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REVIEW

Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA

Parker L Andersen[1], Fang Xu[2], Wei Xiao[1]

[1]Department of Microbiology and Immunology, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E5, USA; [2]Department of Biology, Ningxia Medical College, Yinchuan, Ningxia 750004, China

In addition to well-defined DNA repair pathways, all living organisms have evolved mechanisms to avoid cell death caused by replication fork collapse at a site where replication is blocked due to disruptive covalent modifications of DNA. The term DNA damage tolerance (DDT) has been employed loosely to include a collection of mechanisms by which cells survive replication-blocking lesions with or without associated genomic instability. Recent genetic analyses indicate that DDT in eukaryotes, from yeast to human, consists of two parallel pathways with one being error-free and another highly mutagenic. Interestingly, in budding yeast, these two pathways are mediated by sequential modifications of the proliferating cell nuclear antigen (PCNA) by two ubiquitination complexes Rad6-Rad18 and Mms2-Ubc13-Rad5. Damage-induced monoubiquitination of PCNA by Rad6-Rad18 promotes translesion synthesis (TLS) with increased mutagenesis, while subsequent polyubiquitination of PCNA at the same K164 residue by Mms2-Ubc13-Rad5 promotes error-free lesion bypass. Data obtained from recent studies suggest that the above mechanisms are conserved in higher eukaryotes. In particular, mammals contain multiple specialized TLS polymerases. Defects in one of the TLS polymerases have been linked to genomic instability and cancer.

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DNA damage tolerance

In the presence of spontaneous or carcinogen-induced DNA damage, living cells have to maintain and complete DNA synthesis or risk replication fork collapse. Since the process of DNA licensing is to ensure the genome is duplicated once and only once during each cell cycle, stalled or collapsed replication forks may not be able to restart, which often results in double-strand breaks (DSBs) and causes compromised genome integrity or cell death. In addition to highly conserved DNA repair pathways, all living organisms have evolved schemes to ensure continuation of DNA synthesis in the presence of damage. These schemes were originally termed DNA postreplication repair (PRR) due to observations of transient shortened nascent DNA structures following S phase in response to DNA damage. In bacteria and unicellular yeast, these shortened DNA segments can be measured by an alkaline sedimentation assay [1] or directly observed in electron micrographs [2]. In wild-type cells, these truncated DNA segments were restored to full length following a short recovery time. One typical experiment [1] involved the restoration of the nascent strand following UV exposure in nucleotide excision repair (NER)-deficient cells and was originally assumed to represent a mechanism of DNA repair. However, further investigation revealed that, although the nascent fragments were re-annealed, the original UV-induced pyrimidine dimers, which were responsible for the generation of single-strand gaps, often persisted in the genome [3, 4]. It was argued that the replication-blocking lesion was not necessarily corrected, but rather transiently bypassed and carried over to the next generation. Perhaps it is more beneficial for the organism to tolerate DNA damage rather than to allow replication fork collapse. Since, unlike other DNA repair mechanisms, this pathway does not actually remove damage-induced lesions, we propose to use the term DNA damage tolerance (DDT) to describe this general phenomenon. In both prokaryotes and eukaryotes, DDT is accomplished by alternative mechanisms with rather

Correspondence: Wei Xiao
Tel: +1 306 966 4308; Fax: +1 306 966 4311
E-mail: wei.xiao@usask.ca

different biological consequences and these mechanisms appear to be evolutionarily conserved. In this review, we will focus on recent advances pertaining to cohesive DDT mechanisms mediated by covalent modifications of proliferating cell nuclear antigen (PCNA) in eukaryotes. Readers are encouraged to refer to recent review articles [5-7] for this general field.

DDT in prokaryotes

Early experiments using an NER mutant in *Escherichia coli* demonstrated DNA gap formation in the nascent strand opposite UV-induced thymidine dimers [8], suggesting replication restart downstream of the blocking lesion. Using an alkaline sedimentation assay, it was demonstrated that these gaps were transient [9] and the persistence of lesions in subsequent generations suggested the existence of a DDT mechanism in *E. coli* [4]. This damage tolerance mechanism is thought to be dependent upon the bacterial SOS response, in which single-strand DNA (ssDNA) is recognized and bound by the RecA protein [10], which promotes two parallel pathways. Activated RecA (RecA*) induces the self-cleavage of the LexA repressor that in turn allows the transcription of various genes required for DNA repair and cell survival [11]. SOS induces the expression of both *DinB* and the *umuDC* operon encoding two polymerases, PolIV and PolV, respectively, for translesion synthesis (TLS). In addition, RecA* stimulates the cleavage of the regulatory subunit UmuD to form a fully functional PolV ($UmuD'_2$-UmuC) [12, 13], and the RecA-ssDNA filaments are required for both homologous recombination and TLS [14]. This ssDNA-binding activity of RecA has been suggested to induce fork regression forming a chicken-foot structure, or to act as a primer for TLS allowing DDT and replication restart [15], as illustrated in Figure 1.

