CRL ubiquitin ligases and DNA damage response

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INTRODUCTION

Our genome is under constant attack by exogenous DNA-insulting agents, such as UV light and chemical carcinogens, and by endogenous metabolic products, such as reactive oxidative species. In order to prevent genome instability, a hallmark of cancers, eukaryotic cells have evolved several protective mechanisms of the DNA damage response (DDR), including DNA replication and cell cycle arrest, and DNA repair initiation (Harper and Elledge, 2007; Negrini et al., 2010). During a DDR, cascades of phosphorylation events are initiated by a family of PI3-like family kinases, mainly an ataxia telangiectasia mutated (ATM), and ATM and Rad3-related (ATR) kinases (Harper and Elledge, 2007; Negrini et al., 2010). Recent studies, however, have indicated that ubiquitination also plays important roles in the DDR (Harper and Elledge, 2007; Miesick and Greenberg, 2009; Negrini et al., 2010; Silverman et al., 2012).

Ubiquitin is an 8-kDa protein and can be covalently conjugated to other proteins via an isopeptide linkage between its C-terminal glycine and a primary amino group of its substrates, which is usually from a lysine side chain (Pickart, 2004; Hannah and Zhou, 2009). Ubiquitination is catalyzed by three enzymes, including a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3) (Pickart, 2004; Hannah and Zhou, 2009). The ubiquitination machinery can conjugate a single ubiquitin to one lysine residue of substrates (called mono-ubiquitination), multiple single ubiquitin molecules to multiple lysine residues (called multi-ubiquitination), or multiple ubiquitins in a chain (called poly-ubiquitination) to substrates. Mono-ubiquitination and multi-ubiquitination play important roles in endocytosis, DNA repair, immune response, and transcriptional regulation, etc. Poly-ubiquitination via surface lysine residues of ubiquitin, such as Lysine-48 (K48) or Lysine-11 (K11) often leads to protein proteolysis through the 26S proteasome (Pickart, 2004; Pickart and Eddins, 2004). However, protein poly-ubiquitination does not necessarily drive protein turnover. Poly-ubiqutin chains via Lysine-63 residue (K63) of ubiquitin and linear poly-ubiquitin chains appear to play non-proteolytic functions and are involved in DNA repair, NF-κB activation, and Ras localization and signaling, etc. (Pickart, 2004; Jura et al., 2006; Yan et al., 2009, 2010; Xu et al., 2010).

The human genome contains two E1 with distinctive functions (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007; Lee et al., 2011), dozens of E2s and hundreds of E3 ligases which determine the specificity of substrates for ubiquitination (Pickart, 2004; Pickart and Eddins, 2004). Thus far, more than 600 ubiquitin ligases have been identified in the human genome, although many of them have not been linked to any substrates or biological activities. Based on their domain characteristics, ubiquitin ligases can be separated into two sub-groups, HECT domain E3 ligases and RING finger domain E3 ligases (Pickart, 2004; Pickart and Eddins, 2004). CRLs are modular ubiquitin ligases (Petroski and Deshaies, 2005; Sarikas et al., 2011). CRLs are involved in cell cycle regulation, DNA replication, DNA damage response (DDR), development, immune response, transcriptional regulation, circadian rhythm, viral infection, and protein quality control. One of the main functions of CRLs is to regulate the DDR, a fundamental signaling cascade that maintains genome integrity. In this review, we will discuss the regulation of CRL ubiquitin ligases and their roles in control of the DDR.

Keywords: DNA damage, cullin, Cdc25A, claspin, Cdt1, cyclin D1, ubiquitin ligase, protein degradation

INTRODUCTION OF CRL UBIQUITIN LIGASES

Cullin/RING ubiquitin ligases (CRL) comprise the largest subfamily of ubiquitin ligases. CRLs are involved in cell cycle regulation, DNA replication, DNA damage response (DDR), protein degradation (Petroski and Deshaies, 2005; Sarikas et al., 2011). CRLs are modular ubiquitin ligases (Petroski and Deshaies, 2005; Sarikas et al., 2011). CRLs are involved in cell cycle regulation, DNA replication, DDR, development, immune response, transcriptional regulation, circadian rhythm, viral infection, and protein quality control (Petroski and Deshaies, 2005). This review will focus on the interplay between human CRL ubiquitin ligases and the DDR. We will discuss molecular features of CRLs and how they participate in the DDR by targeting DDR-regulatory proteins for ubiquitination.
The conjugation of NEDD8, named neddylation, is catalyzed by NEDD8-specific E1, E2, and E3 (Dye and Schulman, 2007). The general consensus is that neddylation of cullins is required for activation of CRL ubiquitin ligases. However, untimely neddylation of cullins could drive destruction of substrate receptors via auto-ubiquitination (Cope and Deshaies, 2006). Therefore, the neddylation of cullins is counteracted by the deneddylation activity of a multifunctional protein complex, the COP9 signalosome (CSN) (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wei et al., 2008).

