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Review

Roles of sequential ubiquitination of PCNA in DNA-damage tolerance

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ABSTRACT

Living organisms not only repair DNA damage induced by environmental agents and endogenous cellular metabolites, but have also developed mechanisms to survive in the presence of otherwise lethal lesions. DNA-damage tolerance (DDT) is considered such a mechanism that resumes DNA synthesis in the presence of replication-blocking lesions. Recent studies revealed that DDT in budding yeast is achieved through sequential ubiquitination of DNA polymerase processivity factor, proliferating cell nuclear antigen (PCNA). It is generally believed that monoubiquitinated PCNA promotes translesion DNA synthesis, whereas polyubiquitinated PCNA mediates an error-free mode of lesion bypass. This review will discuss how ubiquitinated PCNA modulates different means of lesion bypass.

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1. Introduction

Proliferating cell nuclear antigen (PCNA) is an auxiliary factor of DNA polymerases and forms the eukaryotic DNA sliding clamp. Due to its cyclical expression profile during the different phases of the cell cycle, PCNA was initially named cyclin to indicate its putative role in regulating cell proliferation [1–3]. PCNA is characterized by its trimeric ring-shaped structure [4]. Three PCNA monomers are associated to form a closed ring consisting of two sides. The topologically identical N and C termini of PCNA monomer are connected on one side while the other side contains several β sheets linked by loops [4]. The ring-shaped structure of PCNA is evolutionarily conserved and belongs to the family of β-clamps [4,5]. Rich in lysine and arginine residues, the inner ring of PCNA is positively charged, which allows for the effective encircling around the negatively charged duplex DNA. Recent studies reveal that PCNA is not only essential for replication in eukaryotes, but also plays critical roles in several DNA damage-responsive pathways [6]. In this review, we summarize recent advances in the understanding of how mono- and polyubiquitinated PCNA functions in DNA-damage tolerance (DDT) in eukaryotes.

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2. PCNA and DNA replication

PCNA is a DNA polymerase processivity factor. It functions as a loading scaffold for the replication machinery through association with various replication-related factors [6]. Three conserved peptide sequences have been identified to mediate the interaction of proteins with PCNA, including the PCNA-interacting peptide (PIP box) [7], the KA box [8], and the ABH2 PCNA-interacting motif (APIM) [9]. During DNA replication, the chaperonin-like replication factor C (RFC) binds to the RNA primer-DNA template junction and loads PCNA onto DNA [10,11]. Upon PCNA loading, Polα is released and Polε is loaded to mediate leading-strand elongation [12]. For the discontinuous lagging strand, firstly the short Okazaki fragments have to be produced by Polα and Polδ [13]. Next, the initiator RNA sequences are removed from the newly synthesized Okazaki fragments by Flap structure-specific endonuclease-1 (FEN-1) [14]. The processed Okazaki fragments are then ligated via DNA ligase I. During this process, PCNA can directly interact with Polδ to stimulate its enzymatic activity while it encircles DNA [15–17]. Similarly, PCNA can also interact with Polε, FEN-1 and DNA ligase I to stabilize their association with DNA and increase their enzymatic activities [18–21].

3. DNA damage and PCNA ubiquitination

Living organisms are constantly challenged by various sources of DNA damage. Environmental agents including radiation and

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chemical mutagens, and endogenous cellular metabolites can cause DNA damage [22]. Some types of DNA damage, such as UV irradiation-induced lesions, may result in interference with DNA replication. This is due to the failure of the highly stringent replicative DNA polymerases to accommodate modified DNA template, resulting in the blockade of the progression of the DNA replication fork. Under normal conditions, most DNA lesions can be removed by DNA repair pathways such as nucleotide excision repair and base excision repair. However, failure in lesion correction by these pathways prior to S phase in the cell cycle could pose severe consequences leading to genome instability or even cell death. Cells have evolved sophisticated lesion-bypass mechanisms to deal with this threat and ensure survival by allowing DNA synthesis in the presence of replication-blocking lesions. These lesion-bypass pathways in the budding yeast *Saccharomyces cerevisiae* belong to the *RAD6* epistasis group [23–25] and have been classified as error-prone translesion synthesis (TLS), error-free TLS and error-free postreplication repair [26] or DNA damage tolerance (DDT). Interestingly, all three bypass pathways require PCNA, and different covalent modifications of PCNA by ubiquitin (Ub) or a small Ub-like modifier (SUMO) determine which tolerance pathway will be utilized in the face of unrepaired lesions [27].

