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Translesion synthesis: Y-family polymerases and the polymerase switch

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ABSTRACT

Replicative DNA polymerases are blocked at DNA lesions. Synthesis past DNA damage requires the replacement of the replicative polymerase by one of a group of specialised translesion synthesis (TLS) polymerases, most of which belong to the Y-family. Each of these has different substrate specificities for different types of damage. In eukaryotes mono-ubiquitination of PCNA plays a crucial role in the switch from replicative to TLS polymerases at stalled forks. All the Y-family polymerases have ubiquitin binding sites that increase their binding affinity for ubiquitinated PCNA at the sites of stalled forks.

1. Introduction

Most types of DNA damage cannot be accommodated in the active sites of the replicative DNA polymerases and consequently provide a block to the progress of the replication machinery. One way to circumvent this block is by "damage avoidance", using recombinational mechanisms to copy genetic information from the undamaged sister duplex. This is the principal mechanism used in *Escherichia coli*. The alternative is to incorporate nucleotides opposite the damage, a process-designated translesion synthesis (TLS). This is quantitatively a minor (but nevertheless important) process in *E. coli*, but is probably the major mechanism employed in mammalian systems. The discovery of a new family of DNA polymerases (the Y-family) in 1999 dramatically changed our perception of TLS. Previously it had been envisaged that, in response to damage, the stringency of replicative polymerases was somehow compromised such that they were now able to replicate past lesions. This long-held view turned out to be completely wrong. It is now evident that TLS is carried out by low stringency polymerases, each of which has a different substrate specificity. In mammalian cells, these polymerases (pols) are polη, ι, κ and Rev1 in the Y-family and polζ, in the B-family. Several other recently discovered DNA polymerases (pols θ, λ, μ, ν) may also have roles in TLS, but this has not yet been firmly established and these polymerases will not be discussed further in this review.

By using one or several TLS polymerases in concert, the cell is able to bypass most types of DNA lesions. The properties of these polymerases have been extensively reviewed [1–9], and readers are referred to these reviews for detailed discussion of polymerase specificities. We will summarise these properties here, laying greater emphasis on their biological roles and recent insights. We will confine the review to eukaryotic polymerases. The reader is referred to the review by Fuchs in this volume for discussion of TLS in bacteria.

The Y-family polymerases all have conserved catalytic domains, in most cases close to their N-termini.

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The C-terminal thirds of the proteins are not conserved between the polymerases and are responsible for important protein–protein interactions that mediate their localisation in the cell and utilisation at blocked replication forks. All the polymerases differ from replicative polymerases in having relatively poor processivity and low fidelity, the latter being partly due to the lack of an associated 3′–5′ proofreading exonuclease activity. All of the Y-family members are stimulated by PCNA, the polymerase sliding clamp accessory protein.

2. TLS polymerases

2.1. DNA polymerase η (polη)

Polη has the unique property of being able to synthesise past DNA containing cyclobutane thymine dimers with similar efficiency to undamaged DNA [10]. In the majority of cases, adenines, the correct nucleotides, are inserted opposite the thymines in the dimer, despite the distortion caused by the cyclobutane ring [10,11]. This ability to replicate efficiently past the major UV photoproduct is likely to be the principal function of polη in vivo. Cells from patients with the variant form of xeroderma pigmentosum (XP-V) are defective in polη [12,13]. They have a greatly increased frequency of UV-induced mutations and an altered UV mutation spectrum. XP-V patients, have, like XP patients defective in nucleotide excision repair, a very high incidence of sunlight-induced skin cancer. These findings are consistent with polη being the normal enzyme for carrying out TLS past cyclobutane pyrimidine dimers (CPDs). In its absence another enzyme does the job, but less efficiently and less accurately. This results in the increased rate of UV-induced mutagenesis and carcinogenesis in XP-V cells. Evidence has been obtained to suggest that in mouse cells this back-up polymerase is probably polι [14]. Mice in which the catalytic domain of polη has been deleted, show, like XP-V patients, an increased frequency of UV carcinogenesis [15]. Polη does not appear to be able to carry out TLS past the other major photoproduct, the pyrimidine(6-4)pyrimidone photoproduct (6-4 PP), either in vitro or in vivo.

Polη is able to carry out TLS past a limited number of other types of damage in vitro, albeit less efficiently than past CPDs. Whether this also happens in vivo has in most cases not yet been established. XP-V cells are sensitive to cisplatin, suggesting that bypass of cisplatin lesions is also normally effected by polη [16,17]. It does not however appear to play a role in replication past 8-oxoguanine [18] or abasic sites [19].

