Genetic evidence for reconfiguration of DNA polymerase θ active site for error-free translesion synthesis in human cells

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The action mechanisms revealed by the biochemical and structural analyses of replicative and translesion synthesis (TLS) DNA polymerases (Pols) are retained in their cellular roles. In this regard, DNA polymerase θ differs from other Pols in that whereas purified Polθ misincorporates an A opposite 1,N6-ethenodeoxyadenosine (εdA) using an abasic-like mode, Polθ performs predominantly error-free TLS in human cells. To test the hypothesis that Polθ adopts a different mechanism for replicating through εdA in human cells than in the purified Pol, here we analyze the effects of mutations in the two highly conserved tyrosine residues, Y2387 and Y2391, in the Polθ active site. Our results that these residues are indispensable for TLS by the purified Pol but are not required in human cells, as well as other findings, provide strong evidence that the Polθ active site is reconfigured in human cells to stabilize εdA in the syn conformation for Hoogsteen base pairing with the correct nucleotide. The evidence that a DNA polymerase can configure its active site entirely differently in human cells than in the purified Pol establishes a new paradigm for DNA polymerase function.

Biochemical and structural studies with translesion synthesis (TLS) DNA polymerases (Pols) have indicated a high degree of specificity in the types of DNA lesions they can replicate through (1). Thus, the ability to accommodate two template residues in its active site provides Polη the proficiency for replicating through the covalently linked cyclobutane pyrimidine dimer (CPD) (2-6). The adoption of a syn conformation by the purine template in the Polι active site for forming a Hoogsteen base pair with the incoming nucleotide (nt) enables it to insert nts opposite DNA adducts which impair Watson-Crick (W-C) base pairing or impinge upon the DNA minor groove (7-10). TLS studies in human cells have corroborated the roles and mechanisms inferred from biochemical and structural studies of Polη, Polι, and other TLS Pols (11-15).

In vitro studies of purified Polθ, an A family Pol, have suggested that in contrast to Polη or Polι, it lacks the specificity for replicating through DNA lesions; and compared to TLS mediated by Pols with high
specificity, Polθ acts in a more error-prone manner (12,13). In human cells, for example, Polθ functions in TLS opposite two very different types of lesions, CPDs and 1,6-ethenodeoxyadenosine (εdA). TLS opposite CPDs occurs either by a Polη-dependent error-free pathway or by an alternative error-prone pathway in which Polθ inserts a nt opposite the 3’ pyrimidine residue of a CPD from which Polκ or Polζ subsequently extend synthesis (13). TLS opposite the εdA adduct, which is generated from interaction of DNA with aldehydes derived from lipid peroxidation (16,17), and which impairs Watson-Crick (W-C) base pairing, operates via two major pathways dependent upon Polη/Polζ and Polθ, respectively, in which the sequential action of Polη and Polζ promotes error-free TLS and Polθ performs error-prone TLS (12). A third pathway dependent upon Rev1 polymerase activity makes a relatively minor contribution (12). Apart from these Pols, no other Pols such as Polη, Polκ (12), or Polν are required for TLS opposite this adduct in human cells.

The ability of Polη to insert nts opposite the εdA adduct by Hoogsteen base pairing and the proficiency of Polζ for extending synthesis from the nt opposite εdA explains the roles these Pols play in TLS through εdA in human cells (9,12). Since Polθ replicates DNA by utilizing classical W-C base pairing, εdA would present a strong block unless the adduct adopts an extrahelical position in the Polθ active site; hence, Polθ replicates through εdA using a mechanism similar to that it uses for TLS through an abasic (AP) site. The observation that purified Polθ replicates through both the εdA and AP lesions by inserting an A is consistent with εdA adopting an ‘AP’ mode in the Polθ active site (12). However, in striking contrast to the extremely error-prone TLS opposite εdA by purified Polθ, Polθ-dependent TLS in human cells operates in a predominantly error-free manner wherein Polθ incorporates over 90% T opposite εdA (12). Such error-free TLS could occur in human cells only if the εdA adduct adopts a syn confirmation in the Polθ active site and forms a Hoogsteen base pair with the T residue.

To test the validity of the hypothesis that Polθ adopts a different mechanism for TLS in human cells than in purified Polθ, in this study we analyze the effects of mutations in the two highly conserved tyrosine residues in Polθ active site on TLS opposite εdA by purified Polθ and on TLS in human and mouse cells. Our results that these mutations affect TLS by purified Polθ in a dramatically different way than they affect TLS in human and mouse cells strongly support the premise that the Polθ active site is configured differently for TLS in human cells than in purified Polθ.

