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Mysterious and fascinating: DNA polymerase ι remains enigmatic 20 years after its discovery

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Abstract

With the publication of the first paper describing the biochemical properties of DNA polymerase iota (polι), the question immediately arose as to why cells harbor such a low-fidelity enzyme which often violates the Watson-Crick base pairing rules? Yet 20 years after its discovery, the cellular function of polι remains unknown. Here, we provide a graphical review of the unique biochemical properties of polι and speculate about the cellular pathways in which enigmatic polι may participate.

Keywords

Replicase; Replication fidelity; Translesion DNA Synthesis; Mutagenesis; Y-family DNA polymerase

Introduction

In order to transfer genetic information from generation to generation timely and accurately, cells should not only be able to copy ideal double helix DNA, but also DNA containing fragments with imperfect structure. The vast majority of endogenously and exogenously generated lesions distort DNA to the extent that hinders progression of the cell's DNA replicase. Numerous mechanisms are utilized to repair the damaged DNA, but a substantial number of DNA defects remains unrepaired by the time they are encountered by a nascent replication fork. The fact that cells are able to complete their genome doubling suggests that the replication machinery is able to overcome these obstacles. How is this achieved in a living organism? For many years, the prevailing concept was that the damage tolerance mechanism, also called post-replicative repair, primarily relies on replication of damaged DNA catalyzed by the cell's main replicase with the assistance of accessory factors. It was proposed that translesion DNA synthesis (TLS) occurs in two discrete steps: i) (mis)insertion opposite a lesion, followed by ii) extension of the resulting base (mis)pair so as to complete bypass of the lesion. When this mechanism was first developed in 1985 using

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Escherichia coli (*E. coli*) as a model organism, it implied that the cell's main replicative polymerase, pol III, catalyzes both steps of TLS, but the elongation step was accomplished with the help of polymerase accessory proteins, UmuD and UmuC (Figure 1A) [1]. The notion that this process was conserved throughout evolution gained traction though the discovery of a family of so-called "mutagenesis proteins" related to *E.coli* UmuC, such as *S. cerevisiae* Rev1 [2], *E.coli* DinB [3] archeal Dbh [4]; and *S.cerevisiae* Rad30 [5]. The two-step model began to evolve in 1999, when several investigators reported intrinsic DNA polymerase activity associated with the mutagenesis proteins (reviewed in [6]). Thus, rather than acting solely by assisting replicative polymerases at the extension step, the TLS polymerases would catalyze both misinsertion *and* extension themselves (Figure 1B) [7]. With the discovery of human homologs of *S.cerevisiae* Rad30 (polη and polι) and *E.coli* DinB (polκ) (reviewed in [6]), it became clear that the mechanism of TLS is conserved throughout evolution (Figure 1C) [8] and gave rise to the "Y-family of DNA polymerases" which are found in all domains of life [9]. Our graphical review focuses on one member of this family, polι, whose cellular function remains enigmatic two decades after its discovery [10]. We speculate on possible roles for polι based on its enzymatic properties, although to date, direct *in vivo* evidence for most of these proposed roles is still lacking.

Unique misincorporation specificity makes polι useful for somatic hypermutation

The most intriguing property of polι which distinguishes it from other DNA polymerases is its fidelity (Figure 2D–F, I & J). The accuracy with which polι incorporates nucleotides is unusually template sequence dependent. Misincorporation frequency is lowest at template A, and highest at the template T, where polι favors incorporation of a wobble G instead of the correct "Watson–Crick" base, A (Figure 2D) with a 3 to 11-fold preference depending on the surrounding sequence context [11]. Furthermore, T and A are inserted opposite T with roughly the same efficiency. It is important to note that polι exhibits a similar pattern of misincorporation opposite thymines and uracils [12, 13]. Whereas preferential misinsertion of G opposite T is highly mutagenic, the same specificity opposite U can reduce mutation frequencies if the uracils in DNA result from cytosine deamination.

While the fidelity of polι is very similar on primed single-stranded and gapped DNA substrates (Figure 2D & E), on DNA templates containing a one nucleotide 5′ overhang, the pattern of nucleotide misincorporation changes (Figure 2F). On these templates, polι has lowest fidelity opposite C, where C and A are misinserted 3 to 8-fold more frequently than the correct base, G [14]. Reduced fidelity was also seen opposite template G, where it inserted C and T with roughly equal efficiency.

The unique fidelity of polι initially suggest that it would be a good candidate as a somatic hypermutase (Figure 3). Indeed, the misincorporation pattern seen for polι *in vitro* using different DNA substrates suggested that it could participate in the hypermutation of immunoglobulin variable genes *in vivo* by generating A/T mutations in the middle (Figures 2D & 3A) [11] and G/C mutations at the end of DNA template (Figures 2F & 3B) [14]. However, analysis of hypermutation in variable genes from mice carrying a naturally

occurring nonsense mutation in exon 2 near the 5′ end of the *Poli* gene that results in negligible expression of a truncated catalytically inactive polymerase [15], did not reveal any changes in the overall frequency and spectrum of base pair substitution in the variable region compared to the mice carrying wild-type *Poli* [16]. The same results were obtained using knock-in mice expressing full-length catalytically inactive polι, suggesting that loss of polι does not contribute to single base-pair hypermutation in mice [17]. Although these data might argue against the participation of polι in hypermutation, they are also consistent with the scenario where another low-fidelity polymerase can readily substitute for missing polι. Furthermore, an essential role for polι in somatic hypermutation was eventually discovered, in that it, along with pol ζ, is required for the generation of tandem mutations in immunoglobulin genes [17]. The proposed mechanism for the generation of tandem mutations is conceptually similar to the two-step model of TLS with polι catalyzing the first errant misincorporation and pol ζ extending the resulting mispair, while simultaneously introducing a second mutation (Figure 3A).

