischer et al., 2011; Groisman, 2006). How CRL4^{CSA}-mediated degradation of CSB impacts TC-NER has yet to be determined. Overall, CRL4A regulates factors that are crucial to the early stages of both global genome- and transcription-coupled-NER.

Spontaneous DNA damage can occur anywhere in the genome. When damage occurs in dense or non-transcribed chromatin, it is necessary to relax the chromatin in order to recruit factors needed for repair processes. Remodeling of chromatin requires damage recognition proteins, histone acetyltransferases, helicases, and polymerases. In a series of reports, one group has shown that CRL4B^{DDB2} monoubiquitinates histone H2A on lysines 119 and 120 at damage loci following UV irradiation — relaxing the nucleosome (while the CUL4A complex is less efficient) (Guerrero-Santoro et al., 2008; Kapetanaki et al., 2006; Lan et al., 2012). Interestingly, CUL4B silencing in primary mouse embryonic fibroblasts does not seem to affect the global genomic repair of CPDs and 6-4PPs. Future work should clarify whether the CRL4A complex can step up to assume the function of CRL4B in histone modification in the early steps of chromatin remodeling during GG-NER when CUL4B protein is absent. Once again, DDB2 appears to direct CRL4 activity towards the damage site. CRL4s can also ubiquitinate histones H3 and H4 in vivo following UV irradiation — in turn, destabilizing nucleosomes, stripping damaged DNA and making lesions more accessible to repair factors such as XPC-RAD23B (Wang et al., 2006).

Even outside the context of DNA damage, CRL4A appears to regulate the deposition of newly-synthesized histone 3 variants into nucleosomes (Han et al., 2013). Two DCAFs, WDR5 and EED, also serve as subunits of histone methylation complexes involved in chromatin-remodeling (Higa et al., 2006). Knockdown of CUL4B or mutation of its NLS stabilizes WDR5 protein and alters epigenetic patterns in the PC12 neuroendocrine cell line (Nakagawa and Xiong, 2011). Of course, massive chromatin remodeling occurs during the process of DNA replication and mitosis. CRL4 also regulates the histone methyltransferase enzyme PR-Set7/SET8, which is required for successful chromatin compaction during G2/M phase (discussed in detail below) (Abbas et al., 2010; Centore et al., 2010; Jorgensen et al., 2011; Oda et al., 2010).

In addition to chromatin remodeling, DNA unwinding via helicase activity is also important for DNA repair. The torsional strain that results from this unwinding must then be relieved by DNA topoisomerases, which are particularly vital during DNA replication. Because cancer cells and bacteria proliferate rapidly, they are highly sensitive to chemical agents that inhibit topoisomerase function. *Camptothecins* (CPTs) inhibit *topoisomerase 1* (TOP1) by stabilizing a TOP1-DNA intermediate structure and preventing the re-ligation of strands of DNA (Kerrigan and Pilch, 2001). These TOP1-DNA-CPT complexes are often resolved by the ubiquitin-proteasome system (Desai et al., 2001). Kerzendorfer et al. showed that lymphoblastoid cell lines derived from men with *CUL4B* mutations have increased sensitivity to CPT and that knockdown of CUL4A and CUL4B reverses TOP1 degradation after CPT treatment (Kerzendorfer et al., 2010).

Upon recognition of DNA damage, cells upregulate repair proteins as well as factors involved in cell cycle arrest and apoptosis. DNA lesions can halt the process of replication and activate the ATR-Chk1 pathway. When activated, Chk1 kinase targets numerous proteins and promotes cell cycle checkpoint activity. Zhang et al. reported that stress caused by CPT treatment induces Chk1 ubiquitination and degradation likely caused by the SCF and CRL4A complexes (Zhang et al., 2005). While Chk1 is also degraded by CRL4A at low levels under normal conditions, replicative stress enhances this activity (Leung-Pineda et al., 2009). In order for DNA repair to occur, cells must be given adequate time to resolve lesions and breakage events. Induction of DNA damage causes upregulation of the *cyclin-dependent kinase* (CDK) inhibitor p21, which acts against S phase entry. In *Cul4a*^{-/-} mouse-derived embryonic fibroblasts, p21 is highly upregulated — slowing cell cycle progression and providing cells with an extended period of time to resolve UV-induced DNA damage (Liu et al., 2009). Ablation of p21 reverses the observed checkpoint effects in *Cul4a*^{-/-} cells and reduces their DNA repair capacity. UV irradiation causes activation of CRL4A complexes, which then promote the degradation of p21 to disengage the G1/S checkpoint. As such, cells with high levels of CUL4A are likely compromised in their ability to launch an effective DNA damage response.

UV-mediated CRL4A activation induces ubiquitination of other key regulators of DNA replication such as the replication licensing factors Cdt1 and Mcm10 as well as the p12 subunit of the DNA replication polymerase δ (Higa et al., 2003; Hu et al., 2004; Kaur et al., 2012; Zhang et al., 2013; Zhong et al., 2003). Two groups have reported that CRL4s also promote the degradation of the essential tumor suppressing transcription factor p53, which

controls the transcription of numerous DNA repair, checkpoint and apoptosis genes (Banks et al., 2006; Thirunavukarasou et al., 2014).

Overall, both CUL4A and CUL4B regulate several aspects of DNA repair, replication and chromatin remodeling. CUL4A is particularly relevant to DNA repair processes because it is activated (via neddylation) by replicative stresses such as UV-irradiation and defective topoisomerase activity. CRL4s control the activity and turnover of multiple DNA damage recognition proteins involved in NER and the G1/S DNA damage checkpoint. Collectively, downregulation of those proteins negatively affects the coordinated NER and the DNA damage checkpoint response. *Cul4a*^{-/-} mice have increased levels of DDB2, XPC and p21 expression and are highly resistant to UV-induced skin cancer, which underscores the importance of CUL4A in suppressing repair capacity (Liu et al., 2009). Taken together, these findings suggest that patients with *CUL4A* amplification or overexpression are likely predisposed to alterations of genomic integrity and susceptible to tumorigenesis.

4.2. Cell cycle progression

CRL4 complexes often target proteins in a context-dependent manner. In DNA repair pathways, CRL4s are recruited to damage foci because of UV-DDB's ability to interact with both UV-induced DNA lesions and the CUL4A scaffold. In addition to DNA repair factors, CRL4s also target proteins that regulate the onset of DNA replication. In the context of cell cycle and DNA replication regulation, CRL4 often utilizes the DCAF protein Cdt2 to target proliferating cell nuclear antigen (PCNA)-bound proteins. PCNA is a homotrimeric ring complex that encircles double-stranded DNA and recruits a variety of factors involved with DNA replication and repair such as polymerases, chromatin-remodeling proteins, minichromosome maintenance and GINS complex proteins (Strzalka and Ziemienowicz, 2010). Over 50 proteins have been shown to interact with PCNA — many of which contain a PIP (PCNA-interacting protein) box motif (De Biasio and Blanco, 2013). PCNA plays an essential role as a docking platform in DNA synthesis and chromatin remodeling and, as such, it is an ideal contact point for regulatory proteins.