Using immunofluorescence, it has proved difficult to visualise any of the TLS polymerases at their endogenous levels. However, in cells mildly overexpressing polη, it is constitutively located in replication factories in the nucleus during S-phase, this localisation depending on several motifs close to the C-terminus of the protein including a zinc finger, bipartitie nuclear localisation signal and C2H2 zinc finger ([20] and Kannouche, Wing and Lehmann, unpublished data). Thus polη is always on hand close to the sites of replication, in case it might be needed to carry out TLS.

Recent evidence has suggested that outside its role in TLS, polη is required for gene conversion in chicken cells [21], and in vitro polη can extend the invading strand in a D-loop structure [22]. Polη may therefore also play a role in some types of recombination, though there is no obvious manifestation of recombination deficiency in XP-V patients. Furthermore, SV40-transformed XP-V cells have an elevated level of UV-induced sister-chromatid exchanges, implying that polη cannot be required for this type of recombination [23].

2.2. DNA polymerase ι

Polι has very low processivity. It is able to insert bases opposite some types of damage, but is not able to extend synthesis further from the inserted base. It has a very high error rate, particularly opposite template T, at which it inserts G in preference to the correct A [24]. Its function remains a mystery. It is, like polη, localised in replication factories and physically interacts with polη [25]. Recent data suggest that when polι is depleted from mouse cells using siRNA, UV mutagenesis is reduced, and this is particularly evident in a polη-deficient background [14]. However, when a different mutation system was used in human 293 cells, no effect of polι depletion on UV mutagenesis was observed [26]. The reason for this discrepancy is not obvious.

No human condition has been found associated with polι deficiency. However, the 129 strains of mice, widely used in the generation of knock-out mice has an ochre mutation close to the N-terminus of the protein and appears to make no functional protein [27]. To date these mice have no unusual phenotype.

2.3. DNA polymerase κ

Polκ is able to carry out TLS past benzo[*a*]pyrene-guanine and other adducts on the N^2 position of guanine both in vitro and in vivo [28–30], but is not required for bypass of AP sites. polκ-deficient mouse cells are sensitive to benzo[*a*]pyrene [28] and methyl methanesulfonate [31]. These cells are also sensitive to UV light despite polκ's inability to carry out TLS past either of the UV photoproducts. Unexpectedly this UV sensitivity is due to a deficiency in NER [32]. Thus polκ seems to have a role in the repair synthesis step of NER aside from its role in TLS. Polκ-deficient mice have been generated in several laboratories but do not show any significant phenotype [28,33,34]. Although exclusively nuclear, polκ, unlike all the other Y-family members, is located in replication factories in only a small proportion of S-phase cells [35].

2.4. Rev1 and polζ

The fourth member of the Y-family, Rev1, differs from the other members in several ways. Although structurally it is a bona fide member of the Y-family, it is not in fact a DNA polymerase, but a dCMP transferase, capable of inserting dCMP opposite either Gs or abasic sites in template DNA [36]. Rev1 deficiency confers very similar phenotypes to deficiencies in DNA polymerase ζ, a heterodimer consisting of the B-family catalytic subunit Rev3 and the regulatory Rev7 subunit. These Rev proteins are required for mutagenesis induced by many types of DNA damage in yeast and, with more limited evidence, in human cells [37–39]. Curiously, however the catalytic

activity of Rev1 is not required for its role in mutagenesis. However, mutation of the catalytic site did affect the nature of the inserted base during TLS past an abasic site [40]. Rev1 also differs from the other TLS polymerases in having a BRCT domain close to its N-terminus. This BRCT domain is important for binding to PCNA [41], and for survival after UV-irradiation in both yeast and chicken cells [41]. Studies in yeast have shown that Rev1 and Rev3 play only a minor role in TLS past CPD, but are required for bypass of 6-4 PP and abasic sites [40,42–44].

In chicken DT40 cells, complete deletion of the *Rev1* gene results in slow growth and sensitivity to a wide variety of DNA-damaging agents [45]. Mouse cells in which the BRCT domain has been deleted are slightly sensitive to UV-irradiation and have a reduced level of UV-induced mutations [46]. Mutations at A–T base pairs are completely absent, suggesting that Rev1 plays a role in TLS past mutagenic lesions at T–T dipyrimidine sites. Attempts to generate a Rev3-deficient mouse have not been successful [47–50], but an embryonic fibroblast cell line has been generated from Rev3-deficient p53+/− embryonic cells, which had also lost the wild-type copy of p53. These cells also grew slowly and were sensitive to UV and cisplatin [51].

