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The Roles of Cullin RING Ligases and the Anaphase Promoting Complex/Cyclosome in the Regulation of DNA Double Strand Break Repair

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<http://dx.doi.org/10.5772/intechopen.70482>

Abstract

Historically, genome maintenance has been viewed as the largely independent activities of (1) ubiquitin ligases driving unidirectional cell cycle progression and, (2) the activity of cellular checkpoints that monitor DNA integrity and DNA replication. It is well established that the DNA damage response (DDR) checkpoint machinery promotes the activation of repair mechanisms in addition to opening a window for repair. Emerging evidence demonstrates an integrated network of the central cell cycle driving E3 ubiquitin ligases and the checkpoint machinery, as well as deubiquitinating enzymes, which intermittently cooperate and antagonize one another to define windows of checkpoint and repair activities to optimize genome stability and cellular health. A growing number of components of the ubiquitin machinery are involved in the DDR. Herein, we focus on the regulation of cell cycle checkpoints and the DNA repair mechanisms for double strand breaks (DSBs) by the coordinated activities of Cullin RING ligases (CRLs) and the anaphase promoting complex/cyclosome (APC/C).

Keywords: DNA repair, deubiquitinating enzymes, E3 ubiquitin ligase, APC/C, Cullin-RING ligase, SCF, homologous repair, non-homologous end-joining

1. Introduction

Our cells face a multitude of DNA damaging insults, both internally and externally derived, on a daily basis. The majority of our cells is not cycling and must simply respond by rapidly repairing the damaged DNA to maintain homeostasis. For those cells that are cycling, however, the precise maintenance of the genome is of even more critical importance to ensure the faithful transmission of identical copies of an undamaged genome to the next generation of

cells. Of critical concern for cycling cells is the precise duplication of one exact copy of the genome followed by the accurate segregation of the two copies. To ensure that these events happen once, only once, and in the proper order, cells utilize the periodic synthesis and ubiquitin-mediated degradation of a host of proteins to control the timely activity of multiple enzymatic activities, such as kinases and polymerases, to drive the unidirectional transit through the cell cycle.

Upon the occurrence of DNA damage, cycling cells must not only sense and respond to the insult, but must also coordinate cell cycle progression with repair. Moreover, as damage can occur during any of the many processes taking place during the cell cycle, proliferating cells have evolved a number of mechanisms for repairing and overcoming damage to maintain the genome. However, the proper selection of the repair mechanism to use is nearly as important as sensing the damage as some mechanism could be highly mutagenic if utilized at the wrong point in the cell cycle or if used without proper control. Such errors would clearly result in the generation of mutations that could lead to a number of human pathologies, most notably cancer. Moreover, the induction of DNA damage is a central therapeutic strategy for treatment of the majority of cancer types. And, while clinically useful, such treatments lack specificity and are often limited by toxicities. The dissection of genome maintenance pathways thus holds the potential to define new therapeutic targets that may ultimately lead to more effective therapeutic strategies. This review will focus on recent advances in our understanding of how the interplay of the major cell cycle-associated ubiquitin ligases, the DNA-monitoring checkpoint machinery, and deubiquitinating enzymes coordinate cell cycle progression with the response to the proper repair of DNA damage. In particular, the focus will be on the use of mechanisms of repairing DNA double strand breaks and stalled replication forks.

2. Modular E3 ubiquitin ligases in the cell cycle and genome repair

2.1. Cullin RING ligases (CRLs)

The cullin family proteins (Cul1, 2, 4A, 4B, 5, and 7) function as the central scaffolds for the assembly of multi-subunit ubiquitin ligases [1]. The cullin C-terminus adopts a globular conformation that provides a docking site for the RING finger proteins Rbx1 or Rbx2. The RING fingers recruit the ubiquitin E2 enzymes and catalyze the transfer of ubiquitin to substrates. At the cullin N-terminus is a helical domain that is the site of interaction with an adapter protein (s), which recruits substrate receptors. In general, each cullin associates with a distinct set of substrate receptors. For example, the CRL1 ligases utilize the adaptor protein Skp1 to interact the F-box family of proteins defined for a Skp1-interacting motif defined in the archetypical F-box, Cyclin F. The CRLs are denoted by the identity of the cullin family member and the associate receptor. For example, CRL1^{Cyclin F} denotes a cullin1-based ligase complexed with the substrate adaptor Cyclin F. The human genome contains nearly 200 cullin-associated substrate receptors thus allowing CRLs to regulate myriad cellular processes [1]. The extent of this functional diversity is exemplified by the fact that a single CRL can have either oncogenic or tumor suppressive activity depending on the substrate adaptor, for example CRL1^{Skp2} and CRL1^{Fbw7}, respectively [2–5].

With nearly 200 E3 ligases regulating an estimated 20% of the human proteome and a growing number of cellular process, it is not surprising that the function of CRLs is highly regulated at multiple levels, including; regulation of substrate receptor availability (e.g., regulated expression and degradation of receptors), activation/inactivation by the reversible neddylation of the cullin subunit, CAND1-mediated exchange of substrate receptors, regulation of substrate-receptor interactions (e.g., post-translational modification of substrates such as phosphorylation and glycosylation) and the activity of deubiquitinating enzymes [1, 6].

2.1.1. CRL1 (a.k.a. SCF) complexes

The Cullin1-based CRL1 ligases are more commonly known as the Skp1-Cullin1-F-box (SCF) ligases. There are nearly 70 F-box proteins in the human genome, although only a subset has been studied in great detail. For the purposes of this review we will utilize the SCF rather than CRL1, nomenclature. Multiple SCF ligases are involved in cell cycle control and the response to and repair of DNA damage. In consideration of space constraints, we will give overviews of two key SCF, rather than CRL1, ligases as more specific examples of the function of this group of enzymes.