Ubiquitination and sumoylation

It is apparent that, in bacterial cells, RecA plays central and multiple roles in DDT. In contrast, the eukaryotic RecA sequence homolog Rad51 and its various paralogs in higher eukaryotes inherit the ssDNA filament formation and homologous recombination activity but do not confer regulatory functions, nor are they required for DDT. Recent studies show that eukaryotes employ a completely different mechanism to coordinate DDT, namely covalent modifications of a substrate by ubiquitin (Ub) and small Ub-like modifier (SUMO), processes not found in bacteria. For this reason, it is important to briefly review the process of ubiquitination. Ub is a highly conserved 76 amino-acid protein that can be specifically attached to the ε-amino group of a Lys residue on a target protein in a three-step manner. Firstly, in an ATP-dependent manner, Ub is linked by a thioester bond to the Ub-activating enzyme (Uba or E1). Ub is then transferred to an active site cysteine residue of a Ub-conjugating enzyme (Ubc or E2), which often operates along with a Ub ligase (E3) for target specificity. Ub is then transferred to the target protein, forming an isopeptide bond between the C-terminus of Ub and the ε-amino group of a Lys residue. Covalently bound Ub is often further modified by sequential addition of Ub molecules to already bound Ub peptides to produce poly-Ub chains. The most characterized function of Ub modification is proteasome-dependent protein degradation of substrates modified with Ub chains joined sequentially at the Lys48 residue of the previous Ub [16]. However, it becomes clear that alternative Ub modifications influence diverse activities [17]. Such specifications may involve distinct linkages, such as Lys63-linked poly-Ub chains, or simple mono-Ub additions [18]. It is generally believed that these alternative Ub modifications differ from Lys48-linked Ub chains in that they often regulate the target protein activity instead of its degradation.

Eukaryotic cells also contain several classes of Ub-like molecules that adopt a Ub-like fold with conserved positioning of C-terminal residues for isopeptide bond formation and target protein modification [19, 20]. Each class employs a specific E2-E3 complex for target conjugation. SUMO is probably the most extensively characterized Ub-like molecule and its conjugation (sumoylation) often alters the target protein activity [21, 22].

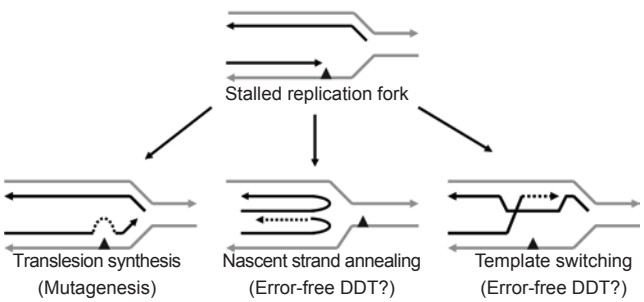

Figure 1 Possible mechanisms of DNA damage tolerance through replicative bypass of stalled replication forks. A triangle on the bottom template strand represents a replication block that stalls the replication machinery. Most organisms possess specialized polymerases capable of translesion synthesis across the block, often associated with an increased mutation rate. An error-free DDT utilizes newly synthesized sister chromatid as a template; however, it is subject to debate whether it is achieved through fork regression followed by nascent strand annealing and synthesis (the chicken-foot model), or through sister chromatid invasion followed by synthesis and Holliday junction resolution (the template switching model).