Ubiquitination is a chemical process by which Ub is covalently attached to the Lys residue of a target protein by three enzymes: Ub-activating enzyme (Uba or E1), Ub-conjugating enzyme (Ubc or E2) and Ub ligase (E3) [28]. Substrate proteins can be modified by a Ub monomer either at one Lys residue (monoubiquitination) or multiple Lys residues (multi-monoubiquitination). Proteins can also be modified by a Ub chain where Ub moieties sequentially link to a previous Ub (polyubiquitination) [29]. However, whether monoubiquitination is the prerequisite for polyubiquitination remains unclear. Although all 7 Lys residues (K6, K11, K27, K29, K33, K48 and K63) in Ub have been shown capable of forming poly-Ub chains [30,31], the physiological significance of some poly-Ub chains in living cells is not fully understood. The most characterized function of Ub modification is the K48-linked poly-Ub chain that targets proteins for degradation by the 26S proteasome [29]. On the other hand, the non-canonical K63-linked poly-Ub chain plays a role in regulating various signaling pathways largely in a proteolysis-independent manner [32]. A paradigm of DNA-damage response through covalent modifications of PCNA was discovered by Stefan Jentsch and his colleagues [33], in which PCNA can be either monoubiquitinated by the E2-E3 complex Rad6–Rad18 at the K164 residue or further modified with K63-linked Ub chain by another E2-E3 complex, Mms2-Ubc13-Rad5 (Fig. 1). Recently, the stepwise PCNA monoubiquitination and polyubiquitination by the two complexes was reconstituted in vitro [34], further confirming the above genetic model. In addition, the same K164 residue of PCNA can also be sumoylated by yet another E2-E3 complex, Ubc9-Siz1 [33,35].

3.1. PCNA monoubiquitination

Rad6 is a multi-functional E2 [36] and its role in DNA-damage response is dependent on its physical interaction with Rad18, a RING finger-containing E3 ligase [37,38]. Several observations provide strong evidence that the Rad6–Rad18 complex is recruited to the stalled replication site to monoubiquitinate PCNA. Firstly, in both yeast and mammalian cells, Rad18 is capable of binding to single-stranded DNA (ssDNA), and this process appears to be mediated by ssDNA-binding replication protein A (RPA) [37,39]. Secondly, Rad18 can form a tight complex with Rad6 through its Rad6-binding domain (R6BD) (residues 371–410 in yRad18 [40] and 340–395 in hRad18 [41]), although the N-terminal RING-finger domain may also independently bind Rad6 [42,43]. Thirdly, purified human Rad18 and Rad6B can efficiently monoubiquitinate PCNA in vitro [41] and ectopic over-expression of *hRAD18* induces PCNA monoubiquitination [44]. Finally, *RAD18* deletion in human HCT116 cells results in the failure of PCNA monoubiquitination in response to DNA damage [45], and Rad6A/6B depletion by siRNA dramatically reduced monoubiquitinated PCNA in human cells [41].

PCNA ubiquitination can be induced by various DNA-damaging agents, such as UV irradiation, methyl methanesulfonate (MMS), mitomycin C (MMC), hydroxyurea (HU) and the bulky adduct-forming genotoxin benzo[*a*]pyrene dihydrodiol epoxide (BPDE) [44,46]. In addition, both types of UV irradiation-generated DNA lesions, cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP), are capable of inducing PCNA monoubiquitination [46]. In contrast, agents that generate double-stranded breaks and block the cell cycle without stalling DNA replication forks, such as bleomycin, camptothecin, nocodazole or ionizing radiation, do not induce PCNA monoubiquitination [46]. This observation is consistent with a model that PCNA monoubiquitination is dependent on Rad18 to recognize ssDNA [37]. Notably, ionizing irradiation results in inconsistent response in different organisms. For example, PCNA ubiquitination is detected in *Schizosaccharomyces pombe* but not in mammalian cells when subjected to ionizing radiation [46–48]. This discrepancy is probably due to the exceptional resistance of *S. pombe* to ionizing radiation that allows very high doses to be used, at which a wide spectrum of DNA lesions may be induced [22].

Replication stresses and spontaneous DNA damage may also induce PCNA ubiquitination. A classic example is that deletion of *POL32*, encoding the non-essential subunit of Polδ in budding yeast, induces PCNA ubiquitination [49]. Monoubiquitinated PCNA was also detected in undamaged cells [50,51], probably due to the high frequency of spontaneous DNA damage or genetic defects in certain types of cultured cells.

