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Regulation of DNA Damage Response Pathways by the Cullin-RING Ubiquitin Ligases

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Abstract

Eukaryotic cells repair ultraviolet light (UV)- and chemical carcinogen-induced DNA strand-distorting damage through the nucleotide excision repair (NER) pathway. Concurrent activation of the DNA damage checkpoints is also required to arrest the cell cycle and allow time for NER action. Recent studies uncovered critical roles for ubiquitin-mediated post-translational modifications in controlling both NER and checkpoint functions. In this review, we will discuss recent progress in delineating the roles of cullin-RING E3 ubiquitin ligases in orchestrating the cellular DNA damage response through ubiquitination of NER factors, histones, and checkpoint effectors.

Keywords

ubiquitin; cullin-RING ligase; nucleotide excision repair

1. Introduction

Every day, an individual human cell suffers an average of 10,000 injuries on its DNA by external and internal culprits. DNA damage elicits concerted cellular responses that stall the cell cycle and activate specific DNA repair mechanisms, which vary according to the type of damage (reviewed in [1]). Failure to extricate DNA lesions disrupts genomic integrity and contributes to the pathogenesis of an array of human diseases, including cancer and premature aging. If the damage is too extensive and cannot be effectively repaired, cells will undergo apoptosis. Recent studies revealed multiple layers of control mechanisms at the transcriptional and post-translational levels that govern the execution of the DNA damage response. Among these, the modification of repair and checkpoint proteins by ubiquitin has gained increasing attention. One of the most exciting recent developments is the regulation of PCNA, the essential processivity factor of polymerases, by ubiquitin and ubiquitin-like modifiers. Elegant genetic and biochemical studies have revealed that mono- or poly-ubiquitin or SUMO conjugation to PCNA dictates the activation of specific repair pathways, inducing either error-prone DNA damage tolerance (lesion bypass) or error-free damage avoidance [2–5]. In this review, we will discuss key repair and checkpoint proteins involved

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in ultraviolet light (UV)-induced DNA damage that are subjected to ubiquitin-dependent regulation by members of the cullin-RING ligase (CRL) family.

2. The cullin-RING ligase family

2.1. CRL structure

Protein ubiquitination entails the covalent attachment of one or more ubiquitin moieties to target proteins via a cascade of enzymatic reactions that involve an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. Post-translational modifications by ubiquitin typically result in the functional alteration of the substrate or proteasomal degradation. While the E1 and E2 enzymes are required for the activation and transfer of ubiquitin, the substrate specificity of any given ubiquitin pathway is conferred exclusively by the E3 ubiquitin ligase. Cullins are elongated modular proteins that serve as scaffolds for assembly of the multi-subunit CRL family of E3 ubiquitin ligase complexes. There are seven cullins in vertebrates, CUL1, 2, 3, 4A, 4B, 5 and 7, that assemble structurally similar but functionally distinct E3 complexes (reviewed in [6]) (see Table 1). The CRL complexes feature a cullin scaffold base that brings together two functional subcomplexes: the RING domain protein Rbx1 (ROC1 or Hrt1) and the E2 ubiquitin-conjugating enzyme at its carboxyl (C-) terminus, and specific adaptor proteins at its amino (N-) terminus. The substrate-binding domain of cullins, through their adaptors, can recruit hundreds of known substrate receptors that specifically target an even larger array of substrates, some of which are involved in DNA repair and DNA damage checkpoint pathways.