Polι is able to catalyze translesion replication past a variety of DNA lesions

Multiple *in vitro* studies suggest that polι can incorporate nucleotides opposite a variety of DNA lesions, while further elongation is either substantially inhibited, or completely abolished (Figures 2J & 4A, B) (reviewed in [10]). For example, it has been shown that polι may, under certain circumstances, facilitate efficient TLS of UV photoproducts (Figure 4) [18, 19]. In support of this idea, mice lacking polι develop mesenchymal cancers in response to UV exposure, and mouse embryonic fibroblasts lacking polι exhibit an altered spectrum of UV-induced mutations. These findings are consistent with suggestions implicating polι in TLS past UV-induced lesions. In particular, it seems likely that while polι only plays a minor role in TLS of UV lesions in normal cells, it is in demand in xeroderma pigmentosum variant [XPV] cells lacking polη, the enzyme responsible for the efficient and accurate bypass of cyclobutane pyrimidine dimers (CPDs). This hypothesis is supported by the fact that the increased frequency of UV-induced mutations in XPV cell lines closely correlates with the relative level of polι expression and the abnormal mutational spectrum generated in cells devoid of polη is strikingly similar to the misincorporation pattern characteristic for polι *in vitro* (Figure 4A–C) [20].

Polι accumulates at UV-induced stalled replication forks and its localization is facilitated, to a large extent, through an interaction with polη [21]. Consequently, in XPV cells lacking polη, polι foci formation is drastically diminished. These findings seem to argue against an involvement of polι in TLS in XPV cells. However, we believe that the remaining number of polι-containing foci generated as a result of recruitment of polι by an interaction with PCNA [22], is sufficient to carry out replication of UV-damaged DNA, although with markedly reduced efficiency and fidelity. Therefore, polι appears to be at least partially responsible for the hypermutable phenotype ultimately resulting in skin cancers associated with an XPV defect.

Polι's unique biochemical characteristic of misincorporating G opposite T or U suggests a potential error-free role in TLS, and in replication of undamaged DNA, if the T or U was once 5-methylcytosine, or cytosine, that had undergone deamination (Figure 4F) [12, 13]. In

fact, hydrolytic deamination is a common problem, especially for growing cells since it is >100 times faster in single- stranded DNA generated during replication and transcription, than in double- stranded DNA. Uracil derivatives are also often produced by cytosine deamination following exposure to DNA damaging agents. In all these cases, recruitment of polι, the only known polymerase favoring misinsertion of G opposite undamaged and damaged U, makes perfect sense and might help to explain why downregulation of its expression leads to cancer development. Indeed, an unusual substrate specificity of polι would play an important role in preventing CG to TA transition mutations that would be generated if the modified base was copied accurately. Therefore, an important biological function of polι could be maintaining genome integrity by restoring the coding properties of cytosines that have undergone spontaneous, or damage-induced deamination.

Studies in several laboratories are consistent with findings suggesting that while polι is able to (mis)insert bases opposite a number of lesions, it often cannot extend primers past the damaged sites (recently reviewed in [10]). However, it should be noted that most of the earlier *in vitro* TLS studies were performed using buffer containing a high concentration of Mg^{2+}, which we now know is not the optimal metal ion cofactor for polι activity [23]. The optimal buffer for *in vitro* replication by polι contains low (50–250 μM) Mn^{2+} (Figure 2H) [23]. Indeed, polι-dependent incorporation opposite various damaged sites and in many cases the overall lesion bypass, was greatly stimulated in the presence of $MnCl_2$ [23]. The biological relevance of these observations remains to be established, but it seems reasonable to presume that although Mn^{2+} is present at a much lower concentration than Mg^{2+} intracellularly, its accumulation through specific import pathways is high enough to activate polι. In addition, the catalytic activity of polι is greatly stimulated by an interaction with PCNA (Figure 2C) [22]. Therefore, it seems plausible to speculate that polι plays a much more prominent role in TLS than previously assumed from *in vitro* studies.

dRP lyase activity makes polι suitable to participate in specialized base excision repair

Another property which sets polι apart from other members of the Y-family polymerases is its intrinsic 5’-deoxyribose phosphate (dRP) lyase activity (Figure 2G) [24, 25]. This property, coupled with the enhanced catalytic activity of polι at short gaps and ability to catalyze strand-displacement DNA synthesis (Figure 2B) [14] prompted us to hypothesize that the enzyme may participate in a specialized form of short- and long-patch BER (Figure 5). Involvement of polι in specialized BER would be particularly advantageous when T is generated by deamination of 5-methylcytosine and the G from the resulting G:T mispair is errantly excised by a DNA glycosylase (Figure 5C) [24]. In this case, a gap- filling reaction catalyzed by polι occurring through frequent misinsertion of G opposite template T would represent a correct event by preventing C:G to T:A transition mutations. In an alternate scenario, if the uracil in DNA arises as a consequence of incorporation of dU opposite template A, after the dU is removed by UNG, then polι’s ability to efficiently and accurately incorporate T opposite template A [11] would also maintain genome integrity (Figure 5B) [24]. Therefore, a role for polι in specialized polβ-independent forms of BER is another

example when the combination of its unique properties makes it exceptionally fit to fulfill specific cellular tasks.