In addition to DNA damage and replication stress, loading of the PCNA homotrimeric ring onto DNA by RFC is a prerequisite for PCNA ubiquitination [52]. Cell-cycle checkpoint signaling pathways are also involved in regulating PCNA ubiquitination. For example, one report indicates that in BPDE-treated human cells, monoubiquitination of PCNA is regulated by the ATR/Chk1 signaling pathway [44]. However, reports from several other groups suggest that PCNA monoubiquitination is independent of ATR, but requires Chk1, Claspin, and Timeless [46,53,54]. Although it was suggested that these factors function in stabilizing stalled replication forks in response to DNA damage, the precise mechanism underlying their regulation of PCNA ubiquitination remains unknown. Tumor suppressor proteins p53 and p21 are also involved in regulating PCNA ubiquitination, probably acting as repressors to inhibit ubiquitin modification of PCNA [55–58].

Monoubiquitination is a reversible process [59]. A class of proteases, also known as deubiquitinating enzymes (DUBs), function as negative regulators by removing the Ub tag from modified proteins. Ubiquitin-specific protease 1 (Usp1) is responsible for deubiquitinating monoubiquitinated PCNA [60]. A high dose of UV irradiation diminishes the level of Usp1 through autocleavage, which correlates with the increase of monoubiquitinated PCNA [46,60,61]. Depleting Usp1 in HeLa cells by siRNA knockdown results in an elevation of monoubiquitinated PCNA upon UV irradiation [51]. These findings may provide an explanation for UV-induced accumulation of monoubiquitinated PCNA. However, the Usp1 loss concomitant with the increased PCNA monoubiquitination was not detected in MMS- or MMC-treated mammalian cells [46]. One possible explanation for this observation is that different DNA-damaging agents employ distinct mechanisms to regulate Usp1 through differentially regulating its partners, such as UAF1 [61]. Interestingly, a significant increase in monoubiquitinated PCNA was also detected in the absence of damage in

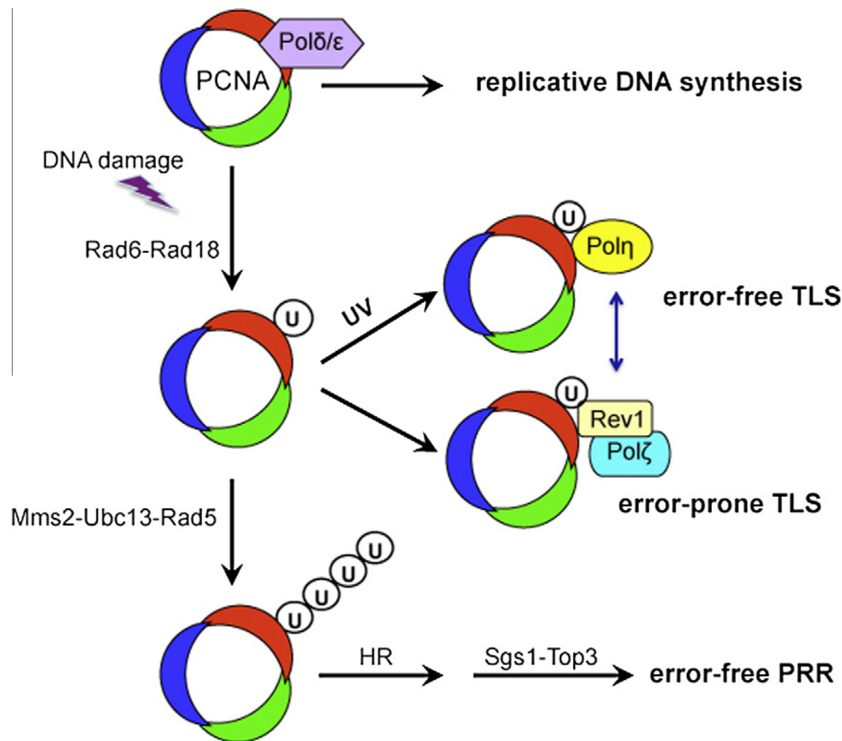

Fig. 1. The role of PCNA in replication and DNA-damage tolerance. PCNA is involved in genomic DNA synthesis as a loading scaffold for the replication machinery. Upon DNA damage, the Rad6-Rad18 ubiquitination complex mediates at least three different lesion bypass pathways, namely Polζ-dependent mutagenic TLS, Polη-dependent error-free TLS and Mms2-Ubc13-Rad5-dependent error-free postreplication repair. All three lesion-bypass pathways appear to require Ub-modified PCNA. Physical and genetic interactions between the two TLS pathways are indicated by a double arrow. This model is primarily based on studies in yeast.

