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Regulatory Role of Ubiquitin in Eukaryotic DNA Translesion Synthesis

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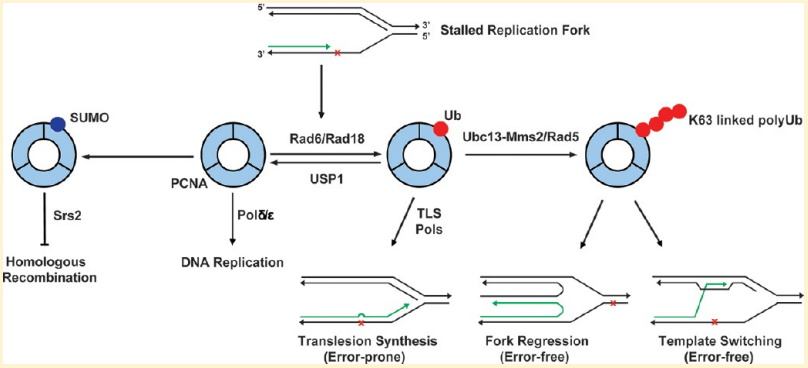

ABSTRACT: Although often associated with proteasome-mediated protein degradation, ubiquitin plays essential non-degradative roles in a myriad of cellular processes, including chromatin dynamics, membrane trafficking, innate immunity, and DNA damage response. The recent progress in understanding DNA translesion synthesis (TLS), an important branch of DNA damage response, has largely been stimulated by the finding that ubiquitination of an essential nuclear protein, proliferating cell nuclear antigen (PCNA), controls precisely how eukaryotic cells respond to DNA damage. Despite the remarkable activity of the TLS polymerases in synthesizing past the damaged nucleotides, they are intrinsically error-prone on the normal DNA template. Therefore, a stringent regulation of the TLS polymerases is essential for the faithful replication of the DNA genome. Here we review the structure and function of the Y-family TLS polymerases and their interactions with ubiquitin and monoubiquitinated PCNA (Ub-PCNA). Driven by the need for monoubiquitinated PCNA in a sufficient quantity and purity, researchers developed both chemical and enzymatic methods for PCNA monoubiquitination, which have propelled our understanding of the structure of Ub-PCNA by X-ray crystallography and small-angle X-ray scattering. Together with studies using a reconstituted polymerase switching assay, these investigations revealed a surprising conformational flexibility of ubiquitin as a modifier on PCNA. Although the molecular details of TLS in cells still need to be deciphered, two working models, polymerase switching and postreplicative gap filling, have been proposed and tested in both *in vitro* and cellular systems. Evidence for both models is discussed herein. Compared to PCNA monoubiquitination, polyubiquitination of PCNA in DNA damage response is much less well understood and will be the subject of a future investigation. Given the close connection of DNA damage response and anticancer therapy, an in-depth understanding of the eukaryotic translesion synthesis and its regulation by ubiquitin will likely provide new opportunities for therapeutic intervention.

Genome integrity is important for the fitness of organisms from all three kingdoms of life. A variety of endogenous and exogenous agents inflict DNA damage and block DNA replication because of the altered structure and property of nucleotide building blocks. A majority of DNA lesions are repaired by the cellular DNA repair proteins; nonetheless, significant numbers of DNA lesions can evade the DNA repair machineries. This presents an acute problem especially in the S phase of the cell cycle, in which the bulk of genomic DNA is replicated. To ensure the timely replication of the genome in the presence of unrepaired DNA lesions, a mechanism that allows the DNA replication machinery to bypass the DNA lesion is beneficial for cell survival. Indeed, in organisms ranging from bacteria to humans, a cellular process known as DNA translesion synthesis (TLS) has been shown to allow the DNA replication machinery to overcome barriers on DNA. TLS is considered largely as an error-prone process because of the low intrinsic fidelity of the specialized DNA polymerases involved.

EUKARYOTIC Y-FAMILY POLYMERASES IN TLS

In eukaryotes, five translesion synthesis polymerases have been identified. Polymerase η (Polη), polymerase ι (Polι), polymerase κ (Polκ), and Rev1 belong to the Y-family of DNA polymerases[1,2] (Figure 1A). The fifth TLS polymerase, Polζ, belongs to the B-family of DNA polymerases. The catalytic domain of Y-family TLS polymerases resembles that of a replicative DNA polymerase with a right-hand-like catalytic core consisting of palm, finger, and thumb domains (Figure

Received: February 14, 2013
Revised: April 2, 2013
Published: May 1, 2013

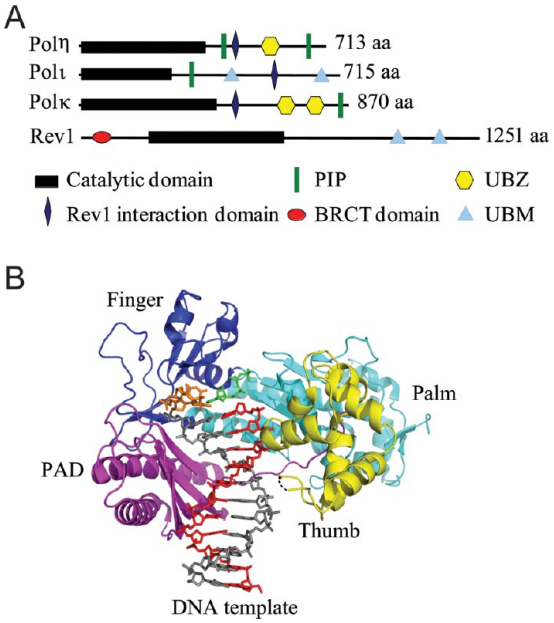

Figure 1. Y-family translesion synthesis polymerases. (A) Cartoon representation of the protein domains of human Y-family TLS polymerases *η*, *ι*, *κ*, and Rev1. Abbreviations: PIP, PCNA-interacting protein motif; UBM, ubiquitin-binding motif; UBZ, ubiquitin-binding zinc finger domain; BRCT, BRCA1 C-terminal domain. (B) Crystal structure of the catalytic domain of yeast Pol*η* with a CPD-containing DNA template [Protein Data Bank (PDB) entry 3MFI]. The catalytic domain of Pol*η* comprises finger (blue), palm (cyan), and thumb (yellow) domains. It also possesses a novel PAD domain (magenta) that interacts with the upstream DNA duplex to stabilize the TLS polymerase ternary complex. The black dashed line depicts the unstructured loop in the thumb domain. The two strands of CPD-containing DNA are colored red (primer) and gray (template). The CPD nucleotides are colored orange, and the incoming dATP is colored green.

1B). However, distinct from the replicative DNA polymerase, TLS polymerases possess a more open active site that can accommodate a damaged DNA template. In addition, TLS polymerases usually contain a novel little-finger domain (or PAD domain) that provides additional interactions with the bound DNA duplex (Figure 1B). These interactions are important for the stability of the TLS polymerase ternary complex given that the finger and thumb domains of TLS polymerases are short and stubby and thus make fewer contacts with the DNA backbone than the replicative DNA polymerases.[3,4]

TLS polymerases usually possess reduced fidelity and processivity compared to those of the replicative DNA polymerases. Because of a relaxed polymerase active site and the absence of a 3′−5′ exonuclease proofreading activity, TLS polymerases often demonstrate a misincorporation frequency 10^3−10^6-fold higher than that of a replicative DNA polymerase.[5−7] Therefore, to avoid excessive mutations, both temporal and spatial regulations of TLS in cells are necessary to limit the action of TLS polymerases to the site of DNA damage.