2.1.1.1. SCF^{Skp2}

SCF^{Skp2} functions as a driver of S-phase and exhibits oncogenic activity in multiple settings. Skp2 activity is regulated by its controlled expression and degradation. In addition, even when the Skp2 protein is present and complexed with Cullin1 and Skp1, its ability to recruit substrates for ubiquitination requires site-specific phosphorylation of its target proteins to create a phosphodegron that is recognized by Skp2. Many Skp2 substrates are phosphorylated in a cell cycle-specific fashion, adding an additional layer of control. Skp2 is predominantly known for its role in driving S-phase entry by degrading the Cdk inhibitors p21 and p27 to drive S-phase entry. It is frequently overexpressed in tumours of varying origins and exhibits oncogenic activity [7].

2.1.1.2. SCF^{βTrCP}

SCF^{βTrCP} is a collective term for two SCF complexes defined by the F-box proteins βTrcp1 and βTrcp2, which are largely, but not exclusively interchangeable. In contrast to the fluctuating levels of Skp2, βTrcp levels are relatively constant throughout the cell cycle, and a major determinant SCF^{βTrCP} activity is the creation of a consensus DpSGxxpS phosphodegron upon substrates. Multiple kinases are involved in the generation of phospho-DSGxxS in substrates, including GSK3β, CK2, Polo-like kinases (e.g., Plk1) and Chk1. Thus, some substrates, for example those directed to SCF^{βTrCP} by Plk1, are degraded in a cell cycle specific manner owing to the regulated expression of Plk1 itself [7].

2.1.2. CRL4 complexes

The cullin 4-based ligases, encompassing cullin 4A or 4B, display almost complete functional redundancy and are generally referred to collectively as CRL4. These ligase complexes incorporate the adapter protein damage DNA-binding 1 (DDB1) and associate with ~25 substrate receptors known as the DDB1 and Cul4 associated factors (DCAFs) [8, 9]. As with the SCF

ligases, the majority of CRL4 complexes have not been studied in detail, yet it is clear that the CRL4 ligases are involved in a multitude of processes, including embryogenesis and haematopoiesis and impact both tumorigenesis and tumour suppression depending on context. CRL4 ligases are best characterized for their roles in cell cycle progression (predominantly controlling replication) and DNA repair. In regard to the latter, CRL4^{CSA} and CRL4^{DDB2} are well characterized for their roles in nucleotide excision repair (NER) in response to UV irradiation [10].

2.1.2.1. CRL4^{Cdt2}

CRL4^{Cdt2} is a central component of the S-phase machinery, which acts to ensure that genome replication is limited to a single round per cell cycle. CRL4^{Cdt2} couples destruction of these targets to replication through a partnership with PCNA, which interacts with a host of proteins to maintain genomic integrity, including licensing factors, helicases, methyltransferase, repair enzymes, and the translesion (TLS) polymerases [11]. The regulated recruitment of these proteins is critical for preparing the genome for faithful transmission to the next generation as spurious engagement of several PCNA-binding proteins has been shown to have deleterious effects [8, 12–17]. Importantly, the majority of these factors engage the same interaction surface on PCNA via a PCNA-interacting protein (PIP)-box motif. Interestingly, the PIP-box of a subset of PCNA-interacting proteins, such as the Cdk inhibitor p21 and the replication licensing factor Cdt1, when bound to PCNA, acts to recruit the CRL4^{Cdt2} leading to the ubiquitination and destruction of these proteins [13, 18]. Notably, these CRL4^{Cdt2}-PCNA-substrate interactions only occur when PCNA is bound to DNA to allow recruitment of additional factors [11]. A number of mechanisms regulate these interactions with PCNA, but a critical determinant is the strength of the PCNA-PIP-box interface.²⁰ The PIP-box of the tumour suppressor p21 has the highest known affinity for PCNA, allowing it to prevent PCNA interactions with other PIP-box proteins [19]. In this way, p21 acts to prevent spurious replication and prevent the inappropriate engagement of the error-prone polymerases, which are able to continue DNA replication despite damaged DNA. However, upon replication blocks such as UV-induced damage, p21 is degraded by CRL4^{Cdt2} to allow TLS. Subsequently, the bypassed sites of damage can be repaired by NER.

2.2. Anaphase promoting complex/cyclosome (APC/C)

The APC/C is a large, multi-subunit E3 ubiquitin ligase conserved from yeast to humans. By targeting a multitude of proteins for destruction by the 26S proteasome, the APC is a major driver of cell cycle progression, as well as regulating many diverse processes including meiosis, TGF β signalling, synaptic maturation and differentiation [20–31]. Although not itself a cullin, the central APC2 subunit bares significant homology to the cullins and like these proteins provides a scaffold for the assembly of the multi-subunit APC/C E3 ligase. APC2 contains a binding site for APC11, the RING finger and catalytic component of the APC/C. The APC/C, like CRLs, is involved in numerous cellular processes. However, in contrast to the CRLs, substrate recognition by the APC/C is mediated by a bipartite receptor made up of the APC/C core component APC10 and one of only two substrate receptor/activator proteins, Cdc20 and Cdh1.

Recognition of substrates is mediated by several cis-acting sequence motifs (degrons). It is generally thought that D-boxes and KEN-boxes are responsible for the destruction of all APC substrates [32, 33]. Indeed, most substrates contain one (often multiple) of these two degrons;

however, there are a growing number of motifs identified as critical for APC/C-mediated ubiquitination in the ever-increasing number of APC/C substrates. Recent structural analyses have identified the molecular basis for the interaction of substrates with Cdc20 and Cdh1, which suggests that non-canonical APC/C degrons interact with the activators in manners analogous to the canonical degrons.