Usp1-depleted cells [51]. This finding demonstrates the important role of Usp1 in repressing PCNA monoubiquitination in normal human cells. Whether Usp1 is responsible for deubiquitinating polyubiquitinated PCNA remains to be determined, although one study did detect an elevated PCNA polyubiquitination upon Usp1 depletion [51].

3.2. PCNA polyubiquitination

PCNA modification with K63-linked Ub chain is mediated by the Mms2-Ubc13-Rad5 complex [33]. Mms2 is a member of the Ubc variant (UEV) family of proteins [62] and shares significant sequence similarity with other Ubcs [63]. Due to the lack of the active Cys residue, Mms2 does not possess Ub-conjugating activity [64]. Ubc13 is the only identified E2 to mediate PCNA ubiquitination with K63-linked poly-Ub chain; this enzymatic activity requires UEV as a co-factor [65]. Rad5 belongs to the SWI/SNF superfamily, and it functions as a RING-domain-containing E3 in the process of PCNA polyubiquitination [33]. The notion that both Rad6–Rad18 and Ubc13-Mms2-Rad5 complexes are required for PCNA polyubiquitination in yeast is supported by several lines of evidence. First of all, PCNA interacts with both Rad5 and Rad18. Moreover, deletion of *RAD6* abolishes not only monoubiquitination but also polyubiquitination on PCNA upon DNA damage [33]. Furthermore, polyubiquitinated PCNA is absent in *UBC13*-, *MMS2*- or RAD5-mutated yeast cells treated with DNA-damaging agents, but monoubiquitinated PCNA remains [33]. Structural analyses revealed the stable Ubc13-Mms2 complex formation through hydrophobic interaction [66,67] and the site-specific mutagenesis analyses of the interface residues revealed the molecular basis by which Mms2 interacts specifically with Ubc13 but not other Ubcs [68]. More interestingly, NMR studies discovered the non-covalent interaction between Mms2 and Ub that facilitates K63-linked di-Ub chain formation with the Ubc13-Ub thiolester [64]. Since functional human homologues of yeast Rad5 [69,70], Ubc13 [71] and Mms2 [62,72,73] have been identified, and polyubiquitinated PCNA is detected in cultured mammalian cells [51,74], it is generally believed that PCNA polyubiquitination is conserved throughout the eukaryote kingdom.

4. Roles of PCNA ubiquitination in DNA-damage tolerance

DNA-damage tolerance (DDT) is a molecular mechanism that does not restore damaged DNA to the correct sequence but allows completion of DNA replication across damaged DNA. A group of specialized DNA polymerases, termed TLS polymerases, is responsible for the DDT process. These specialized polymerases are capable of accommodating damaged DNA sites [75], and are evolutionarily conserved between prokaryotes and eukaryotes [76]. Most TLS polymerases belong to the Y family of DNA polymerases although some A, B and X family polymerases are also able to mediate TLS in certain circumstances [77,78]. These Y-family polymerases exhibit relaxed active sites for DNA distortions and are capable of accommodating damaged DNA and mediating nucleotide insertion opposite lesions. However, they do not have the $3' \rightarrow 5'$ proofreading exonuclease activity associated with replicative polymerases; hence, the replication fidelity is relatively low [75,79]. In mammals, four Y-family TLS polymerases are known, including polymerase eta (Polη, Rad30 in yeast), polymerase kappa (Polκ), polymerase iota (Polι) and Rev1 [79,80]. Interestingly, all of these polymerases contain one or two Ub-binding domains (UBM or UBZ), which are involved in mediating their interaction with ubiquitinated PCNA [81], and three of them, including Polη, Polκ and Polι, also contain PIP box and Rev1 binding domains [82–85] (Fig. 2). On the other hand, Rev1 interacts with PCNA via its