Results

Conserved tyrosine residues in Polθ fingers domain

The O-helix in the fingers domain is conserved among A-family Pols. Within the O-helix, the Y2391 residue in human Polθ is conserved among all the eukaryotic, prokaryotic, and phage A-family DNA polymerases, whereas Y2387 in human Polθ is conserved in both the eukaryotic A-family Pols Polθ and Polν, but it is not conserved in E. coli PolI, Taq polymerase, or T5 Pol (Figure 1). The ternary crystal structures of human Polθ have revealed that the Y2387 residue contacts the β-phosphate of the incoming nt and Y2391 lies beneath the template residue (18).

Indispensability of Y2387 and Y2391 for TLS through εdA by purified Polθ

To better understand the ability and mechanism of purified Polθ for TLS through εdA, we carried out in vitro DNA synthesis assays on DNA substrates that harbor a single εdA lesion with the (1708-2590) WT Polθ protein and the Y2387A and Y2391A mutant Polθ proteins. For comparison, we also examined synthesis on DNA containing an AP site, in the form of a tetrahydrofuran
moiety. The Polθ (1708-2590) protein affects TLS opposite εdA and AP site similarly as the full length Polθ (kindly provided by Richard Pomerantz).

We first performed assays with DNA substrates containing a running start primer, where DNA synthesis initiates 3 nt before the lesion (Figure 2A). We analyzed DNA synthesis by (1708-2590) WT Polθ, and the Y2387A and Y2391A mutant Polθ proteins, each at three different protein concentrations (0.2 nM, 1 nM, and 10 nM) on undamaged DNA and on the εdA and AP site containing DNA substrates. The Y2387A mutation exhibited a strong deleterious effect on DNA polymerase activity of Polθ. On undamaged DNA, DNA synthesis by 10 nM Polθ Y2387A protein was about the same as that for 0.2 nM WT Polθ, suggesting that it is at least ~50 fold less efficient for polymerase activity. Importantly, Y2387A Polθ lacked the ability to incorporate a nt opposite εdA or opposite an AP site even at high protein concentrations (Figure 2A). On undamaged DNA, the Y2391A Polθ protein exhibited a moderate decline in DNA polymerase activity, but not as severe as the Y2387A Polθ. We estimate a reduction in catalytic efficiency of ~10 fold, based on the similar DNA synthesis by 1 nM WT Polθ versus 10 nM of the Y2391 mutant. Even though Y2391A Polθ is less efficient in DNA synthesis, it inserts a nt opposite εdA and the AP site. However, there is a complete lack of extension of synthesis opposite from either lesion (Figure 2A).

Next we qualitatively assessed the fidelity of nucleotide incorporation opposite εdA by including only a single nucleotide in the assays, rather than all four. For these assays on undamaged DNA, we used 10-fold more mutant protein than WT protein because DNA synthesis is reduced by the Y2387A and Y2391A mutations. On undamaged DNA, WT Polθ incorporates T opposite A most efficiently, as do the Y2387A and Y2391A mutant proteins (Figure 2B). In the presence of all four dNTPs, Y2387 also incorporates a C at about 20% compared to T. Y2391A Polθ is also error-prone as indicated by the number of doublets and altered DNA ladder as compared to WT Polθ. Opposite εdA, the WT protein can incorporate A or G, but an A is incorporated the most and in the presence of all 4 dNTPs, only an A is incorporated, and Polθ extends synthesis to the end of the template (Figure 2B). At the same protein concentration, the Y2387A Polθ protein is unable to incorporate nt opposite εdA or AP site. Opposite both the εdA and AP lesions, nucleotide incorporation by Y2391A Polθ is reduced compared to the WT protein; it primarily inserts a G but an A is also inserted with a reduced proficiency. And, as was seen in the running start assay (Figure 2A), Y2391A Polθ is completely devoid in extending synthesis past εdA, or the AP site (Figure 2B).