COP9 signalosome was initially found to be involved in plant photo morphogenesis and was later identified as a conserved complex in eukaryotes (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wei et al., 2008). CSN is comprised of eight subunits, which are CSN1–8 in order of decreasing molecular weight. The CSN complex participates in multiple biological events, including transcriptional regulation, cell division, and development, etc. (Tateishi et al., 2001; Lykke-Andersen et al., 2003; Panattoni et al., 2008). Part of the multi-functionality of CSN is linked with the neddylation system, with its isopeptidase activity to remove NEDD8 conjugation. This deneddylation activity is attributed to the metalloprotease motif of CSN5 but the whole CSN complex is required for the reaction (Cope et al., 2002). Indeed, conditional silencing of CSN5 in HEK293 cells increased the neddylation of cullins. Consequently, expression of multiple F-box proteins, but not the cullins, was decreased (Cope and Deshaies, 2006). The reduced expression of F-box proteins depends on Cul1 and the proteasome, further supporting an auto-ubiquitination and self-destruction mechanism (Cope and Deshaies, 2006). These data explain why accumulation of CRL substrates has been observed in cells where either CSN or the NEDD8 conjugation system is inactivated (Tateishi et al., 2001; Lykke-Andersen et al., 2003; Cope and Deshaies, 2006; Panattoni et al., 2008; Choo et al., 2011). It is clear that expression of some substrate receptors is not affected by neddylation, however (Cope and Deshaies, 2006).

Neddylation of cullins positively regulates the E3 ligase activity of CRLs by at least three mechanisms. First, neddylation of cullins enhances their interaction with ubiquitin-activated E2. It has been suggested that neddylation of Cul1 can increase the affinity between ubiquitin-activated E2 and Rbx1 (Kawakami et al., 2001). Using fluorescence resonance energy transfer (FRET) technology, Saha and Deshaies (2008) observed that neddylation of Cul1 can enhance Dc34 binding to SCF ubiquitin ligase.

Second, neddylation positively regulates the ubiquitination activity of CRL ubiquitin ligases by enhancing ubiquitin transfer to substrates from the active E2 site and by positioning the active E2 site closer to ubiquitin accepting sites of substrates. Neddylation has been shown to stimulate the Dc34-dependent ubiquitination activity of SCF by more than 10-fold (Saha and Deshaies, 2008). The $k_{cat}$ for ubiquitin transfer is enhanced by NEDD8 conjugation (Saha and Deshaies, 2008). According to the structural analysis of the SCF$^{Skp2}$ ubiquitin ligase, Cul1 holds the Skp1–Skp2 and Rbx1 subunits more than 100 Å apart (Zheng et al., 2002b). A gap of 50 Å between the active cysteine of E2 and the leucine-rich repeat of Skp2 exists even after E2 joins SCF$^{Skp2}$ (Zheng et al., 2002b). This gap was confirmed by additional SCF structural studies (Wu et al., 2008).

FIGURE 1 | Architecture of CRL1 (SCF) and CRL4 ubiquitin ligases. SCF ubiquitin ligases contain Cul1, Rbx1, Skp1, and F-box proteins which function as substrate receptors; whereas CRL4 has Cul4, Rbx1, DDB1, and DCAF proteins as substrate receptors. Both SCF and CRL4 can be activated by neddylation. F-box and DCAF proteins are substrate specificity factors.
meiosis (Chen et al., 2001a; Ferguson et al., 2005). Cdc25B and Cdc25C are dispensable in mouse development, except for G2/M transition (Boutros et al., 2006). However, both Cdc25B and Cdc25C complete this process in the nucleus during the entry. Cdc25A has two close homologs, Cdc25B and Cdc25C. It and cyclin A/Cdk2, and enables the G1/S transition and mitotic sine by Cdc25A activates cyclin/Cdk kinases, mainly cyclin E/Cdk2.

In the following sections, we will discuss the roles of individual ubiquitin ligases that are important for DDR.

The CRL family is one example of ubiquitin ligases that mediate many cellular signaling events (Petroski and Deshaies, 2005). In the following sections, we will discuss the roles of individual CRL ubiquitin ligases in control of DDR.

SCFβTRCP IN DNA DAMAGE CHECKPOINT CONTROL

SCFβTRCP IN DDR-INDUCED Cdc25A UBQUITINATION AND TURNOVER

Cdc25A is a dual phosphatase that targets phosphorylated tyrosines and serines/threonines for de-phosphorylation (Karlsson-Rosenthal and Millar, 2006; Ray and Kiyokawa, 2007, 2008; Rudolph, 2007). Removal of inhibitory phosphate on tyrosine by Cdc25A activates cyclin/Cdk kinases, mainly cyclin E/Cdk2 and cyclin A/Cdk2, and enables the G1/S transition and mitotic entry. Cdc25A has two close homologs, Cdc25B and Cdc25C. It was proposed that Cdc25B initiates the activation of cyclin B/Cdk1 and Cdc25C completes this process in the nucleus during the G2/M transition (Boutros et al., 2006). However, both Cdc25B and Cdc25C are dispensable in mouse development, except for meiosis (Chen et al., 2001a; Ferguson et al., 2005). Cdc25B and Cdc25C double knockout mice are viable, but females are sterile, as are Cdc25B knockout females (Ferguson et al., 2005). In contrast, Cdc25A is essential for early embryo development (Ray et al., 2007a). In summary, Cdc25A is an essential gene for mouse development and is one of the major regulators of cell division (Molinari et al., 2000; Zhao et al., 2002; Ray and Kiyokawa, 2007, 2008). The Cdc25A protein level is tightly regulated via transcription and proteasome degradation in a cell cycle-dependent manner (Ray and Kiyokawa, 2007).

Over-expression of Cdc25A has been observed in multiple cancers (Kristjánsdóttir and Rudolph, 2004; Boutros et al., 2006). Studies in Cdc25A knockout mice indicated that Cdc25A is a rate-limiting oncogene that restricts tumorigenesis induced by the HER2/neu–RAS oncogenic pathway (Ray et al., 2007a). Consistent with this observation, MMTV-Cdc25A transgenic mice dramatically promote murine mammary tumorigenesis in cooperation with oncogenic RAS or neu (Ray et al., 2007b). Over-expression of Cdc25A in human cells can result in aberrant mitotic events, compromised DDR and destabilized chromosomes, such as chromosomal breaks at fragile sites (Cangi et al., 2008). Therefore, it is critical to maintain appropriate amounts of Cdc25A in human cells.