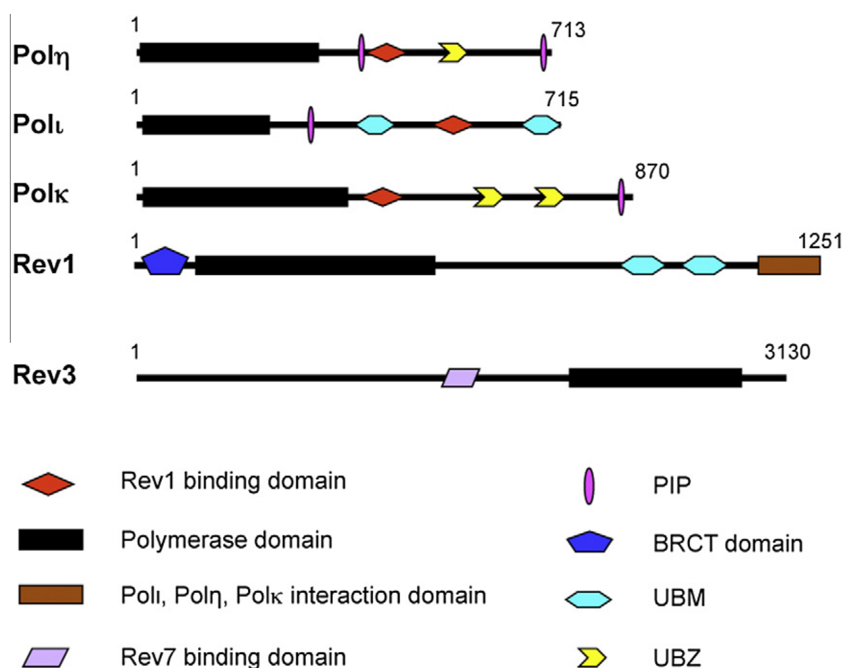

Fig. 2. Schematic diagram illustrating the functional domains of human TLS polymerases. Domains are depicted as boxes with different shapes and colors. Numbers indicate the protein length. Note that Rev3 is not in scale with other Y-family polymerases, and that little is known about its functional domains other than a B-family polymerase domain and a Rev7-binding domain. UBM, Ub-binding motif; UBZ, Ub-binding zinc finger; PIP, PCNA interacting peptide; BRCT, BRCA1 C-terminal domain.

N-terminal BRCT domain [86], and with other TLS polymerases as well as Rev7 through its C-terminus [87–89].

In spite of the relaxed active sites, TLS polymerases exhibit substrate specificity, and different polymerases may be responsible for specific lesions. For example, Polη mediates error-free TLS to bypass UV-induced CPDs [90–92]. Polι cannot mediate *cis-syn* T<>T dimer bypass; however, it efficiently inserts deoxynucleotides opposite 6-4 PP and abasic sites [93,94]. Polζ can read through different types of lesions, but with poor efficiency in nucleotide insertion, whereas it is efficient in nucleotide extension [93,95].

The DDT pathways can be either mutagenic or error-free, which is dependent on the type of PCNA ubiquitination as well as the polymerases employed. The currently established model is that Ub-modified PCNA mediates a switch from replicative DNA polymerases to TLS polymerases in response to DNA damage, and different types of PCNA ubiquitination confer distinct responses: monoubiquitinated PCNA promotes Polζ-dependent (mutagenic) or Polη-dependent (error-free) TLS, whereas polyubiquitinated PCNA promotes Mms2-Ubc13-Rad5-dependent error-free DDT [33,35,96]. The critical role of PCNA ubiquitination in DDT was initially reported in 2002 [33] and subsequently confirmed and extended to form a paradigm model of DDT (Fig. 1).

4.1. Polη-dependent error-free TLS

Compared with other TLS polymerases, Polη is the most efficient TLS polymerase capable of reading through UV-induced T<>T dimers in a largely error-free manner [97–99]. Yeast *rad30* mutant cells display an increased sensitivity to UV irradiation [100,101]. Human cells derived from xeroderma pigmentosum variant (XP-V) patients contain a defective mutation in *XPV/POLH*, indicating that Polη plays a critical role in preventing onset or growth of human cancers through promoting error-free TLS in response to UV irradiation [90,102]. The underlying mechanism is that in the absence of Polη, cells tend to utilize less efficient and highly mutagenic means of lesion bypass [78,103].

It is of great interest to understand whether other Y-family polymerases also have preferred substrates to mediate an error-free mode of TLS. To this end, a recent study showed that Polκ and Polζ coordinated to promote error-free bypass of thymine glycol-induced lesions with much higher efficiency than Polη [104].

The activity of Polη is dependent on monoubiquitinated PCNA, which orchestrates the switch between replicative DNA polymerases and Polη at the lesion sites and possibly stimulates Polη's enzymatic activity [105]. One mechanism underlying this switch is that the conserved Ub-binding domain of Polη enhances its binding to monoubiquitinated PCNA [81]. In response to DNA damage, Polη forms nuclear foci [106,107] that co-localize with PCNA [83,107] and other Y-family polymerases [48,108] at the lesion sites in a Rad18-dependent manner [41]. In an affinity-pulldown experiment, Polη preferentially binds monoubiquitinated PCNA from UV-irradiated HeLa cell extract whereas Polδ does not [41]. Interestingly, the PCNA-K164R mutation leads to a much higher UV sensitivity than Polη depletion [46], demonstrating the indispensability of PCNA ubiquitination in response to UV irradiation. This may be attributed to two factors. Firstly, some other TLS polymerases may play redundant roles with Polη [103]. This notion is supported by findings that Rev1 co-localizes with Polη at DNA-damage sites in mammalian cells [48,108] and that different TLS polymerases compete for binding with Rev1 in vitro [87]. Secondly, PCNA can also be polyubiquitinated at the same residue, which may lead to an error-free mode of lesion bypass [33].