DDT in *Saccharomyces cerevisiae*

Eukaryote DDT has been most extensively characterized in the budding yeast *S. cerevisiae*. Initially an alkaline sedimentation assay was employed to demonstrate the existence of DDT activity in yeast, and genetic analyses indicate that, in the absence of NER (*rad1*), UV-induced ssDNA gaps cannot be filled in the *rad6* or *rad18* mutants [23]. *RAD6* was the founding member of the PRR and mutagenesis pathway, one of the three major radiation repair pathways. However, historically, *RAD* genes that do not belong to either of the well-defined *RAD3* (NER) or *RAD52* (homologous recombination repair (HRR)) group were assigned to the *RAD6* pathway [24, 25]. The *rad6* mutant exhibits a mutator and slow growth phenotype, is defective in UV-induced mutagenesis, and becomes extremely sensitive to killing by UV and a variety of DNA damaging agents [25, 26]. The *rad6* diploid is also defective in sporulation [27]. *RAD6* was found to encode an E2 enzyme (Ubc2) [28] and its Ub conjugation activity is absolutely required for all of its functions [29]. The C-terminal polyacidic tail of Rad6 is required for its ability to polyubiquitinate histone H2B *in vitro* [30] and *in vivo* [31]. However, deletion of the entire Rad6 C-terminal tail has little effect on its role in DNA repair and UV-induced mutagenesis, and affects only sporulation [32]. The N-terminal 15 amino-acid sequence is nearly identical among all Rad6 homologs [33-36]; deletion of the first 9 amino acids from Rad6 ($rad6_{\Delta 1\text{-}9}$) abolishes sporulation, reduces cell survival after UV treatment, but surprisingly increases spontaneous and UV-induced mutagenesis [37]. Furthermore, the N-terminus of Rad6 is also required for N-end rule protein degradation [29, 37, 38]: while the full-length Rad6 interacts with the E3 protein Ubr1, the $Rad6_{\Delta 1\text{-}9}$ protein is unable to form a complex with Ubr1 [37]. Rad6 is known to form a stable complex with Rad18 [39], and this complex displays Ub conjugation (from Rad6), ssDNA-binding and ATPase (from Rad18) activities [40]. However, Rad18 had not been defined as an E3 until the RING finger motif was discovered [41, 42] and found in Rad18, and the physical interaction of Rad18 with the substrate Pol30 (PCNA) was demonstrated [43]. Like *rad6*, the *rad18* mutant is extremely sensitive to killing by UV and a variety of DNA damaging agents, and displays a mutator phenotype [44]; however, unlike *rad6*, *rad18* displays a signature spontaneous GC-to-TA mutation increase [45] and does not display slow growth and sporulation defects [26]. Hence, Rad6 appears to be a multi-functional E2 with different partners, and its DDT activity is exclusively achieved through interaction with Rad18 [5].

Further genetic analysis has demonstrated that the *RAD6* pathway can be divided into two parallel pathways, one being error-prone and another error-free. The error-prone or mutagenesis pathway was first discovered through genetic screens of *rev* mutants incapable of reverting the *arg4-17* and *lys1-1* alleles in response to UV irradiation [46, 47]. *REV1* was cloned and found to encode a 112-kDa protein [48] with deoxycytidyl transferase activity [49], whereas *REV3* and *REV7* encode two subunits of a non-essential DNA polymerase, Polζ, capable of bypassing thymine dimers more efficiently than Polα [50]. The *rev* mutants exhibit moderate sensitivity to a variety of DNA damaging agents, but with strongly compromised mutability [51]. Thus, the yeast mutagenesis pathway relies on a non-essential DNA polymerase to bypass DNA replication blocks, or TLS, at the cost of increased mutagenesis.

The *rad6* and *rad18* mutations are epistatic to *rev* mutations; however, it is apparent that TLS is not the only pathway operated by *RAD6-RAD18*, since the *rad6* or *rad18* mutants are much more sensitive to DNA damaging agents than the *rev* mutants [26]. An error-free branch within the *RAD6* pathway had been proposed but not convincingly demonstrated until the identification and functional characterization of *MMS2* [52]. The *mms2* mutant is moderately sensitive to a broad range of DNA damaging agents, and epistasis analysis places *MMS2* within the *RAD6* pathway. However, unlike *rev3*, the *mms2* mutant displays a massively increased spontaneous mutation rate and this increase is dependent on *REV* functions. Furthermore, the *mms2* and *rev3* mutations are synergistic with respect to DNA damage sensitivity and the double mutant is comparable to that of the *rad18* single mutant [52, 53]. Based on these analyses, a model was proposed in which the *RAD6* pathway is composed of two independent subpathways: one is mediated by TLS that requires *REV1*, *3* and *7*, whereas the other is mediated by error-free PRR that requires *MMS2* [52]. *MMS2* encodes a protein homologous to Ubc but lacking the active Cys residue [52]. It turns out that Mms2 forms a stable complex with a true Ubc, Ubc13, and that the Mms2-Ubc13 complex specifically catalyzes the formation of Lys63-linked Ub chains [54]. Indeed, the *ubc13* mutant displays phenotypes indistinguishable from those of the *mms2* mutant [55]. The cognate E3 for Mms2-Ubc13 turns out to be Rad5, another RING-finger protein that interacts with both Ubc13 and Rad18 [56]. *RAD5* encodes a protein with DNA helicase and zinc-binding domains [57] and DNA-dependent ATPase activity [58]. Hence, at least two E2-E3 complexes, namely Rad6-Rad18 and Mms2-Ubc13-Rad5, are required for DDT in yeast. In addition, *RAD5* has been reported to promote instability of simple repetitive sequences [57] and to inhibit non-homologous end-joining of DSBs [59]. Indeed, Rad5 is involved in double-strand break repair independent of its ubiquitination activity [60].