Cdc25A is an unstable protein and its protein levels are mediated by phosphorylation and the ubiquitin–proteasome pathway (Bernardi et al., 2000; Mailand et al., 2000, 2002; Falck et al., 2001; Donzelli et al., 2002, 2004; Shimuta et al., 2002; Zhao et al., 2002; Busino et al., 2003; Goloudina et al., 2003; Jin et al., 2003, 2008; Sørensen et al., 2003; Xiao et al., 2003; Kasahara et al., 2010). In response to DNA damage, Cdc25A is phosphorylated by the checkpoint kinase, Chk1, and subjected to proteasome-dependent degradation (Zhao et al., 2002; Goloudina et al., 2003; Jin et al., 2003, 2005; Sørensen et al., 2003; Xiao et al., 2003; Donzelli et al., 2004). The consequence of Cdc25A turnover is persistent Cdk phosphorylation and cell cycle arrest at S-phase upon DDR (Busino et al., 2003; Jin et al., 2003). IR-induced Cdc25A turnover in interphase was shown to depend on Cul1, suggesting the involvement of the SCF E3 ligase (Donzelli et al., 2002). In a screen to identify the substrate receptor of the SCF complex, the F-box proteins, βTRCP1 and βTRCP2, two homologs of human βTRCP, were found to be interacting with Cdc25A. Knockdown of both βTRCP1 and βTRCP2 by siRNA or expression of a dominant-negative βTRCP1 blocked DDR-induced Cdc25A proteolysis (Busino et al., 2003; Jin et al., 2003). In addition, depletion of both βTRCP1 and βTRCP2 results in hyperactive Cdk2 kinase activity and radio-resistant DNA synthesis, which are characteristics of a defective intra-S-phase checkpoint (Busino et al., 2003; Jin et al., 2003).

The recognition of ubiquitin substrates by F-box proteins often requires post-translational modification, usually phosphorylation, on target proteins (Petroski and Deshaies, 2005). Chk1 kinase activity is indispensable for in vitro ubiquitination of Cdc25A catalyzed by SCFβTRCP (Jin et al., 2003). Upon DDR, the Serine-296 (S296) site of Chk1 is autophosphorylated after it is phosphorylated at Serine-345 (S345) by ATR (Kasahara et al., 2010). Chk1 phosphorylated at S296 is further recognized by 14-3-3 gamma. 14-3-3 gamma not only tethers Chk1 in the nucleus, but also bridges the interaction between Chk1 and Cdc25A (Kasahara et al., 2010). Therefore, 14-3-3 gamma is important for Chk1-mediated
Cdc25A turnover via the ubiquitin–proteasome pathway (Kasahara et al., 2010). Several Chk1 phosphorylation sites on Cdc25A were mapped, but only mutation on Serine-76 (S76) abolishes Cdc25A ubiquitination (Jin et al., 2003, 2008). Surprisingly, peptide scanning and mutagenesis revealed that S76 alone was not sufficient for direct binding with βTRCP (Jin et al., 2003). Adjacent to S76, there is a DSGFCLDSP sequence (residues 81–89) which resembles the classic phospho-degron motif in other βTRCP substrates (Cardozo and Pagano, 2004). Serine-88 is dispensable for SCFβTRCP–mediated Cdc25A ubiquitination (Jin et al., 2003). Although Aspartic acid-87 could mimic a phosphorylated-serine, S82 (S82) is required for Cdc25A ubiquitination (Jin et al., 2003). Phosphorylation of these residues recruits SCFβTRCP to subsequently phosphorylate S79 and S82 residues. Phosphorylation appears to play a minor role in DDR-induced degrada-

tion of Cdc25A (Jin et al., 2003; Reinhardt et al., 2007). Therefore, p38MAPK and MK2 are potential candidates for drug targets to kill cancer cells that are defective in p53 function. However, how p53 affects the selection of checkpoint pathways in Cdc25A phosphorylation has yet to be determined.

Chk1 and SCFβTRCP mediate Cdc25A turnover in the S and G2 phases of cell division. At late G1-phase, Cdc25A stability is not controlled by Chk1, although Cdc25A ubiquitination is still SCFβTRCP–dependent. Kang et al. (2008) found that GSK3β phosphorylates S76 of Cdc25A at late G1-phase. Interestingly, S76 phosphorylation requires prior phosphorylation at Threonine-80 (T80) by PLK3 (Kang et al., 2008). A strong correlation between GSK3β inactivation and Cdc25A over-expression was observed in some human tumors, further supporting the negative role of GSK3β in Cdc25A production (Kang et al., 2008). However, under DDR, checkpoint kinases, such as Chk1 and MK2, are in charge of S76 phosphorylation of Cdc25A (Jin et al., 2003; Reinhardt et al., 2007).