It's been 20 years since the discovery of the mysterious and fascinating polι. Let's hope it's not another 20 years before its true cellular function is uncovered!

Acknowledgments

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Abbreviations-:

pol	DNA polymerase
TLS	translesion DNA synthesis
BER	base excision repair
E. coli	Escherichia coli
XPV	xeroderma pigmentosum variant
dRP	5'-deoxyribose phosphate
CPD	cyclobutane pyrimidine dimer
AP	apurinic/apyrimidinic

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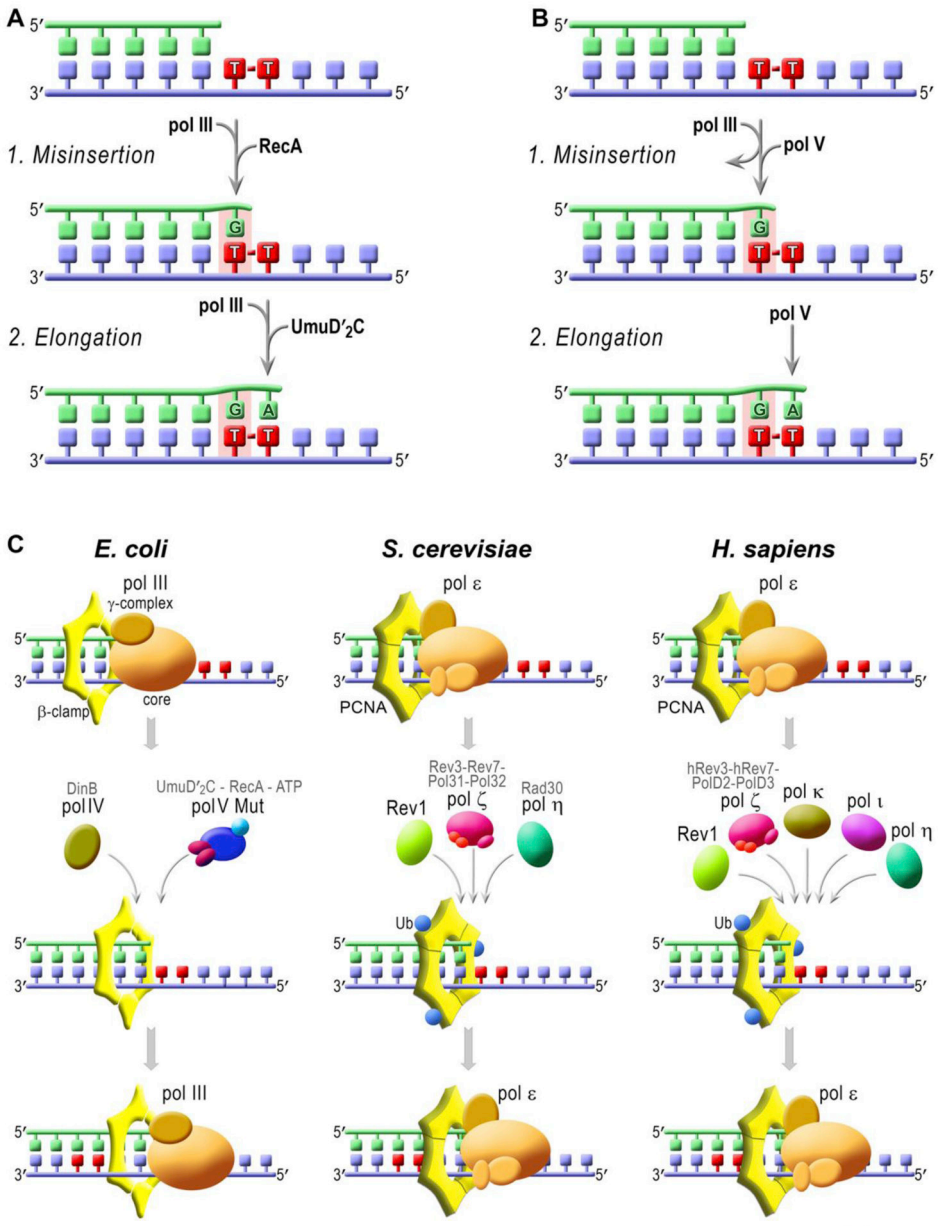

Figure 1. Evolution of the two-step model for error-prone translesion DNA synthesis.