APC^{Cdc20}, essential for cell division and viability, is indirectly inhibited by clinically relevant agents (*e.g.* paclitaxel, an anti-cancer drug Taxol), and has received substantial evaluation for pharmacological manipulation. In contrast, APC^{Cdh1} activity is not required for viability, although increasing data demonstrate a role for APC^{Cdh1} in genomic stability and tumor suppression [34, 35]. Indeed, many APC^{Cdh1} substrates (*e.g.* Cyclin A, Skp2, Aurora A, Plk1, and Id2) are associated with oncogenesis, and the regulation of the stability of these substrates has been extensively linked to highly malignant cancers [36]. However, increased Cdh1 activity is also deleterious to cells.

APC/C activity must be tightly controlled and this is accomplished by several mechanisms. First, the activators are regulated at the level of expression with both Cdc20 and Cdh1 accumulating during S and G2 phases. At the end of mitosis, Cdc20 is then degraded by APC/C^{Cdh1}. APC/C^{Cdh1} activity remains high in G1 and its inactivation is critical for commitment to S-phase. Down regulation of APC/C^{Cdh1} activity involves APC/C-mediated degradation of its primary E2 enzyme, UbcH10, Cdk-mediated phosphorylation of Cdh1 which antagonizes its binding to the APC/C holoenzyme, degradation of Cdh1, and the interaction of APC/C with Emi1. Binding of Emi1 prevents substrate engagement and ubiquitination activities and is critical for inhibition of APC/C^{Cdh1}.

2.3. Crosstalk between CRL and APC/C ligases

There is increasing understanding that crosstalk between the CRL ligases and APC/C ligases is required for efficient cell cycle. For example, Skp2 is a substrate of APC/C^{Cdh1} and as cells near the G1/S transition, Cyclin E-Cdk2 complexes initiate the inactivation of APC/C^{Cdh1}, which promotes early accumulation of APC substrates such as Cyclin A and Skp2 (and the activation of SCF^{Skp2}) which promotes further Cdk activity as well as the expression of Emi1, leading to rapid abrogation of APC/C^{Cdh1} activity [37–47]. Then, as cells transit S and G2 the accumulation of the APC/C^{Cdh1} Plk1 leads to the SCF^{βTrCP}-mediated degradation of Emi1 at the G2/M transition to allow APC/C to become active in mitosis [48–51]. Recently, it was discovered that in addition to APC/C-mediated degradation of Cdh1 in late G1, Plk1 also directs the SCF^{βTrCP}-mediated degradation of Cdh1 as cells enter S-phase [52]. SCF^{Fbw7} via its ability to target and regulate the levels of Cyclin E and Plk1 adds another input to this regulatory circuit [53].

3. DNA damage responses

3.1. The double strand break response

The generation of double strand breaks (DSBs) is of potentially grave consequence to cells at any stage of the cell cycle and must be dealt with immediately. In response to DSBs the MRE11-RAD50-NBS1 (MRN) complex and the inactive dimers of the ATM kinase localize to

the damaged site, resulting in the autophosphorylation and activation of ATM monomers (**Figure 1**). The MRN-dependent activation of ATM is facilitated by the non-degradative, K63 linked ubiquitination of NBS1 by SCF^{Skp2} [54]. Phosphorylation of histone H2AX by ATM leads to the recruitment of the checkpoint mediator MDC1, which recruits additional MRN-ATM complexes, to amplify the checkpoint signal, and promotes ubiquitination of histone H2AK15, by the concerted actions of the RNF8 and RNF168 ubiquitin ligases [55–61]. The PR-Set7 and MMSET methyltransferases are also recruited to sites of DNA damage where they catalyse methylation of histone H4K20 [62–66]. Together the ubiquitination of H2A and the methylation of H4 provide a high-affinity binding sight for the checkpoint mediator 53BP1 at sites of damage [67, 68]. 53BP1 further stimulates ATM activity by interacting with MRN complexes and sets the stage for repair. While ATM provides local regulation of the DDR, global regulation is carried out by the effector kinase Chk2, which is activated by ATM. Chk2 phosphorylates numerous proteins, including Cdc25 family phosphatases (to promote/maintain inhibitory phosphorylation of Cdks and cell cycle arrest), p53, and the repair protein BRCA1.

Figure 1. Activation of the ATR and ATM kinase cascades upon DNA damage. Left—Single stranded DNA, generated by blocks to DNA replication or the resection of double strand breaks (DSBs) is coated by RPA, which acts to recruit the ATR-ATRIP heterodimer. The RAD9-RAD1-HUS1 [1] complex is loaded by RAD17. 9-1-1 recruits the ATR activator TopBP1. The mediator protein, Claspin then recruits Chk1 to the site of damage where it is activated by ATR to effect the checkpoint. Right—The induction of a DSB leads to the direct binding of the MRE11-RAD50-NBS1 (MRN)—ATM complex, which phosphorylates histone H2AX (grey spheres represent the histone octamer). The checkpoint mediator MDC1 binds to the phosphorylated histone and is then bound by another MRN-ATM complex. Phosphorylation of MDC1 by ATM recruits the E3 ubiquitin ligase RNF8, which in conjunction with RNF168, ubiquitinates histone H2K15. The ubiquitin ligase SCF^{Skp2} also promotes the MRN-ATM complex formation. The methyltransferases MMSET and PR-SET catalyse methylation of histone H4K20. The H2K15-Ub and H4K20me marks are recognized by 53BP1 leading to further stimulation of ATM activity and the ultimate induction of cell cycle arrest and DNA repair by the effector kinase Chk2.