S82 of Cdc25A is a primary phosphorylation site in the phospho-degron motif of Cdc25A. Several kinases have been reported to phosphorylate S82 to trigger Cdc25A turnover (Melixetian et al., 2009; Honaker and Piwnica-Worms, 2010; Piao et al., 2011). In a screen with a shRNA library, Melixetian et al. (2009) identified NIMA (never in mitosis gene A)-related kinase 11 (NEK11) as a gene that is involved in the G2/M checkpoint. Silencing NEK11 prevents ubiquitin-dependent proteolysis of Cdc25A, in both unsynchronized and DNA damaged cells. NEK11 phosphorylates S82 of Cdc25A during DDR. Moreover, NEK11 is a downstream effective kinase of the checkpoint pathway and is activated via phosphorylation at Serine-273 of NEK11 by Chk1. Thus far, there is no evidence to show whether NEK11 is capable of phosphorylating S79 of Cdc25A, although phosphorylation of S79 is important for S82 phosphorylation of Cdc25A and Cdc25A recognition by βTRCP. In a separate study, Honaker and Piwnica-Worms (2010) reported that casein kinase 1 alpha (CK1α) phosphorylates Cdc25A at both S79 and S82 during interphase and under genotoxic stress. In the same study, the authors claimed that NEK11 could not phosphorylate S82 of Cdc25A in vitro. However, it is unclear whether any priming kinase was included in the in vitro kinase assay. Paradoxically, Melixetian et al. (2009) found that NEK11 can phosphorylate S82 of Cdc25A in vitro, but the phosphorylation was independent of S76 phosphorylation. In contrast to the Honaker and Piwnica-Worms (2010) report, Piao et al. (2011) observed CK1ε directly phosphorylated S82 without any prior phosphorylation of Cdc25A, and silencing CK1ε stabilized
cellular Cdc25A in HEK293 cells. These conflicting results reveal the complexity of phosphorylation-regulated Cdc25A proteolysis (Figure 2). They may also reflect the fact that Cdc25A stability is regulated by various signaling pathways and that different genetic background may select distinctive kinases to create a phosphodegron motif for Cdc25A. Further experiments are needed to clarify these discrepancies. Nevertheless, the consensus is that S76 is a priming site for generation of a phospho-degron sequence surrounding S79 and S82.

In addition to residues surrounding S82, other serine residues also contribute toDDR-induced Cdc25A degradation. PLK3 phosphorylates Serines-513 and -519 of Cdc25A in vitro (Myer et al., 2011). The role of PLK3 in Cdc25A stability was studied in PLK3 knockout mice where the G1/S checkpoint was found to be defective (Myer et al., 2011). PLK3-mediated Cdc25A stabilization did not translate into a significant increase in tumorigenesis in vivo (Myer et al., 2011), however, suggesting that PLK3-mediated Cdc25A turnover may not be a major pathway to control in vivo functions of Cdc25A.

**SCF**<sup>βTRCP</sup> IN CLASP IN UBQUITINATION AND TURNOVER DURING DDR RECOVERY

While SCF<sup>βTRCP</sup>-mediated Cdc25A degradation is critical for the execution of the DNA damage checkpoint, SCF<sup>βTRCP</sup>-controlled Claspin turnover is important for checkpoint recovery. Claspin associates with the replication fork and is one of the major checkpoint mediators for Chk1 activation by ATR in response to replication stress (Chini and Chen, 2003; Kumagai and Dunphy, 2003; Lee et al., 2003; Lin et al., 2004). Claspin is a periodically synthesized protein and its expression peaks at S/G2 and is then degraded after cells transit into mitosis. In unsynchronized cells, the mitotic degradation of Claspin depends on SCF<sup>βTRCP</sup>, which binds to Claspin through a conserved DpSGxxpS phospho-degron motif (Cardozo and Pagano, 2004). Phosphorylation of these two serine residues is necessary for ubiquitination of Claspin. Alanine substitutions of these serine residues abolish the association of Claspin with SCF<sup>βTRCP</sup> and the poly-ubiquitination of Claspin (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Apparently, PLK1 is the kinase that phosphorylates these serines, because PLK1 is necessary for in vitro ubiquitination and siRNA depletion of PLK1 suppresses mitotic Claspin degradation (Mairland et al., 2006; Mamely et al., 2006).

In G2, Claspin quickly accumulates after UV damage, suggesting a post-transcriptional regulatory mechanism. The level of Claspin correlates well with activation of Chk1, represented by phosphorylation of Serine-317 of Chk1 (Chini et al., 2006). Chk1 phosphorylates Threonine-916 of Claspin and is important to maintain Claspin protein stability (Bennett and Clarke, 2006; Chini and Chen, 2006; Chini et al., 2006). Although Chk1 affects Claspin turnover, apparently, the stability control of Claspin is independent of ATR (Chini and Chen, 2006; Chini et al., 2006; Yang et al., 2008). Conversely, Claspin is also a stabilizer of Chk1 protein (Yang et al., 2008). During the recovery from replication stress, Chk1 activity is gradually diminished, concomitantly with the reduction of Claspin level. When serine residues in the phosphodegron of Claspin are mutated into alanine, Claspin is no longer a substrate of the SCF<sup>βTRCP</sup> ubiquitin ligase (Mairland et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Consequently, the activation of checkpoint pathway is extended and the percentage of mitotic cells is reduced (Mairland et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Therefore, SCF<sup>βTRCP</sup>-dependent turnover of Claspin is critical for cells to terminate the checkpoint pathway once the damage is repaired (Mairland et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006).

In summary, the SCF<sup>βTRCP</sup> ubiquitin ligase plays important roles in multiple steps of DDR via proteolytic control of multiple substrates which functions at different steps of DDR. Obviously, distinct protein kinases have been shown to create a conserved phospho-degron motif on different substrates. Nevertheless, as the substrate receptor, βTRCP is a key player to maintain genome integrity. Consistent with this, mutations of βTRCP1 have been identified in human cancer (He et al., 2005). The inhibitory role of βTRCP1 in tumorigenesis has been established (Kudo et al., 2004; Bhatia et al., 2011), suggesting that βTRCP1 is a bona fide tumor suppressor.