One of the first proteins to be discovered as a bona fide CRL4 substrate is the Cdt1 DNA replication licensing factor, which promotes formation of the pre-replication complex. To prevent repeated origin firing and re-replication events in each cell cycle, Cdt1 is either inhibited by Geminin or degraded by the ubiquitin-proteasome system. Following up on Zhong et al.'s finding that *cul-4* interference stabilizes Cdt1 in *Caenorhabditis elegans*, several groups reported that CRL4-mediated degradation of Cdt1 depends on PCNA and that ablation of the Cdt1-PCNA interaction via PIP box mutation promotes re-replication (Arias and Walter, 2006; Higa et al., 2006; Nishitani et al., 2006; Senga et al., 2006; Zhong et al., 2003). Notably, CRL4 degrades Cdt1 in S phase during a healthy mitotic cycle and not only in the instance of DNA damage and stress. Cdt2 (encoded by the *DTL* gene in humans) was identified as the DCAF, or substrate receptor, required for CRL4- and PCNA-dependent Cdt1 degradation (Higa et al., 2006; Jin et al., 2006; Sansam et al., 2006). Importantly, Cdt1 is only stabilized when both CUL4A and CUL4B are targeted simultaneously with RNA interference, indicating that this is an overlapping and likely crucial function for the two CRL4 complexes.

In addition to inhibiting Cdk2 and Cdk1 kinase complexes, p21 and the other members of the Cip/Kip family influence other cellular processes such as transcription, apoptosis, senescence and cytoskeletal organization (Starostina and Kipreos, 2012). Although p21 and other CDK inhibitors may actually promote tumorigenesis in certain contexts, p21 enforces cell cycle checkpoints, curbs cellular proliferation and is generally considered a tumor-suppressor protein (Besson et al., 2008). During the G1/S transition and G2/M checkpoint, p21 is targeted for ubiquitination and degraded by the proteasome by SCF^{Skp2} and APC/^{C^{Cdc20}}, respectively. In 2008, several groups demonstrated that p21 exhibits a similar pattern of post-translational degradation as Cdt1 in that it is also degraded in S phase by CRL4^{Cdt2} complexes in a PCNA-dependent manner (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). While both CRL4 complexes appear to target p21 for degradation, loss of CUL4A alone stabilizes p21 in *Cul4a*^{-/-} mouse skin cells and causes p21-dependent delays in cell cycle progression in mouse embryonic fibroblasts (Liu et al., 2009). RNAi-mediated knockdown of *Cul4b* results in massive accumulation of p21 in XEN extra-embryonic endoderm mouse stem cells (which express extremely low endogenous levels of CUL4A) (Liu et al., 2012). In certain contexts, either CUL4A or CUL4B can serve as the primary regulator of p21 expression.

During the processes of genomic replication and mitosis, chromatin must be extensively remodeled. To achieve changes in the contour and compaction of chromatin, histone subunits are post-translationally modified in several ways to alter the nucleosome's structure and its interactions with chromatin-binding proteins. Among the numerous histone modification events that have been characterized, methylation of lysine 20 in histone H4 has been associated with gene silencing, mitosis, DNA damage and tumorigenesis (Pesavento et al., 2007). Monomethylation of H4 Lys20 appears to be solely performed by the histone methyltransferase PR-Set7/SET8. Loss of PR-Set7 results in mouse embryonic lethality and accumulation of cells in G2/M phase (Oda et al., 2009). Due to PR-Set7's observed degradation during S phase and its possession of two PIP-boxes, several groups investigated whether PR-Set7 was also regulated by the CRL4^{Cdt2} complex in a PCNA-dependent manner. When the PCNA-PR-Set7 interaction is disrupted by mutation of the PIP box degon, PR-Set7 cannot be degraded during S phase — leading to aberrant chromatin compaction and arrest in late G2 phase (Abbas et al., 2010; Centore et al., 2010; Jorgensen et al., 2011; Oda et al., 2010). Another group reported a key role for CRL4 in G2 phase by showing that knockdown of Cdt2 causes G2 arrest due to accumulation of Chk1, which is degraded by CRL4^{Cdt2} in a PCNA-independent manner (Huh and Piwnicka-Worms, 2013).

Zhang et al. determined that DNA polymerase δ , which participates in DNA replication as well as repair, is also regulated by CRL4 ligases in a similar manner (Zhang et al., 2013). Building on work showing that the p12 subunit of the Pol δ heterotetramer contains a PIP box and is sharply downregulated after UV irradiation, they showed that knockdown of CUL4A and Cdt2 shields p12 from UV-induced degradation and that PIP box mutations stabilized p12 during S phase (Zhang et al., 2013). This data further reinforces CRL4's role as a regulator of DNA replication during typical cell cycle progression as well as during times of stress.

CRL4 ligases do not solely utilize Cdt2 and PCNA for the ubiquitination and degradation of cell cycle regulators. CRL4^{DCAF1} complexes degrade the DNA replication factor Mcm10, which is crucial for recruiting DNA polymerase α , promoting replication initiation and elongating newly-synthesized strands (Kaur et al., 2012). The mechanism is unclear but CRL4A also promotes the degradation of another member of the Cip/Kip family of cyclin-dependent kinase inhibitors: p27 (Miranda-Carboni et al., 2008). The tumor suppressor protein RASSF1A, which negatively regulates the anaphase-promoting complex, is degraded by the CRL4A complex during mitosis (Jiang et al., 2011). CRL4 complexes also target tumorigenic cell cycle regulators. CRL4^{TRUSS} downregulates the c-myc and N-myc transcription factors, which promote cell cycle entry and progression (Choi et al., 2010). Also, knocking down CUL4B via RNAi or mutating its NLS stabilizes Cyclin E (Zou et al., 2009). However, it is possible that tissue-specific and temporal factors affect this specific relationship as Cyclin E levels are not changed by *Cul4b* knockout in mouse brain tissue (Chen et al., 2012).

All of these data underscore how CRL4 complexes – often in a Cdt2- and PCNA-dependent manner – promote cell cycle progression but also act against re-replication. These findings also illustrate how excessive CRL4 activity can potentially cause uncontrolled cell proliferation due to eradication of several key cell cycle checkpoint and chromatin-remodeling proteins.