A. The initial model explaining UV-induced mutagenesis was published in 1985 [1]. It was proposed that in *E. coli*, the copying of DNA containing a UV-induced thymine–thymine cyclobutane pyrimidine dimers is catalyzed by the replicative polymerase, pol III. While the first step of misincorporation opposite the 3'T of CPD is facilitated by the RecA protein, the second, elongation step, is carried out with the assistance of the UmuDC proteins, the active form of which was subsequently shown to be a complex of UmuD'$_2$C. Mispaired bases here and in other Figures are marked by pink rectangles. **B**. A revised model for UV-mutagenesis in *E. coli.* A T-T CPD is a kinetic block to replication by pol III. According to the updated model, both steps of TLS consisted of nucleotide (mis)insertions opposite the 3'T and 5' the T of the dimer are readily performed by the UmuD'$_2$C complex, named pol V [7]. Pol V-

introduced misincorporations are fixed as mutations by pol III resuming chromosomal duplication once the kinetic block to replication has been overcome. **C**. The current two-step model for TLS in *E. coli*, *Saccharomyces cerevisiae* and *Homo sapiens* [8]. Similar to *E. coli*, duplication of damaged DNA in other organisms is catalyzed by specialized translesion polymerases after replication by polε is hindered by the lesion. The polymerases most efficient in TLS are those belonging to the Y-family that are unevenly distributed among three kingdoms of life, and eukaryotic polζ from the B-family of DNA polymerases. Prokaryotes possess two Y-family DNA polymerases, pol IV and pol V, while eukaryotes have four members of this family, pols η, ι, κ and Rev1. Replicative bypass in *S. cerevisiae* involves three major TLS polymerases, pols η, ζ and Rev1, whereas humans utilize two additional enzymes, pols ι and κ. Similar to prokaryotes, the efficiency and fidelity of TLS in eukaryotes is determined by the lesion type and TLS polymerase(s) recruited to stalled replication fork through an interaction with ubiquitinated PCNA. For example, polη catalyzes both steps of TLS past T-T cyclobutane pyrimidine dimers very efficiently and accurately. In the absence of polη, CPDs can be replicated much less efficiently and in a highly error-prone manner by sequential action of polι catalyzing an insertion step and polζ or polκ catalyzing an extension step. After bypass is achieved, polε resumes chromosomal duplication.

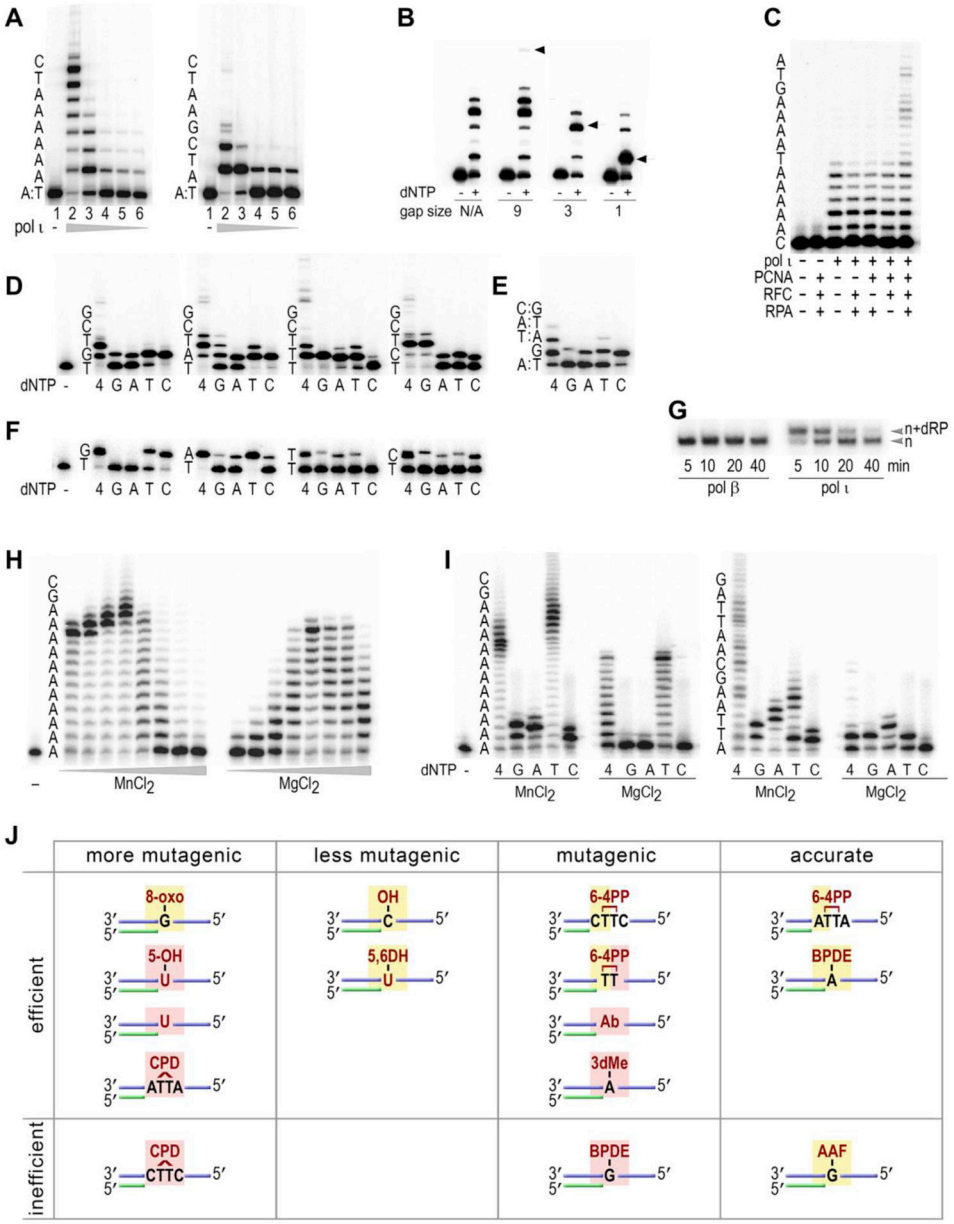

Figure 2. Unique biochemical properties of polι.