**CRL4<sup>Cdt2</sup> IN DNA DAMAGE RESPONSE**

DNA replication is initiated from the sequential recruitment and assembly of regulatory proteins onto specialized chromosome regions known as DNA replication origins. During the late M-phase and G1-phase, pre-replicative complex (pre-RC) needs to be assembled onto origins. Origins then become "licensed" and can further recruit DNA polymerase for replication at S-phase (Dutta and Bell, 1997; Arias and Walter, 2007). The assembly of pre-RC is a stepwise process that starts with binding of origin recognition complex (ORC) to an origin. ORC then recruits Cdc6 and Cdt1, two DNA replication licensing factors which are required for loading of MCM complex. To prevent re-replication, where origins fire more than once during a single cell cycle, it is essential to dissemble the pre-RC complex immediately after replication is initiated (Dutta and Bell, 1997; Arias and Walter, 2007). In Xenopus and mammals, Cdt1 can be inactivated through binding to its inhibitor, Geminin, or via ubiquitination-mediated proteolysis (Wohlschlegel et al., 2000; Nishitani et al., 2001; Tada et al., 2001; Arias and Walter, 2005). Inhibition of Geminin or over-expression of Cdt1 stimulates DNA re-replication (Vaziri et al., 2003; Zhu et al., 2004; Takeda et al., 2005). Cdt1 transgenic mice develop thymic lymphoblastic lymphoma in the absence of p53 (Seo et al., 2005) and over-expression of Cdt1 and Cdc6 promotes malignant events by inducing genome instability and by abrogating antitumor barriers, suggesting their oncogenic functions (Liontos et al., 2007). Over-expression of Cdt1 and/or Cdc6 has been observed in certain cancers (Karakaidos et al., 2004). Therefore, DNA replication licensing machine could be a good therapeutic target for cancer cures.

**CRL4<sup>Cdt2</sup> IN DDR-INDUCED Cdt1 UBQUITINATION AND TURNOVER**

In the unperturbed cell cycle, Cdt1 is degraded after replication is initiated at G1-/S-phase (Nishitani et al., 2001). Walter and colleagues demonstrated that Cdt1 degradation in Xenopus egg extract requires replication initiation (Arias and Walter, 2005). Depletion of Cdc45 or RPA, which are essential for origin firing, abolished the chromatin-associated Cdt1 ubiquitination and degradation (Arias and Walter, 2005). Two E3 ligases, SCFSkp2 and CRL4<sup>Cdt2</sup>, are identified to mediate cell cycle-dependent degradation of Cdt1 (Higa et al., 2003; 2006; Li et al., 2003; Zhong et al.,
CRL4Cdt2, since the absence of Skp2 does not compromise Cdt1 degradation (Arias and Walter, 2006; Kondo et al., 2004; Senga et al., 2006). The DDR-induced ubiquitination of Cdt1 is solely dependent on SCFSkp2, which could potentially promote DNA repair. Two E3 ligases, SCFSkp2 and CRL4Cdt2, are implicated in regulating p21 proteolysis under DNA damage conditions (Bornstein et al., 2003; Soria et al., 2006; Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). It has been shown that degradation of p21 after low dose UV irradiation (<40 J/m²) depends on ATM and Skp2. The interaction between Skp2 and p21 has been detected and is further increased after DNA damage (Bendjennat et al., 2003). Mutation of all lysine residues impaired UV-induced p21 degradation and cells exhibited defects in DNA repair. This implies that p21 ubiquitination promotes its degradation to prompt the accumulation of PCNA on chromatin for DNA repair process (Chen et al., 1996).

Under low dose UV irradiation, p21 undergoes proteolysis, which could potentially promote DNA repair. Two E2 enzymes, Cdc34 and CRL4Cdt2, are involved in regulating p21 proteolysis under DNA damage conditions (Bornstein et al., 2003; Soria et al., 2006; Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). It has been shown that degradation of p21 after low dose UV irradiation (<40 J/m²) depends on ATM and Skp2. The interaction between Skp2 and p21 has been detected and is further increased after DNA damage (Bendjennat et al., 2003). Mutation of all lysine residues impaired UV-induced p21 degradation and cells exhibited defects in DNA repair. This implies that p21 ubiquitination promotes its degradation to prompt the accumulation of PCNA on chromatin for DNA repair process (Chen et al., 1996).

Although both UV and IR can trigger CRL4Cdt2-dependent Cdt1 proteolysis, the phosphorylation event of Cdt1 is only observed in UV damaged cells (Kondo et al., 2004). IR-induced degradation of Cdt1 is not dependent on either ATM or ATR checkpoint pathway, whereas UV-induced degradation is sensitive to the inhibitor of ATM and ATR, caffeine (Higa et al., 2003; Kondo et al., 2004). Although Cdt1 is phosphorylated after UV irradiation, none of the phosphorylation events are located at the N-terminus, where the degron motif is localized (Kondo et al., 2004; Senga et al., 2006). However, it is still unclear why caffeine, as the inhibitor of ATM and ATR, only affects UV-mediated Cdt1 proteolysis. How ATM and ATR regulate UV-controlled Cdt1 degradation is still unclear. Further lost-of-function investigations on ATM and ATR are necessary to validate their roles in UV-triggered Cdt1 proteolysis. Moreover, whether and how the phosphorylations of Cdt1 at its C-terminus are linked to Cdt1 ubiquitination and turnover are yet to be determined. Recent study from Cook’s group indicated that p38MAPK and c-Jun N-terminal kinase (JNK) can phosphorylate Cdt1 both during unperturbed G2 phase and under stress condition (Chandrasekaran et al., 2011). These results explained why Cdt1 becomes a stable protein when cells move into G2 and mitosis.
UbcH8 in poly-ubiquitin chain generation on p21 remains to be determined. Moreover, like Cdc34, UBE2G E2s contain an acidic sequence that is required for poly-ubiquitin chain formation (Petroski and Deshaies, 2005). One important question is whether UBE2G, like Cdc34, needs assistance from another E2, such as UbcH5, as a priming subunit to boost its poly-ubiquitination capability. Another critical question is whether the CRL4DDB2 ubiquitin ligase employs Cdc34, UbcH8, or UBE2G as E2 enzyme to conjugate poly-ubiquitin chains on Set8/PR-Set7, another critical substrate of CRL4DDB2 under DDR (Abbas et al., 2010; Centore et al., 2010; Oda et al., 2010; Jørgensen et al., 2011). Understanding these questions is important to better comprehend protein poly-ubiquitination by CRL ubiquitin ligases under DDR.