4.3. Embryogenesis

Initial efforts to characterize the expression patterns and functional roles of the CUL4 proteins in mammals proved to be challenging. In the first reported model of *Cul4a* knockout, homozygous *Cul4a* mutant embryos did not progress past 7.5 days post coitus (Li et al., 2002). However, in this model, Cre-mediated excision of *loxP*-flanked *Cul4a* exon 1 also caused deletion of the 5' exon 1 of *Pcid2*, which is located on the complementary strand. The *Pcid2* gene product, which contains protein domains that are homologous to subunits of the ribosome, the 26S proteasome and COP9 signalosome, is crucial for survival and appears to be the basis for the observed developmental defects (Hofmann and Bucher, 1998; Liu et al., 2009). In 2009, Liu et al. reported an alternate strategy for targeting the *Cul4a* locus in which exons 17–19 were flanked by *loxP* sites — deletion of this region prevents ROC1/RBX1 binding and ablates E3 ligase function (Liu et al., 2009). Germline knockout of *Cul4a* using this approach showed that mice with total loss of CUL4A function were viable and showed no clear health or developmental defects aside from male infertility (discussed in detail below). This is presumably due to compensatory effects of CUL4B expression and activity — as knockdown of *Cul4b* in *Cul4a*^{-/-} cells halted proliferation and rendered the cells inviable (Liu et al., 2009). Furthermore, germline knockout of *Ddb1*, which encodes a core component of both CRL4A and CRL4B ligase complexes, causes embryonic lethality (Cang et al., 2006). A third *Cul4a* knockout mouse was reported in 2011 in which exons 4–8 of the *Cul4a* locus are flanked with *loxP* sites (Kopanja et al., 2011). This report showed that *Cul4a* is important for spermatogenesis and confirmed that the gene is largely dispensable for mouse embryogenesis and development.

In 2012, data from three independently-generated *Cul4b* transgenic mouse lines showed that complete deletion of *Cul4b* expression causes embryonic lethality (Chen et al., 2012; Jiang et al., 2012; Liu et al., 2012). The defects primarily result from attenuated proliferation and increased apoptosis in extra-embryonic tissues — including the placenta and yolk sac (Liu et al., 2012). While CUL4A has overall low levels of expression throughout the embryo, CUL4B is highly expressed in several extra-embryonic structures (Liu et al., 2012). CUL4B is only essential within these tissues as restricting *Cul4b* knockout to the epiblast produces live mice (Chen et al., 2012; Liu et al., 2012). Although loss of CUL4B expression has little effect on the embryo proper (presumably due to compensation by CUL4A), it does produce minor neural and behavioral problems, which are discussed in more detail below.

4.4. Hematopoiesis

Hematopoietic stem cells (HSCs) have the ability to self-renew and produce identical, multipotent daughter HSCs or undergo differentiation to form any and every cell type in the blood system. Hematopoiesis is a tightly regulated, context-dependent process that relies on several intrinsic and extrinsic factors. Many *HOX* genes, which are highly conserved regulators of animal development, are expressed during the early stages of hematopoiesis within progenitor cells and undergo downregulation as cells differentiate (Argiropoulos and Humphries, 2007). *HOX* genes are also upregulated in certain leukemias and are frequently implicated in oncogenic fusion events that produce overactive and stabilized gene products (Chung et al., 2006).

HOXA9 is highly expressed in primitive CD34⁺ HSCs and is down-regulated as cells undergo terminal differentiation (Sauvageau et al., 1994). Deletion of *Hoxa9* in mice causes several defects in hematopoiesis including: large reductions in total leukocytes and lymphocytes, reduced spleen and thymus mass and diminished G-CSF response (Lawrence et al., 1997). Also, *Hoxa9*^{-/-} mouse-derived bone marrow cells (BMCs) demonstrate poor growth in vitro and perform poorly in competitive transplantation assays (Lawrence, 2005). Conversely, HOXA9-overexpressing BMCs expand more readily in vitro but demonstrate some defects in B cell lineage differentiation (Thorsteinsdottir et al., 2002). HOXA9 can also promote leukemogenesis when co-overexpressed with leukemia-associated transcription factor Meis1 (Kroon et al., 1998). Several *HOX* genes – including *HOXA9* – have been identified in leukemogenic translocation events involving the *NUP98* gene, which encodes a nuclear pore protein. The resulting fusion gene product contains the N-terminal portion of NUP98, which is able to recruit transcriptional regulators and histone modifying enzymes, and the homeodomain (HD)-bearing C-terminus of HOXA9. Introduction of this fusion protein into myeloid cells enhances transcription of HOXA9 target genes at greater rates than wild-type HOXA9 alone (Ghannam et al., 2003). In an attempt to identify CUL4A substrates, Zhang et al. performed a yeast two-hybrid screen and discovered an interaction between CUL4A and HOXA9 (Zhang et al., 2003). Further characterization showed that the HD is required for binding to the CUL4A machinery and serves as a degradation signal. Mutation of helix 1 within the HD of HOXA9 ablates CUL4A binding and ubiquitination (Zhang et al., 2003). The NUP98-HOXA9 fusion product, which connotes greater proliferative, self-renewal and engraftment capacity in HSCs, is also more resistant to CUL4A-mediated degradation (Chung et al., 2006; Moore et al., 2007).

HOXB4 is another member of the HOX family of transcription factors that is highly expressed in primitive human and murine blood cells (Sauvageau et al., 1994). Ablation of *Hoxb4* in mice allows for normal hematopoiesis but reduces proliferation capacity - while HOXB4 over-expression promotes HSC self-renewal and expansion (Antonchuk et al., 2001; Brun, 2004; Sauvageau et al., 1995). HOXB4 overexpression in supportive stromal cells promotes human HSC expansion in vitro and enhances hematopoietic reconstitution of *nonobese diabetic-severe combined immunodeficiency* (NOD-SCID) mice without promoting leukemia (Amsellem et al., 2003). Since both HOXB4 and HOXA9 promote HSC self-renewal, Lee et al. investigated whether CRL4 also induces ubiquitination and proteolysis of HOXB4. A conserved LEXE motif in helix 1 of the HD within HOX proteins was defined as a degron for multiple HOX proteins — including HOXB4 (Lee et al., 2013). Mutation of the LEXE motif in HOXB4 renders the protein resistant to CRL4-mediated degradation and promotes proliferation of HSCs while maintaining the stemness of transduced adult CD34⁺ cells in NOD-SCID/IL-2R γ^{null} recipient mouse bone marrow (Lee et al., 2013).

These data collectively indicate that CRL4s likely play a significant role in regulating the self-renewal and differentiation of hematopoietic stem cells by targeting HOXA9 and HOXB4 for ubiquitination and degradation. Future work is needed to identify the DCAF substrate receptor and any other potential co-factors that may be required for CRL4-mediated ubiquitination of these and other HOX family proteins.