A. Processivity of polι. Replication reactions reconstituted *in vitro* were carried out using 0.4–40 nM polymerase [11]. Here and in all other panels, the immediate template sequence context is shown on the left-hand side of the figure. As expected for a TLS polymerase, polι is an extremely distributive enzyme. While it normally inserts up to three nucleotides in one binding event, the pattern of primer extension changes depending on the DNA substrate sequence context. The greatest activity occurs on the template consisting of short homopolymeric runs of As. By comparison, on templates with a random DNA sequence, polι pauses after replicating template T. **B**. Polι-dependent replication using different DNA substrates. The ability of polι to utilize primed single-stranded and gapped DNA templates is compared [14]. The gap size is indicated below each reaction. N/A corresponds to the

reactions using primed single-stranded DNA. For the gapped substrates, an arrow at the right-hand side of each reaction pair indicate the size of the expected full-sized product. Polι appears to be somewhat more active on gapped substrates than on DNA templates with recessed 3' ends. The most robust reactions are seen with single-nucleotide gapped DNA. Polι is also able to catalyze limited strand displacement of a downstream primer. **C**. Effect of PCNA on the activity of polι. The catalytic activity of polι is stimulated by PCNA in the presence of RFC and RPA [22]. **D**. Fidelity of murine polι on primed single stranded DNA. The properties of human and murine polι are very similar. Both enzymes are extremely error-prone, and their fidelity is unusually template sequence dependent. The misincorporation frequency is lowest at template A, and highest at template T, where it favors the incorporation of a wrong G instead of the correct A. Furthermore, misinsertion of T opposite T occurs with roughly the same efficiency as A opposite T. **E**. Fidelity of polι on a 1 bp gapped substrate. The pattern of nucleotide incorporation by polι is very similar during primer extension of the recessed single stranded DNA and during gap filling. **F**. Fidelity of DNA synthesis of murine polι at the end of a template. The pattern of nucleotide misincorporation on DNA templates containing a one nucleotide 5′ overhang differs significantly from that on the recessed template (compare with panel D), in this case, polι has lowest fidelity opposite C, where it clearly favors the misincorporation of C. Reduced fidelity is also seen opposite template G, where it inserts the correct C and the wrong T with roughly the same efficiency. **G**. Deoxyribose phosphate lyase activity of polι. As shown by a time-course study, polι is able to excise a dRP group although less efficiently than polβ. **H**. Effect of divalent metal activator on the catalytic activity of polι. A dose-response analysis was performed using 0.05–5 mM $MnCl_2$ or $MgCl_2$. Unlike most DNA polymerases, including Y-family polη and polκ, whose catalytic activities are mediated by Mg^{2+}, polι prefers to utilize manganese over magnesium [23]. Furthermore, the greatest activity was seen with quite low levels (50–250 μM) of Mn^{2+}. Even with Mg^{2+}, peak activity was observed in a much lower and narrower concentration range (0.1–0.5 mM) than reported for the optimal performance of other polymerases (usually 5–8 mM). **I**. Effect of divalent metal activator on the fidelity of polι. Like other polymerases, polι has much lower fidelity in the presence of $Mn^{2+}$when incorporating nucleotides opposite template A [23]. However, replacement of Mg^{2+} by Mn^{2+} increased the fidelity of polι at template T, such that the hallmark preference for dGTP incorporation disappeared. Nevertheless, polι retained its extremely error-prone behavior at this position, since it favored the incorporation of the correct base A only with ~2.3-fold preference over the G misincorporation. **J**. Efficiency and fidelity of polι-catalyzed TLS past various DNA lesions. The outcome of replicative bypass depends on the type and structure of the lesion, template sequence context, and type of the damaged template base [10, 12, 13, 18, 19]. Pink boxes indicate a preference for error-prone nucleotide incorporation. Preferentially non-mutagenic nucleotide incorporation catalyzed by polι is shown in yellow, even when this preference is marginal. The lesions shown are: cyclobutane pyrimidine dimer (CPD); 6–4 thymine–thymine pyrimidine–pyrimidone photoproduct (6–4PP); benzopyrene diol epoxide (BPDE); 7,8- dihydro- 8- oxoguanine (8-oxoG), 5- hydroxycytosine (5- OHC); acetyl amino fluorene (AAF); 3-deaza analog of N3-methyl-adenine (3dMeA); uracil (U); 5- hydroxyuracil (5- OHU); 5,6- dihydrouracil (5,6-DHU); and abasic site (Ab).