The C-terminal 150 aa of mammalian Rev1 are able to interact with the other three Y-family polymerases as well as the Rev7 subunit of polζ [52–55]. This suggests that Rev1 may have a role as a platform during TLS (see below for further discussion).

2.5. *TLS polymerases and the immune system*

A major feature of the generation of immune diversity is a phase of somatic hypermutation in target cells. The first stage of hypermutation is the deamination of cytosine to uracil by AID in the variable regions of immunoglobulin genes. Uracils are removed from DNA by the enzyme uracil glycosylase, and insertion of bases other than G opposite from the resulting abasic site is thought to be the source of hypermutation at C–G base pairs [56].

However, SHM is also found at A–T base pairs and this is proposed to result from an error-prone base excision repair process on excision of the abasic site. Models for the generation of SHM invoke roles for the Y-family polymerases. No defect in SHM has been found in polι-deficient [57] or polκ-deficient mice [33,34] or polκ polι double mutant mice [58]. SHM is found at normal levels in XP-V patients and polη-deficient mice [59,60]. However, the mutation spectrum is altered. There is a reduction in the mutations at A–T sites and a compensating increase at G–Cs. This has led to the suggestion that polη is involved in the process that generates hypermutation at A–T sites. This correlates with the spectrum of mutations generated when polη was used to replicate an immunoglobulin gene in vitro [61]. This spectrum did not change further in a double mutant polι polη mouse.

In the chicken DT40 system, immune diversity is generated largely by gene conversion and to a lesser extent by point mutations. As mentioned above, the former appears to involve polη [21], whereas the latter is largely dependent on Rev1 [45].

3. Polymerase switching

In order for the Y-family polymerases to be able to carry out TLS, the replicative polymerase must first be displaced and replaced with the appropriate TLS polymerase. This process is referred to as the polymerase switch (reviewed in [62]). Work with *E. coli* has highlighted the central role of the polymerase-associated sliding clamp (the β subunit of polIII) in this process (e.g. [63]; see also article by Fuchs in this issue). Similarly in eukaryotic cells, the homotrimeric sliding clamp PCNA plays a major role in switching between replication and TLS. This switching is activated by the post-translational modification of PCNA by ubiquitination.

3.1. *Ubiquitination of PCNA*

It has long been known from genetic studies in *Saccharomyces cerevisiae* that the Rad6, Rad18, Mms2, Ubc13 and Rad5 proteins are involved in the replication of damaged DNA. Rad6 and Rad18 are required for all replication-associated processes, both error-free and -prone, whereas the other proteins specifically control an error-free branch of replication past damage [64]. Subsequent biochemical studies revealed that Rad6 and a heterodimer of Mms2 and Ubc13 are E2 ubiquitin conjugating enzymes and that Rad18 and Rad5 are E3 ubiquitin ligases. Mms2–Ubc13 is able to form ubiquitin chains linked via lysine-63 rather than the classical lysine-48 linkage used to target proteins for degradation. The target of these ubiquitination reactions was revealed in 2002 by Hoege et al., who showed that, following exposure of cells to methyl methanesulfonate, PCNA became mono-ubiquitinated on lysine-164 by Rad6 and Rad18, and subsequently the mono-ubiquitinated PCNA became polyubiquitinated via Lys-63 linkage in a reaction mediated by Mms2–Ubc13 and Rad5 [65]. Genetic studies suggested that mono-ubiquitination of PCNA mediated the switch to translesion synthesis, whereas polyubiquitination channelled the DNA into an error-free damage avoidance pathway that remains uncharacterised [66].

In mammalian cells the major modification of PCNA in response to UV-irradiation is mono-ubiquitination [67]. Polyubiquitination is barely detectable, but has been revealed at a level about 20-fold lower than mono-ubiquitination [68]. PCNA is mono-ubiquitinated in response to UV-irradiation as well as to a variety of other DNA-damaging agents (MMS, mitomycin C, cisplatin, H_2O_2, benzo[*a*]pyrene-diolepoxide ([67,69–71] and our unpublished data)) that result in stalling of the replication fork.