The N-terminus of all cullins folds into three α -helical repeat bundles, designated cullin repeats (CRs), and each CR contains 5 α -helices [7]. The second and fifth α -helices of CR1 form direct contacts with cullin-specific adaptors, and the specificities of the adaptor interactions are determined by the amino acid residues at the respective binding interfaces, which differ among cullins [6,7]. The cullin adaptors further select different classes of substrate receptors by means of specific protein-protein interaction motifs. The various compositions of these adaptor subcomplexes allow CRLs to bind numerous substrates with distinct specificities (see Table 1). For example, in the substrate-recruiting module of the Skp1-CUL1-F-box protein (SCF) complex, the Skp1 adaptor binds to CUL1 at its N-terminal CR1 region [7]. Skp1 also interacts with one of ~70 F-box-bearing proteins that function as substrate receptors in mammals [8,9]. These receptors contain additional protein-protein interaction domains (e.g. WD40 or leucinerich repeats) responsible for interaction with substrates. Other CRLs feature different arrangements of adaptors and substrate receptors: CUL2 and CUL5 bind to the elongin B/C adaptor that interacts with SOCS-box-bearing substrate receptors [10] while CUL3 binds substrates via *bric-a-brac*, *tramtrack*, and *broad* (BTB)-bearing proteins, which are structurally similar to elongin C [11–13]. Unlike in other cullin CRLs, both the adaptor and the substrate receptor functions are collectively conferred by a single BTB protein. These cullin adaptors share a common BTB fold, a protein-protein interaction module in their tertiary structure (reviewed in [14]). One adaptor that structurally deviates from those of other CRLs is the DDB1 (UV-Damaged DNA Binding protein 1) adaptor of the CUL4A and CUL4B CRLs. As shown in Figure 1, DDB1 is comprised of three consecutive WD40 β -propeller domains (designated BPA, BPB and BPC) instead of the BTB fold [15] and recruits a subclass of WD40 domain-containing substrate receptors that share one or two WDXR signature motifs located within the short linkers connecting two β -strands of a WD40 β -propeller blade [16–19] (reviewed in [20]).

2.2. CRL regulation

Overexpression or amplification of several cullins has been observed in human cancers, implicating the need for precise control of CRL activities [21]. Just as CRLs regulate proteins by covalent modification, they too are subjected to modifications at the post-translational level, which in turn regulate CRL E3 activity. NEDD8, a ubiquitin-like protein, is covalently attached to a conserved lysine residue in the C-terminal cullin homology domain. NEDD8 modification, or neddylation, activates CRL complexes through multiple mechanisms. Recent structural and biochemical studies have revealed that neddylation induces a conformational change at the Rbx1-cullin interface that enables the Rbx1-associated E2 ubiquitin-conjugated enzyme to move closer to the adjacent substrate, thus facilitating ubiquitin transfer [22]. Neddylation also promotes the initial recruitment of ubiquitin-charged E2 to the Rbx1-cullin complex, thereby enhancing poly-ubiquitination of the substrate [23]. Furthermore, Nedd8 conjugation to Cul1 prevents recognition by the cullin inhibitor CAND1, which sequesters cullins to prevent them from assembling with Rbx1 and cullin-specific adaptors [24–26]. As such, cullin neddylation effectively excludes CRL association with CAND1 and facilitates assembly of productive CRLs. Thus, neddylation of cullins serves as a critical “on-switch” that facilitates ubiquitin transfer to substrates and prevents inhibition of CRLs.

Conversely, the “off-switch” of cullin deneddylation is executed by the JAMM metalloprotease activity of the CSN5 subunit of the COP9 signalosome, or CSN [27–30]. In addition, the CSN complex recruits the deubiquitinating enzyme UBP12, which is able to reverse the autoubiquitination of CRL components by disassembling polyubiquitin chains to prevent proteasomal degradation [31]. Therefore, CSN deactivates CRLs, but can also prevent the self-destruction of active CRLs. As discussed in the next section, CSN regulation of CUL4A-DDB1 plays an important part in the nucleotide excision repair process.

3. Regulation of global genomic repair by the CUL4A-based CRLs

Many types of DNA damage that induce DNA strand distortions are resolved by the process of nucleotide excision repair (NER). These include UV-induced photolesions such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), as well as bulky adducts induced by chemical carcinogens such as polycyclic aromatic hydrocarbons (PAHs) derived from tobacco smoking and environmental pollutants. In eukaryotic cells, NER is a multistep process that employs over 30 proteins to carry out the distinct steps of recognizing DNA damage, incising the 5' and 3' ends of the lesion to remove damaged DNA, filling in the gap via DNA polymerase activity, and attaching the newly synthesized DNA to the parental DNA using DNA ligase (reviewed in [32,33]). There are two sub-pathways of NER that vary according to the location of the DNA damage and the sensors of damage detection: transcription-coupled NER (TC-NER) repairs DNA lesions that occur on actively transcribed segments of the genome and are recognized by the RNA polymerase holoenzyme; global genomic NER (GG-NER) repairs DNA damage that occurs on the non-transcribed, complementary strands of transcribed genes as well as inactive chromatin (reviewed in [34]). Damage detection in the GG-NER pathway is dependent upon the xeroderma pigmentosum C (XPC)/HHR23B complex and the heterodimer of UV-damaged DNA binding proteins 1 and 2 (DDB1 and DDB2). While the XPC/HHR23 complex is sufficient to carry out damage recognition in the *in vitro* reconstituted NER reactions, DDBs display a higher affinity for CPDs and 6-4PPs than XPC/HHR23 and enhance the excision activity of both CPDs (17-fold) and 6-4PPs (2-fold) *in vitro* with purified repair factors [35,36]. Additionally, UV-DDBs were shown to arrive at UV-induced lesions prior to XPC recruitment following UV irradiation, and to promote the recruitment of XPC/HHR23 to the damage sites *in vivo* [37–40]. Thus, DDBs appear to play a critical role in initiating GG-