4.5. Spermatogenesis

CUL4A and *CUL4B* have high sequence homology and are likely the result of a gene duplication event that occurred before the appearance of mammals. When Zhong et al. characterized CUL4 cellular and physiological function in nematodes (which only express a single cullin 4 gene) using RNAi techniques, they found that *cul-4* mRNA is widely expressed in larvae and that levels are particularly high in proliferating tissue (Zhong et al., 2003). They also found that interfering with *cul-4* expression in *C. elegans* causes DNA replication defects (due to improper Cdt1 regulation as described in detail above) as well as premature meiosis activity and sperm formation in germ cell lineages. Similar findings were reported last year by a group researching *Cul4* expression during Chinese mitten crab development (Wang et al., 2014b). Despite the functional redundancy of CUL4A and CUL4B, their patterns of expression often differ spatially and temporally — highlighting key, non-overlapping roles for each protein in mature and developing tissues. Although *Cul4a*^{-/-} mice do not show obvious developmental or health defects, *Cul4a*^{-/-} males are infertile and develop smaller testes (Kopanjan et al., 2011; Yin et al., 2011). During spermatogenesis, CUL4B is expressed in pluripotent spermatogonia cells and late-stage, differentiated secondary spermatocytes and spermatids — while CUL4A is highly expressed in the pachytene and diplotene stages of primary spermatocyte development (Yin et al., 2011). During these latter stages, recombination occurs and crossover events must be resolved by DNA repair factors. Failure to resolve these interchromosomal structures can result in meiotic arrest and apoptosis. Both of the groups investigating sperm development in *Cul4a*-deficient mice found that primary spermatocytes could not resolve dsDNA breaks and underwent extensive apoptosis (Kopanjan et al., 2011; Yin et al., 2011). Similarly, *CUL4B*-

deficient human males typically exhibit hypogonadism (see below for more information on clinical relevance) (Kerzendorfer et al., 2011).

4.6. Physiological functions and tumorigenesis

CUL4A and CUL4B individually demonstrate higher expression in certain tissues — suggesting that they may regulate certain substrates with tissue-specific biological implications. CUL4B was shown to promote the ubiquitination of the reactive oxygen species scavenging protein peroxiredoxin III (Li et al., 2011b). Recently, Chen et al. reported that interference with CUL4^{CRBN} expression via RNAi or dominant-negative constructs stabilizes the voltage-gated chloride channel protein CLC-1, which is expressed in skeletal muscle cells and is sometimes mutated in patients with myotonia congenita (Chen et al., 2015). Moreover, CUL4B has been found to assemble a noncanonical CUL4 complex independent of any DCAF substrate receptor. In 2007, Ohtake et al. showed that the unique N-terminal region of CUL4B can form a distinct E3 ubiquitin ligase complex consisting of CUL4B and the dioxin receptor AhR that promotes degradation of estrogen receptor α in a ligand-dependent manner (Ohtake et al., 2007).

CUL4 complexes induce the degradation of numerous tumor suppressor proteins — many of which are described above (Table 1). The p150^{Sal2} transcription factor, which promotes quiescence and demonstrates tumor suppressor activity, is degraded by the CUL4^{RBBP7} complex (Sung et al., 2011). Further evidence of potential CUL4 tumorigenic activity is indicated by the finding that CUL4 is potently inhibited by the Merlin/NF2 tumor suppressor protein (Li et al., 2010). When mutations prevent Merlin from binding to CUL4^{DCAF} complexes, the LATS1 and LATS2 kinases of the Hippo signaling pathway are more readily degraded — resulting in increased cell proliferation and tumorigenesis (Li et al., 2014). CUL4^{FBW5} may also promote mTOR signaling by downregulating TSC2 protein (Hu et al., 2008). CUL4A was reported to degrade another negative regulator of mTOR activity — REDD1/DDIT4 — but REDD1 expression may be regulated by a CUL4-independent negative feedback mechanism involving mTORC1 (Katiyar et al., 2009; Tan and Hagen, 2013).

However, CUL4 complexes also target proteins that are generally associated with promoting tumorigenesis. For example, CUL4A targets proto-oncogenic transcription factors c-Jun, ETV1, ETV4 and ETV5, which promote cell proliferation and/or suppress apoptosis, via DET1 and the DCAF protein COP1 (Vitari et al., 2011; Wertz et al., 2004). Moreover, HOXA9, Cyclin E, c-myc and N-myc are also targeted by CUL4s. The relevance of CUL4A and CUL4B in human cancers is discussed in greater detail in the next section.

5. Clinical relevance

5.1. Cancer

CUL4A is located in the chromosomal region ch13q34, which is amplified or lost in a subset of numerous cancers. In nearly all cases, *CUL4A* is gained or deleted along with *TFDPI*, *CDC16*, *GAS6*, and *LAMP1*, which makes it difficult to disambiguate the specific importance of *CUL4A* copy number variation from the other genes in this locus (Abba et al., 2007; Melchor et al., 2009; Yasui et al., 2002). Point mutations in *CUL4A* are less frequent

than copy number variations and do not show any distinct pattern (Cerami et al., 2012; Gao et al., 2013). Hu et al. reported that CUL4B is upregulated on a transcriptional or protein level in numerous cancers (Hu et al., 2012). Interestingly, 19/91 pancreatic cancer samples have an identical truncating mutation in *CUL4B* that occurs at Ser143 (Cerami et al., 2012; Gao et al., 2013). The functional significance of this event has not been determined as of this date.

One of the earliest investigations of the role of CRL4s in cancer was the initial characterization of human CUL4A in normal mammary and breast cancer cell lines (Chen et al., 1998). As compared to normal mammary cells, there are much higher levels of *CUL4A* mRNA expression in tumor-derived cells as well as a few amplification events (Chen et al., 1998; Hu et al., 2012). Ch13q34 amplifications sometimes occur in breast cancers and are more likely to be found in p53-deficient, BRCA1-deficient and basal like breast cancers (Abba et al., 2007; Melchor et al., 2009) (Table 2). Unfortunately, accurate quantification of CUL4A protein expression using immunohistochemical (IHC) methods is not possible due to the lack of any unique, conventional epitopes that aren't also present in CUL4B. Although the specific upregulation of CUL4A versus CUL4B cannot be determined, analysis in lymph node-negative invasive breast tumors showed that patients with strong CUL4 expression had poorer overall and disease-free survival (Schindl et al., 2007). *CUL4A* is also a transcriptional target of the oncogenic Wnt/beta-catenin signaling pathway and induces p27 degradation in Wnt10b-stimulated MCF7 cells — suggesting a possible mechanism for CUL4A contribution to breast carcinogenesis (Miranda-Carboni et al., 2008). Also, Wang et al. reported that high *CUL4A* expression correlates with metastasis and overexpression of CUL4A promotes epithelial-to-mesenchymal transition (EMT) (Wang et al., 2013).