Likewise treatment with hydroxyurea, which results in halting of fork progression by depleting the cell of deoxyribonucleotides, triggers ubiquitination of PCNA. In contrast, ionising radiation, bleomycin, and neocarzinostatin, which prevent initiation of replicon firing rather than slowing down of forks, do not trigger PCNA ubiquitination, nor do actinomycin D, daunorubicin or nocodazole ([67,70] and our unpublished results).

In *S. pombe* PCNA is mono-, di-, and tri-ubiquitinated in a normal S-phase and this is much greater in damaged cells [72]. Curiously in *Xenopus laevis* egg extracts incubated with sperm chromatin, PCNA was both sumoylated and mono-

ubiquitinated during replication of undamaged chromatin. When damaged chromatin was used as template, the PCNA became di-ubiquitinated [73]. In chicken DT40 cells, the major modification of PCNA is mono-ubiquitination, like in mammalian cells. Interestingly however, this is only partially dependent on Rad18, unlike in mammals and the yeasts [74], implying that in the chicken cells there is another E3 ligase that can modify PCNA. The reasons for the different modifications of PCNA in damaged and undamaged cells in different organisms remain to be established.

3.2. *Control of PCNA ubiquitination*

PCNA mono-ubiquitination thus appears to result from replication fork stalling. Blocking of the replication machinery is likely to generate single-stranded regions of DNA, where the replication-associated helicase continues to progress along the template, but the replication machinery itself is blocked. In both yeast [65] and human cells [67,75], mono-ubiquitination of PCNA is dependent on Rad18. Rad18 binds to single-stranded DNA [76], so the exposed single-stranded regions of DNA at the stalled forks may be sufficient to activate the E3 ligase activity of Rad18. Stalled replication forks are thought to trigger two other signalling pathways, namely the cell cycle checkpoint mediated by ATR and the mono-ubiquitination of FANCD2. It appears that these three signals are independent of each other. Checkpoint responses can be abrogated either in yeast by using appropriate mutant strains or in mammalian cells by using siRNA. In *S. pombe* the *rad3* gene is the homolog of *S. cerevisiae MEC1* and human ATR [77]. The ubiquitination of PCNA in response to DNA damage was identical in wild-type and *rad3* deletion strains of *S. pombe* [72]. Similarly depletion of ATR from human cells had no significant effect on PCNA mono-ubiquitination (Kannouche, unpublished data). Mutation of PCNA lys164 to arg prevents its ubiquitination, but in *S. pombe*, this has no effect on activation of the checkpoint response [72]. Most Fanconi anemia cell lines are unable to ubiquitinate FANCD2 in response to damage, but the mono-ubiquitination of PCNA is similar to that in normal cells [78].

Several recent findings suggest that a stalled replication fork might not be the only trigger for PCNA ubiquitination. UV-induced PCNA ubiquitination was found in human cells held in either G0 or G2 [70] (and our unpublished observations), and in *S. pombe* cells maintained in G2 [72]. Furthermore, after exposure of human cells to H_2O_2, PCNA ubiquitination reaches maximum levels 30 min after treatment (SB, PLK and ARL, unpublished observations), in contrast to agents such as UV which generate maximum PCNA levels after several hours, by which time many blocking lesions have been encountered. Finally, following treatment of *S. cerevisiae* cells held in G1 with nitrogen mustard, which generates interstrand cross-links, PCNA ubiquitination was observed following incision and unhooking of the cross-link [79]. This process generates a gap opposite a lesion (in this case the unhooked cross-link), a structure which may resemble a blocked replication fork and may trigger PCNA ubiquitination by a similar mechanism. In the other cases discussed above, the nature of the inducing signal remains to be established.

At the time of writing, the only mammalian mutant in the PCNA ubiquitination pathway is a mouse RAD18 knock-out cell line. RAD18$^{-/-}$ ES cells had a normal growth rate but were sensitive to UV, MMS, cisplatin and mitomycin C, but not to ionising radiation [80]. These responses correlate well with the stimulation of PCNA mono-ubiquitination by these agents. Post-replication repair, the generation of high molecular weight daughter strands in UV-irradiated cells, was somewhat reduced in the RAD18$^{-/-}$ cells. UV and MMS-induced mutagenesis were slightly reduced, whereas the levels of SCEs induced by various agents were increased [80]. The mutagenesis responses are quite different from those of *rad18* mutants of *S. cerevisiae*, in which UV mutagenesis is abolished [64].