3.2. Mechanisms of DSB repair

Cells with DNA damage in the form of double strand breaks (DSBs) predominantly use two mechanisms to repair these lesions (**Figure 2**) [69, 70]. The least error-prone of these mechanisms, homologous recombination (HR), utilizes the non-damaged sister chromatid as a template to inform repair of the damaged DNA and is thus limited to S and G2 phases of the cell cycle, where the sister template is available [69]. Indeed, damage incurred during S-phase, whether DSB, interstrand cross-links, or collapsed replication forks rely heavily on HR for repair. The alternative repair pathway, non-homologous end-joining (NHEJ), as the name suggests, involves the sequence-independent ligation of broken DNA ends. Although, some NHEJ (alt-NHEJ or microhomology-mediated, mmNHEJ) do utilize very small regions of homology to identify DNA ends for ligation, canonical NHEJ has no requirement for any

Figure 2. Mechanisms of DSB repair. Upon induction of DSBs, KU proteins are recruited to the broken DNA, protecting the ends. MRN complexes are then recruited. In the presence of BRCA1 and CtIP, the DNA ends are resected, recruiting additional nucleases, including EXO1. Resection leads to the removal of KU proteins. SCF^{Cyclin F} prevents excessive resection by targeting EXO1 for degradation. ssDNA generated by resection is coated by RPA. The BRCA1, BRCA2, PALB2 complex then stimulates the replacement of RPA with RAD51, which promotes strand invasion of the sister chromatid template, leading to homology directed repair of the break. PALB2 function is negatively regulated by the CRL3^{KEAP} ligase and promoted by USP11. In the absence of resection, DNA-PKcs is recruited by the KU proteins, which leads to the recruitment of additional factors, including XRCC4, which is stimulated by SCF^{Fbw7} ligase activity, and DNA ligase IV, which ultimately joins the DNA fragments together.

sequence homology in the selection of ends to be ligated and NHEJ is thus potentially error prone and mutagenic [71]. A key step in NHEJ is the rapid recruitment of Ku70/80 proteins to the severed DNA ends, which function to hold the broken fragments together, limiting the mutagenic potential of this mechanism (**Figure 2**) [72, 73]. Ku70/80 recruits the DNA-PKcs to form the functional DNA-dependent protein kinase, which directs NHEJ. Small gaps in the broken DNA are filled by polymerase μ in to generate blunt ends, which are then ligated by DNA ligase IV in conjunction with XRCC4 [70, 74]. NHEJ is further stimulated by the K63-linked ubiquitination of XRCC4 by SCF^{Fbw7} [74]. The end-joining process is rapid and likely of relatively little genetic consequence [75]. Small deletions could readily occur [71]. If, however, the damage is extensive, processing of DNA ends in an ATM and MRN dependent manner is required, which may lead to larger deletions and in the case of multiple damage sites can produce mutagenic events on the scale of chromosomal rearrangements [71].

In contrast to blunt-ended ligation of NHEJ, the use of the sister chromatid as a template for HR requires the formation of a synapse between the damaged DNA and the undamaged sister (**Figure 2**). Synapse formation requires resection of the DNA from at the site of the break to generate ssDNA. Resection is driven by stimulation of the nuclease activity of the MRN complex by CtIP [76–79]. The ability of CtIP to drive resection is controlled by the balance of BRCA1 and 53BP1 on the chromatin [79–86]. The presence of 53BP1 forms a barrier that limits the accessibility of chromatin to HR-driving nucleases (**Figure 3**). A major role of BRCA1 in HR is to antagonize the binding of 53BP1 to chromatin to allow resection and repair. Indeed, loss of 53BP1 function in BRCA1 mutant cells improves resection and overall genomic stability (**Figure 2**) [81, 85]. BRCA1 recruitment to damaged chromatin is multifactorial and it is thought that BRCA1-CtIP-MRN complex accesses chromatin directly while BRCA1-PALB2-BRCA2 complexes promote the loading of Rad51 on the resected DNA [78, 79, 87]. Rad51 functions to coat the ssDNA and facilitates synapse formation with the template DNA. In contrast the BRCA1-A (BRCA1-MERIT40-BRCC36-BRCC45-ABRAXAS) complex is recruited

Figure 3. 53BP1 and BRCA1 determine the choice between DNA repair mechanisms. Homologous recombination, takes place in S- and G2 phases after DNA replication. Histones (gray circles) of newly replicated DNA lack methylation at H4K20, which weakens the interaction with 53BP1. Moreover, MMS22-TONSL binds the non-methylated H4, which may further antagonize 53BP1, and promotes RAD51 function, which is antagonized by FBH1. BRCA1 is then able to complex with CDK phosphorylated CtIP to drive end resection, preventing NHEJ and setting the stage for HR. BRCA1 further blocks 53BP1 binding, in part by competing for the histone H2K15-Ub marks when complexed with RAP80. This complex also limits resection and is antagonized by the deubiquitinase USP37. In G1 and G2 upon *de novo* H4K20 methylation, 53BP1 is recruited to chromatin to form a barrier to resection. ATM phosphorylation of 53BP1 recruits RIF1 and PTIP, which antagonize BRCA1 and promote recruitment of additional factors to promote repair by NHEJ.

to chromatin by the RNF168-mediated ubiquitination of histones, via the ubiquitin-binding RAP80 protein (**Figure 3**) [88–90]. Interestingly, formation of this complex limits the access of BRCA1 to the damaged DNA, suppressing resection [88].

The critical distinction between these two pathways is the dependency of HR on the resection of the broken DNA end to generate ssDNA to form the synapse with the template DNA. In addition, because NHEJ relies on the ligation of blunt DNA ends and once resection is initiated NHEJ cannot be used. Thus, regulation of resection is central to the choice between mechanisms of repair. Moreover, the inappropriate induction of resection can also give rise to the use of alt-NHEJ. Given that cells that are HR-deficient, (e.g., BRCA1 mutant cells) exhibit sensitivity to DSBs suggests that NHEJ is either too mutagenic or simply does not function efficiently during S-phase, when HR normally predominates. These two possibilities are not mutually exclusive. In addition to DSB repair, HR is also critical for the stabilization and restart of replication forks after prolonged replication stress. As discussed below, multiple layers do, in fact, limit the use of NHEJ during S-phase.