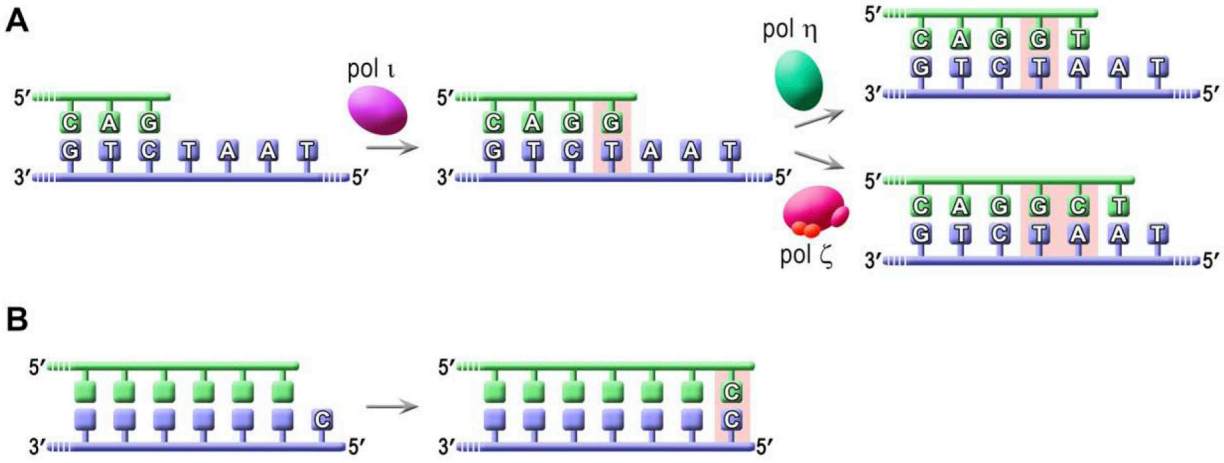

Figure 3. Extraordinarily unfaithful behavior makes polι a candidate for the introduction of nucleotide substitutions in the variable region of immunoglobulin genes.

A. Generation of single T:G and TA:GC tandem mismatches on the recessed DNA template by the sequential action of polι and either polη, or polζ. **B**. Generation of polι-catalyzed C:C mismatches at the end of DNA template. The pattern of nucleotide incorporation depends on the structure and sequence of a DNA substrate, suggesting that polι could be responsible for the different nucleotide changes found in the Ig mutational spectra. Thus, preferential misincorporation of G opposite template T on the recessed and gapped DNA templates (A) suggests that polι can contribute to the A to G substitution, one of the most common errors occurring during somatic hypermutation. If the next, elongation step is catalyzed by the error-prone polζ, then tandem T-A to G-C mutations arise. Such a scenario has been shown to occur *in vivo* [17]. It has been reported that most somatic mutations occur within 1–2 bases of a double-strand breaks. Very efficient and the highly error-prone activity of polι at the end of DNA templates (Figure 2F) hints that it could be responsible for some of these mutations. Specifically, C-G to G-C transversions can result from polι favoring misinsertion of C opposite C on the DNA substrate containing a one nucleotide 5′ overhang.