NER for certain types of DNA lesions, such as CPDs. In humans, DDB2 mutations are responsible for all known cases of the sun-sensitive and cancer-prone disease xeroderma pigmentosum (complementation group E), thus demonstrating the crucial role of DDBs in the repair process [41].

DDB2 is believed to make direct contact with DNA, while DDB1 is required for high-affinity interaction of the DDB1-DDB2 heterodimer with damaged DNA (designated UV-DDB activity) [42]. As previously mentioned, DDB1 also serves as the adaptor of the CUL4 ubiquitin ligase complex by bridging CUL4A or CUL4B and the DDB1 and CUL4A-associated factors (DCAF)-type substrate receptors [16,17]. Interestingly, DDB2 was also identified as a DCAF [16,17], which suggests that, in addition to its role in recognizing UV-damaged DNA, DDB2 also potentially recruits cellular proteins for ubiquitination by the CUL4 CRLs. Initial insight into the functional relationship between NER and ubiquitination of DDBs came from biochemical studies on the physical interactions between DDBs and CUL4A [43] and the finding that DDB2 is subjected to ubiquitination and degradation by ectopic CUL4A expression [44,45], resulting in an overall reduction of UV-DDB activity [44]. Physical association of DDB2 with DDB1-CUL4A is required for DDB2 ubiquitination, as the XPE-associated R273H mutation of DDB2, which is deficient for DDB1 and CUL4A binding, is rendered insensitive to CUL4A-mediated degradation [44,45]. Therefore, targeted degradation of DDB2 by CUL4A is considered a typical case of auto-ubiquitination, as previously defined for CRLs and other types of E3 ubiquitin ligases [46–48].

Upon UV irradiation, the DDB heterodimer immediately binds to DNA damage sites on chromatin to initiate NER. DDBs remain on DNA for 0.5–3 hours, and then are removed from DNA in a CUL4A- and proteasome-dependent manner [38,39]. Specifically, DDB2, the rate-limiting factor of UV-DDB activity, is ubiquitinated and targeted for degradation by CUL4A [39]. The temporal order of binding to damaged DNA and proteolytic removal of DDB by CUL4A reveals an active dynamic control over the damage recognition step of NER.

Recent studies have begun to shed light on the functional role of the temporal association of DDBs and CUL4A on DNA damage sites during the damage recognition step of NER. As the first detectable DNA repair proteins to arrive at UV-induced lesions, DDBs have been postulated to actively participate in remodeling the damage sites to set the stage for subsequent NER reactions. Chromatin remodeling is achieved largely through modifications of histones that include acetylation, methylation, phosphorylation and ubiquitination. Notably, the DDB-CUL4 complex was shown to specifically catalyze the ubiquitination of histones H3 and H4 in response to UV irradiation, leading to enhanced recruitment of XPC to the damaged foci and facilitating the repair process (Fig. 2) [49]. Histone H2A was also found to be ubiquitinated by the DDB-CUL4 ubiquitin ligase [50,51]. Considering that GG-NER is assigned to recognize DNA lesions embedded in the tightly-bound chromatin structure, the observed histone ubiquitination may trigger epigenetic modifications at the lesion sites that render them more accessible for subsequent NER factors.