3.3. The replication stress checkpoint

Cells encounter a multitude of intrinsic and extrinsic barriers in attempting to achieve accurate DNA replication. To ensure that replication is error free, eukaryotes possess a conserved checkpoint that monitors replication progress. Upon replication stress (e.g., stalled replication fork, nucleotide deficiency, DNA damage), extensive regions of ssDNA are formed, which are coated by Replication Protein A (RPA) which mediates the recruitment of the apical kinase ATR to the DNA [91]. The Rad17 protein then promotes the loading of a protein complex including the ATR activator, TopBP1, and the checkpoint mediator, Claspin, that then recruits the effector kinase Chk1, which is ultimately phosphorylated by ATR at S317 and S345 that allow Chk1 to adopt an open, active conformation. In turn, Chk1 phosphorylates many proteins, including the Cdk-activating Cdc25 phosphatases, the CDK inhibitory kinase WEE1, and the key HR protein Rad51 [92]. Notably, phosphorylation of Cdc25A by Chk1 leads to SCF^{βTrCP}-mediated degradation. Chk1 ultimately controls origin firing and entry into mitosis as well as promoting replication fork restart and repair, which is predominantly dependent upon the HR machinery.

In the absence of Chk1 recruitment and activation, cells undergoing replication stress maintain high levels of Cdk activity and, continue to fire origins. Under these conditions, replication forks may be prone to stalling and will likely collapse to form DSBs leading to chromosomal abnormalities. These cells are thus highly sensitive to additional replication stress. Importantly, high levels of replication stress are associated with high rates of proliferation during early development and expression of multiple oncogenes (e.g., Cyclin E, c-Myc) [93–98]. Chk1 activity is essential for embryonic development and it follows that surviving the process of transformation requires Chk1 function to survive with abnormal levels of replication stress [99]. As a result, transformed cells are highly dependent on the ATR-Claspin-Chk1 pathway for survival and are sensitive to agents that either induce additional stress or inhibit this critical checkpoint [94, 97, 98]. Indeed, mice possessing an extra copy of Chk1 are more susceptible to oncogenic stimuli. Intriguingly, premature Chk1 activation may drive S-phase entry and failure to down-regulate Chk1 activation is also detrimental.

3.4. Crosstalk between the ATM-Chk2 and ATR-Chk1 axes

As described above, it would seem that the ATM-Chk2 and ATR-Chk1 pathways function in isolation, depending on cell cycle stage and type of insult. However, there is clear cross-talk between the two and, at least in some cell types, the G2 DDR is dramatically weakened, if not abrogated, in the absence of Chk1 function. Resection of damaged DNA ends upon initiation of the HR process yields ssDNA similar to replication stress, which is also coated by RPA and serves as a scaffold upon which to activate the ATR-Chk1 cascade.

4. CRLs and APC/C in DNA damage checkpoint responses

4.1. The G2 DNA damage checkpoint

Initial evidence that Cdh1 possesses a function in the DDR was obtained from chicken DT40 cells in which Cdh1 gene had been deleted [100]. Surprisingly, these Cdh1 knock-out cells were unable to maintain a G2 arrest in the presence of DNA damage. This result was unexpected as APC/C^{Cdh1} is largely thought to be inactive in S and G2 cells due to Cyclin A- and Cyclin E-Cdk-mediated phosphorylation of Cdh1, which both prevents its binding to the APC/C holo-enzyme and, at least at the G1/S transition promotes the creation of a phosphodegron that is recognized by SCF^{βTrCP} leading to Cdh1 degradation [40, 41, 52, 53, 101]. In addition, Emi1, which binds to the APC/C with high affinity and prevents ubiquitination of substrates, is maximally expressed from late G1 through early mitosis [44, 46, 47, 49–51, 102]. Moreover, key APC/C substrates, including Cyclin A, Cyclin B, and Skp2 remain stable during a G2 arrest [103]. Indeed, nearly all APC/C targets tested do remain stable upon DNA damage in G2, with the exception of Plk1 [103]. This is an important distinction as APC/C substrates can have dramatically different impacts on the checkpoint. Plk1 dampens the checkpoint by phosphorylating 53BP1 and Chk2 to inhibit ATM signalling (**Figure 4**) [104]. In addition, Plk1 catalyzes the SCF^{βTrCP}-mediated inhibition of ATR-Chk1 signalling (**Figure 4**) [105–109]. Down-regulation of Plk1 protein levels upon DNA damage was demonstrated to be the result of APC/C^{Cdh1} activation (**Figure 4**) [103]. A critical question stemming from these studies is how APC/C^{Cdh1} targets only Plk1 under these conditions.

The studies in both chicken and human cells indicate that active APC/C^{Cdh1} complexes form upon DNA damage in G2 [100, 103]. Previous analyses had suggested the existence of an Emi1-free pool of the APC/C during interphase [44]. Consistent with this idea, an increased APC/C-Cdh1 association was detected upon damage whereas changes in the abundance of either Emi1 protein or in amount of Emi1-bound APC/C were not observed upon DNA damage [103]. Given that Cdk activity is diminished upon DNA damage (**Figure 4**), these data suggest that a pool of APC/C exists that is independent of Emi1 and regulated largely by inhibitory phosphorylation of Cdh1. The failure to phosphorylate Cdh1 may result in the dephosphorylation and activation of this pool of Cdh1 due to a shift in the balance of kinase phosphatase activities brought about by the inactivation of Cdks by the DDR. In addition, it has been shown that specific release of the Cdc14B phosphatase from the nucleolus upon DNA damage contributes to the dephosphorylation of Cdh1, promoting APC/C^{Cdh1} formation [103].