**CRL4DDB2 AND CRL4CSA IN DNA REPAIR CONTROL**

Nucleotide excision repair (NER) is a versatile repair mechanism to remove the damaged DNA lesions from the genome, including UV-induced cyclobutane pyrimidine dimers (CPD), 6–4 pyrimidine–pyrimidone photoproducts, and a variety of bulky adducts (Gillet and Schärer, 2006). It is a gap-filling process executed by DNA polymerase and ligase following the excision of a 24–32 DNA base oligonucleotide, where the lesion localizes. There are two sub-types of NER, global genome repair (GGR), and transcription-coupled repair (TCR), which differ in the mechanism of damage detection. GGR occurs throughout the whole genome, whereas TCR is restricted in the active transcribing regions. Mutations in the NER pathway are responsible for several rare inheritable diseases, such as xeroderma pigmentosum (XP) and Cockayne syndrome (Bootsma et al., 1998).

**CRL4DDB2 IN NUCLEOTIDE EXCISION REPAIR**

Xeroderma pigmentosum patients are hypersensitive to UV and are pre-disposed to skin cancer. Cells from XP patients are defective in repair of UV-induced DNA damage (Friedberg, 1995; Friedman et al., 1995). In XP complementation group E (XP-E), the DDB2 gene is mutated. DDB2 encodes a WD-40 repeat protein that was originally identified as one subunit of a heterodimer with DDB1 (Dualan et al., 1995). The DDB1–DDB2 heterodimer binds to damaged DNA and is involved in NER. The crystal structure of DDB2–DDB1 dimer explains its high affinity toward UV-induced DNA lesion and its central role in NER. The hairpin motif of DDB2 inserts into the minor groove of a DNA duplex and thereby flips out the pyrimidine dimer to be recognized by the binding pocket of DDB2 (Scrima et al., 2008). The DNA lesion recognition ability of DDB2 is essential for subsequent recruitment of repair proteins to the damage site. Consistent with the clinical implication of DDB2 in human disease (XP-E), DDB2 knockout mice are susceptible to UV-induced lesions, and to impair the recruitment of XPC and the subsequent removal of CPD from the genome (El-Mahdy et al., 2006). Moreover, XPC ubiquitination may accelerate DDB2 turnover (Sugasawa et al., 2009). Therefore, it seems likely that a UV-induced DNA lesion is handed over from DDB2 to XPC for DNA repair and that this process depends on the poly-ubiquitination capability of the CRL4DDB2 ubiquitin ligase (Sugasawa et al., 2009). Whether XPC ubiquitination triggers DDB2 degradation via auto-ubiquitination is still debatable. However, a recent study from Arabidopsis suggested that DET1 is the substrate receptor of CRL4 that targets DDB2 for ubiquitination and degradation (Castells et al., 2011). Therefore, it will be interesting to see whether DET1 is responsible for UV- and Cu4A-mediated DDB2 ubiquitination and proteolysis in human cells. Using a chemical inhibitor, Zhao et al. (2008) demonstrated that p38MAPK is required for UV-induced DDB2 ubiquitination and proteolysis. The same group also found that DDB2 is phosphorylated by p38MAPK after UV treatment. However, the actual phosphorylation sites of DDB2 are yet to be identified. Moreover, it is still unclear whether phosphorylation of DDB2 under UV damage plays any critical roles in DDB2 ubiquitination and degradation. It is also unknown whether p38MAPK phosphorylates DDB2 directly or via its downstream kinases, such as MK2. Answering these questions will help understand the concise mechanism of NER under DDR.

In addition to XPC, histone proteins, H2A, H3, and H4, are conjugated with a single ubiquitin by CRL4DDB2 in response to UV irradiation. In XP-E patient-derived lymphoblastoid cells, UV-induced mono-ubiquitination of H2A is impaired (Kapetanaki et al., 2006; Oda et al., 2010; Jørgensen et al., 2011). Understanding these questions is important to better comprehend protein poly-ubiquitination by CRL ubiquitin ligases under DDR.
proposed that ubiquitination of histone proteins by CRL4 DDB2 DNA lesions (Takedachi et al., 2010). Although K244E maintains (Groisman et al., 2003). Similar to CRL4DDB2, the ubiquitin ligase associating with the stalled RNA polymerase II (RNA pol II) with Cul4 and Rbx1. In response to UV damage, CSA is found binds to DDB1 and is able to assemble an E3 ligase complex (Petroski and Deshaies, 2006). However, inconsistent with this hypothesis, histone ubiquitination is not sufficient to destabilize the nucleosome in vitro (Takedachi et al., 2010). Therefore, one potential function of histone ubiquitination is to recruit other DNA repair factors to lesion DNA. One such factor could be XPA whose association with lesion DNA is enhanced by the ubiquitination activity of CRL4DDB2 (Takedachi et al., 2010). However, XPA does have an obvious ubiquitin-binding motif, suggesting that additional factors might be involved in the recruitment of XPA. CRLs usually conjugate poly-ubiquitin chains on their substrates (Petroski and Deshaies, 2005). How CRL4DDB2 is regulated to conjugate mono- versus poly-ubiquitin chains on its substrates remains enigmatic.