DDBs display over 100-fold higher affinity to CPDs than the XPC/HHR23 complex [35]. Using repair-deficient XPA cells that express CPD- or 6-4PP-specific photolyases to differentiate specific types of photolesions, it was demonstrated that the XPC/HHR23 complex associates with 6-4PPs independently, but requires DDBs to recognize CPDs *in vivo* [52]. Subsequent studies demonstrated a direct physical interaction between DDB2 and XPC using affinity-purified recombinant proteins [53]. Strikingly, the DDB-CUL4A CRL induces poly-ubiquitination of XPC, resulting in a modest increase in XPC binding to DNA (Fig. 2) [53]. Conversely, ubiquitination of DDB2 abrogates its ability to bind DNA

photolesions (Fig. 2). Alteration of the DNA-binding properties of DDB2 and XPC upon ubiquitination by CUL4A suggests a mechanism by which DDBs are displaced by XPC from the damage loci. However, given that there is only 2-fold increase in XPC affinity to both damaged and undamaged DNA following its poly-ubiquitination, the exact role of XPC ubiquitination during the stepwise damage recognition in NER is yet to be determined. The fate of ubiquitinated XPC is also a matter of debate. While Sugawara et al. observed no degradation of XPC following its ubiquitin modification, but rather disassembly of the polyubiquitin chains [53], Wang and colleagues presented evidence to suggest that XPC, like DDB2, is also degraded by the proteasome in a ubiquitin-independent manner [54]. Further studies will determine what functional role XPC ubiquitination plays during dynamic DNA damage recognition, the rate-limiting step of NER.

4. Regulation of CUL4A-based CRL activity during GG-NER

Given the multiple ubiquitination events orchestrated by CUL4A during damage recognition, it is necessary that cells implement delicate control mechanisms to ensure the proper execution of CUL4A activity. While the temporal control of CUL4A CRL activity remains largely elusive, recent studies implicate known cullin regulators as well as novel activators that control CUL4A function during NER.

4.1. COP9 signalosome/CSN

The stoichiometric binding of the CSN with the CUL4A-DDB1-DDB2 complex under normal conditions is believed to suppress CUL4A CRL activity [37]. Upon UV irradiation, CSN dissociates from the DDB2 complex, in parallel with increased neddylation and activation of CUL4A. Silencing of CSN5 by siRNA resulted in the reduction of unscheduled DNA synthesis, indicating that CSN, a cullin inhibitor, plays an overall positive role in augmenting NER [37]. However, the reduced NER activity in CSN knockdown cells cannot be simply attributed to defects of UV-induced CUL4A activation, as CSN5 inactivation prior to UV treatment is expected to elevate CUL4A activity that may result in the premature destruction of DDB2, thus compromising UV-DDB activity.

4.2. c-Abl and Arg non-receptor tyrosine kinases

Increased CUL4A neddylation is insufficient to trigger degradation of chromatin-bound DDB2 in mouse embryonic fibroblast (MEF) cells deficient for the c-Abl and Arg non-receptor tyrosine kinases [39]. c-Abl was recently identified as a potent activator of CUL4A for DDB2 ubiquitination and degradation under normal conditions and following UV irradiation [39]. Interestingly, the tyrosine kinase activity of c-Abl is completely dispensable for its stimulatory effect on CUL4A E3 activity. In MEF cells deficient for both c-Abl and Arg (designated DKO cells), DDBs remain associated with UV-damaged DNA, as CUL4A-mediated DDB2 degradation post-UV is completely abrogated. These findings were recapitulated with the expression of a dominant negative CUL4A mutant that inactivates endogenous CUL4A activity. While the biochemical basis of robust CUL4A activation by c-Abl is unclear, silencing of the cullin inhibitor CAND1 using siRNA rescued the loss of UV-induced DDB2 degradation on chromatin, suggesting that c-Abl may function in part to relieve the suppressive effect of CAND1 on the CUL4A CRL.

Surprisingly, DKO MEFs displayed dramatically elevated GG-NER activity compared to wild-type MEFs [39]. Retroviral-mediated reconstitution of c-Abl or the kinase-deficient mutant resulted in reduction of GG-NER activity. These studies indicated that degradation of DDB2 on chromatin following UV irradiation does not serve to recruit subsequent NER factors to the damage site and ensure the continuation of NER reactions -- as previously predicted. Instead, CUL4A-mediated degradation of DDB2 on damaged chromatin likely

represents a mechanism to terminate damage recognition despite the presence of unrepaired DNA lesions. Future studies featuring CUL4A knockout or a degradation-resistant DDB2 mutant are necessary to define precisely the physiological role of CUL4A-mediated DDB2 ubiquitination in NER.