Several X-ray crystal structures of the Y-family TLS polymerases in complex with DNA have revealed unique features of this family of polymerases in bypassing specific lesions. Pol*η* can bypass the UV-induced *cis-syn* cyclobutane pyrimidine dimer (CPD) with an efficiency and accuracy similar to those of the undamaged DNA.[5,8] Crystal structures of Pol*η* bound to a CPD-containing DNA revealed that Pol*η* acts as a "molecular splint" in stabilizing the damaged DNA in a normal B-form DNA conformation.[3] In addition, the open active site of Pol*η* can accommodate CPD with a favorable configuration that allows a normal Watson−Crick base pairing with the correct incoming nucleotides.[3,4] Notably, Pol*η* cannot bypass the UV-induced pyrimidine-pyrimidone (6−4) photoproduct [(6−4) PP] because of the more severe distortion in (6−4) PP. Nonetheless, Pol*η* can still incorporate a G opposite the 3′ thymine in (6−4) PP.[9] Pol*ι*, another TLS DNA polymerase, is known to incorporate a C opposite an 8-oxoguanine (8-oxo-G) lesion in an error-free manner. Pol*ι* can restrict the 8-oxo-G base to a *syn* conformation, which prevents its Hoogsteen edge from pairing with an incorrect incoming dATP and promotes the formation of a stable base pair with a dCTP.[10] Pol*ι* can also incorporate nucleotides opposite an abasic site or the 3′-thymine of (6−4) PP but lacks the ability to extend the DNA product past the lesion.[11] Compared to other Y-family polymerases, the active site cleft of Pol*κ* is more constrained by the finger domain in a closed position and an additional "N-clasp" domain.[12] As a result, Pol*κ* cannot incorporate nucleotides opposite most of the lesions. Nonetheless, Pol*κ* can effectively extend from the nucleotides inserted by other DNA polymerases opposite the lesions.[13] A fourth TLS polymerase, Rev1, is a dCMP transferase. It can insert a C opposite the lesion by utilizing the Arg324 in the catalytic domain to form hydrogen bonds with the incoming dCTP.[14] In addition, Rev1 plays a role in regulating the activities of other TLS polymerases and promoting polymerase switching via its ability to interact with multiple TLS polymerases.[15−19]

The Y-family DNA polymerases are multidomain proteins. Besides the catalytic core domain, the C-terminal region of TLS polymerases usually contains domains and motifs that are essential for the regulation of TLS polymerases (Figure 1A). Notably, all eukaryotic TLS polymerases contain a PCNA-interacting protein (PIP) motif, which allows the interaction of TLS polymerases with proliferating cell nuclear antigen (PCNA). PCNA is a toroid-shaped protein that can encircle duplex DNA and interacts with many proteins in DNA transactions, including the replicative and TLS DNA polymerases.[20,21] Another important feature of the C-terminal region of TLS polymerases is the presence of one or two ubiquitin-binding domains (UBDs), including the ubiquitin-binding zinc finger domain (UBZ) or ubiquitin-binding motif (UBM) (Figure 1A). These domains have been shown to be required for the recruitment of TLS polymerases to the site of DNA lesion in response to DNA damage.

PCNA AND ITS INTERACTION WITH THE Y-FAMILY POLYMERASES

PCNA is involved in a number of nuclear processes, including DNA replication, cell cycle control, and DNA repair. PCNA, initially found to be a processivity factor of the replicative DNA polymerases,[22] also interacts with many other proteins in DNA transactions, including the TLS polymerases.[23−25] With the exception of Rev1, all Y-family polymerases (Pol*η*, -*ι*, and -*κ*) interact with PCNA through a noncanonical PIP motif.[23,24,26−29] Rev1 interacts with PCNA through a BRCA1 C-terminal (BRCT) domain near the N-terminus of Rev1[30] (Figure 1A). The canonical PIP motif, found in FEN1 (flap endonuclease 1), p21, and the p66 subunit of Pol*δ*[31−33] (Figure 2A), can be described as QXXhXXaa (where h and a represent

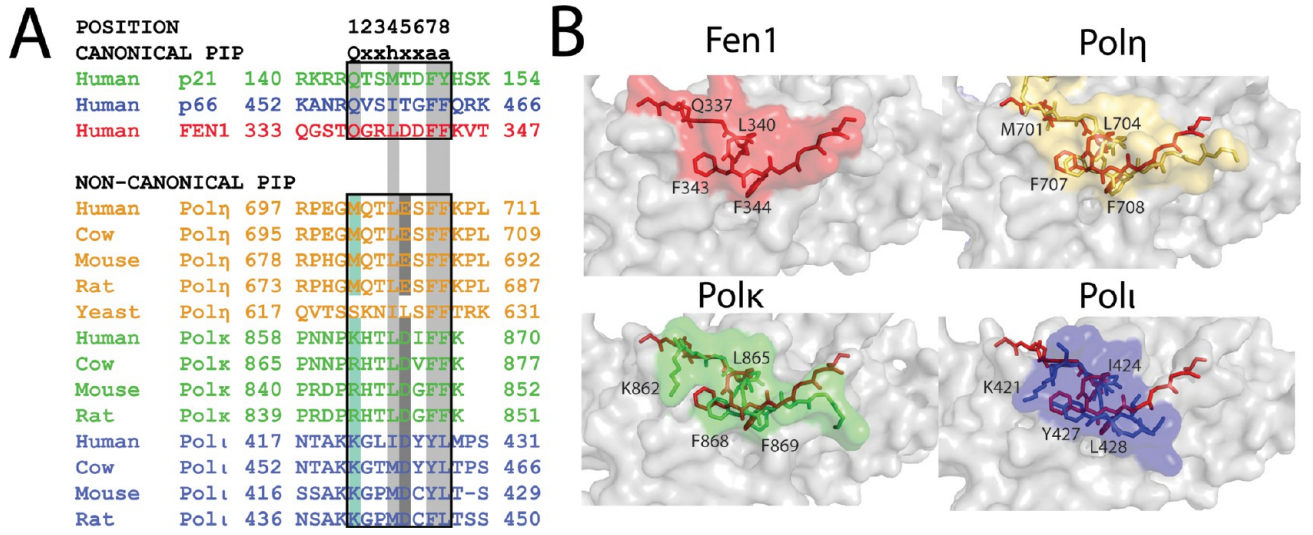

Figure 2. PIP motifs in Y-family DNA polymerases. (A) Sequence alignment of the canonical PIP motifs from p21, p66, and FEN1, and the noncanonical PIP motifs from Y-family polymerases *η*, *κ*, and *ι*. The canonical and noncanonical PIP sequences are boxed. **Q** stands for the conserved glutamine, **h** for hydrophobic residues, and **a** for aromatic residues. The conserved acidic residue in higher eukaryotes that forms a hydrogen bond with His44 of PCNA is shaded dark gray at position 5. Other conserved residues of the canonical and noncanonical PIP box are shaded light gray. (B) Comparison of the binding of the canonical and noncanonical PIPs to PCNA. A stick model of the canonical PIP sequence of FEN1 (PDB entry 1U7B) and its surface are colored red, with PCNA shown as a gray surface. The residues of human FEN1 PIP at positions 1, 4, 7, and 8 are labeled. The noncanonical PIPs of human Pol*η* (PDB entry 2ZVK), human Pol*κ* (PDB entry 2ZVL), and human Pol*ι* (PDB entry 2ZVM) are colored yellow, green, and blue as a stick model and as a surface on the PCNA, respectively. The canonical PIP of FEN1 (PDB entry 1U7B, colored red) is overlaid on each of the PCNA surfaces. The residues of the noncanonical PIP in human Pol*η*, -*κ*, and -*ι* at positions 1, 4, 7, and 8 are labeled.