CRL4CSA IN NUCLEOTIDE EXCISION REPAIR
Besides CRL4DDB2, the CRL4CSA ubiquitin ligase is also actively involved in DNA repair (Groisman et al., 2003). In this complex, CSA is a substrate specific factor. CSA is also a WD-40 repeat protein and has 40% sequence homology with DDB2 from the second to the fifth WD-40 repeat. Similar to DDB2, CSA directly binds to DDB1 and is able to assemble an E3 ligase complex with Cul4 and Rbx1. In response to UV damage, CSA is found associating with the stalled RNA polymerase II (RNA pol II) (Groisman et al., 2003). Similar to CRL4DDB2, the ubiquitin ligation activity of CRL4CSA is inhibited due to the binding of CSN (Groisman et al., 2003). CSA, a DNA-dependent ATPase, is the only substrate identified for CRL4CSA thus far. CSA preferentially associates with elongating or stalled RNA pol II, and is potentially involved in the elongation step of RNA synthesis. Both CSA and CSB are mutated in Cockayne syndrome patients and involved in transcription-coupled DNA repair (Li et al., 2011). CSA fibroblasts exhibit hypersensitivity to UV irradiation and a defect in resumption of RNA synthesis after damage (Bregman et al., 1996; Balajee et al., 1997). Moreover, poly-ubiquitination and degradation of stalled RNA pol II in response to UV is also impaired in CSB cells (Bregman et al., 1996). CSA is a member of the SNF2-like family, which possesses chromatin-remodeling activity. CSA is believed to push away the stalled RNA pol II or to rearrange the chromatin structure so that the repair proteins can get access to the lesion (Groisman et al., 2006). The key function of CSA is to recruit histone acetyltransferase p300, NER proteins, and the CRL4CSA ubiquitin ligase to lesion DNA (van Goor et al., 1997; Fousteri et al., 2006); whereas, CSA is required to recruit XAB2, the nucleosomal binding protein HMGN1, and TFIIIS in cooperation with CSB. If the lesion cannot be repaired, CSB then initiates poly-ubiquitination and degradation of stalled RNA pol II (Svejstrup, 2003). Three hours after damage, CSB itself is eliminated via CRL4CSA-mediated poly-ubiquitination and proteasome-dependent degradation (Groisman et al., 2006). The degradation of CSB is necessary for transcription to resume at a normal rate, and is coordinated with the dissociation of the CSN from the CRL4CSA ubiquitin ligase (Groisman et al., 2003), indicating that the ubiquitination function of CRL4CSA was inhibited during DDR, but activated after DNA repair was accomplished. However, how the CSN is released from the CRL4CSA ubiquitin ligase is still unclear.

In summary, CRL4CSA and CRL4DDB2 are two important ubiquitin ligases that not only function as ubiquitin ligases to ubiquitinate their cognate substrates, but also play distinct roles in recruiting critical DNA repair factors to lesion DNA upon DDR. Multiple mutations have been identified for DDB2 and CSA from human patients. Interestingly, most of these mutations fail to form ubiquitin ligases with other subunits of CRL4 (Jin et al., 2006). As a consequence, CRL4 ubiquitin ligases cannot be recruited to damage sites to assist DNA repair. Therefore, these disease-derived mutations all affect activities of CRL4CSA and CRL4DDB2 ubiquitin ligases, further supporting their fundamental roles in NER.

SCF\(\text{Fbxo}4\) AND SCF\(\text{Fbxo}31\) IN CYCLIN D1 UBIQUITINATION
Cdk kinase activity is the master regulator that controls cell cycle progression. Cdk kinase activity can be modulated via phosphorylation, through binding to the inhibitors, or by association with cyclin co-factors. The level of cyclin proteins fluctuates during the cell cycle through periodic transcriptional activation or proteolysis. Cyclin D1 is expressed in G1-phase and its presence activates Cdk2 for entrance into S-phase. To prevent cells from entering into S-phase and propagating erroneous DNA, cyclin D1 is degraded via the ubiquitin–proteasome pathway after encountering genotoxic stress (Pagano et al., 1994; Diehl et al., 1997, 1998). Cyclin D1 is over-expressed in various malignant neoplasms, suggesting its role as an oncogene (Malumbres and Barbacid, 2001). MMTV-cyclin D1 transgenic mice develop mammary adenocarcinomas, indicating its oncogenic role in breast cancer (Wang et al., 1994; Hosokawa et al., 2001). Therefore, cyclin D1 expression is well controlled during cell division. Dysregulation of cyclin D1 can lead to genomic instability (Ponimaskin et al., 2008). Cyclin D1 is an unstable protein. Its ubiquitination and degradation depends on phosphorylation of Threonine-286 (T286), which is mediated by GSK3\(\beta\) kinase (Diehl et al., 1997, 1998). Four F-box proteins, including Fbxo4, Fbxw8, Fbxo31, and Skp2, have been reported to recognize cyclin D1 and mediate its ubiquitination (Yu et al., 1998; Lin et al., 2006; Okabe et al., 2006; Barbash et al., 2009; Santra et al., 2009).