4.2. Polζ-dependent mutagenic TLS

Polζ belongs to the B family of polymerases and consists of two subunits, Rev3 and Rev7 [109]. Different from Polη, Polζ was identified as capable of mediating mutagenic DNA synthesis that bypasses UV-induced CPDs [109]. The central role of Polζ in error-prone TLS was further confirmed by examining individual yeast TLS polymerase mutants, in which the *rev3* mutation is epistatic to *rev7* and *rev1*, and is absolutely required for UV-induced mutagenesis [110]. Human Rev3 protein is twice the size (352 kDa) of the yeast homolog (Fig. 2), and the two proteins are only 29% identical in sequence [111]. Nevertheless, antisense suppression of *REV3* in cultured human cells abolished UV-induced mutagenesis,

similar to what is observed in yeast [111]. The indispensability of Polζ in mutagenesis can be explained by the unique role of Polζ as the mispair extender during TLS, while the role of translesion insertion can be served by multiple redundant TLS polymerases [93]. This two-polymerase TLS model becomes a core component of the polymerase switch model [112].

4.3. Regulation of TLS by monoubiquitinated PCNA

Ubiquitinated PCNA is involved in regulating mutagenic TLS through coordinating with different TLS polymerases. For example, Rev1 requires ubiquitinated PCNA to bypass lesions induced by various DNA-damaging agents, such as MMS, 4NQO, and UV irradiation [113]. Rev1 is capable of directly binding to unmodified and monoubiquitinated PCNA through its N-terminal BRCA1 C-terminal (BRCT) domain and C-terminal ubiquitin-binding motifs (UBMs) (Fig. 2), and its affinity to monoubiquitinated PCNA is higher than to unmodified PCNA [86,113]. Moreover, monoubiquitinated PCNA promotes Rev1-mediated DNA synthesis across an abasic site with about fivefold higher efficiency than unmodified PCNA [52]. It was found that mutation in the UBMs, but not the BRCT domain, abolished the focus formation with monoubiquitinated PCNA at the DNA-damage site, suggesting that UBMs play a unique role in the TLS process [113]. On the other hand, the Rev1 enzymatic activity is dispensable for TLS [95,114], suggesting that Rev1 serves as a scaffold to mediate protein interactions in TLS. Indeed, the C-terminal 100 amino acids of Rev1 is sufficient to interact with Polη, Polι, Polκ as well as Rev7 [82–85], and a Rev1-interacting motif has been recently defined [115] (Fig. 2). In undamaged human cells, *RAD18* over-expression alone is sufficient to induce an interaction between monoubiquitinated PCNA and Polκ [44]. Whether this interaction requires additional factors such as Rev1 remains unclear.

Direct support of the critical role of PCNA ubiquitination in TLS came from the creation and characterization of the PCNA-K164R mutants. In budding yeasts, PCNA ubiquitination is essential for the DNA-damage tolerance pathway, as demonstrated by the observation that the *pol30-K164R* mutation is epistatic to mutations in all members of the error-prone and error-free lesion bypass pathways [33]. In the chicken DT40 B cell line, the $PCNA^{K164R}$ mutation renders cells hypersensitive to DNA-damaging agents and reduces hypermutation at the Ig locus [116], In two independent $PCNA^{K164R}$ transgenic mouse models, selected reduction of A/T mutations in B-cell Ig genes were observed [117,118], which is consistent with the hypothesis that during somatic hypermutation, PCNA ubiquitination is required for the recruitment of Polη and Polκ.