hydrophobic and aromatic residues, respectively). The conserved Gln inserts its side chain into a "Q-pocket" on the PCNA surface. In the noncanonical PIP motif of human Y-family polymerases, the conserved Gln is replaced with a Met in Pol*η* and a Lys or Arg in Pol*κ* and Pol*ι*. These substitutions play a role in modulating the PCNA binding affinity in light of the fact that mutagenesis of the first PIP residue to Gln in the PIP box increases the affinity in both yeast and human Pol*η*.[34] Another feature of the noncanonical PIP in Y-family polymerases is that the conserved acidic residue, Asp or Glu, at position 5 forms a hydrogen bond with H44 of PCNA. The H44A mutation of PCNA greatly weakened its interactions with Pol*η* and Pol*ι*.[34] Given that PCNA interacts with multiple DNA polymerases in TLS, modulating the PCNA binding affinity of the polymerases by diverging the PIP sequences suggests a potential mechanism for coordinating the actions of the replicative and the specialized DNA polymerases in TLS.

Despite the differences in sequence, the noncanonical PIP motifs in the TLS polymerases bind to PCNA like the canonical PIP motif with the exception of Pol*ι* (Figure 2B). Both human Pol*η* and Pol*κ* PIP motifs form a 3_{10} helix and bind PCNA through hydrophobic residues at positions p4, p7, and p8.[34] The PIP motif of human Pol*ι* adopts an unusual *β*-bend-like structure stabilized by intramolecular hydrogen bonding interaction.[34] This *β*-bend results in a high degree of shape complementarity, which facilitates the binding of Pol*ι* to PCNA.

■ NONDEGRADATIVE FUNCTION OF UBIQUITINATION IN TLS

Ubiquitin is an 8.6 kDa protein that can be covalently linked to target proteins through an isopeptide bond between the ubiquitin C-terminal carboxylate and the side chain *ε*-amino group in the target protein. Ubiquitin as a signal protein was first linked to the proteasome-mediated protein degradation,[35] and this process requires a K48-linked polyubiquitin chain conjugated to the lysine side chain on a target protein. Remarkably, polyubiquitin chains linked through each of the seven ubiquitin lysine residues (K6, K11, K27, K29, K33, K48, and K63) have been identified.[36] Moreover, a linear ubiquitin chain assembled from ubiquitin in a head-to-tail fashion has also been described.[37] Monoubiquitination, in which a single ubiquitin moiety is used to modify a protein, is also common in cells. Monoubiquitination and polyubiquitination through the non-K48 linkages are often associated with nondegradative functions in cells for the regulation of protein interaction, localization, and enzymatic activity.[38] The conjugation of ubiquitin to a target protein and the subsequent growth of the polyubiquitin chain are achieved through a three-enzyme cascade that involves the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3).[35] Like many other post-translational modifications, ubiquitination is a reversible process. The isopeptide bond between ubiquitin and a target protein or within a polyubiquitin chain can be cleaved by a class of enzymes known as deubiquitinating enzymes or deubiquitinases (DUBs). Close to 100 DUBs are encoded by the human genome, suggesting the important roles of this class of enzymes in cellular processes.[39]

A prominent example of the nondegradative function of ubiquitin is PCNA ubiquitination in the DNA damage response. Following DNA damage, stalling of the replication fork at the lesion site uncouples the MCM (minichromosome maintenance) helicase and the DNA polymerase activities, which in turn leads to the single-stranded DNA region at the replication fork. It has been suggested that RPA (replication protein A) binds to the single-stranded DNA and recruits the Rad6/Rad18 complex for the monoubiquitination of PCNA at Lys164.[40,41] PCNA can be further polyubiquitinated at the

dx.doi.org/10.1021/bi400194r | *Biochemistry* 2013, 52, 3217−3228

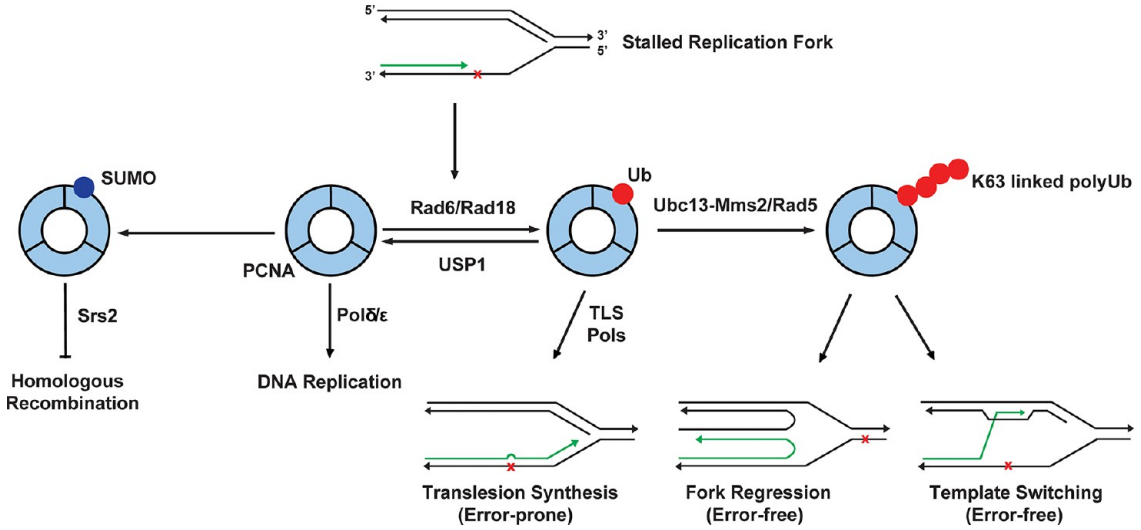

Figure 3. Post-translational modification of PCNA by ubiquitin and SUMO in DNA damage response. Following DNA damage, PCNA is monoubiquitinated at Lys164 by Rad6 and Rad18. Monoubiquitinated PCNA promotes the recruitment of the TLS polymerases to the DNA damage site to conduct translesion synthesis. Monoubiquitinated PCNA can be further polyubiquitinated by Ubc13-Mms2/Rad5 at the same Lys164 residue on PCNA with a K63-linked polyubiquitin chain. Polyubiquitination of PCNA acts as a signal of error-free lesion bypass, including fork regression and template switching. Ubiquitination of PCNA is a reversible process. The ubiquitin moiety on PCNA can be removed by a deubiquitinase, USP1 in human cells. In normal DNA replication, *Saccharomyces cerevisiae* PCNA is SUMOylated at Lys164 to recruit the helicase Srs2, which inhibits the untimely homologous recombination.

same lysine residue by Ubc13-Mms2/Rad5 through a Lys63-linked polyubiquitin chain.[42] Genetic studies have suggested that monoubiquitination of PCNA mediates DNA translesion synthesis and polyubiquitination of PCNA is required for the error-free damage avoidance pathway[43] (summarized in Figure 3). However, how the switching from PCNA monoubiquitination to polyubiquitination is controlled in the DNA damage response remains largely unclear.