Sequential modifications of PCNA

PCNA (encoded by *POL30* in budding yeast) forms a homotrimer which circles the DNA and operates as a scaffold, often termed a processivity factor, to assemble a multitude of proteins required for DNA unwinding and synthesis, cell cycle progression and chromatin structure maintenance [7]. The involvement of this DNA-polymerase sliding clamp in DDT was first suggested by the isolation and characterization of the *pol30-46* allele [61]. *pol30-46* is epistatic to *rad6* and *rad18*, but synergistic with *rev3*. The *pol30-46* mutant is normal in UV-induced mutagenesis and DNA synthesis but displays significantly reduced PRR activity as judged by the alkaline sedimentation assay [61].

PCNA can be either ubiquitinated or sumoylated in budding yeast [43]. In response to DNA damage, PCNA is modified by a single Ub on the Lys164 residue and this process is dependent on the Rad6-Rad18 complex [43]. Ub modification appears to be limited to the PCNA that has been loaded onto DNA by replication factor C [62], suggesting that PCNA is monoubiquitinated only at stalled replication forks. In wild-type cells, polyubiquitinated PCNA was also observed upon DNA damage, and this modification is also at the Lys164 residue, linked through the Lys63 Ub chain, and requires functional *MMS2*, *UBC13* and *RAD5* [43]. Hence, it is conceivable that the two ubiquitination complexes Rad6-Rad18 and Mms2-Ubc13-Rad5 sequentially ubiquitinate PCNA. Interestingly, the identical residue can also be targeted for sumoylation; the fraction of sumoylated PCNA increases during S phase as well as during extensive DNA damage, and this process requires yet another E2-E3 complex Ubc9-Siz1 [43, 63]. It is noted that PCNA can also be sumoylated at the Lys127 residue [43], and this specific modification does not appear to affect the DDT activity, but is required for the establishment of sister chromatid cohesion during S phase [64].

The discovery of PCNA covalent modifications imposes several functional implications. Firstly, it predicts that the *pol30-164R* mutation is epistatic to all DDT pathway mutations. Indeed, Pol30-164R cannot be ubiquitinated and the *pol30-164R* mutation suppresses the severe sensitivity of *rad6* and *rad18* mutations [43]. Secondly, it predicts that monoubiquitinated PCNA promotes TLS, which was subsequently demonstrated [63]. Thirdly, the above model suggests that polyubiquitinated PCNA promotes error-free DDT. To date, this prediction has not been explored. Finally, it indicates that the Pol30-K164 sumoylation plays a role in the regulation of DDT. Interestingly, the *pol30-164R* mutant is less sensitive to DNA damage than *rad6*, *rad18* or the *mms2 rev3* double mutant, suggesting that the Pol30-K164 sumoylation sensitizes cells to DNA damage. This model is further strengthened by analyzing the effects of *siz1* mutation that specifically affects sumoylation but not ubiquitination (*UBC9* is an essential gene), and is reminiscent of the *srs*2 (*s*uppression of *r*ad *s*ix) mutation that was initially isolated by its ability to suppress the severe damage sensitivity of *rad6* mutants [65]. Srs2 possesses a 3′ to 5′ DNA helicase activity [66, 67] that is crucial for recombination [67] and suppression of DDT defects [68, 69]. Genetic data indicate that Srs2 negatively regulates recombination [70, 71] possibly by reversal of intermediate recombination structures [72-75]. Indeed, the DNA strand exchange mediated by Rad51 is inhibited by Srs2 through disruption of the Rad51-ssDNA filaments [76, 77], and it turns out that sumoylated PCNA has increased affinity for Srs2 [78, 79] and represses the Rad52-dependent recombination pathway [80]. These observations collectively support the hypothesis that Srs2 serves as a molecular switch between homologous recombination and DDT [6], and further confirm that the sensor for this switch is the state of PCNA modification.