Due to CUL4A's extensive involvement in regulating DNA repair and cell cycle progression, excessive CUL4A activity may promote carcinogenesis in tissues which receive high levels of UV irradiation or exposure to genotoxic, DNA adduct-forming chemicals such as benzo[a]-pyrenes (these adducts are also resolved via NER). In 2009, Liu et al. reported that skin-specific *Cul4a*^{-/-} mice are highly resistant to UV-induced skin tumors — likely due to increased expression of UV lesion recognition proteins DDB2 and XPC as well as the Cdk inhibitor p21, which enhance GG-NER activity and prolong the G1/S checkpoint, respectively (Liu et al., 2009). In the colorectal epithelium, cells turn over quickly and are exposed to various DNA adduct-forming molecules found in charcoal, fried food, and cigarette smoke. Analysis of a large cohort of colorectal cancer patients in China showed that tumor cells have higher CUL4B expression than surrounding normal mucosal cells and that high CUL4B expression (both mRNA as well as protein) correlates with tumor invasiveness, metastasis, more advanced tumor stages and poorer prognosis (Jiang et al., 2013). Another study showed that CUL4B protein and transcript levels are higher in colorectal cancers (Hu et al., 2012). Although direct involvement of CUL4A in colorectal cancer has not been reported, it is a downstream target of the Wnt/beta-catenin axis, which is a major driver of this malignancy (Miranda-Carboni et al., 2008). In the lung (which is exposed to carcinogenic pollutants and tobacco smoke), *CUL4A* and *CUL4B* transcripts and CUL4B protein were found to be expressed at higher levels in cancers than normal tissue (Hu et al., 2012; Wang et al., 2014a). Furthermore, in tumor cell engraftment studies using nude mice, Wang et al. showed that non-small-cell lung cancer (NSCLC) cells expressing an

anti-*CUL4A* shRNA construct grew more slowly than the empty vector control cells and had lower levels of EGFR expression. In addition, Cre-driven overexpression of *CUL4A* in mice can induce hyperplasia and tumorigenesis in the lungs (Li et al., 2011a; Yang et al., 2014). *CUL4A* was found to be amplified in a small group of primary malignant pleural mesothelioma (MPM, which is associated with occupational exposure to asbestos) samples (Hung et al., 2011). MPM cells derived from those samples grow more slowly in vitro after knockdown of *CUL4A* mRNA (Hung et al., 2011). The tumor-suppressive effects of Merlin/NF2, which regulates cell cycle progression, are due to inhibition of the CRL4^{DCAF1} complex and mutations that affect the Merlin–DCAF1 interaction have been found in mesothelioma and Schwannoma cells (Li et al., 2014; Li et al., 2010).

CUL4A and *CUL4B* have been investigated in other cancers as well. Ch13q34 amplifications occur in a subset of hepatocellular carcinomas and enforced overexpression of human *CUL4B* in mice accelerates chemically-induced liver tumorigenesis (Yasui et al., 2002; Yuan et al., 2015). Components of CRL4 complexes including *CUL4B*, DDB1 and ROC1 are upregulated in ovarian cancers (Hu et al., 2012; Pan et al., 2013). *CUL4A* transcript is often upregulated in prostate cancers and knockdown of *CUL4A* decreases 22RV1 cell viability, proliferation and tumor growth in mouse engraftment models (Ren et al., 2012). *CUL4B* is more highly expressed in cervical cancers — particularly in high grade tumors (Yang et al., 2013). Expression of an anti-*CUL4B* shRNA in esophageal and cervical cancer cell lines decreases the resulting tumor burden in engrafted mice as compared to those expressing non-targeting shRNAs (Hu et al., 2012; Yang et al., 2013). In addition to those cancers, Hu et al. showed that *CUL4B* protein is more highly expressed in stomach and pancreatic cancers and that *CUL4B* transcript is higher in thyroid cancers (Hu et al., 2012).

Due to the observed upregulation of *CUL4* proteins in several cancers and their targeting of several tumor suppressor proteins, they could be classified as oncogenes in certain contexts. As a result, there have been efforts to develop therapies directed against CRL4 and other components of the ubiquitin proteasome system. Perhaps the most successful of these drugs are proteasome inhibitors (such as bortezomib/Velcade), which are now established agents used to treat multiple myeloma and mantle cell lymphoma. Moreover, the NEDD8 E1 enzyme inhibitor MLN-4924 has the ability to decrease CRL activity by preventing cullin neddylation and has been investigated in 8 clinical trials to date (Nawrocki et al., 2012; Soucy et al., 2009). However, those agents have fairly broad effects on the overall ubiquitin proteasome system and are not specific for CRL4. The only known CRL4 E3 ligase-targeting compounds are thalidomides, which were originally used to treat pregnancy-induced nausea (morning sickness) but were subsequently discontinued because of the teratogenic deformities they cause in fetuses. Interestingly, thalidomide was repurposed due to its ability to suppress inflammation in patients with erythema nodosum leprosum, which led to its reclassification as an immunomodulatory drug (IMiD) and eventual approval by the FDA for treatment of that condition (Pan and Lentzsch, 2012). Currently, IMiDs are being developed as treatments for numerous malignancies — particularly myelomas. When Ito et al. utilized thalidomide-bound beads to identify the cellular target(s) of thalidomide, they uncovered CRBN along with other components of CRL4 complexes (Ito et al., 2010). The importance of CRBN as a target was confirmed when it was shown that CRBN is required

for the anti-myeloma effects of IMiDs (Zhu et al., 2011). In 2014, two groups reported the crystal structure of IMiD-bound CRL4^{CRBN} complexes (Chamberlain et al., 2014; Fischer et al., 2014). Although initially described as inhibitors of CRBN, IMiDs actually promote the CRL4^{CRBN}-mediated degradation of two Ikaros transcription factors, IKZF1 and IKZF3, which are highly expressed in B cell malignancies (Kronke et al., 2014; Lu et al., 2014). Therefore, this is an example of redirecting CRL4 activity rather than inhibiting it. Currently, there are no available specific CRL4 inhibitors. An alternative approach is to target relevant proteins in CRL4-overactive cells such as Wnt pathway components, cyclin-dependent kinases and histone modifiers. Use of genotoxic agents may also exploit the impaired DNA repair capacity of CRL4-overactive cells.

5.2. Viral pathogenesis

Despite their typically tiny size and minimal nature, viruses are capable of sophisticated manipulations of their hosts. In addition to inducing the expression of their own proteins, viruses can cripple or even hijack endogenous proteins to promote viral infection, survival and proliferation. DDB1 was first implicated in viral pathogenesis when it was shown to interact with hepatitis B protein X (HBx), which competitively inhibits DDB2 from interacting with DDB1 by binding to the same pocket (Bontron, 2002; Lee et al., 1995; Li et al., 2010). Although the precise mechanisms have not been uncovered, DDB1–HBx interactions can promote viral replication, induce cell death and potentially disrupt endogenous CRL4 activity (Guo et al., 2014; Leupin et al., 2005).