Monoubiquitination of PCNA is required for the recruitment of TLS polymerase in DNA translesion synthesis. Monoubiquitination of PCNA enhances the interaction between PCNA and TLS polymerases and serves to recruit the TLS polymerases to the DNA damage site.[27,44−46] In cells, Polη prefers to bind monoubiquitinated PCNA and form nuclear foci following DNA damage, which is abolished in the RAD18$^{-/-}$ cells.[45] Mutations in the Polη UBZ domain that disrupt its interaction with ubiquitin greatly reduced the nuclear foci formation of Polη in response to DNA damage.[46,47] Similar observations were made for Polι and Polκ, again suggesting the important role of PCNA monoubiquitination in recruiting the TLS polymerases to the DNA damage site.[46,48,49] The effect of monoubiquitinated PCNA on the incorporation of the nucleotide opposite the DNA lesion catalyzed by TLS polymerases remains unclear given the different effects observed in the reconstituted translesion synthesis assays using an abasic (AP) site-containing DNA.[50,51]

Besides PCNA, another PCNA-like sliding clamp, the 9−1−1 complex (Rad9−Rad1−Hus1 in humans and Ddc1−Mec3−Rad17 in *Saccharomyces cerevisiae*), is also involved in DNA damage response and is known to be ubiquitinated. The 9−1−1 complex can function as a DNA damage sensor in checkpoint signaling.[52,53] It interacts with and stimulates the activity of a number of DNA repair proteins.[54−58] In addition, the 9−1−1 complex also interacts with Polκ in *Schizosaccharomyces pombe*[59] and Polζ in *S. cerevisiae*,[60] thus suggesting a potential role of the 9−1−1 complex in error-prone TLS. Another study revealed that the 9−1−1 complex may also play a dual role in error-free DNA damage tolerance through template switching and homologous recombination.[61] It was reported that the 9−1−1 complex is monoubiquitinated at Lys197 of the Rad17 subunit by the Rad6/Rad18 complex in *S. cerevisiae* to promote the checkpoint activation and SOS-like transcriptional regulation following DNA damage.[62] However, another study reported that ubiquitination of the 9−1−1 complex is independent of DNA damage and the Rad6/Rad18 complex and may lead to proteasomal degradation.[63] Thus, the function of ubiquitination of the 9−1−1 complex in DNA damage response remains to be defined.

Notably, both Polη and Polι were found to be monoubiquitinated in cells.[47] Polη is monoubiquitinated at four lysine residues close to its C-terminus (K682, K686, K694, and K709),[64] while the ubiquitinated residues in Polι remain to be determined. Monoubiquitination of Polη precludes its interaction with PCNA, and upon DNA damage, Polη is deubiquitinated.[64] These observations support a regulatory role of Polη monoubiquitination in DNA damage response. A more recent study demonstrated a physical interaction between Polη and Polι mediated by ubiquitin and the UBDs in the two TLS polymerases.[65] Further studies are needed to understand the significance of this interaction in TLS.

■ PREPARATION OF MONOUBIQUITINATED PCNA

Enzymatic ubiquitination of PCNA was initially achieved in a reconstituted assay that included purified ubiquitin pathway enzymes and DNA replication proteins.[50,51] Despite the success with the enzymatic system, the overall yield is low because of the requirement of a large excess of purified enzymes and the low catalytic efficiency of the Rad6/Rad18 ubiquitin ligase complex. The ability to produce monoubiquitinated PCNA in sufficient quantities is the key to the biochemical and biophysical investigations of the DNA damage tolerance pathways. To date, three different methods for nonenzymatic monoubiquitination of PCNA have been developed. One method utilized chemical ligation via intein chemistry and

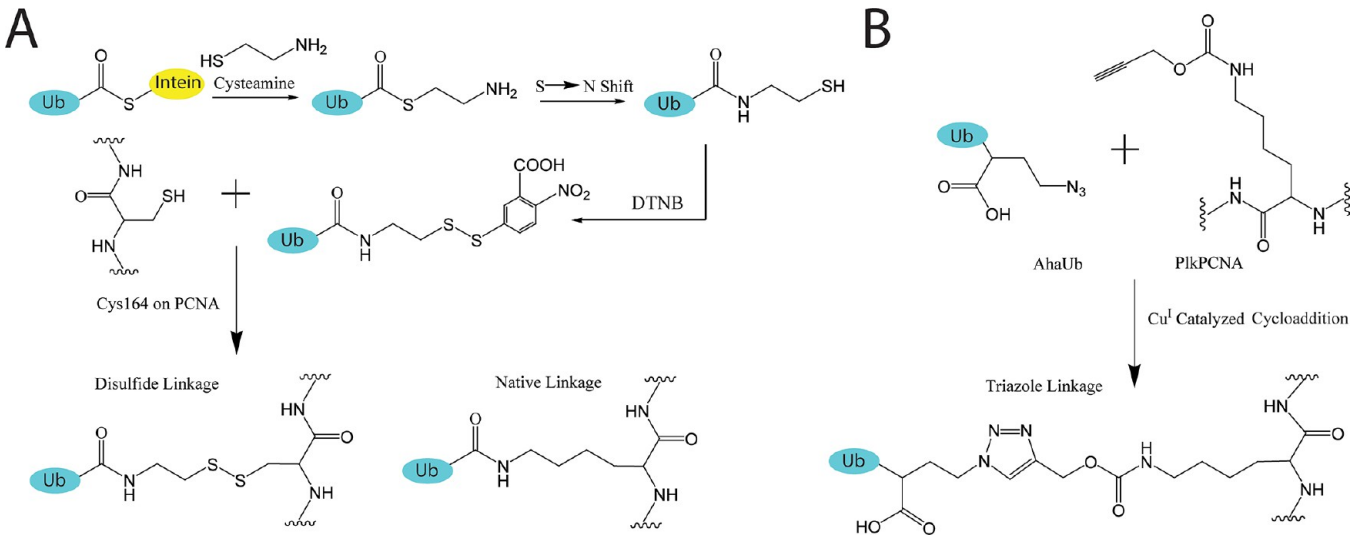

Figure 4. Chemical approaches for PCNA monoubiquitination. (A) Ubiquitin is activated at the C-terminus by the formation of a thioester in an intein fusion. Introduction of cysteamine induces the cleavage of ubiquitin followed by an intramolecular S-to-N shift to generate a modified ubiquitin (Ub-SH) with a thiol introduced at its C-terminus. Ub-SH was then activated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The conjugation of Ub to K164C PCNA through a disulfide exchange reaction affords chemically monoubiquitinated PCNA. (B) PlkPCNA and AhaUb undergo Cu^{I}-catalyzed Huisgen azide–alkyne cycloaddition leading to a site-specific triazole linkage and generation of a mimic of monoubiquitinated PCNA. The native linkage in monoubiquitinated PCNA is shown for comparison.