Activation of Rad18 is not the only event that controls the ubiquitination of PCNA. The de-ubiquitinating isopeptidase (DUB) USP1 is able to remove the ubiquitin from mono-ubiquitinated PCNA [78]. In response to UV-irradiation, USP1 is degraded by an autocleavage mechanism, and this is correlated with the appearance of ubiquitinated PCNA. Expression of a non-degradable form of USP1 decreased the level of UV-induced PCNA ubiquitination. Depletion of USP1 by siRNA resulted in an increased mutation frequency in the *supF* gene of a transfected UV-irradiated plasmid. These data suggest that USP1 keeps PCNA ubiquitination at a low level in undamaged cells to prevent the unwanted employment of TLS polymerases in undamaged cells. We have found that following DNA-damaging treatments, PCNA remains ubiquitinated for many hours, even if the damage has been removed (our unpublished data), and correspondingly, following its cleavage, USP1 remains at a low level for many hours.

PCNA ubiquitination after UV-irradiation also seems to be regulated by p53 and p21. However, two different experimental designs gave apparently conflicting results. Soria et al. noted a correlation between p21 degradation in response to DNA damage and PCNA ubiquitination. When they overexpressed myc-tagged p21, which was not degraded following irradiation, PCNA ubiquitination was suppressed. This suppression was not mediated by direct interaction with PCNA, since a mutant p21 defective in binding to PCNA gave a similar inhibition of PCNA ubiquitination [70]. The second study assessed the effect on PCNA ubiquitination of depressing p53 and p21 levels using siRNA. Reducing the concentrations of either protein reduced the level of PCNA ubiquitination [81]. Thus it appears that either reducing or overexpressing p21 levels can inhibit UV-induced PCNA ubiquitination.

3.3. *Mechanism of the polymerase switch*

Mono-ubiquitination of PCNA increases its affinity for polη, polι, polκ and Rev1 [67,71,75,82,83]. All these Y-family polymerases have novel ubiquitin binding motifs designated UBM (Polι and Rev1) or UBZ (polη and polκ). These motifs provide a mechanism to explain the increased affinity of the polymerases for ubiquitinated PCNA [82]. Furthermore, in vitro studies have shown that the ability of polη to carry out TLS past an abasic site by either polη or Rev1 is stimulated much more by ubiquitinated than by unmodified PCNA, whereas on an undamaged template, synthesis is stimulated by PCNA irrespective of its ubiquitination status [84]. Taken together, these findings provide a persuasive model