In addition to HBx, DDB1 binds to protein V, which is expressed in simian virus 5 (SV5) and other paramyxoviruses (Lin et al., 1998). DDB1 is required for SV5-V-mediated degradation of STAT1, which is a vital factor in the antiviral interferon response (Andrejeva et al., 2002). It was later reported that SV5-V and type II human parainfluenza virus protein V can function as atypical substrate adaptors for CRL4-mediated STAT protein degradation (Precious et al., 2005; Ulane et al., 2005). Interestingly, one of the earliest structures of the CRL4A complex included the SV5-V protein, which helped researchers to visualize how endogenous DCAFs and other substrate receptors are oriented within CRL4 complexes (Li et al., 2006).

Human immunodeficiency viruses (HIVs) also usurp CRL4 ligases to promote viral pathogenesis. DCAF1 is also known as VPRBP due to its initial characterization as a cytoplasmic protein that binds to the HIV-1 protein Vpr (Zhang et al., 2001). In 2007, a deluge of reports described how Vpr binds to DCAF1 and utilizes CRL4^{DCAF1} to promote G2 arrest and apoptosis (Belzile et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Schrofelbauer et al., 2007; Wen et al., 2007). Schröfelbauer et al., following up on previous work, showed that CRL4^{DCAF-Vpr} targets the uracil DNA glycosylase UNG2/SMUG1 for degradation, which also was confirmed by another group (Ahn et al., 2010; Schrofelbauer et al., 2007; Schrofelbauer et al., 2005). Le Rouzic et al. reported that Vpx, which is a Vpr-related protein expressed by HIV-2 and simian immunodeficiency virus (SIV), also binds with CRL4^{DCAF1} (Le Rouzic et al., 2007). Evidently, the interaction between Vpx and CRL4^{DCAF1} is the basis for HIV-2's and SIV's ability to efficiently infect human myeloid cells (Srivastava et al., 2008). The CRL4^{DCAF-Vpx} complex targets SAMHD1, which is an

antiviral deoxynucleoside triphosphohydrolase that blocks reverse transcription and is expressed in myeloid cells (Ahn et al., 2012; Hofmann et al., 2012; Hrecka et al., 2011).

Due to lentiviral and paramyxoviral reliance on CRL4 activity, it may be worthwhile to develop and utilize specific inhibitors of this E3 ligase to combat certain infections. However, the potential inhibitory effect of HBx on CRL4 activity should also be taken into consideration, as it could exacerbate infections in certain cases.

5.3. X-linked intellectual disability

Approximately 10% of intellectual disability (previously known as mental retardation) cases in males are caused by defects in the X chromosome (des Portes, 2013). *X-linked intellectual disability (XLID)* has been associated with 82 different genes and is a highly variable condition — about 215 distinct syndromes have been described. XLID has been broken down into two fairly broad categories: non-syndromic XLID, where patients solely exhibit intellectual disabilities, and syndromic XLID, in which other physiological and behavioral abnormalities also manifest. Clinicians and researchers have made significant efforts to identify the genetic causes of XLID in families where several individuals suffer from the condition. In 2000, Cabezas et al. reported a syndromic form of XLID in which patients had numerous clinical features including: short stature, prominent lower lip, small testes, lower leg muscle wasting, kyphosis, joint hyperextensibility, abnormal gait, tremor and poor motor coordination (Cabezas et al., 2000). Linkage analysis revealed that the individuals in this family who suffered from XLID (all males besides a female carrier with mild clinical features) shared a genetic sequence that mapped to Xq24-25, which encompasses the *CUL4B* gene. A massive XLID study conducted by Tarpey et al. uncovered eight distinct mutations in the *CUL4B* gene (5 out of 8 mutations resulted in truncations or aberrant splicing) (Tarpey et al., 2007). Importantly, the XLID patients with *CUL4B* mutations exhibited highly similar symptoms to those in the Cabezas et al. study (Tarpey et al., 2007). Other reports have identified truncations, aberrant splicing and deletions in *CUL4B* in XLID patients presenting similar clinical features to those previously described (Badura-Stronka et al., 2010; Isidor et al., 2010; Londin et al., 2014; Zou et al., 2007). Recently, 9 novel mutations in *CUL4B* were identified and several *CUL4B*-associated cerebral defects were reported including: malformations of cortical development, ventriculomegaly and diminished white matter volume (Vulto-van Silfhout et al., 2015).

Research performed on multiple mouse models has shown that *CUL4B* is indispensable for extra-embryonic tissue development (Chen et al., 2012; Jiang et al., 2012; Liu et al., 2012). Chen et al. performed an extensive analysis on the neural and behavioral effects of *Cul4b* loss to determine if transgenic mice can serve as appropriate research models for the investigation of XLID. Behavioral studies demonstrated that *Cul4b*^{-Y} males exhibit phenotypes associated with XLID such as impaired learning and memory (Chen et al., 2012). *Cul4b*^{-Y} mice also have reduced complexity in dendrites and decreased spine density in neurons within the hippocampus — a structure which is crucial for spatial learning and memory (Chen et al., 2012). In addition, these mice are more sensitive to chemically-induced seizures, which suggests greater susceptibility to epilepsy (another characteristic of XLID) (Chen et al., 2012). Although the mice did not demonstrate many of the other clinical

features found in syndromic XLID, they appear to be useful models for studying this disease.

6. Concluding remarks

CUL4A and CUL4B have a high degree of sequence homology and share numerous essential E3 ligase functions in the cell due to their mutual dependence on DDB1 and DCAFs. Loss of either CUL4A or CUL4B is typically compensated by the expression and activity of the remaining paralog. Loss of both CUL4 proteins or their substrate adaptor DDB1 results in loss of cell proliferation, apoptosis and embryonic lethality — underscoring the importance of CUL4 function (Cang et al., 2006; Liu et al., 2009). Gene-targeting mouse models have been tremendously informative on delineating the unique functions and needs for these cullin proteins. CUL4A can be activated by the onset of cellular stresses such as UV irradiation and is a transcriptional target of the Wnt signaling pathway. As such, it likely contributes to tumorigenesis in certain contexts. While *Cul4a*^{-/-} mice are viable, males are rendered infertile by meiotic defects and sperm progenitor apoptosis. CUL4B plays a key role in the development of extra-embryonic tissue and hippocampus formation. In humans, *CUL4B* defects cause intellectual disability and numerous other physiological and behavioral abnormalities. Both CUL4s are key regulators of the mitotic cycle, DNA repair and chromatin remodeling, which may be the reason that they are altered in cancers and hijacked by viruses. As we gain further insight into the specific and overlapping functions of CUL4A and CUL4B, we expect that appropriate therapeutic agents and strategies will be developed to combat cullin 4 dysregulation in cancer, embryogenesis and viral infection.