When cells progress into S-phase, the T286 on cyclin D1 is phosphorylated by GSK3\(\beta\). Phosphorylated T286 and surrounding residues constitute the phospho-degron motif that is recognized by Fbxo4, a specificity factor in the SCF\(\text{Fbxo}4\) ubiquitin ligase (Diehl et al., 1997, 1998; Lin et al., 2006). Thus, phosphorylated cyclin D1 at T286 is an unstable protein that is subjected to poly-ubiquitination by SCF\(\text{Fbxo}4\) and then degradation by the 26S proteasome (Lin et al., 2006). Importantly, the ubiquitination of cyclin D1 by SCF\(\text{Fbxo}4\) requires a small heat-shock protein, αB crystallin (Lin et al., 2006). Over-expression of Fbxo4 and αB crystallin can stimulate cyclin D1 ubiquitination and accelerate its degradation, whereas knockdown of either of them can block cyclin D1 ubiquitination and increase its protein stability (Lin...
et al., 2006). In consistent with these observations, Fbxo4 and αB crystallin expression is reduced in subset of primary human tumors that overexpress cyclin D1 (Lin et al., 2006). In addition to Fbxo4, another F-box protein Fbxw8 was also identified as an E3 ligase for cyclin D1 in HCT116 cancer cell line. Fbxw8 can associate with either Cul1 or Cul7 to assemble an SCF or SCF-like complex, respectively (Okabe et al., 2006). Fbxw8 also recognizes the degron sequences surrounding phosphorylated T286. However, the phosphorylation of T286 in this case is mediated via the MAPK pathway rather than by GSK3β in the cell context examined (Okabe et al., 2006). The access of cyclin D1 to its cognate E3 ligase is spatially regulated. Similar to Fbxo4, majority of Fbxw8 is localized in the cytoplasm during G1- and S-phase, separating away from the nucleus localized cyclin D1. In S-phase, cyclin D1 is translocated into the cytoplasm, where it is now accessible to E3 ligase for subsequent ubiquitination and proteolysis. The phosphorylation of cyclin D1 by GSK3β at T286 is required for its nuclear export (Lin et al., 2006).

In contrast to Fbxo4 and Fbxw8, Fbxo31 is specifically dedicated to damage-induced degradation of cyclin D1 (Santra et al., 2009). In response to IR or other genotoxic stress, Fbxo31 is targeted by the checkpoint kinase ATM for phosphorylation, which results in the accumulation of Fbxo31. Fbxo31 binds to the same phospho-degron motif on cyclin D1 as Fbxo4 and Fbxw8 and mutation of T286 abolishes the interaction between cyclin D1 and all three F-box proteins. However, blocking of the MAPK pathway but not GSK3β prevents this damage-induced cyclin D1 degradation (Santra et al., 2009). It should be noted that the characterization of these four F-box proteins were done in different cell lines. Whether these four E3 ligases are involved in regulating cyclin D1 simultaneously or exclusively from each other remain to be determined. The Fbxo4 knockout mice develop various tumors whose cyclin D1 expression is enhanced (Vaites et al., 2011). However, a recent study in knockout mice of these ubiquitin ligases indicated that none of these four ubiquitin ligases is required for cyclin D1 proteolysis in mouse embryonic fibroblasts (Kanie et al., 2012), further supporting cell type-specific regulation of cyclin D1 stability. Therefore, it is intriguing that how cells choose among different ubiquitin ligases reacting to different stimuli for cyclin D1 destruction if they all recognize the same phospho-degron motif. Nevertheless, loss of heterozygosity of Fbxo31 has been reported in multiple cancers (Launonen et al., 2000; Lin et al., 2001; Härkönen et al., 2005; Kumar et al., 2005; Huang et al., 2010; Kogo et al., 2011). Inactive mutations of Fbxo4 are also identified in 15% of esophageal tumors (Barbash et al., 2008). These data support the idea that Fbxo31 and Fbxo4 are tumor suppressors.

CONCLUSION AND PROSPECTIVE

Here we discuss how CRL ubiquitin ligases mediate protein ubiquitination and turnover to enforce unidirectional signal transduction in the DDR pathway. Many of the substrate receptors of CRL are still uncharacterized. Growing evidence suggests that more CRL ubiquitin ligases will be found to mediate the DDR pathway and more DDR-related proteins will be identified as ubiquitin substrates of CRLs. Thus far, the main research focus has been on dissecting the roles of CRL1 (SCF) and CRL4 ubiquitin ligases in DDR. Emerging evidence suggests that other culin-related ubiquitin ligases are also involved in DDR (Ribar et al., 2007; Yasukawa et al., 2008; Blackford et al., 2010). Further investigation is needed to explore the functions of culin-related ubiquitin ligases other than CRL1 and CRL4 in DDR. Moreover, several groups have reported knockout mice models of Cul4A and suggest an important role of Cul4A in guarding the genome stability (Kopanja et al., 2009; Liu et al., 2009). The homolog of Cul4A, Cul4B shares significant sequence homology with Cul4A and has some redundant functions with Cul4A (Higa et al., 2003; Hu et al., 2004). Considering the distinct knockout phenotype of Cul4A in mice and cell lines (Nishitani et al., 2001; Karakaidos et al., 2004), it is conceivable that Cul4A and Cul4B play distinct roles in genome integrity maintenance via regulating protein stability of different substrates. However, Cul4B also plays significant roles in DDR (Kerzendorfer et al., 2010). In fact, degradation of p27 and p53 seems to depend on Cul4A solely (Barsky et al., 2006; Higa et al., 2006). Therefore, more investigation is required to unravel the relationship between Cul4A and Cul4B and to advance our understanding of Cul4 as genome guardian.

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