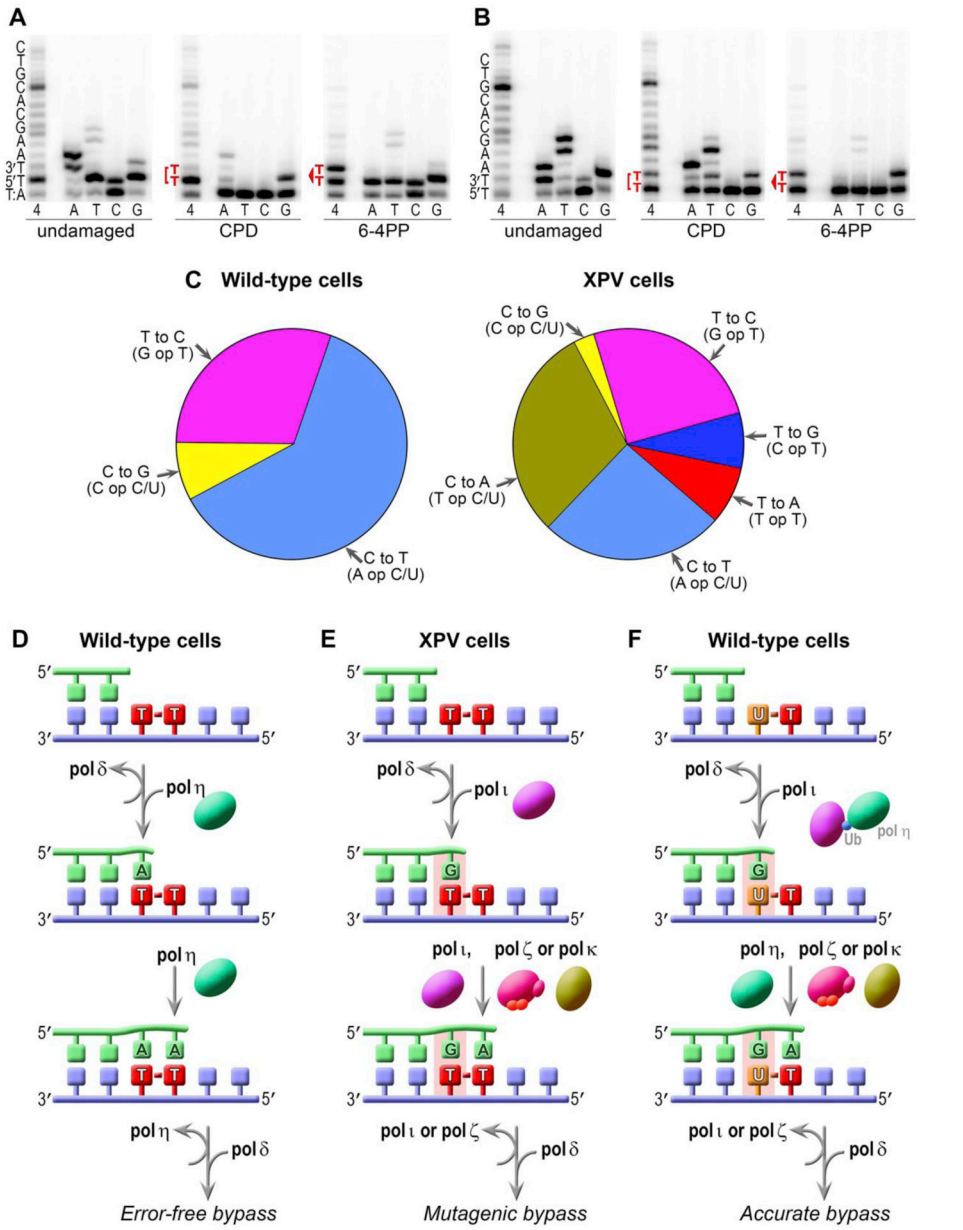

Figure 4.

Potential involvement of polι in translesion DNA synthesis past UV-induced lesions *in vivo*. **A**. Reconstitution of polι-catalyzed primer extension reactions on undamaged and CPD- or 6–4PP-containing templates with primer termini juxtaposed to the 3'T of the lesion. **B**. Extension of the correctly paired primers (A:3'T) on templates with primer termini juxtaposed to the 5'T of CPD or 6–4PP on undamaged templates and on CPD- or 6–4PP-containing templates. **C**. Spectra of UV-induced mutations in wild-type and XPV cells. **D**. Model of polη-catalyzed error-free bypass of T-T CPD adducts in wild-type cells. **E**. Model of the mutagenic TLS in XPV cells with polι-catalyzing misinsertion of G opposite the 3'T of CPD followed by extension of the resulting mispaired primers by polζ or polκ. **F**. Model of error-prone TLS that leads to restoration of correct base pairing in wild-type cells with

polι misinserting G opposite the 3'U generated by damaged cytosine deamination and polζ or polκ extending the resulting mispaired primers. Recruitment of polι in wild-type cells occurs through interaction with polη [21]. Monoubiquitination of either polι or polη enhances the interaction between these two polymerases. *In vitro* studies suggest that incorporation of nucleotides opposite the 3'T of 6–4PP is more efficient than opposite the 3'T of CPD, but elongation past the lesion is much more efficient for CPDs [13, 19]. Polι-catalyzed TLS past CPDs is most efficient when it occurs by incorporation of G opposite the 3'T. But extension of the resulting mispaired primers by polι is very inefficient (not shown) and requires recruitment of another TLS polymerase, such as pols η, ζ, or κ. Polι itself efficiently extends correctly paired primers (A:3'T), but this extension is highly inaccurate. On the other hand, polι can reduce UV-induced mutagenesis if it would participate in the bypass of a U-T CPD, when U is generated by cytosine deamination (F). Analysis of misincorporation patterns on the damaged DNA templates (A, B) suggests that the nucleotide selection specificity of polι closely correlates with changes in the mutation spectrum recovered from the cells lacking polη (XPV) (A-C). Thus, the appearance of high levels of T to A and C to A mutations corresponds to the characteristic for polι misincorporation of T opposite T and T opposite U generated by cytosine deamination, whereas the reduction in C to T mutations is due to misincorporation of G opposite U generated by cytosine deamination.