Notably, the model that monoubiquitinated PCNA promotes mutagenic TLS has been challenged by several observations. Firstly, in budding yeast, whether the Polη Ub-binding motif plays a role in cellular response to UV irradiation in vivo [119,120] and whether monoubiquitinated PCNA stimulates TLS polymerase activity in vitro [52,121,122] have been subjected to debate. In vitro reconstitution of the yeast DNA synthesis reaction demonstrated that PCNA monoubiquitinated on all three monomers does not enhance affinity for or stimulate the TLS activity of Y-family polymerases [122]. Secondly, human Polη contains a major and a minor PIP motif; inactivation of both motifs abolishes its PCNA-binding activity, its accumulation in UV-induced nuclear foci as well as the stimulation of DNA synthesis by PCNA. In contrast, mutations in the UBZ domain have no adverse effect in the above assays [84]. The physical interaction between Polη and PCNA is further complicated by a recent finding that Polη itself can be monoubiquitinated at a C-terminal nuclear localization signal (NLS) region which directly interacts with PCNA, and that its monoubiquitination decreases after UV irradiation [123]. Thirdly, monoubiquitinated PCNA does not seem to be crucial to mutagenic TLS in chicken DT40 cells. Elevated PCNA monoubiquitination does not result in the induction of mutagenesis upon DNA damage, indicating that PCNA monoubiquitination may not be sufficient for mutagenic TLS induction [124]. Although the $PCNA^{K164R}$ mutation results in an increased sensitivity to DNA-damaging agents and decreased mutagenesis at the Ig locus and is epistatic to *rad18*, it is not epistatic to *rev1* mutation [116,125], nor is *rad18* epistatic to *polK* [126] or *rev1* [127] in response to DNA damage. The *rev1 PCNA-K164R* double mutant displays sixfold and eightfold decrease in mutagenesis in comparison with the corresponding single mutants, suggesting that roles of Rev1 and monoubiquitinated PCNA do not largely overlap, at least in DT40 cells [116]. Consistent with this notion, it was reported that in DT40 cells, PCNA monoubiquitination and Rev1 function in distinct steps of lesion bypass [125]. Together these findings indicate that covalent modifications of Y-family polymerases influence their association with PCNA and/or monoubiquitinated PCNA and that the role of PCNA monoubiquitination is dispensable in mutagenic TLS in certain eukaryotic organisms.

Recently, a strategy of artificially fusing PCNA with Ub was utilized to study the role of monoubiquitinated PCNA in DNA-damage tolerance. In this strategy, a K164R-mutated PCNA is fused to a Ub at either the N- or C-terminus and expressed in yeast cells. As expected, expression of the PCNA-Ub fusion genes led to elevated resistance to UV and MMS compared to the PCNA-K164R transformant; however, conflicting results have been reported as to whether this resistance is dependent on TLS polymerases [120,128,129].

4.4. Rad5-mediated error-free lesion bypass pathway

The error-free lesion bypass pathway is thought to utilize undamaged sister chromatid as the template to carry out limited DNA replication [25]. It is also responsible for cellular resistance to chronic low-dose UV treatment in yeast cells [130]. Although detailed molecular events underlying this pathway are not well understood, extensive genetic studies in yeast have identified several genes that play central roles in this pathway, including *MMS2, UBC13* and *RAD5* [63,65,131,132].

Rad5 is a multi-functional protein. Mutations either in the Rad5 ATPase or RING domain cause elevated UV sensitivity [133,134], suggesting that both the ATPase and ubiquitin ligase activities of Rad5 are required for DNA-damage tolerance. Rad5 interacts with both Rad18 and Ubc13 [132] as well as PCNA [33], and these interactions recruit the Ubc13-Mms2 complex to the Rad6–Rad18 complex at the DNA-damage site [132]. It can be envisaged that the recruited Ubc13-Mms2 complex replaces Rad6 to mediate K63-linked poly-Ub chain formation on a monoubiquitinated PCNA substrate. Mutations in either *MMS2* or *UBC13* result in defective error-free PRR comparable to that of a Ub-K63R substitution mutation [63,65,131,135]. Ultimate evidence supporting the role of K63-linked polyubiquitin chain-modified PCNA in the error-free lesion bypass pathway comes from direct demonstration that deletion of *RAD5, UBC13* or *MMS2* abolishes DNA damage-induced PCNA poly-Ub chain formation without affecting its monoubiquitination [33].

Polyubiquitinated PCNA was also detected in cultured mammalian cells upon UV irradiation [74]. Similar to yeast, human cells also employ K63-linked poly-Ub chain-modified PCNA to protect cells from mutagenesis [74]. Furthermore, two mammalian Rad5 homologs, SHPRH [69,70] and HLTF [72,73], appear to be required for such a poly-Ub chain formation in cultured human cells. In contrast, deletion of both *Hltf* and *Shprh* genes in a transgenic mouse model does not affect PCNA ubiquitination at the K164 residue [136], suggesting the existence of an alternative E3 ligase.