The current model of yeast DDT through covalent modifications of PCNA is depicted in Figure 2.

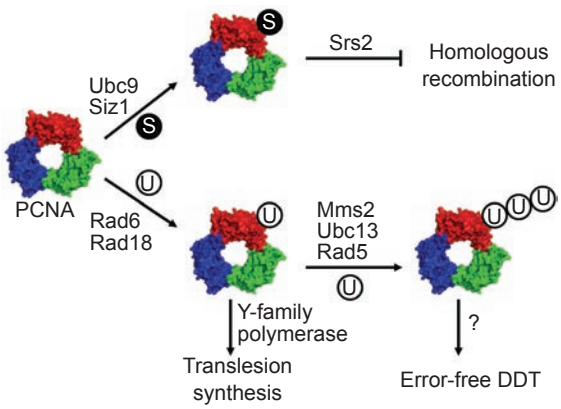

Figure 2 DNA damage tolerance through covalent modifications of PCNA, a model well established in the lower eukaryote *S. cerevisiae* and presumably conserved in higher eukaryotes. The PCNA homotrimer is illustrated in different colors and can be modified by either SUMO (S) or Ub (U) at the same Lys164 residue [43]. In the latter case, monoubiquitinated PCNA can be further polyubiquitinated by the E2-E3 complex Mms2-Ubc13-Rad5 to form Lys63-linked chains. Sumoylated PCNA is known to recruit the Srs2 helicase to disrupt the Rad51-ssDNA filament and prevent inappropriate homologous recombination; monoubiquitinated PCNA enhances affinity for Y-family polymerases to facilitate TLS, whereas polyubiquitinated PCNA is thought to promote error-free DDT, although it is unclear at present how this is achieved. Note that it is subject to debate whether just one or all three subunits of PCNA are modified to execute its functions. Modification of only one subunit is shown for simplicity.

Y family DNA polymerases

Error-prone TLS can occur by the regular replicative polymerases or specialized, error-prone polymerases. Replicative polymerases include PolI, PolII and PolIII in prokaryotes, Pol1(α), Pol2(ε) and Pol3(δ) in yeast, and Polα, Polε and Polδ in higher eukaryotes. Errors can arise by simple incorrect base-pairing and/or lack of proofreading. Frameshift mutations often occur in regions of repeated nucleotide sequences likely from slippage of the template strand. In addition, certain nucleotide repeats can readily form secondary structures that become recombination hotspots and fragile sites in the DNA, among which triplet repeats can also provide sources of extensive amino-acid expansion in the coding region [81]. Furthermore, replicative polymerases may be required for extension from nucleotide insertion by a low-fidelity polymerase, thus stabilizing a potential mutation.

Essentially all TLS polymerases except one (i.e., Polζ) are Y-family polymerases that lack a 3′-5′ proofreading exonuclease activity and contain relatively non-restrictive active sites compared with the replicative polymerases [82] (Figure 3). Surprisingly, although members of this family of proteins have been studied for many years and implicated in mutagenesis or TLS, it was only at the end of the last century when they were reported as a novel class of DNA polymerases. This review is not intended to cover a comprehensive analysis of each Y-family polymerase. Readers are referred to excellent review articles that cover this topic [82-86] and an accompanying review article (McCulloch and Kunkel in this issue) discussing the fidelity of eukaryotic DNA polymerases. Rather, this section aims to provide an in-depth analysis of current literature on how monoubiquitinated PCNA leads to TLS. In addition, an accompanying review article (Gan *et al*. in this issue) provides an in-depth analysis of eukaryotic Polζ.

E. coli contains two Y-family polymerases, PolIV and PolV; both are DNA damage inducible and belong to the SOS regulon. PolIV has an extremely low affinity for the naked primer-template substrate and heavily relies on the β clamp (a bacterial functional homolog of PCNA) to load onto DNA [87, 88]. *In vitro* studies indicate that PolIV and its archeal homolog Dpo4 are relatively faithful polymerases at the incorporation step and the low fidelity primarily results from poor discrimination between correct and incorrect incoming nucleotides at the binding stage and the capacity to elongate mismatched primer template, which results in –1 frameshift mutations [89, 90]. Hence, PolIV promotes mutagenesis through three distinct mechanisms: replication error, TLS and incorporation of base analogs.