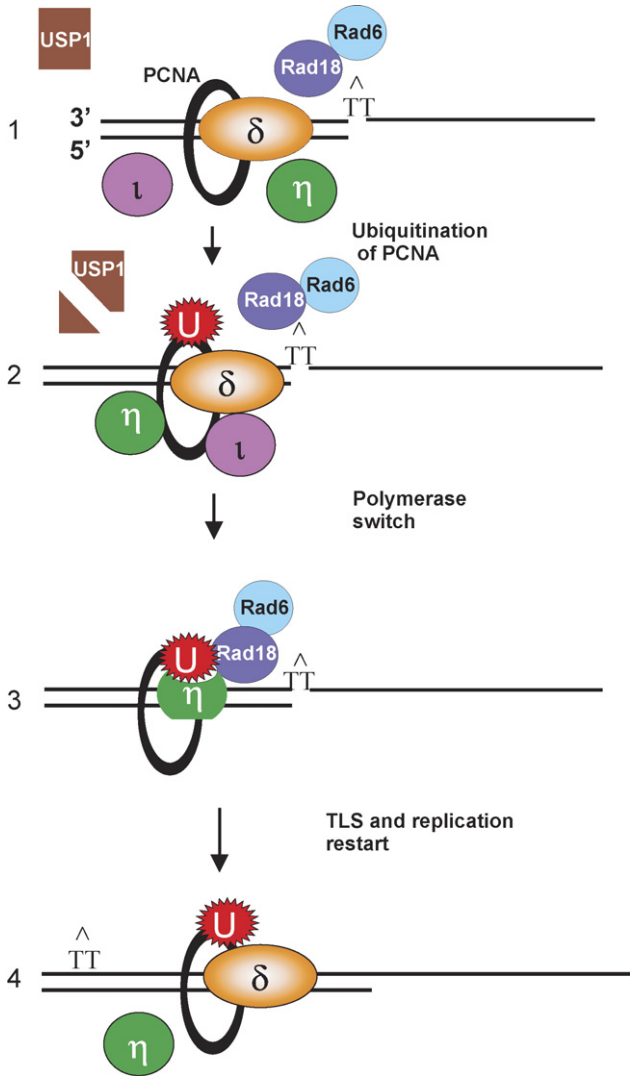

Fig. 1 – Model for translesion synthesis. (1) The replication machinery including PCNA and polδ is stalled at a CPD. As a result of recruitment of Rad18–Rad6 and cleavage of USP1, PCNA is mono-ubiquitinated (2). For clarity, only one PCNA monomer is shown to be ubiquitinated, although in reality, all three monomers of one trimer are probably ubiquitinated [67]. (3) This increases the affinity for Y-family polymerases, in this case polη, which carries out TLS and then dissociates (4).

for polymerase switching at the site of a lesion (Fig. 1). In the first step, the lesion blocks the passage of the replication machinery. This exposes single-stranded regions of DNA, which activate Rad18, and concomitantly USP1 is cleaved. Rad18 binds Rad6 and this E2–E3 complex mono-ubiquitinates PCNA. The ubiquitin moiety strengthens the binding of the TLS polymerases to PCNA at the site of the blocked replication machinery, increasing the opportunity for one of them to bypass the lesion if it is an appropriate substrate. The ubiquitin binding domains are crucial for the biological functions of polη and Rev1 and for localisation of polη, polι and Rev1 [82,83,85].

3.4. *Polyubiquitination of PCNA*

In *S. cerevisiae*, Rad18 is able to bind not only to itself and Rad6 but also to the Rad5 E3 ligase [86]. This will presumably recruit Rad5 to the blocked replication machinery and enable polyubiquitination of PCNA to be effected by Mms2–Ubc13 and Rad5. Genetic studies have indicated that this mediates an error-free process, which has been proposed to be some kind of template-switch copy choice mode of recombination that is independent of the Rad52 pathway (e.g. see [87]). Very little is known of the mechanism of this pathway. Mammalian orthologs of Mms2 and Ubc13 have been identified and treatment of human cells with antisense constructs to MMS2 led to an increased UV-induced mutation frequency, consistent with a role of MMS2 in an error-free process [88]. The ortholog of Rad5 designated SHPRH has only recently been identified and overexpression of this protein together with other proteins in the polyubiquitination pathway results in polyubiquitination of PCNA in response to MMS treatment [89]. Under normal conditions, polyubiquitinated PCNA can only be detected with the most sensitive assays and its level is much less than that of mono-ubiquitinated PCNA [68]. The exact function of this modification remains obscure.

4. Choice of polymerase and site of TLS

An important question that has been the subject of extensive debate concerns the selection of the polymerase to carry out TLS past a blocking lesion. Does each polymerase have a cognate lesion, or do the polymerases compete by mass action and the enzyme best able to do the job wins the competition? Several findings tend to favour the latter hypothesis. (1) There are weak interactions between the different polymerases themselves; (2) there are weak interactions between the polymerases and PCNA, which are increased when PCNA is ubiquitinated (these interactions are summarised in Fig. 2); (3) the homotrimeric ring structure of PCNA could allow up to three polymerases to interact with PCNA simultaneously

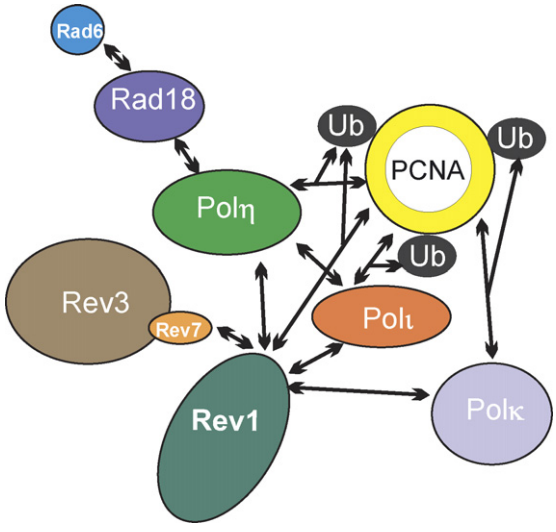

Fig. 2 – Interactions between different proteins involved in TLS.