Figure 4. Interplay between Plk1 kinase, ubiquitin machinery and DNA damage checkpoint activity. The circuitry in the main figure depicts the crosstalk between APC/C, SCF ligases and the checkpoint. Plk1 is a critical factor in checkpoint recovery, silencing both the ATM and ATR cascades. Plk1 phosphorylates and inhibits both 53BP1 and Plk1 to allow Cdk1 activity. Similarly, Plk1 triggers the SCF^{βTrCP}-dependent destruction of Claspin and FANCM to silence the ATR-Chk1 axis. Plk1 activity may also contribute to the silencing of APC/C^{Cdh1} activity upon replication stress and during recovery from APC/C^{Cdh1} activation during the G2 DDR. In G2 APC/C^{Cdh1} targets Plk1 for degradation, while USP28 prevents it from targeting Claspin. USP28 also stabilizes 53BP1 after DNA damage as well, possibly from APC/C^{Cdh1} (represented by, "?"). Chk1 activation requires Claspin function, which is protected from SCF^{βTrCP}-mediated degradation by USP29 and USP7. USP20 stabilizes both Claspin and Rad17 to promote Chk1 activity, possibly from APC/C^{Cdh1} ("?") as they are both substrates of the ligases. ATR and Chk1 prevent checkpoint recovery by inhibiting the Plk1 activators Aurora and Bora. Irreversible checkpoint activation is prevented by the degradation of active Chk1 by SCF^{Fbx6}. USP7 prevents the complete destabilization of Chk1. The inset shows a potential feedback loop between ATR-Chk1 and the Fanconi pathway. FANCM promotes Chk1 activation (indirectly via ATR). Chk1 promotes FANCM-promoted FANCD2 monoubiquitination. In turn, FANCD2-Ub promotes the CRL4^{Cdt2}-mediated degradation of Chk1. USP1 deubiquitinates FANCD2, stabilizing Chk1. The negative feedback loop favors silencing of Chk1 due to the inactivation of USP1 upon DNA damage.

However, whether this Cdc14B contributes to the G2 checkpoint activity or DNA repair functions of APC/C^{Cdh1} is unclear and could be influenced by cell type [110–112].

Activation of this pool of APC/C^{Cdh1} may not be sufficient to target the majority of APC/C substrates. As dephosphorylated Cdh1 localizes to the nucleus, substrates such as Cyclin B, which are localized in the cytoplasm in G2 would be likely to remain safe from this pool of APC/C^{Cdh1} [113]. How other nuclear APC/C substrates (e.g., Cyclin A) remain stable is an open question. One potential mechanism by which APC/C substrates may evade degradation is via the antagonistic activity of DUBs. Indeed, there is evidence that USP28 activity prevents APC/C^{Cdh1}-mediated degradation of Claspin after DNA damage (Figure 4) [103, 114]. Given the apparently small size of the APC/C pool activated by DNA damage, it is likely that selective targeting of APC/C substrates may also be achieved by specific localization of DNA damage-activated APC/C^{Cdh1}, for example to sites of DNA damage where proteins such as Claspin and Plk1 are expected to be found. This idea remains to be tested, but it is worth noting that APC/C^{Cdh1} is found on chromatin in S-phase and APC/C^{Cdh1}-mediated regulation of Cdc7-Dbf4 activity appears to be via specific targeting of the chromatin-bound fraction of the

kinase [115]. Thus it is possible that the apparent substrate-specificity may be due to limited access to substrates in conjunction with antagonism by DUBs. In keeping with this idea, it is worth noting that Plk1 levels are diminished, but not abolished by damage-activated APC/C^{Cdh1}, perhaps reflecting degradation of a pool of Plk1 in the vicinity of the sites of damage, where phosphorylation of key substrates such as Claspin and 53BP1 will eventually be phosphorylated to promote checkpoint recovery [103, 104]. In addition, 53BP1 has recently been identified as an APC/C substrate and, intriguingly, was also identified as an USP28 substrate raising the possibility that it too may be protected from APC/C at sites of DNA damage, but idea has not been tested (**Figure 4**) [114, 116].

4.2. The replication stress checkpoint

Whereas it is clearly established that APC/C^{Cdh1} controls entry into S-phase, multiple recent lines of evidence suggest that the E3 is also a key regulator of the replication stress response as well. However, in contrast to its role as a positive regulator of the G2 DNA damage checkpoint, APC/C^{Cdh1} appears to be a negative regulator of the replication stress checkpoint, as it targets two critical regulators of the checkpoint, Rad17 and Claspin, which are central to the activation of Chk1 (**Figure 4**) [103, 117, 118]. Indeed, in the absence of Cdh1, failure to degrade Claspin leads to unscheduled Chk1 activation, which is associated with premature S-phase entry [117]. Given the importance of these proteins for the stress response, both UV-irradiation and induction of replication stress by treatment with hydroxyurea lead to the degradation of Cdh1 and, at least in the case of UV, to the stabilization of Rad17 [115, 118]. Activation of Chk1 then feeds back to further enhance its own activation by triggering Cdh1 destruction [115]. Claspin stability is also dependent on Chk1 activity, suggesting that down-regulation of Cdh1 contributes to this arm of a Chk1 auto-amplification loop as well [119]. Notably, Claspin stability is also dependent on context dependent DUB activity as well. ATR activation leads to the stabilization of USP20, which promotes Claspin stability during S-phase [120, 121]. USP20 has also been demonstrated to stabilize Rad17, suggesting perhaps that this DUB may antagonize APC/C^{Cdh1}-mediated destruction of these proteins to promote ATR-Chk1 function (**Figure 4**) [122, 123]. USP9x has also been identified as a DUB for Claspin during replication stress and USP7 has been found to counteract the degradation of Claspin by SCF^{βTrCP}, but not APC/C^{Cdh1}, during replication stress as well [123, 124]. A similar role has been demonstrated for USP29 (**Figure 4**).