Meanwhile, it is noticed that others have reported failure in the detection of polyubiquitinated PCNA in UV-, HU- [48] or MMS-treated human cells [33]. Thus, whether polyubiquitination of PCNA is universally required for DDT in mammalian cells remains to be elucidated.

Although the detailed mechanism underlying how the PCNA poly-Ub signals error-free lesion bypass remains unclear, it was proposed that polyubiquitinated PCNA coordinates with Rad5 to disrupt the activity of replication inhibitors or replicative polymerase and thus allows error-free DNA synthesis in the presence of replication-blocking lesions [133]. This may be achieved by one of two events [25]. Replication fork regression followed by nascent strand annealing and DNA synthesis (a chicken-foot model) gained support by the demonstration that the Rad5 helicase activity is required for fork regression in vitro [137]. On the other hand, it is generally believed that error-free lesion bypass is mediated by template switch and Holliday junction resolution. A recent study [138] demonstrates that the completion of yeast error-free PRR requires the homologous recombination complex including Rad51, Rad52 and Rad54, as well as the Sgs1 helicase, which provides strong support for the template switch model [139]. It should be noted that the above two models are not necessarily mutually exclusive. It remains plausible that fork regression followed by sister chromatid invasion and resolution may allow for error-free bypass. Alternatively, error-free PRR may employ two parallel modes of lesion bypass.

4.5. Regulation of different modes of lesion bypass

One interesting question is how eukaryotes manage the mutagenic TLS and error-free bypass process when facing DNA damage. Clearly different lesions induced by DNA-damaging agents may play a critical role in determining the means of lesion bypass. For example, Polη is a preferred error-free bypass pathway for UV-induced thymine dimers, but it plays little if any role in bypassing other types of DNA damage. In mammalian cells, it has been debated whether Polη and Polζ constitute two separate TLS pathways, since conflicting observations have been reported as to whether the Rev1 nuclear focus formation is dependent on Polη [48,108]. In chicken DT40 cells, deletion of the *POL*η gene rescues the severe sensitivity to a broad range of DNA-damaging agents as well as a growth defect in the *pol*ζ null cells [140], which is apparently different from that of yeast [100] and mammalian cells [103].

Early studies have clearly demonstrated that yeast Polζ and Mms2-Ubc13 mediate two alternative pathways and respond to a broad range of DNA damage; simultaneous inactivation of the two pathways results in very strong (10^3–10^4 fold) synergistic effects [63,131,135]. Surprisingly, it was recently found that Rad5 is also required for Polζ-dependent TLS [141], suggesting a cross-talk between TLS and error-free PRR pathways.

5. Perspective

Investigation of roles of PCNA ubiquitination in DDT is critical to our understanding of cellular responses to DNA damage and to correlate with disease development, particularly cancer. Recent years have witnessed great advances on this front; however, a number of questions remain to be answered. For example, what determines the ubiquitination mode of PCNA upon DNA damage, and how does polyubiquitinated PCNA mediate the error-free DDT process? Although sequential PCNA ubiquitinations have been reconstituted in vitro [34], a reliable in vivo model to study PCNA-mediated DDT remains elusive. Nevertheless, by demonstrating in a yeast model that RAD18-mediated DDT can function in G2 [49] and be separated from genome replication [142], recent studies have successfully challenged a conventional belief that DDT acts at the replication fork and suggest that the postreplicative ssDNA gaps may be preferred substrates for DDT.

Surprisingly, the PCNA-K164 residue is not the only site for ubiquitination. It was recently reported [143] that yeast PCNA can be ubiquitinated at the K107 residue in response to DNA ligase I deficiency, and that this ubiquitination is dependent on Rad5, Mms2 and Ubc4, but independent of Rad6, Rad18 and Ubc13. Furthermore, yeast PCNA can also be sumoylated at two residues. Sumoylation at K164 occurs in untreated cells and is dependent on the Ubc9-Siz1 complex [33,35], which helps to recruit a Srs2 DNA helicase [144,145] and inhibit unwanted homologous recombination [146,147], while sumoylation at K127 prevents binding of an essential cohesion factor Eco1 (Ctf7) to PCNA [148]. It is of great interest to understand how the above PCNA modifications are orchestrated in model organisms and whether these mechanisms are conserved in humans. It is noted that several studies reported the detection of an elevated PCNA level in human cancers [149–151] and PCNA has been deemed a target of autoimmune diseases, such as systemic lupus erythematosus (SLE) [152–154]. Since UV irradiation and treatment with other DNA-damaging agents have been known to contribute to the onset of human cancers and SLE, the involvement of PCNA in DNA-damage response may provide underlying mechanisms for these diseases.

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