(see [90,91]); (4) the residence time of polη and polι in replication foci is very short ([92] and Sabbioneda et al., unpublished results). Taken together these results suggest a dynamic scenario with the different polymerases binding briefly to PCNA, somewhat longer if it is ubiquitinated. If PCNA is sited at a blocking lesion, an associated polymerase will attempt to replicate past the lesion, and if the lesion can be accommodated in its active site it will carry out TLS successfully. Alternatively, it may be able to insert nucleotides opposite the lesion but not to extend from the lesion, as found for polι with certain lesions in vitro. In this case, a second switch would be necessary to a polymerase that can extend from a nucleotide inserted opposite a lesion, a possible role for polζ. If the polymerase cannot accommodate the lesion in its active site, it will dissociate from the PCNA.

The ability of Rev1 to bind all other TLS polymerases as well as to ubiquitinated PCNA (Fig. 2), together with its role in TLS being independent of its catalytic activity has led to the suggestion that it too may play a role in polymerase switching by acting as a platform for different polymerases [62,93]. Its biological properties suggest that it may have a role in switching events that involve polζ, for example in a two-stage event with one polymerase carrying out the insertion opposite the lesion, and a second switching event facilitating extension from the inserted base by polζ.

A further important question pertains to the location of TLS relative to the replication fork. Most current models envisage TLS occurring at the stalled fork (Fig. 3A). However, early results and very recent data suggest that this may not be the case. The replication fork might proceed, leaving a gap opposite the lesion, with the gaps being sealed subsequently (Fig. 3B). This was in fact the original model for replication past DNA damage proposed in 1968, based on results from *E. coli* [94]. Recently short single-stranded regions located several kilobases behind the replication fork have been visualised in electron micrographs of UV-irradiated yeast cells. The number of these gaps that could be visualised was increased in mutant strains lacking TLS polymerases [95]. These data suggest that gaps were indeed left behind the fork on both leading and lagging strands. Such a model necessitates replication restart beyond the lesion on both leading and lagging strands. Mechanistically this is easily accommodated by discontinuous synthesis on the lagging strand. However, it has been demonstrated that replication restart can also be effected beyond a blocked replisome on the leading strand, at least in an *E. coli* in vitro system [96]. Some of the gaps left behind the replication fork may even persist into G2, providing a possible explanation for the recent observation that the level of Rev1 mRNA in *S. cerevisiae* increases 50-fold in G2 [97].

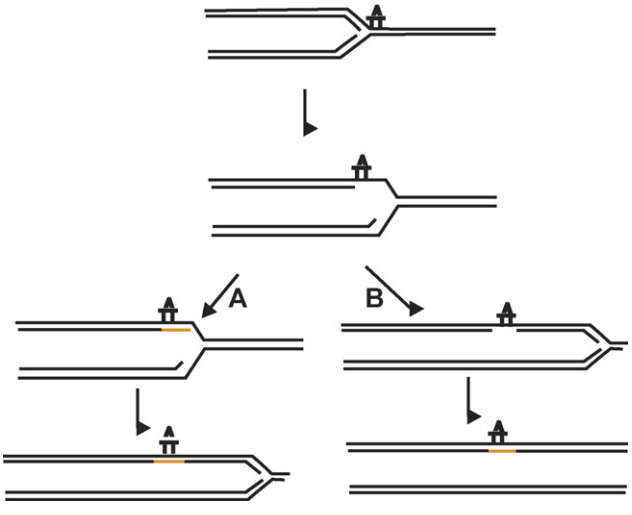

Fig. 3 – TLS at or behind the fork. Following blocking of the replication machinery by a CPD, model A shows TLS occurring at the site of the blocked fork followed by replication restart. Model B shows replication restarting beyond the blocked fork leaving a gap, which is subsequently sealed by TLS after the fork has proceeded downstream.

5. Concluding remarks

Developments in recent years have dramatically increased our knowledge of the mechanism of TLS. However, our understanding is still very rudimentary and many questions remain. For example: (1) What are the functions in vivo of polι, polκ, Rev1 and polζ, in vivo? (2) Although we have gained insights into the control of PCNA ubiquitination, its de-ubiquitination, or lack of it, remains enigmatic. (3) As discussed above, are polymerases selected for TLS purely by mass action, or is there some more positive discrimination? (4) How is polyubiquitination induced and/or prevented, and what is the precise nature of the error-free pathway that it is thought to mediate? (5) Does TLS occur at or behind forks and are there differences between leading and lagging strands? (6) What is the role that polη plays in generating mutations at A–T sites during somatic hypermutation and do other polymerases also play a role?

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