4.3. p38 stress-induced kinase

While the PTK activity of c-Abl is dispensable for enhancement of CUL4A-dependent DDB2 ubiquitination, recent studies suggest that the catalytic activity of the p38 serine/threonine MAP kinase is required for efficient NER, as inhibition of p38 by the pharmacological inhibitor SB203580 compromised the efficiency of CPD removal [55]. These findings coincided with the effect of SB203580 in reducing UV-induced ubiquitination and degradation of DDB2. Additionally, histone H3 acetylation and recruitment of XPC and the TFIIH complex to CPD lesions were perturbed upon SB203580 treatment, while DDB2 binding to damaged DNA remained unaffected. Further investigation will delineate the biochemical mechanism by which the p38 MAP kinase pathway regulates DDB2 degradation and CPD repair. In this regard, it is interesting to note that p38 can be activated by c-Abl in a kinase-independent manner [56].

5. Regulation of transcription-coupled NER by CUL4A-based CRL

As opposed to the GG-NER pathway that employs XPC-HHR23 and the UV-DDB complex as damage sensors, recognition of DNA lesions on actively transcribed segments of the genome is carried out by the RNA polymerase II complex (RNAPII_o) (reviewed in [32]). Upon encountering the lesion, the PolII complex stalls and initiates a series of events that are mediated by the Cockayne Syndrome protein B (CSB) subunit. CSB is a SWI/SNF-like ATPase that translocates to the damage sites, in part through its interaction with the stalled RNAPII_o, and serves as a coupling factor for TC-NER by orchestrating the assembly of TC-NER-specific as well as the core NER factors p300 acetyltransferase and Cockayne Syndrome protein A (CSA) [57]. CSA, in turn, recruits the XAB2 scaffold protein that displays enhanced binding to RNAPII_o after UV exposure [58], the HMGN1 nucleosome binding protein, and the TFIIS transcription cleavage factor that purportedly helps RNAPII_o to restart elongation after removal of the damage [57]. Although distinct mechanisms are evoked for damage detection, the subsequent TC-NER repair processes for lesion removal and gap filling appears identical to GG-NER (reviewed in [32]).

CSA shares some sequence homology with DDB2, as both contain the tandem WDXR motif that binds to CUL4A-DDB1. As a result, both assemble similar CRL complexes [16–19,37] (reviewed in [20]). Upon UV irradiation, the entire CUL4A-DDB1-CSA CRL is recruited to DNA damage sites in a CSB-dependent manner [57]. In contrast to GG-NER, in which the CSN dissociates from DDB2-containing CRLs on damaged chromatin post-UV, the CSN complex was found together with CSA-containing CRLs following UV irradiation. As a result, the CUL4A-DDB1-CSA ubiquitin ligase is kept in an inactive state during the early steps of TC-NER [37,57]. While the E3 ubiquitin ligase activity of the CUL4A-DDB1-CSA complex is not directly involved in TC-NER initiation, the CSA-containing CRL is activated at 3 hours after a high UV dose (25 J/m²) to trigger abrupt CSB degradation, leading to the proposal that CSA-mediated degradation of CSB plays a role in the resumption of transcription elongation after lesion removal [59]. However, reduction of CSB levels at low UV dose (5 J/m²) as early as 1 hour after exposure was also observed prior to the recovery of UV-inhibited RNA synthesis [60]. Future studies are anticipated to address UV-induced CSB degradation in relation to the linear kinetics of restoration of transcriptional activity [60].

6. Regulation of DNA damage checkpoint pathways by CRLs

In response to genotoxic assault, the cellular DNA damage checkpoint pathways are activated to halt the cell cycle at defined checkpoints and allow time for repair of DNA lesions (reviewed in [32] and references therein). DNA lesions are first detected by damage sensors such as ATM, ATR, the Rad17-RFC complex and the 9-1-1 complex, which collectively activate signal transduction pathways that employ the Chk1 and Chk2 kinases. The Chk signal transducers induce cell cycle arrest by phosphorylation of two critical effectors: inhibitory phosphorylation of the Cdc25 phosphatase results in CDK inactivation, while the activating phosphorylation of the p53 tumor suppressor stabilizes p53 and increases transcription of p53 target genes, including the cyclin-dependent kinase inhibitor p21/CIP1/WAF-1. This in turn inactivates cyclin-dependent kinases to block the cell cycle transitions. The DNA damage checkpoint pathways have been extensively reviewed. Here we will focus specifically on CRL-mediated regulation of checkpoint proteins during DNA damage-induced checkpoint activation, as well as during the subsequent resumption of the cell cycle after damage clearance.