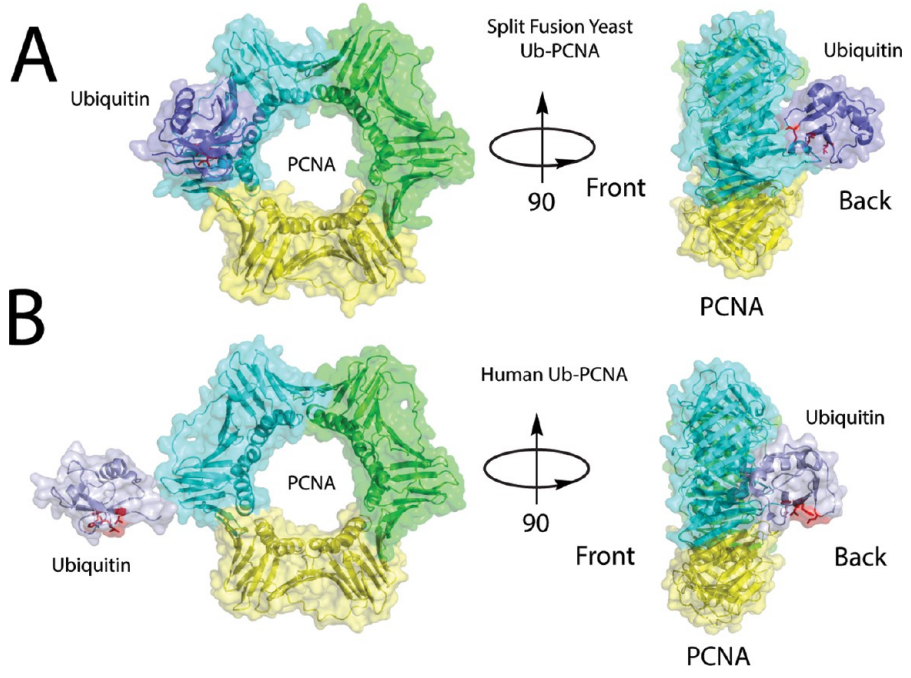

Figure 5. Crystal structures of monoubiquitinated PCNA. (A) Crystal structure of the yeast split PCNA-Ub fusion (PDB entry 3L0W). The hydrophobic patch on ubiquitin formed by L8, I44, H68, and V70 is colored red. The monoUb-PCNA structure is shown in a front view (left) and a side view (right). The front and back sides of PCNA are indicated. (B) Crystal structure of human Ub-PNCA (PDB entry 3TBL). The hydrophobic patch on ubiquitin formed by L8, I44, H68, and V70 is colored red. MonoUb-PCNA is shown in a front view (left) and side view (right). The front and back sides of PCNA are indicated.

disulfide exchange (Figure 4A).[44] In this method, a PCNA mutant was generated with a unique cysteine introduced to replace K164 that undergoes monoubiquitination. Ubiquitin is activated at its C-terminus by the formation of a thioester through intein chemistry. Introduction of cysteamine cleaves ubiquitin from the ubiquitin–intein fusion protein to form the ubiquitin–cysteamine species, which then undergoes an intramolecular S-to-N shift to generate a modified ubiquitin with a thiol at its C-terminus (Ub-SH). Ub-SH is then ligated to cysteine 164 on PCNA through disulfide exchange. The reaction generated milligrams of monoUb-PCNA with 89–94% ubiquitinated PCNA subunits in the final product. Compared to enzymatic ubiquitination, this method also has the advantage of allowing monoubiquitination of PCNA at several other selected residues.[44] The method was readily adapted for SUMOylation of PCNA at positions 127 and 164.[44]

dx.doi.org/10.1021/bi400194r | *Biochemistry* 2013, 52, 3217–3228

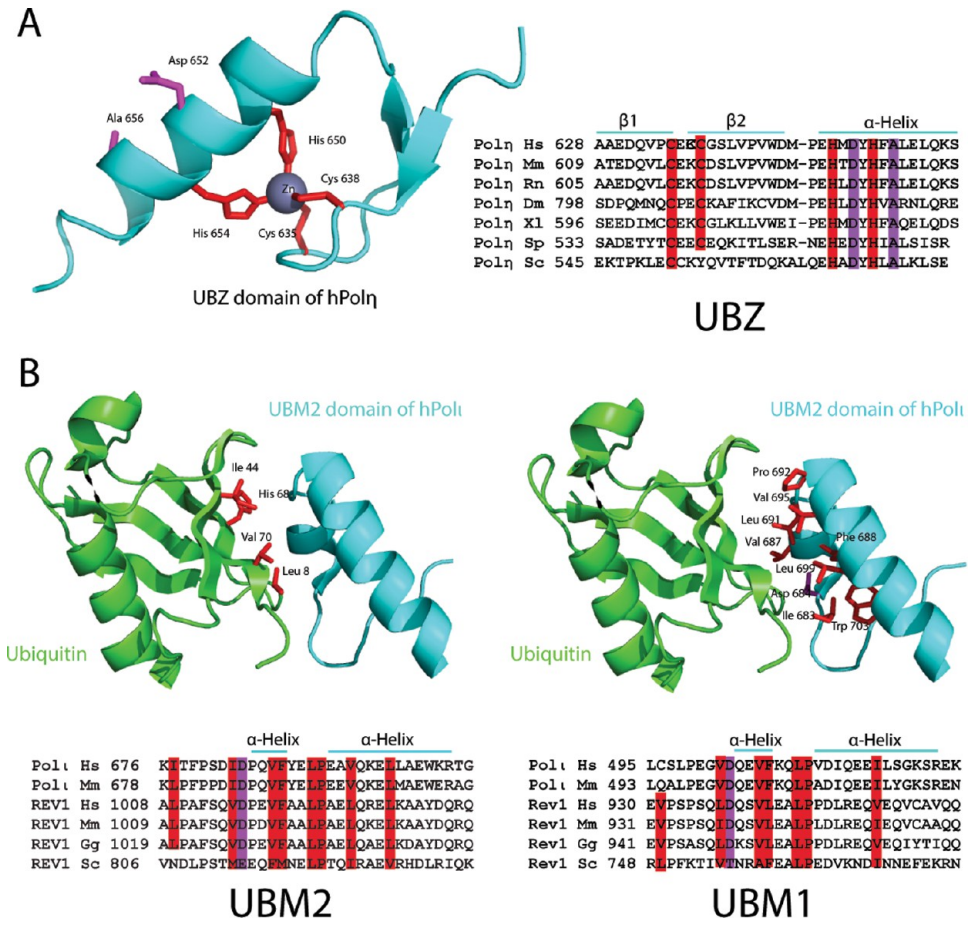

Figure 6. UBZ and UBM domains in TLS polymerases. (A) UBZ domain in human Pol*η*. NMR structure (left) of Pol*η* UBZ. The conserved histidines and cysteines that coordinate the zinc ion are colored red. The conserved residues D652 and A656 that contribute to ubiquitin binding are colored purple. Sequence alignment (right) of Pol*η* UBZ from *Homo sapiens* (Hs), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Drosophila melanogaster* (Dm), *Xenopus laevis* (Xl), *Sc. pombe* (Sp), and *S. cerevisiae* (Sc). The conserved residues are highlighted. Yeast Pol*η* diverges from the CxxC motif conserved in higher eukaryotes with the two cysteines juxtaposed next to each other. (B) UBM domain of human Pol*ι*. NMR structure (top) of Pol*ι* in complex with ubiquitin (PDB entry 2KHW) with ubiquitin hydrophobic residues (left) and Pol*ι* hydrophobic residues (right) highlighted and colored red. D684 is colored purple. Sequence alignment (bottom) of Pol*ι* UBM1 and UBM2 from *H. sapiens* (Hs) and *M. musculus* (Mm) and REV1 UBM1 and UBM2 from *H. sapiens* (Hs), *M. musculus* (Mm), *Gallus gallus* (Gg), and *S. cerevisiae* (Sc). Conserved hydrophobic residues are highlighted in red.