The mechanism for Chk1-mediated degradation of Cdh1 is not well-defined, but in the case of HU-induced stress, APC/C-mediated destruction has been implicated [115]. However, degradation induced by UV exposure, which would presumably be augmented by Chk1 as well, involves a region of Cdh1, which is not known to mediate interactions with the APC/C, but does lie between two regions of the Cdh1 N-terminal domain (NTD) that make critical contacts with the APC/C and are negatively regulated by phosphorylation [125, 126]. Thus, Chk1 may directly or indirectly alter the association of Cdh1 with the APC/C to promote its degradation. In addition, the region containing the UV-responsive degron in Cdh1 also encompasses the SCF^{βTrCP} phosphodegron [53, 115, 125]. Notably, while phosphorylation by Plk1 has been identified as critical for creating this phosphodegron there are additional phosphorylation events, mediated by unknown kinases, which contribute to recognition by

SCF^{βTrCP} [53]. It is tempting to speculate that Chk1 directly or indirectly promotes the SCF^{βTrCP}-mediated destruction of Cdh1 as well.

Chk1 itself is also targeted for destruction. Upon activation, Chk1 adopts an open conformation, which exposes degrons that are recognized by SCF^{Fbw6} and CRL4^{Cdt2} ubiquitin ligases (**Figure 4**) [127–129]. DUB activity also plays a role in the maintenance of Chk1 levels. Surprisingly, there are few examples of Chk1 stabilization by DUBs in comparison to their involvement stabilizing Claspin to promote Chk1 activation. To date, only USP7 and USP1 have been implicated in the maintenance of active Chk1 levels. USP7 directly deubiquitinates Chk1 and this activity is enhanced by ATM activation [124, 130, 131]. Whether ATR may also promote USP7-mediated Chk1 activity is not clear. Active Chk1 levels are indirectly protected by USP1 via its ability to antagonize the ubiquitination FANCD2, which induces CRL4-mediated degradation of Chk1 (**Figure 4**) [132]. USP1 is also an APC/C^{Cdh1} substrate adding another level of complexity to the Chk1-Cdh1 feedback loop [133].

The relationship between Chk1 and USP1 also begins to lend some insight into how the feedback loop is faulted to allow checkpoint recovery (**Figure 4**). First, the ATR-Chk1 axis promotes FANCD2 ubiquitination, which would begin to induce down-regulation of active Chk1 [132, 134–136]. Second, USP1 activity is inhibited by multiple mechanisms after UV-damage or the induction of replication stress [137–140]. Thus as the damage or stress-inducing events are resolved and ATR signalling is diminished, active Chk1 becomes susceptible to degradation, which would allow the accumulation of Cdh1 protein. Stabilization of Cdh1 leads to degradation of Rad17 to further inhibit activation of additional Chk1 [118]. Diminished activity of ATR and Chk1 promotes the stabilization of Bora and allows Aurora A activity, respectively, which are critical for Plk1 activation (**Figure 4**) [108, 134, 141]. Plk1, in turn, phosphorylates FANCM and Claspin to promote their SCF^{βTrCP}-mediated degradation to further silence ATR and Chk1 activity, respectively, and further promote loss of Chk1 activity (**Figure 4**) [109, 142]. A key remaining question is how APC/C activity is then restrained to allow normal cell cycle progression. The increase in Plk1 activity also triggers SCF^{βTrCP}-mediated degradation of Wee1, preventing the inhibitory phosphorylation of Cdks [143, 144]. A straightforward mechanistic model is that increased Cdk activity following stabilization of Cdc25A levels and loss of Wee1 promote increased Cdk-mediated inhibitory phosphorylation of Cdh1 to return to normal levels of APC/C^{Cdh1} activity. It is currently unclear, however, why Cdh1 is able reaccumulate during checkpoint recovery despite rising activity of the SCF^{βTrCP}-targeting kinase, Plk1.

5. CRLs and APC/C influence the selection of DSB repair mechanism

Given their many roles in the regulation and execution of checkpoints that monitor the integrity of DNA, it is not surprising that the CRL and APC/C ligases also have roles in regulating DNA repair pathways. SCF^{Fbw7} has been demonstrated to promote NHEJ by catalyzing K63-linked ubiquitination of XRCC4 to enhance its interaction with Ku70/80 complex [74]. However, for the most part the concerted efforts of these ligases do not appear to exert a predominant effect on the decision between NHEJ and HR repair pathways at the moment of damage. Rather their activities appear to promote proper and efficient use of NHEJ and HR.

A critical distinction between these two pathways is the dependency of HR on the resection of the broken DNA end to generate ssDNA that forms a synapse with the template DNA.

APC/C^{Cdh1} activity is required for faithful repair, possibly independent of its checkpoint role. Indeed, APC/C^{Cdh1} regulates multiple components of these pathways. Recently, it was shown that CtIP levels are regulated by APC/C^{Cdh1} upon mitotic exit and after DNA damage, thus limiting the potential for attempting HR in G1, which would likely be mutagenic, and limiting the potential frequency of HR upon damage in G2 (Figure 5) [145]. Interestingly, failure to

Figure 5. Cell cycle ligases set the stage for the selection of NHEJ and HR in G1 and S phases, respectively. During G1 (upper panel) APC/C^{Cdh1} mediates the degradation of pro-HR factors, CtIP, USP37 and RAP80, whereas pro-NHEJ factors MMSET and PR-SET remain stable. Together these factors promote the recruitment of 53BP1 to chromatin favoring NHEJ. The anti-HR factor FBH1 also remains stable. During S-phase (lower panel) replication coupled, CRL4^{Cdt2}-mediated degradation of FBH1 favors HR. Similarly, CRL4^{Cdt2} targets the methyltransferase MMSET2 and PR-SET7 for destruction. PR-SET7 is also targeted for destruction by SCF^{Skp2} and SCF^{βTrCP}. This destruction hinders the recruitment of 53BP1 to chromatin favoring HR. Finally, Cdk activity, positively regulated by SCF^{Skp2} and the downregulation of APC/C^{Cdh1} activity in S-phase further promotes the use of HR. The activities of SCF^{Skp2} and SCF^{Fbx044} limit BRCA1-CtIP-mediated resection are depicted in both phases, although the cell cycle-dependence of these events is not clear. Similarly, the potential for APC/C^{Cdh1}-mediated regulation of PR-SET7 is depicted, but remains unclear.