6.1. CRLs and checkpoint activation

In response to UV or ionizing radiation, two checkpoint signaling pathways are activated: first, the ATR-Chk1-Cdc25 (UV response) or ATM-Chk2-Cdc25 (IR) signaling pathways initiate a rapid G1/S arrest, while the ATR/ATM-Chk1/Chk2-p53 pathway is fully mobilized several hours after DNA damage to ensure sustained arrest (reviewed in [61] and references therein). During the initiation phase, Cdc25 inactivation allows for the Wee1-mediated inhibitory Tyr phosphorylation event on Cdk2 of the CyclinE/A-Cdk2 complex, and thus resulting in a block in DNA replication. DNA damage induces rapid Chk-dependent phosphorylation and nuclear exclusion of the Cdc25A phosphatase, thus preventing de-phosphorylation of Cdk2. To ensure the irreversibility of Cdc25A inactivation, SCF^{βTrCP} specifically recognizes and targets phospho-Cdc25A for ubiquitination and proteasomal destruction [62,63]. Cells unable to degrade Cdc25A are defective in cell cycle arrest [64]. Therefore, a CUL1-based CRL is required for radiation-induced G1/S checkpoint initiation.

The rapid initiation phase is followed by p53-mediated transcriptional upregulation of p21 that serves to maintain the G1/S arrest [61]. The maintenance phase is initiated by Chk-dependent p53 phosphorylation, which prevents p53 degradation by blocking the access of the Mdm2 ubiquitin ligase to p53. Therefore, two distinct ubiquitination apparatuses are tuned differentially to ensure the proper execution of checkpoint activation.

While the Cdc25 and p53 effectors are mobilized to block Cdk2 activation and cell cycle progression, eukaryotic cells invoke a second mechanism to ensure that DNA replication origins are switched off in the presence of DNA damage. During unperturbed cell cycle progression, DNA replication is initiated when the pre-replication (pre-RC) complex assembles at the replication origins just before S-phase to “license” the origin to initiate DNA replication. The pre-RC complex is comprised of a six-member origin recognition complex (ORC), the MCM2-7 DNA helicase complex, and two replication-licensing factors, Cdc6 and Cdt1 (reviewed in [65]). Each replication origin fires exactly once per cell cycle. Unsolicited formation of the pre-RC can induce re-replication, thereby disrupting genomic integrity. Mounting evidence suggests that ubiquitin-dependent degradation of Cdt1 following origin firing is critically important to prevent premature re-assembly of the pre-RC. In this regard, both the CUL4- and CUL1-based CRLs have been shown to be redundantly involved in Cdt1 turnover [66–70]. Distinct degrons within the N-terminus of Cdt1 are specifically recognized by the Cdt2 (a DCAF) substrate receptor of CUL4 CRL in conjunction with PCNA, or the F-box-containing Skp2 substrate receptor of the CUL1 CRL

SCF^{Skp2} [17,68,70,71]. The CUL4-dependent degron of Cdt1 contains a PCNA interaction domain (PIP box) and specifically mediates Cdt1 turnover during S phase. In contrast, Cdt1 phosphorylation at the CUL1-dependent degron, a Cy-motif required for CDK/cyclin association, triggers its binding and ubiquitination by SCF^{Skp2} in both S and in G2 phase, and is thought to ensure that Cdt1 is suppressed in G2 when the CUL4 machinery is not active [68].

In response to UV or ionizing radiation, ubiquitin-mediated Cdt1 degradation is initiated, ensuring that no cells inflicted with DNA damage can license the replication origin and duplicate DNA. Here the CUL4 CRL is specifically employed to destroy chromatin-bound Cdt1 through the PIP box-containing CUL4 degron [72,73]. PCNA is necessary for radiation-induced Cdt1 turnover on chromatin, suggesting that the CUL4 CRL is recruited to the assembled pre-RC that was engaged in initiating DNA replication prior to radiation exposure. As such, Cdt1 degradation is believed to effectively switch off DNA replication and enact the DNA repair machinery. Cdt1 levels recover post-irradiation, coinciding with the resumption of S phase entry [72]. Although Cdt1 is a critical proteolytic target whose degradation is triggered by DNA damage, proteolysis of Cdt1 following γ -irradiation appears independent of known checkpoint pathways involving ATM/ATR or Chk1/Chk2 [72]. It is currently unclear how the CUL4 CRL is transiently activated to degrade Cdt1, although recruitment of the CUL4 CRL to chromatin-bound Cdt1 and PCNA is considered a novel, phosphorylation-independent mechanism for activation of CUL4 CRLs [74]. This model is supported by further evidence as described below.