PolV plays a critical role in the most characterized damage-induced mutagenesis pathway. Like PolIV, DNA synthesis by PolV is strictly distributive, requires additional cofactors such as RecA, SSB and the β clamp, and can efficiently bypass essentially all lesions tested to date [91]. PolV accounts for the vast majority of UV-induced mutagenesis in *E. coli* [91]. Due to its notorious substrate plasticity, PolV must be placed under strict regulation; indeed PolV activity is controlled at both transcriptional and post-translational levels [81].

Budding yeast also contains two Y-family polymerases. Rev1, the first characterized eukaryotic Y-family member, is a deoxycytidyl transferase that inserts a dCMP efficiently opposite a template abasic site and is probably responsible

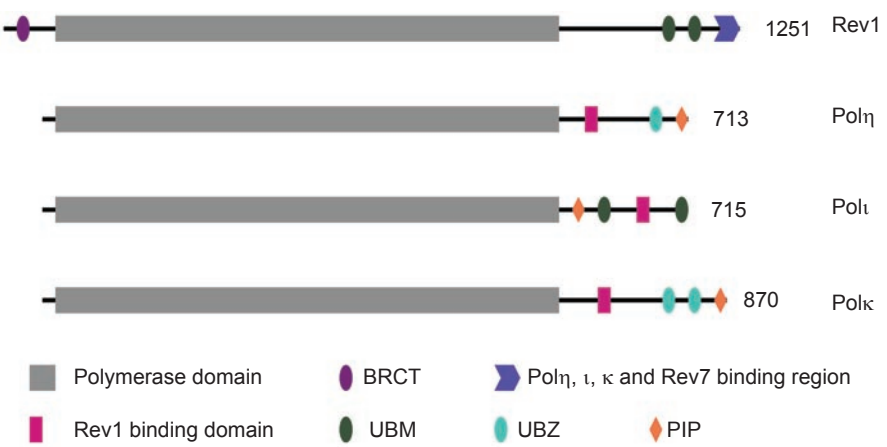

Figure 3 Functional domains of Y-family polymerases. Only four well-defined human Y-family polymerases are illustrated with the relative position of their functional domains indicated. Numbers indicate total amino acids of each protein. Note that the polymerase domain can be divided into subdomains based on structural analysis, which makes each of them specialized for lesion recognition and bypass. Other domains are considered to perform regulatory roles. Figure adapted from [82]. Abbreviations used: BRCT, BRAC1 C-terminal domain; UBM, Ub-binding motifs; UBZ, Ub-binding zinc fingers; PIP, PCNA interacting peptide.

for 60-85% of the bypass events at a specific abasic site *in vivo* [49, 92]. It can also insert dCMP across template G or A, albeit to a lesser extent [49]. Structural analysis indicates that Rev1 does not facilitate base pairing between the template G and the incoming dCTP. Instead, the G is evicted from the DNA helix and the dCTP pairs with a protein "template" Arg residue [93], which ensures base selection in a DNA template-independent manner. The yeast *rev1* mutant displays a complete loss of mutagenesis activity comparable to that of *rev3*, which cannot be explained by its dCMP transferase activity. Indeed, analysis of site-specific mutations confirms that the Rev1 enzymatic activity is not essential for TLS, but its BRCA1 C-terminal (BRCT) domain [94, 95] and/or a polymerase-associated domain (PAD) [96] are required for protein interactions. The C-terminal 100 amino acids of human Rev1 are sufficient to interact with all other TLS polymerases [97] (Figure 3), implying a scaffold role of Rev1 in TLS. The Rev1 structure and functions appear to be highly conserved in higher eukaryotes. Experimental reduction of *REV1* expression in cultured human cells results in a decrease in UV-induced mutagenesis [98].

Polη in yeast is encoded by *RAD30*, whose inactivation [99] or mutations in the corresponding mammalian xeroderma pigmentosum variant (*XPV*) gene [100, 101] lead to an increased susceptibility to UV-induced DNA damage. Polη is able to correctly incorporate AA opposite *cis-syn* thymine-thymine dimers [102] with kinetics comparable to that of the opposite undamaged template [103]. This insertion fidelity is thought to be achieved through an induced-fit mechanism similar to replicative polymerases [104]. However, for other types of lesions including those induced by UV, such as cyclobutane pyrimidine dimers and TT (6-4) photoproducts, Polη has reduced affinity, poor incorporation rates or low fidelity [105]. Hence, Polη appears to be highly specialized and the only known "error-free" Y-family polymerase when bypassing thymine dimers.