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The corresponding Gene Wiki entries for this review can be found here: <https://en.wikipedia.org/wiki/CUL4A>
<https://en.wikipedia.org/wiki/CUL4B>

Abbreviations

6-4PP	6-4 photoproduct
BMC	bone marrow cell
Cdk	cyclin-dependent kinase
CPD	cyclopurimidine dimer
CPT	camptothecin
CRBN	Cereblon

CRL4	cullin RING ligase
CS	Cockayne syndrome
CSN	COP9 signalosome
CUL4A	cullin 4A
CUL4B	cullin 4B
DCAF	DDB1-CUL4-associated factor
EMT	epithelial-to-mesenchymal transition
GG-NER	global genome NER
HBx	hepatitis B protein X
HD	homeodomain
HIV	human immunodeficiency virus
HSC	hematopoietic stem cell
IHC	immunohistochemistry
IMiD	immunomodulatory drug
MPM	malignant pleural mesothelioma
mTOR	mammalian target of rapamycin
NER	nucleotide excision repair
NLS	nuclear localization signal
NOD-SCID	nonobese diabetic-severe combined immunodeficiency
NSCLC	non-small-cell lung cancer
PCNA	proliferating cell nuclear antigen
PIP	PCNA-interacting protein
RING	really interesting new gene
SCF	Skp1, CUL1 and F-box
SIV	simian immunodeficiency virus
SV5	simian virus 5
TC-NER	transcription-coupled NER
TCGA	The Cancer Genome Atlas
UV	ultraviolet

UV-DDB	UV-damaged DNA binding
XLID	X-linked intellectual disability
XP	xeroderma pigmentosum

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Fig. 1.

CRL4 complex subunits and regulators. A. The CRL4A complex recruits substrates to the N-terminal portion of CUL4A via the DDB1 adaptor and various DCAFs/substrate receptors while the C-terminal end binds ROC1/RBX1 to recruit ubiquitin-bearing E2 enzymes. Conjugation of a NEDD8 molecule appears to promote flexibility and E3 ligase activity. B. The CRL4B complex is very similar to CRL4A aside from the extended N-terminus of CUL4B, which contains a nuclear localization signal and may have other effects in substrate recruitment.

Table 1

Putative CRL4 substrates. The substrate names and a brief description of their cellular functions are presented in the far left columns. The specific DCAF or other substrate receptor required for CRL4-induced degradation is also listed when applicable. Experimental evidence abbreviations: IP = immunoprecipitation, IF = immunofluorescence, CS = crystal structure data, IvvU = in vivo ubiquitination, IvtU = in vitro ubiquitination, KO = gene knockout in mammalian tissue, M = mutagenesis, DN = use of dominant negative CUL4 construct, OE = CUL4 overexpression, HL = half-life analysis, Y2H = yeast 2-hybrid, GF = gel filtration, EM = electron microscopy. N/A = not applicable since substrate is also a DCAF. Substrate of non-endogenous CRL4 complex.[†]

Substrate	Function(s)	DCAFs/substrate receptors	CRL4 regulator	Experimental evidence	References
DDB2	DNA repair	N/A	CUL4A	RNAi, IF, CS, KO	Li (2006), Liu et al. (2009), Fischer et al. (2011)
XPC		DDB2		IP, IvvU, IvtU, KO	Sugasawa et al. (2005), Liu et al. (2009), Fei et al. (2011)
Chk1		Cdt2		IP, IvvU, IvtU, RNAi, M	Zhang et al. (2005), Leung-Pineda et al. (2009), Huh and Pwnica-Worms (2013)
CSB		CSA		IvtU	Groisman, 2006; Fischer et al., 2011
TOP1	DNA topoisomerase	Unknown	CUL4A, CUL4B	RNAi, KO, IvvU	Kerzendorfer et al. (2010)
Histone 2A	Nucleosome component	DDB2	Primarily CUL4B	IP, M	Kapetanaki et al. (2006)
Histone 3		Unknown		IvvU, RNAi	Wang et al. (2006)
Histone 4		Unknown			
Mcm10	DNA replication factor	DCAF1	CUL4A, CUL4B	IP, RNAi, IvvU, OE	Kaur et al. (2012)
Cdt1	DNA replication licensing factor	Cdt2, PCNA	CUL4A, CUL4B	IP, M, IF, HL, RNAi	Higa et al. (2006), Nishitani et al. (2006), Aria and Walter (2006), Jin et al. (2006), Sansam et al. (2006)
Pol δ, p12 subunit	DNA polymerase subunit	Cdt2, PCNA	Primarily CUL4A	RNAi, HL, M	Zhang et al. (2013)
PR-Set7/SET8	Histone monomethylase	Cdt2, PCNA	CUL4A, CUL4B	IF, IvvU, IvtU, HL, RNAi, M	Abbas et al. (2010), Centore et al. (2010), Oda et al. (2010), Jorgensen et al. (2011)
WDR5	H3K4 methyltransferase complex subunit	N/A	CUL4B	RNAi, IvvU, IvtU	Nakagawa and Xiong (2011)
p21/CIP/WAF1	Cyclin-dependent kinase inhibitor	Cdt2, PCNA	CUL4A, CUL4B	RNAi, IP, IvvU, IvtU, Y2H, IF, M, KO	Abbas et al. (2008), Kim et al. (2008), Nishitani et al. (2008), Liu et al. (2009)
p27/Kip1		Unknown		RNAi, DN, OE, KO	Miranda-Carboni et al., 2008; Kerzendorfer et al. (2010)
Cyclin E	Cell cycle regulator	Unknown	CUL4B	RNAi, M, HL	Zou et al. (2009)
RASSF1A	Mitotic regulator	Unknown	CUL4A	IP, RNAi, OE, IvvU, M	Jiang et al. (2011)
c-myc	Transcription factor, cell	TRUSS	CUL4A	IP, HL, OE, M	Choi et al. (2010)