Importantly, the chemically monoubiquitinated PCNA was shown to be functionally equivalent to the enzymatically monoubiquitinated PCNA in a polymerase exchange assay.[44]

Another chemical approach utilizing click chemistry was developed to generate monoubiquitinated PCNA (Figure 4B).[66] Here, unnatural amino acid azidohomoalanine (Aha) and a pyrrolysine analogue (Plk) were incorporated into ubiquitin and PCNA, respectively. Aha was incorporated in place of the ubiquitin C-terminal glycine by mutating the codon for glycine to methionine and expressing the mutant ubiquitin using Met auxotrophic *Escherichia coli* B834(DE3) in a minimal medium containing Aha. Plk was incorporated into PCNA at position 164 by amber codon suppression using a pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CAU}$ pair from *Methanosarcina barkeri*. PlkPCNA and AhaUb were then ligated via the Cu^{I}-catalyzed Huisgen azide−alkyne cycloaddition leading to a monoUb-PCNA linked by a triazole. The resulting monoUb-PCNA was shown to bind Pol*η* with an affinity higher than that of unmodified PCNA, as observed for the native monoUb-PCNA.[66]

Besides chemical ubiquitination, a split PCNA fusion approach was also developed for the generation of mono-ubiquitinated PCNA.[67] In this method, PCNA was split into two separate polypeptides at the site of ubiquitination. Ubiquitin was fused to the N-terminus of the C-terminal half of the PCNA polypeptide (residues 165−258) by a diglycine linker. The two polypeptides were co-expressed and self-assembled into a functional PCNA subunit in *E. coli*. This method was used to generate a monoUb-PCNA mimic that led to the first X-ray crystal structure of monoubiquitinated PCNA and later structures of SUMOylated PCNA.[67,68]

Notably, other enzymatic approaches were also developed for PCNA monoubiquitination. One study exploited UbcH5c (a ubiquitin conjugating enzyme) and RNF8 (a ubiquitin ligase) in monoubiquitinating human PCNA at Lys164 and allowed the structural determination of human monoUb-PCNA.[69] Later, UbcH5c with an S22R mutation was used to generate K164 monoubiquitinated human PCNA.[70] The S22R mutation in UbcH5c was introduced to prevent polyubiquitination of PCNA and facilitate the enzymatic monoubiquitination of PCNA.

STRUCTURE OF MONOUBIQUITINATED PCNA

The structures of monoubiquitinated and SUMOylated PCNA have been determined by X-ray crystallography.[67,68,71] For both human and yeast monoUb-PCNA, the structures of PCNA and ubiquitin remain virtually unchanged compared to those of the free proteins, arguing against a potential allosteric modulation in the recruitment of Y-family polymerases in TLS. Instead, ubiquitin likely provides a new hydrophobic surface for PCNA's interaction with TLS polymerases.[48,72] A surprising observation from the crystal structure of the yeast monoUb-PCNA is that the ubiquitin moiety was docked to the back side of the PCNA ring, opposite the side where DNA synthesis transpires (Figure 5A). The hydrophobic patch on ubiquitin centered on Ile44 is buried in this structure. Given that the same hydrophobic surface on ubiquitin binds to the UBZ domain in Polη, the ubiquitin moiety on PCNA must rotate and expose this surface to engage Polη. Interestingly, a more recent crystal structure of human monoUb-PCNA showed that ubiquitin on PCNA was in a radially extended conformation (Figure 5B).[71] Together, the two monoUb-PCNA structures suggest a flexible conformation of ubiquitin on PCNA. The flexibility of the ubiquitin moiety on PCNA has also been revealed by the finding that PCNA chemically monoubiquitinated at position 164, 127, 107, or 44 was indistinguishable in eliciting polymerase switching between Polη and the replicative Polδ in an *in vitro* polymerase exchange assay.[44] The ability of ubiquitin to elicit polymerase switching at different positions on PCNA indicates that the ubiquitin moiety is likely mobile in adopting conformations amenable to its interaction with TLS polymerases.

The flexibility of the ubiquitin moiety on PCNA was further supported by two independent solution structure studies of monoUb-PCNA using small-angle X-ray scattering (SAXS).[70,73] In one study, the solution structure of yeast monoUb-PCNA was obtained using the chemically ubiquitinated PCNA and the split PCNA-ubiquitin fusion construct as described above.[73] The scattering curve of both monoUb-PCNA species differed from the predicted scattering curve of the yeast monoUb-PCNA crystal structure in which ubiquitin was docked at the back side of the PCNA ring.[73] This observation suggested that the solution structure of monoUb-PCNA is distinct from that observed in the crystal structure determined using the split PCNA-ubiquitin fusion. Molecular dynamics simulation combined with a minimal ensemble search (MES) approach was used to fit the SAXS data. The data were best fit using an ensemble of ubiquitin conformations, including the crystallographic conformation with ubiquitin on the back side of PCNA, the MD-simulated conformation with ubiquitin in a concave surface between two PCNA subunits, and an extended conformation. A later SAXS study of human monoUb-PCNA also found that ubiquitin on PCNA possesses a large degree of flexibility.[70] In a nuclear magnetic resonance (NMR) chemical shift perturbation study of human monoUb-PCNA, little interaction between ubiquitin and PCNA was observed beyond the C-terminal tail of ubiquitin.[70] Together, the functional and structural studies of monoUb-PCNA argue for a remarkable ability of ubiquitin to adopt multiple conformations once conjugated to a target protein. This flexibility of ubiquitin as a modifier likely contributes to a high degree of plasticity of the resulting multiprotein complex for translesion synthesis. The flexible conformation of ubiquitin on PCNA is particularly important for the recruitment of the Y-family polymerases to the DNA damage site. Further study will be needed to uncover the functional significance of the other conformations of ubiquitin on PCNA in TLS.

INTERACTION OF UBIQUITIN WITH UBD IN TLS POLYMERASES

In TLS polymerases, the ubiquitin-binding domains (UBDs) play an essential role in mediating the interaction between TLS polymerases and ubiquitin. Evidence suggests that mono-ubiquitination of PCNA increases the affinity between monoUb-PCNA and the TLS polymerases.[27,45−47,49,65,74−76] Remarkably, UBDs are found in all Y-family polymerases, and Polι, Polκ and Rev1 possess more than one UBD (Figure 1A).