As the major downstream effector of the G1/S DNA damage checkpoint pathway, p21 is also under proteolytic control when cells are exposed to UV irradiation. In response to UV irradiation at $<40 \text{ J/m}^2$ and ATR activation, p21 is degraded by the SCF^{Skp2} ubiquitin ligase [75]. However, work from others suggested that Skp2 is dispensable for UV-induced p21 turnover [76,77]. Recent studies revealed that the same CUL4-based CRL complex utilized in Cdt1 degradation, which is comprised of CUL4A, DDB1 and Cdt2, also functions in conjunction with PCNA to target p21 for ubiquitin-dependent degradation in cells irradiated with UV at 20 J/m^2 [77,78].

While it is generally accepted that the accumulation of p21 is a critical step in checkpoint execution and G1/S arrest in response to DNA damage, the role of p21 in NER remains a matter of debate. Upon UV irradiation, p21 co-localizes with PCNA at damage foci [79]. Bendjennat and colleagues proposed that degradation of p21 in response to low doses of UV serves to relieve its suppressive effect on PCNA and thus favors NER [75]. However, there are conflicting reports as to whether p21 stimulates, inhibits, or has no effect on NER [75,80–87]. A role of p21 in the translesion synthesis (TLS) pathway has also been suggested in which p21 binding to PCNA contributes to the suppression of error-prone TLS and reduction of mutation load [88]. Future studies should delineate the specific roles of UV-induced p21 degradation by CRLs in regulating the DNA damage checkpoint, NER and translesion synthesis.

6.2. CRLs in checkpoint termination

Upon completion of DNA repair, eukaryotic cells turn off the checkpoint response pathway in order to resume the cell cycle. While the signals instructing checkpoint disengagement remain largely elusive, recent studies uncovered that ubiquitin-dependent destruction of checkpoint proteins plays a vital role in the termination of checkpoint signaling. One critical checkpoint mediator that is promptly destroyed during checkpoint recovery is Claspin, an adaptor protein that bridges ATR binding to Chk1 and is critical for ATR-dependent Chk1 phosphorylation and checkpoint activation [89,90]. During recovery from DNA damage checkpoint arrest, Claspin is phosphorylated by the Plk1 serine/threonine kinase.

Phosphorylated Claspin is the preferred target for degradation by the CUL1-based SCF^{βTrCP} ubiquitin ligase, leading to Chk1 inactivation and recovery from checkpoint arrest [91–93]. Importantly, Claspin destruction abrogates Chk1-dependent phosphorylation of Cdc25A, which prevents recognition by SCF^{βTrCP} and, in turn, activates CDK to resume cell cycle progression.

The involvement of SCF^{βTrCP} in checkpoint recovery is further underscored by its role in targeting the degradation of another checkpoint kinase, Wee1, which blocks the G2/M transition during checkpoint activation [94]. The Wee1 kinase functions by inhibiting Cdk1 via phosphorylation of Tyr15 within its ATP binding site, thereby contributing to G2/M checkpoint arrest. Once the DNA damage is repaired, Plk1 catalyzes phosphorylation of Wee1, priming Wee1 for recognition by the SCF^{βTrCP} ubiquitin ligase and subsequent destruction [94]. As such, SCF^{βTrCP} serves as a molecular switch that orchestrates multiple proteolytic events during the recovery from checkpoint arrest. This E3 ligase complex promotes Claspin and Wee1 destruction to terminate checkpoint signaling and CDK inhibition and, at the same time, disengages Cdc25A destruction to permit its activation of CDK and cell cycle resumption. Plk1 is a major factor in recovery, as it facilitates Wee1 and Claspin recognition by SCF^{βTrCP}. While temporal regulation of Plk1 in relation to checkpoint activation and termination remains to be determined, Bassermann and colleagues recently uncovered that Plk1 itself is subjected to ubiquitin-dependent degradation by the APC/C^{Cdh1} ubiquitin ligase in response to genotoxic stress during G2, thus ensuring execution of the G2 checkpoint [95]. Moreover, the recently identified deubiquitinating enzyme USP28 removes ubiquitin chains formed on Claspin, thus offering another tier of protection against Claspin degradation [95,96]. While the interplay of several ubiquitin ligases converges on checkpoint disengagement, SCF^{βTrCP}-mediated Claspin degradation appears to be the rate-limiting step for recovery of genotoxic stress-induced G2 arrest [93].