Mammals contain two additional Y-family polymerases. Polι is the only known DNA polymerase to date that violates the Watson-Crick base-pairing rule [106]. It relies on Hoogsteen base pairing as opposed to typical Watson-Crick base pairing and thus operates with very low fidelity [107]. This mechanism may facilitate read-through of replication-blocking minor groove purine adducts [108]. *In vivo*, uracil derived from cytosine deamination may be the desired target of Polι as it inserts a G opposite a template U [109].

Polκ is thought to be involved in the elongation step following mismatched bases or following damaged bases [110-112], and reads through bulky adducts such as modifications by benzo[*a*]pyrene diol epoxide (BPDE) [113, 114]. When nucleotides containing dG-N^2-BPDE, the most potent carcinogenic compound produced by industrial and cigarette smoke, are used as template, Polκ can bypass the adduct with much higher efficiency than Polη or Polι by correctly inserting C opposite the bulky lesion [114]. However, when undamaged DNA or DNA containing some common lesions is used as template, Polκ exhibits extraordinarily low fidelity [115-117].

In summary, although each of its members has distinct base-pair specificity and specialized functions, Y-family polymerases are highly conserved (Figure 3) and generally allow significantly reduced base-pair fidelity and thus result in elevated mutagenic potential. The low fidelity of these polymerases suggests that their activities must be restricted to highly selective conditions in order to limit mutational events.

Regulated access of Y-family polymerases to the damage site

Because of the high probability of TLS polymerases being mutagenic, it is expected that these polymerases are tightly regulated, probably at different levels. At the transcriptional level, an example is that human and mouse *POLK* promoters contain xenobiotic responsive elements (XREs) that can be induced by polycyclic aromatic hydrocarbons (PAHs), among which benzo[*a*]pyrene is the most characterized [118]. Hence, *POLK* is induced in response to specific DNA damage that can be bypassed by Polκ. The second regulatory mechanism is damage-induced accumulation at the replication foci stalled at DNA damage. Polη forms such foci in response to UV irradiation, and mutations with a functional polymerase motif but lacking the domain for relocalization into the damage foci are found in the XPV patient [119]. Polι physically interacts and colocalizes with Polη to the damage-induced nuclear foci [120], suggesting that Polι plays a role in bypassing UV-induced lesions. In contrast, BPDE treatment specifically induces Polκ foci formation but not Polη foci [121], indicating lesion-specific recruitment of the cognate Y-family polymerase. The mechanism of this lesion-specific recruitment is currently unknown.

Perhaps the most exciting advance in recent years is the discovery that all eukaryotic Y-family polymerases contain both PCNA interacting peptide (PIP) and Ub-binding domains, including Ub-binding motifs (UBMs) or Ub-binding zinc fingers (UBZs) (Figure 3). Polη specifically interacts with monoubiquitinated but not unmodified PCNA [122], and the Ub-binding domains are essential for the accumulation of Polι and Polη in replication foci. Similarly, the damage-induced foci formation and UV resistance of Rev1 also requires UBMs [123]. Unlike other Y-family polymerases, Rev1 does not contain a PIP motif; instead, a recent study

suggests that Rev1 utilizes its BRCT domain to interact with PCNA [95]. Supporting this notion is the observation that the damage-induced foci formation of Polη [122, 124] and Polκ [125] is dependent on functional Rad18, presumably because Rad18 is required for the generation of mono-ubiquitinated PCNA. An ultimate support perhaps comes from an *in vitro* study [126], in which PCNA was found to be only ubiquitinated when appropriately loaded onto DNA. The ubiquitinated PCNA shows similar functional interactions as unmodified PCNA with replication factors such as Fen1, Lig1, RFC, Polδ and Polε, but, in addition, is able to activate Polη and Rev1 [126].