Substrate	Function(s)	DCAFs/substrate receptors	CRL4 regulator	Experimental evidence	References
N-myc	cycle and proliferation				
ETV1	Prostate cancer-associated	COP1	N/A	IP, IvvU, M, OE, RNAi, KO	Vitari et al. (2011)
ETV4	transcription factor			IP, OE, KO	
ETV5				IP, OE	
c-Jun	Stress-inducible transcription factor	DET1, COP1	CUL4A	IP, M, HL, IvvU, RNAi	Wertz et al. (2004)
p53	Transcription factor, DNA damage response	Cdt2, PCNA, MDM2	CUL4A, CUL4B	RNAi, IP, IvvU, IvtU, M	Banks et al. (2006), Thirunavukarasou et al. (2014)
MDM2	E3 ubiquitin ligase	Cdt2, PCNA	CUL4A	RNAi, IP, M	Banks et al. (2006)
HOXA9	Transcription factor,	Unknown	CUL4A	IvvU, M, Y2H, IP, RNAi, OE	Zhang et al. (2003)
HOXB4	hematopoiesis	Unknown		IvvU, M, KO	Lee et al. (2013)
LATS1	Kinase, Hippo tumor	DCAF1	CUL4A, CUL4B	IvvU, IvtU, IP, HL, IF	Li et al. (2014)
LATS2	suppressor pathway				
p150 ^{Skl2}	Transcription factor, neural development	RBBP7	CUL4A, CUL4B	IP, DN, RNAi, IvvU	Sung et al. (2011)
TSC2	Inhibitor of mTOR pathway	FBW5	CUL4A, CUL4B	IP, RNAi, IvvU, IvtU, M, OE	Hu et al. 2008
ERα	Estrogen receptor	AhR	CUL4B	IP, DN, RNAi, IvvU, IF, IvtU	Ohtake et al. (2007)
Peroxisredoxin III	Antioxidant protein	Unknown	Primarily CUL4B	RNAi, IP, IvvU, IvtU	Li et al. (2011b)
CLC-1	Voltage-gated chloride channel	CRBN	CUL4A, CUL4B	RNAi, IvvU, IP, DN	Chen et al. (2015)
STAT1 [†]	Transcription factor, interferon response	Paramyxovirus protein V	CUL4A	IP, GF, EM, IvtU	Precious et al. (2005), Ulane et al. (2005)
UNG2/SMUG1 [†]	DNA uracil glycosylase	DCAF1, Vpr	CUL4A, CUL4B	RNAi, IP, IvtU, IvvU	Schrofelbauer et al. (2007), Ahn et al. (2010)
SAMHD1 [†]	Deoxynucleoside triphosphohydrolase	DCAF1, Vpx	CUL4A, CUL4B	IP, M, RNAi, IF	Hrecka et al. (2011), Ahn et al. (2012), Hofmann et al. (2012)
IKZF1 [†]	Transcription factor,	CRBN, IMiDs	CUL4A, CUL4B	RNAi, IP, IvtU, IvvU	Lu et al. (2014), Kronke et al.,
IKZF3 [†]	hematopoiesis				2014

Table 2

CRL4 disease and development studies. Summary of the studies performed on CRL4 core proteins and DCAFs in cancer, therapy, viral infection and mammalian development. In this table, the described human cancer samples are limited to primary tumors and surrounding tissue. Red color represents upregulation or amplification and green color represents downregulation, loss of function or deletion. GA = genetic analysis; GOF = gain of function; H = hyperplasia; IHC = immunohistochemistry; IVT-DR = in vitro drug response; IVT-PA = in vitro phenotype analysis; IVV-DR = in vivo drug response; IVV-PA = in vivo phenotype analysis; KO = knockout; RNAi = RNA interference; TA = transcriptional analysis; WB = Western blotting; XG = human xenograft studies in immunodeficient mice; ↑T = increased tumor growth; ↓T = decreased tumor growth.

Condition or process	Clinical and human cell line data	Mouse model experimental data	References
Cancer			
Breast cancer	13q34 (<i>CUL4A</i>) (GA and TA)	ch8A1 (<i>Cul4a</i>) (GA and TA)	Abba et al. (2007), Chen et al. (1998), Hu et al. (2012), Melchor et al. (2009)
Cervical cancer	<i>CUL4B</i> (IHC)	<i>CUL4B</i> RNAi XG (↓T)	Hu et al. (2012), Yang et al. (2013)
Colorectal cancer	<i>CUL4B</i> (GA, IHC, TA and WB), survival analysis		Hu et al. (2012), Jiang et al. (2013)
Esophageal cancer	<i>CUL4B</i> (IHC, TA and WB)	<i>CUL4B</i> RNAi XG (↓T)	Hu et al. (2012)
Kidney cancer	<i>CUL4B</i> (IHC)		Hu et al. (2012)
Liver cancer	13q34 (<i>CUL4A</i>) (GA and WB)	<i>CUL4B</i> overexpression (↑T)	Yasui et al. (2002), Yuan et al. (2015)
Lung cancer	<i>CUL4A</i> (TA), <i>CUL4B</i> (IHC and TA), IVT-DR	<i>Cul4a</i> overexpression (↑H, ↑T), <i>CUL4A</i> RNAi XG (↓T)	Hu et al. (2012), Li et al. (2011a), Wang et al. (2014a), Yang et al. (2014)
Mesothelioma	<i>CUL4A</i> (GA and WB)		Hung et al. (2011), Li et al. (2014)
Ovarian cancer	<i>CUL4B</i> (TA), <i>DDI1</i> (IHC), <i>ROC1</i> (IHC)	<i>Roc1</i> + 2 RNAi, <i>Cdt2</i> RNAi XG (↓T)	Hu et al. (2012), Pan et al. (2013)
Pancreatic cancer	<i>CUL4B</i> (GA and IHC)		Cerami et al. (2012), Gao et al. (2013), Hu et al. (2012)
Prostate cancer	<i>CUL4A</i> (TA), IVT-DR	<i>CUL4A</i> RNAi XG (↓T)	Ren et al. (2012)
Schwannomas	<i>NF2</i> (GA)	<i>Vprbp</i> RNAi engraftment (↓T)	Li et al. (2010)
Skin cancer		<i>Cul4a</i> KO (↓T)	Liu et al. (2009)
Stomach cancer	<i>CUL4B</i> (IHC)		Hu et al. (2012)
Thyroid cancer	<i>CUL4B</i> (TA)		Hu et al. (2012)
Chemotherapy			
IMiD responsiveness	IVT-DR, IVV-DR, CRL4 ^{CRBN} GOF		Ito et al., 2010; Zhu et al., 2011
Viral infection			
Hepatitis B	IVT-PA		Guo et al. (2014), Leupin et al. (2005)
HIV	CRL4 ^{DCAF1} GOF, IVT-PA		Hrecka et al. (2011), Schrofelbauer et al. (2007), Srivastava et al. (2008)
Paramyxoviruses	CRL4 GOF, IVT-PA		Andrejeva et al. (2002), Ulane et al. (2005)
Development			
Embryogenesis		<i>Cul4b</i> KO, IVV-PA	Chen et al. (2012), Jiang et al. (2012), Liu et al. (2012)
Spermatogenesis		<i>Cul4a</i> KO, IVV-PA	Kopanja et al. (2011), Yin et al. (2011)
X-linked intellectual disability	<i>CUL4B</i> (GA), IVV-PA	<i>Cul4b</i> KO, IVV-PA	Badura-Stronka et al. (2010), Cabezas et al. (2000), Chen et al. (2012), Isidor et al. (2010), London et al. (2014), Tarpey et al. (2007), Vulto-van Silfhout et al. (2015), Zou et al. (2007)