down-regulate CtIP levels by APC/C^{Cdh1} leads to increased resection and inefficient repair, potentially due to interference with the use of NHEJ as well [145]. Although it remains to be tested, it would stand to reason that degradation of Cdh1 upon replication stress may also lead to enhanced stabilization of CtIP to promote HR. In contrast to limiting resection by targeting CtIP, APC/C^{Cdh1} also targets the HR-limiting factor RAP80, which localizes BRCA1 to regions flanking DSB in an ubiquitin-dependent manner, but represses BRCA1-mediated HR [88, 146]. APC/C-mediated destruction limits RAP80 expression during G1, presumably to diminish competition for H2AK15-Ub binding with 53BP1 at DSBs to promote the use of NHEJ in the absence of a homologous template (**Figure 5**). During S and G2, BRCA1-dependent HR is thought to involve the degradation of RAP80 and, although the activation of APC/C^{Cdh1} by DSBs in G2 suggests that it may be, it remains to be determined whether the APC/C^{Cdh1} is involved in this destruction event. In addition, APC/C^{Cdh1} and SCF^{βTrCP} cooperate to limit the expression of USP37 to S-phase and early G2 (**Figure 5**). USP37, along with the related USP26, has been shown to antagonize RAP80 to promote BRCA1-dependent HR [147–149]. SCF^{Skp2} is also required for efficient HR, in part via promotion of checkpoint signaling [54]. In addition, SCF^{Skp2} and SCF^{Fbxo44} ubiquitinate BRCA1 to control the extent of resection (**Figure 5**) [150]. The balance of CRL3^{Keap} and USP11 activities also regulates HR by targeting PALB2 (**Figure 2**) [151]. CRL4^{Cdt2} catalyzes the degradation of FBH1, which negatively regulates Rad51 function to limit HR prior to replication-dependent generation of the template. Interestingly, the interaction of FBH1 with PCNA may promote the use of TLS [152–156]. The APC/C may also contribute to the use of HR by antagonizing the expression of the NHEJ-promoting protein 53BP1 [116]. However, it is not clear whether APC/C impacts NHEJ activation via regulation 53BP1. Interestingly, a proteomic screen identified several additional pro-NHEJ factors in association with APC/C^{Cdh1} [145]. Yet, it remains to be determined whether these are substrates of the ligase.

In addition to restricting the use of HR to S-phase and G2 by regulating the levels of key HR factors to these phases, the coordinated efforts of APC/C and CRL ligases also limit the use of NHEJ during S-phase. The methyltransferases PR-SET7 and MMSET promote NHEJ by directing recruitment methylating H4K20 to recruit 53BP1 (**Figure 5**) [62–65]. Whereas global H4K20 methylation is not significantly altered by the induction of DSBs, de novo methylation of H4K20 at sites of damage has been demonstrated to mediate recruit 53BP1 and promote NHEJ. Importantly, histones deposited during replication lack H4K20 methylation. Multiple ligases, APC/C^{Cdh1}, SCF^{βTrCP}, SCF^{Skp2}, and CRL4^{Cdt2} restrict expression and activity of the methyltransferase PR-Set7 to G2, mitosis, and early G1 (**Figure 5**) [66, 157–161]. In addition, CRL4^{Cdt2} targets MMSET for replication-coupled degradation (**Figure 5**) [162]. Thus, with little capacity to generate NHEJ promoting methylation marks, DSBs occurring in S-phase, and likely early G2 as well, are not permissive for the recruitment of NHEJ factors allowing relatively uncontested access to the damaged sites by the HR machinery. In addition, the deposition of histones lacking H4K20 methylation marks in newly replicated DNA recruits MMS22L-TONSL complex, which directly promotes HR (**Figure 5**) [163–166]. Finally, the window of kinase activities, (cyclin-Cdk activity in particular) opened to promote the transition into and through S-phase also catalyze the phosphorylation of multiple components of the HR machinery, which promote the activity of this pathway [77, 78, 167, 168].

6. Conclusion

For many years, the importance of the CRL and APC/C ligases in cancer and genome stability has been appreciated. It was long thought that these roles were attributed to their ability to control cell cycle transitions, particularly their abilities to regulate one another. As discussed herein, we have more recently begun to elucidate that these ligases possess more direct, highly regulated and interconnected roles in the response to and repair of DNA damage as well.

While alterations in the mechanisms controlling genome stability lead to disease such as cancer, the induction of DNA damage is a tested and potent anti-cancer strategy. Moreover, manipulating these pathways has obvious therapeutic potential. Indeed, recent advances in inhibitors of DNA checkpoint and repair proteins (e.g., Chk1) suggest that manipulating the DDR response offers a therapeutic advantage over DNA damage based therapies alone. However, these strategies have faced challenges in translation. As we move ever closer to the realization of personalized medicine, it is of increasing importance that we understand not only the full cadre of players in a given pathway, but also those regulating it as well. Only with this knowledge can we fully appreciate the impact of altering that pathway, whether in dissecting pathophysiological changes of disease or in the development of potential therapeutic manipulations. We are increasingly successful in targeting components of the ubiquitin proteasome system and there are now small molecules capable of inhibiting specific SCF complexes with potential for substrate specificity. Similar accomplishments have been made in the targeting of the APC/C as well as DUBs, including USP1 and USP7. Finally, while we have focused on the role of these ligases in the major responses to DNA damage and the impact they have on DSB repair, there is mounting evidence that the activities of these enzymes impact multiple damage response and repair pathways. Thus, as we increase our understanding of the how these components of the ubiquitin machinery impact the choice and efficient use of DNA repair mechanisms we also increase our opportunities for improved therapeutic options.

Acknowledgements

We thank Monica Venere for helpful discussions. This work was supported by NIH grants RO1GM112895 and RO1GM108743, to MKS.

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