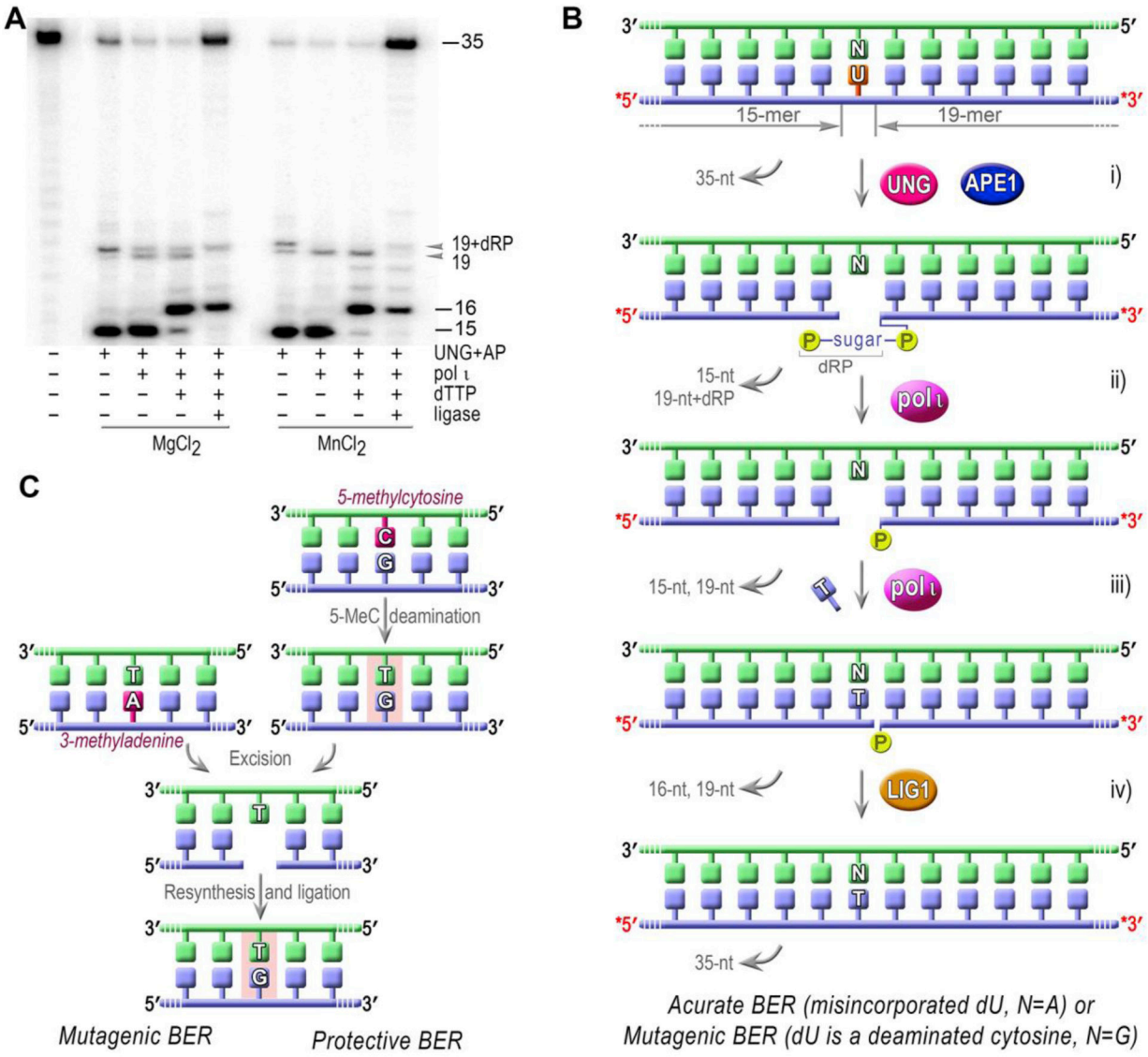

Figure 5. Potential involvement of polι in BER.

A. Reconstitution of BER *in vitro*. **B**. Schematic representation of BER reconstituted *in vitro*. A DNA strand (35-bp oligonucleotide with a uracil (shown in orange) at position 15. The nucleotide incorporated during BER is shown in blue. The strand subject to BER is ^{32}P-labeled on both 3'- and 5'-termini (marked by red asterisk). The sizes of the reaction products are indicated for each step of the pathway. Reconstituted BER consists of the following steps: i) excision of the uracil by uracil DNA glycosylase (UNG) and incision of the phosphodiester bond on the 5′ side of the resulting abasic site by apurinic/apyrimidinic endonuclease (APE1); ii) removal of the dRP group by polι; iii) incorporation of dTTP opposite template A by polι; iv) ligation of the nick by DNA ligase I (LIG1). **C**. Model of polι-dependent BER of 5-methylcytosine and 3-methyladenine. Due to its dRP lyase activity (Figure 2G) and an increased efficiency on gapped DNA templates (Figure 2B), polι emerged as a candidate for the role of an enzyme responsible for the replacement of the excised nucleotide(s) during short- and long-path BER [24, 25]. In some cases, participation

of polι in BER while being highly error- prone, may actually be much less mutagenic than if re-synthesis would be performed by another polymerase. For example, the recruitment of polι for BER would be advantageous when a G:T mismatch generated by deamination of 5-methylcytosine has to be maintained until the T is eventually excised and replaced by C (see panel C). The parental genotype has a chance to be restored, if after removal of the undamaged guanine by a glycosylase, polι would keep inserting G opposite T. On the other hand, the same specificity of polι can lead to harmful consequences for the cell, if the polymerase is targeted for BER of a lesion such as 3-methyladenine. The preference for incorporating G rather than A opposite T in this case would destabilize the genome. The consequences of polι's involvement in BER of uracils depends on whether dU was errantly inserted during replication, or resulted from cytosine deamination (panel B). Replacement of uracils excised from an A:U base pair will be relatively accurate because polι is most faithful while incorporating nucleotides opposite the A (f_{inc} =1–2×10^{-4}) [11]. In contrast, when uracils are generated by cytosine deamination, their replacement will be highly mutagenic, since misinsertion of T opposite G is the third most frequent error made by polι (f_{inc}=0.13) after misincorporation of G or T opposite T [11].