7. Conclusions and perspectives

The CRL families assemble hundreds of E3 ubiquitin ligases in a combinatorial manner to target a wide range of cellular targets for ubiquitination, thereby controlling diverse cellular functions. Much progress has been made recently in the identification of critical effectors and regulators of the DNA repair and DNA damage checkpoint pathways that are subjected to CRL-mediated ubiquitination and degradation, with an extensively characterized role for the CUL4A-based CRL during the DNA damage recognition steps of nucleotide excision repair.

Although the NER pathway has been extensively characterized and reconstituted *in vitro* using purified components [97,98], the regulatory mechanisms that govern the rate-limiting step of damage recognition, especially chromatin remodeling that enables access to damage foci and the sequential recruitment of NER factors under *in vivo* conditions, remain largely elusive. Multiple CUL4A substrates have been described, and ubiquitin modifications of histones have begun to shed light on cellular events that are difficult to recapitulate *in vitro* due to technical limitations. Moreover, little is known about the temporal order by which these ubiquitination events occur, and the cellular pathways that relay the damage signal and ensure the proper sequence of events. Cells respond to non-lethal doses of DNA damage by activating both DNA repair and DNA damage checkpoint pathways, which can function independently of each other, but are believed to be coordinated by unknown mechanisms to achieve efficient and timely repair of DNA lesions.

Understanding the physiological and pathological roles of CRLs in the DNA damage response requires the use of knockout mice. However, most CRL components are essential for cell growth and survival, and the embryonic lethality of knockout animals precludes

analysis of DNA repair and checkpoint phenotypes in their absence. It is therefore necessary to design and implement novel strategies and approaches to further our understanding of CRL-mediated ubiquitination events in the DNA damage response, to identify pathological conditions associated with their alterations in human diseases, and to explore their value as potential therapeutic targets for pharmacological intervention.

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Figure 1.

The CUL4 CRL. CUL4 is the cullin scaffold of the complex. At the N-terminal substrate-binding domain, CUL4 binds the BPB domain of the DDB1 adaptor, which then recruits WDXR motif-bearing DCAF substrate receptors through the BPA-BPC propellers of DDB1. At the catalytic C-terminal end, CUL4 associates with Rbx1, which recruits an E2 ubiquitin-conjugating enzyme carrying activated ubiquitin. Upon CUL4 activation by covalent Nedd8 conjugation, the substrate is brought into close proximity to the E2 enzyme for ubiquitin ligation to occur.

Figure 2. Schematic diagram of the proposed CUL4A CRL-mediated ubiquitination events around DNA damage foci during the damage recognition step of GG-NER. Upon UV irradiation, the UV-DDB heterodimer immediately binds to DNA lesions embedded in condensed chromatin. Association of CUL4A-Rbx1 to the DDB1-DDB2 heterodimer results in the formation of an active CRL that ubiquitinates target proteins, including histones 3, 4, and 2A, which remodels the chromatin to increase accessibility of damage sites, the XPC subunit of the XPC-hHR23B complex to enhance its affinity for damaged DNA, and the DDB2 itself to dislodge it from the damage site for proteasomal degradation. R, Rbx1.

Table 1
Cullin family of ubiquitin-protein ligases

Cullin-RING family ubiquitin ligases. There are 7 cullins identified in mammals. Different cullins utilize the same Rbx1 as the adaptor to recruit E2, but use different adaptors to recruit distinct classes of substrate receptors and degrade a wide range of cellular proteins.

Cullin	Ring domain	Connector	Substrate receptor	Substrates
CUL-1	Roc1	Skp1	F-box proteins	IκB, β-catenin p27, Cyc E, c-myc
CUL-2	Roc1	Elongin B/C	VHL/SOCS box	HIF-α
CUL-3	Roc1	BTB	BTB	MEI-1/Katanin Free Cyc E
CUL-4A CUL-4B	Roc1	DDB1/DET1???	COP1, DDB2???	DDB2, HoxA9, c-Jun, Cdt1, XPC
CUL-5	Roc1	Elongin B/C	VHL/SOCS box	?
CUL-7	Roc1	Skp1	Fbx29	?