The structures of Polη UBZ and Polι UBM have been reported[48,72,77] (Figure 6). The human Polη (hPolη) was shown to be a classical C_2H_2 zinc-finger domain with a zinc ion sandwiched between an α-helix and two antiparallel β-strands through coordination with the conserved cysteine and histidine residues (Figure 6A). A model of hPolη UBZ bound with ubiquitin was generated on the basis of an NMR chemical shift perturbation study.[72] The interaction sites were mapped to the exposed surface of the α-helix in Polη UBZ and the Ile44 hydrophobic patch on ubiquitin. Intriguingly, the UBZ domain in *S. cerevisae* Polη (scPolη) shows a divergence from the conserved CXXC motif with the two cysteine residues juxtaposed next to each other in the primary sequence (Figure 6A). Studies have shown that scPolη UBZ can bind ubiquitin in a zinc-independent manner, in contrast to the absolute requirement of zinc binding in the hPolη UBZ−ubiquitin interaction.[78,79]

The binding affinity of Polη UBZ for ubiquitin was measured by NMR titration and a surface plasmon resonance (SPR) binding assay.[72,79] Both studies revealed a low affinity between UBZ and ubiquitin (K_d values of 73−81 μM for hPolη UBZ and 15 μM for scPolη UBZ). In contrast, the entire C-terminal region of scPolη that encompasses both UBZ and PIP demonstrated a higher binding affinity (K_d of 0.4 μM).[79] The SPR binding study also revealed a striking difference in the ubiquitin binding kinetics between the entire C-terminal region of scPolη and the isolated UBZ domain.[79] These observations suggest other possible ubiquitin-binding elements in the scPolη C-terminal region and a possible conformation change in this region in ubiquitin binding.

Polι and Rev1 contain two conserved UBM domains located in their C-terminal regions.[47,74] In Polι, both UBM domains are functional in binding ubiquitin and are important for localization of Polι to the DNA damage and replication foci.[47,48,77] In mouse REV1, both UBM1 and UBM2 were found to be required for ubiquitin binding, and deletion of either domain greatly decreased the level of formation of nuclear foci of REV1 in cells.[74] Notably, the yeast REV1 requires only UBM2 for ubiquitin binding and for its cellular function.[48,74,80,81] The Polι UBM domain comprises two α-helices connected by a short loop containing a Leu-Pro motif (Figure 6B).[48,77] These two helices contribute hydrophobic residues for interactions with ubiquitin. This contrasts with the UBZ domain, which interacts with ubiquitin through a single α-helix. Moreover, the human Polι UBM2 domain binding to Ub depends on a hydrophobic patch centered on L8 on ubiquitin, instead of I44 as observed for Polη UBZ−ubiquitin interaction.

dx.doi.org/10.1021/bi400194r | *Biochemistry* 2013, 52, 3217−3228

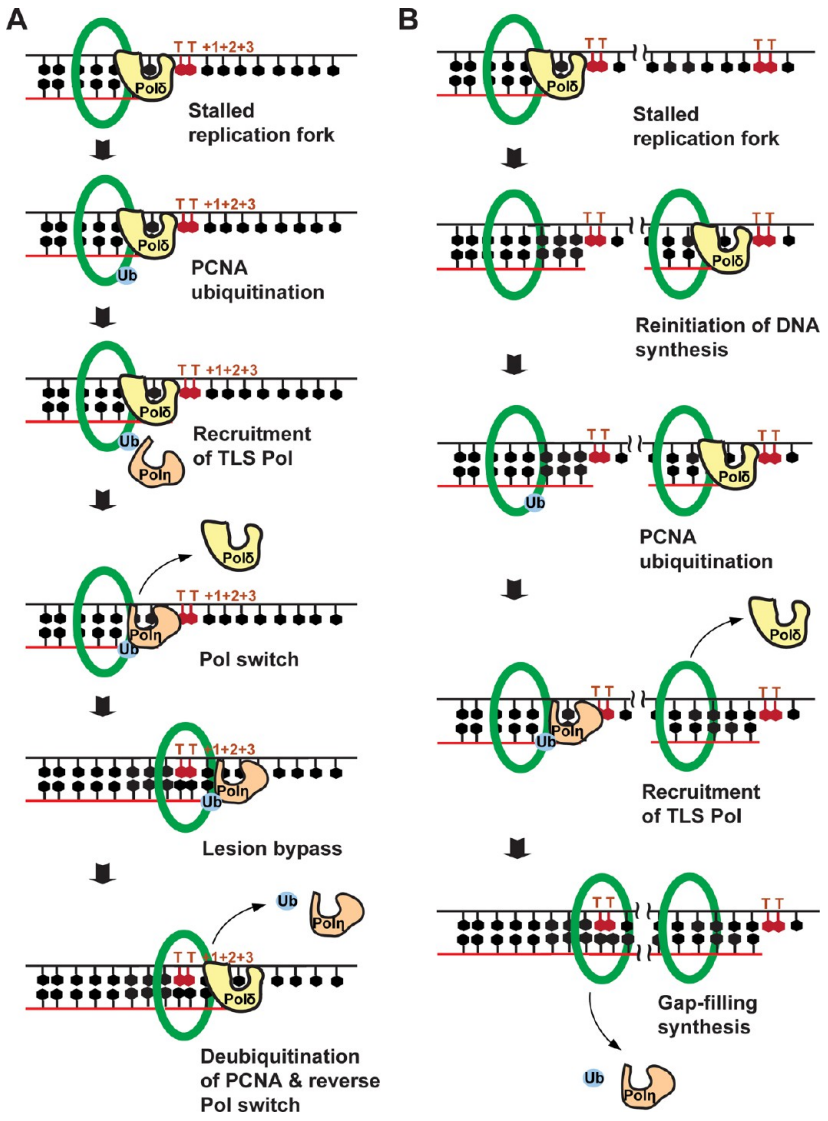

Figure 7. Models of DNA translesion synthesis. (A) Polymerase switching model in TLS through a CPD lesion. When Polδ stalls at the lesion site, monoubiquitination of PCNA facilitates the recruitment of Polη to replace Polδ and conduct lesion bypass. Following lesion bypass synthesis, the binding affinity of Polη for the DNA template is reduced 3 bp downstream of the lesion. Deubiquitination of PCNA likely contributes to the reverse polymerase switching and helps to restore Polδ and resume normal DNA synthesis. (B) Postreplicative gap-filling model. When Polδ encounters a CPD lesion, a new round of DNA synthesis is started downstream of the lesion, which results in a DNA gap. PCNA is monoubiquitinated to recruit the TLS polymerase to conduct gap-filling DNA synthesis.

■ TWO MODELS OF DNA TRANSLESION SYNTHESIS

Despite considerable progress in understanding the structure and function of TLS polymerases and monoubiquitinated PCNA in TLS, the molecular details of TLS remain to be fully elucidated. Two models of eukaryotic translesion synthesis have been proposed, i.e., the polymerase switching model and postreplicative gap-filling model. The polymerase switching model involves a sequential polymerase switching regulated by reversible monoubiquitination of PCNA (Figure 7A). Briefly, when the replicative polymerase encounters a lesion, the stalled DNA synthesis triggers monoubiquitination of PCNA. Ubiquitin on PCNA serves to recruit the TLS polymerases to the DNA damage site to replace the replicative DNA polymerase for lesion bypass synthesis. Following the synthesis, the replicative DNA polymerase is restored to continue the normal DNA synthesis, likely signaled by the deubiquitination of PCNA. In the postreplicative gap-filling model, it is proposed that when the replicative DNA polymerase encounters a DNA lesion, a new round of DNA synthesis is started downstream of the lesion. The PCNA at the damage site is monoubiquitinated and serves to recruit the TLS polymerase to fill the gaps (Figure 7B).