Although the above studies provide a paradigm for the restriction and recruitment of TLS polymerases to the damage site, the overall model is challenged by other reports. One study shows that unmodified PCNA is sufficient to stimulate DNA synthesis by Polκ, primarily by reducing the *Km* to enhance correct nucleotide incorporation [127]. The direct challenge came from the *in vitro* reconstitution of the DNA synthesis reaction, in which PCNA monoubiquitinated on all three monomers does not enhance affinity for any polymerases examined, nor does it enhance TLS activity by Y-family polymerases [128]. Furthermore, a recent report [129] showed that mutations in the UBZ motif of yeast Polη did not impair its *in vivo* or *in vitro* TLS functions. The authors suggested an alternative model in which PCNA monoubiquitination may disrupt its interactions with a protein(s) that inhibits binding to the TLS polymerases. To date no such candidate protein has been identified, although we notice a recent report [130] that Mgs1, a protein with homology to *E. coli* RuvB and eukaryotic clamp loader protein RFC, as well as DNA-dependent ATPase activity and DNA-annealing activities [131, 132], associates with PCNA and appears to repress the *RAD6* pathway in the absence of exogenous damage. Other concerns with the above paradigm include the stability of monoubiquitinated PCNA, particularly in mammalian cells, that extends past the expected time required to bypass the damage [122], which would allow persistent TLS with unnecessarily increased mutation rates. In addition, hydroxyurea treatment, which depletes the nucleotide pool and induces replication fork stalling, also results in PCNA monoubiquitination [120]. The stalled replication fork after this treatment is unlikely to benefit from TLS, raising doubt that mono-Ub is at the heart of polymerase switching.

PCNA modification may not be the only means of promoting TLS; DNA damage checkpoints have been implicated in TLS. For instance, Rad9 of the *S. pombe* 9-1-1 complex, which forms a PCNA-like heterotrimeric clamp, associates with Mms2, and a mutant form of Rad9 incapable of interaction promotes mutagenesis in a TLS-dependent manner [133]. In budding yeast the phosphorylation by protein kinase Mec1 induces the re-localization of Rev1 and Polξ to sites of DNA double-strand breaks independently of mono-Ub PCNA [134]. Furthermore, the budding yeast 9-1-1 clamp physically interacts with the Rev7 subunit of Polζ and is partially required for spontaneous mutagenesis in a Polζ-dependent manner [135]. We wish to emphasize that the above observations did not directly conflict with the PCNA-TLS model.

Error-free DDT

Despite the advances made with PCNA and TLS in the past years, little is known about the molecular events leading to error-free DDT following PCNA polyubiquitination. Apparently, the error-free bypass has to utilize newly synthesized sister chromatid as a template, and, much like PCNA mono-Ub, poly-Ub of PCNA may provide a signal to initiate the process. Two possible models, namely template switching and replication fork regression, have been proposed [5]. Template switching involves homologous sister chromatid invasion/cohesion, high-fidelity DNA synthesis and the subsequent resolution of a Holliday junction (Figure 1). Fork regression (Figure 1) is thought to operate much as it does in bacteria, requiring ssDNA binding protein and RecA to produce a characteristic chicken-foot structure [15]. Experimental evidence to support a chicken-foot structure in eukaryotes came from a recent report [136] that yeast Rad5 has a DNA helicase activity that facilitates replication fork regression. In contrast, several recent reviews [137-139] suggest that the DNA damage checkpoint acts to prevent stalled replication fork regression, while error-free DDT is mediated by template switching. Alternatively, the two error-free DDT models may not be mechanistically different as they appear. Regardless of the mode of reaction, it is abundantly clear that the error-free DDT process is highly conserved in the entire eukaryotic kingdom, from yeast to human. Sequence and functional homologs of all proteins involved in error-free DDT, including Mms2 [140], Ubc13 [141] and Rad5 [142, 143], have been found in mammals, plants and other higher eukaryotes [144, 145]. For a few limited examples, suppression of the above genes resulted in phenotypes reminiscent of the corresponding yeast mutants [143, 146, 147].

DDT, genomic instability and cancer

Studies in the yeast model have clearly demonstrated the significance of DDT in maintaining genomic stability. The two branches within DDT, with one being highly mutagenic and another error-free, are likely kept to a dynamic balance in wild-type cells. However, in yeast cells defective in error-free DDT, spontaneous mutation rates can be elevated

by 30-fold, which would be viewed as a predisposition to cancer. It was postulated that error-prone TLS may constitute a major source of genomic instability and cancer [148], although direct evidence is rather lacking.

Perhaps the best studied example of TLS and tumorigenesis is the discovery of mammalian Polη, whose gene was found mutated in all XPV patients examined. Polη co-localizes with Rev1 [149], Polι [120] and mono-Ub PCNA [124], suggesting that mutations in these genes may also be associated with cancer. The level of translesion polymerases in normal and matched tumor cell lines has been investigated. Several lung cancer cell lines were found to overexpress Polκ, suggesting a role in promoting genomic instability and cancer [150]. In another study, however, transcript levels of TLS polymerases η, ι, κ and ζ are significantly reduced in various lung, stomach and colorectal cancers [151]. Clearly, more research is required to establish roles of DDT in tumorigenesis and carcinogenesis.

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