Evidence of polymerase switching between the replicative DNA polymerase and the TLS polymerase was first reported in a human cell-free system.[82] A CPD-containing plasmid was used as the DNA template in a simian virus 40 (SV40) replication system. The DNA synthesis was stalled at the CPD lesion site in the XPV cell extracts, and the stalled DNA synthesis was restored after the addition of purified Polη, suggesting successful polymerase switching between the replicative DNA polymerase and Polη.[82] Besides the cell-free system, polymerase switching between Polδ and Polη has been reconstituted in both yeast and human *in vitro* systems with purified recombinant proteins. In yeast, polymerase switching occurs between Polδ and Polη under a condition that mimics

dx.doi.org/10.1021/bi400194r | *Biochemistry* 2013, 52, 3217−3228

the stalling of the Polδ−PCNA holoenzyme at the DNA lesion site.[76] It was shown that monoubiquitination of PCNA is required for effective polymerase switching between Polδ and Polη. In the human system, polymerase switching between Polδ and Polη on a UV-irradiated DNA template was stimulated by monoubiquitination of PCNA and dependent on the interaction between Polη UBZ and ubiquitin on PCNA.[83]

Besides the essential role of monoubiquitination of PCNA in polymerase switching, other factors may also regulate polymerase switching. It was shown that when human Polη is 3 bp downstream of the lesion, the CPD nucleotides lose favorable hydrogen bonding with the Polη DNA-binding site and face more steric clashes with Polη.[3] This observation suggests that Polη may lose binding affinity for the DNA template downstream of the CPD lesion, which may facilitate the reverse switching of Polδ to replace Polη (Figure 7A). An electrophoretic mobility shift study has shown that the Polη−DNA complex was destabilized once Polη incorporated two nucleotides downstream of the CPD lesion.[84] Furthermore, deubiquitination of PCNA likely plays an important role in regulating the reverse switching between Polδ and Polη. Ubiquitin on PCNA was found to prevent Polδ from gaining access to the growing DNA end in the presence of Polη, supporting the notion that deubiquitination of PCNA is likely required to restore Polδ after lesion bypass synthesis by Polη.[76] More recently, a new PDIP38 protein in humans was found to interact with both Polδ and Polη and thus may serve as a polymerase switching regulator.[85]

In the postreplicative gap-filling model, DNA replication machinery conducts a new round of DNA synthesis downstream of the lesions with the formation of single-stranded DNA gaps opposite the lesion. Following UV irradiation, gaps can be found in both leading and lagging strands on replicated DNA in yeast and human cells.[86−88] During gap-filling synthesis, monoubiquitinated PCNA was thought to recruit TLS polymerase. It was shown that gap-filling DNA synthesis occurs in the G2/M phase, and DNA synthesis was completely inhibited by knockout of the RAD18 gene.[89] Genetic studies in yeast and DT40 cells also showed that postreplicative gap filling is dependent on the monoubiquitination of PCNA.[90,91] Although monoubiquitination of PCNA is indispensable in the polymerase switching and postreplicative gap-filling models, the requirement and timing of deubiquitination of PCNA in both TLS models have not been clearly defined. It should be noted that the two models of translesion synthesis described above may not be mutually exclusive and may operate in different phases of the cell cycle.

■ POLYUBIQUITINATION OF PCNA IN DNA DAMAGE RESPONSE

In addition to monoubiquitination, PCNA was also found to be polyubiquitinated in response to DNA damage in both yeast and human cells.[42,92−95] Notably, the polyubiquitin chain is linked through K63 on ubiquitin and thus is not associated with protein degradation. Compared to monoubiquitination of PCNA, the function of polyubiquitination of PCNA is less well understood.

Early genetic studies linked polyubiquitination of PCNA at Lys164 to the error-free pathway of DNA damage response through a template switching mechanism. In yeast, using a plasmid containing two (6−4) PP located on two complementary DNA strands, it was found that half of the replicated plasmids undergo recombination and the process was dependent on Rad18 and Rad5, suggesting an important role of polyubiquitination of PCNA in the tolerance of (6−4) PP.[96] A similar conclusion was reached in another yeast genetic study pointing to polyubiquitination of PCNA as a signal for template switching.[97] However, the molecular details of how polyubiquitination of PCNA mediates template switching remains to be defined.

Recently, the discovery of a new polyUb-PCNA binding protein, ZRANB3 (zinc finger, RAN-binding domain containing 3), shed more light on the role of polyubiquitination of PCNA in the error-free lesion bypass pathway. ZRANB3 possesses a DNA annealing helicase activity and can be recruited to DNA damage sites to stabilize the stalled replication fork.[98] It has been shown that ZRANB3 interacts with polyubiquitinated PCNA and restarts the stalled replication fork. ZRANB3 can translocate and regress stalled replication forks to allow access to the newly synthesized sister chromatid strand by other DNA transaction proteins that promote error-free DNA synthesis.[99] In addition, ZRANB3 may prevent unnecessary recombination by resolving inappropriate D-loops at the stalled replication fork.[99] Moreover, ZRANB3 has a structure-specific endonuclease activity that can cleave the branched DNA structure and induce a DNA break in the double-strand region. The cleavage will generate an accessible 3′-OH group in the template, which can be extended by DNA polymerase.[100] All these activities of ZRANB3 promote the error-free lesion bypass.

In yeast, polyubiquitination of PCNA can recruit Mgs1, the budding yeast Werner helicase-interacting protein 1 (WRNIP1/WHIP) homologue, to the site of DNA damage. Mgs1 prefers to bind polyubiquitinated PCNA, and the interaction depends on the UBZ domain in Mgs1.[101] The exact function of Mgs1 remains to be determined. One possibility is that once recruited to the stalled replication fork, Mgs1 can disrupt the interaction between Polδ and PCNA and facilitate the release of Polδ. Another model suggested that the polyubiquitin chain can bind and disengage the TLS polymerase from PCNA, thus allowing other protein factors to bind to the replication fork following lesion bypass synthesis.[92]

■ FUTURE DIRECTION

To date, much progress has been made in understanding translesion synthesis and the important role of PCNA ubiquitination in DNA damage response. The structure and function of TLS polymerases and monoubiquitinated PCNA have been subjected to extensive studies. However, the high-resolution structure of a TLS polymerase complex comprising one of the several TLS polymerases, Ub-PCNA, and DNA is still lacking. Such a structure is crucial for our understanding of the regulatory role of ubiquitin in TLS. Several methods for generating sufficient amounts of monoubiquitinated PCNA have been developed in recent years. This will facilitate the structural determination of the Ub-PCNA−polymerase complex. In addition, understanding the role of deubiquitination of PCNA in TLS is also important given the involvement of USP1, a DUB targeting PCNA, in normal DNA damage response. Polyubiquitination of PCNA and its function in DNA damage response remain to be fully elucidated. Future studies are needed to address the exact role of polyubiquitination of PCNA in error-free lesion bypass synthesis. To that end, methods for generating polyubiquitinated PCNA with defined chain linkage and length are needed for the structural and functional studies of polyubiquitinated PCNA in DNA damage

response. The knowledge obtained in studying PCNA ubiquitination and its role in DNA damage response will provide important insights into the nondegradative functions of ubiquitin in many cellular processes.

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Funding

This work was supported by a grant from the National Science Foundation (MCB-0953764) to Z.Z.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We apologize to colleagues whose work we could not include because of space limitations.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on May 1, 2013. The Abstract graphic and Figure 3 were replaced and the revised version was reposted on May 3, 2013.