Next, we examined the effects of Y2387A Y2391A double mutation on DNA synthesis by Polθ on undamaged and εdA containing DNAs (Figure 3). In contrast to the individual Polθ Y2387A and Y2391A mutant proteins, the double Y2387A Y2391A mutant Polθ is severely deficient in polymerase activity. When Polθ Y2387A Y2391A is assayed on the undamaged DNA substrate at a 5-fold molar excess of protein over DNA the polymerase only incorporates 4 nts (Fig 3, lane 8), whereas the Polθ Y2387A single mutant protein is able to synthesize up to ~17 nt in assays containing equimolar protein:DNA concentrations (Fig 2A, lane 7). Not surprisingly, on the εdA and AP containing DNA substrates, Polθ Y2387A Y2391A behaves similarly to Polθ Y2387A, and no nt incorporation is observed opposite either lesion. Thus, the reduced catalytic activity of the Polθ Y2387A Y2391A mutant appears to be an additive effect of each of the Y to A mutations.

Y2387 and Y2391 are dispensable for Polθ-mediated TLS through εdA in human cells

Our findings, that the Y2387A and Y2391A mutations inactivate purified Polθ's
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ability to replicate through the εdA lesion, and that there is a strong concordance in the pattern of TLS and nt incorporation opposite the εdA and AP lesions by the purified WT Polθ and the Y2387A and Y2391A mutant Polθ proteins (Figure 2), have suggested that the Y2387 and Y2391 residues modulate TLS through εdA adopting an ‘AP’ mode in the active site of purified Polθ. Thereby, by predominantly inserting an A opposite the adduct, purified Polθ conducts extremely error prone TLS through εdA. In human cells, however, Polθ-mediated TLS through εdA is largely error free as the correct nt T is inserted in over 90% of TLS products (12). Since T insertion opposite εdA could occur only if the adduct adopts a syn conformation and forms a Hoogsteen base pair with T(9), the Y2387 and Y2391 residues may play little or no role in TLS through εdA in human cells since these residues effect the ‘AP’ mutagenic mode of TLS through εdA.

To determine the contribution of Y2387 and Y2391 residues to TLS in human cells, we analyzed the effects of Y2387A and Y2391A mutations in Polθ (1708-2590) on TLS opposite εdA carried on the leading strand template of a duplex plasmid in which bidirectional replication ensues from a replication origin (Figure 4). As shown in Table 1, in WT HFs expressing genomic Polθ, TLS opposite εdA occurs with a frequency of ~25%. TLS is reduced to ~14% in Polθ-depleted cells carrying the empty vector or carrying an siRNA sensitive WT Polθ (1708-2590). TLS is restored to WT levels in Polθ-depleted cells harboring siRNA resistant WT Polθ (1708-2590). Thus, the effect of Polθ (1708-2590) on TLS opposite εdA is the same as that of genomically expressed Polθ. Importantly, in Polθ-depleted cells expressing Y2387A or Y2391A mutant Polθ (Figure 5A), TLS occurs at WT levels (Table 1). In the absence of Polθ, TLS opposite εdA is performed primarily by the Polθ/Polζ-dependent error free pathway which requires Rev1 as a scaffolding component, and by a relatively minor pathway which requires Rev1 polymerase activity (12). Hence, in the absence of Rev1, both the Polθ/Polζ and Rev1 polymerase dependent pathways become inactive and only the Polθ-dependent pathway remains functional, whereas in the absence Rev1 and Polθ, all the TLS pathways are inactivated (12). In HFs co-depleted for Rev1 and Polθ where TLS would be abolished as indicated by the near absence of TLS in Rev1−/− MEFs depleted for Polθ or in Polθ−/− MEFs depleted for Rev1 (12) (see Table 2), expression of siRNA resistant WT Polθ raises TLS to ~11%; and importantly, expression of siRNA resistant Y2387A or Y2391A mutant Polθ also raises TLS to WT Polθ levels (Table 1). Thus, in contrast to their indispensability for TLS by purified Polθ, the Y2387A or Y2391A mutations have no perceptible effect on TLS in human cells.

In biochemical assays, Y2387A mutant Polθ is completely defective in TLS through εdA whereas Y2391A mutant Polθ can insert nts opposite εdA but fails to extend synthesis (Figure 2). That raised the possibility that in human cells, pursuant to nt insertion opposite the lesion site by Y2391A Polθ, another polymerase extends synthesis. Since Polζ is a proficient extender of synthesis from the nt inserted opposite the εdA lesion by Polθ (9), and also opposite from a large variety of other distorting DNA lesions including the AP lesion (19,20), we determined whether such a Polζ role could account for proficient Y2391A-mediated TLS in human cells. However, our results that TLS occurs at the same level (~13%) in HFs co-depleted for Rev3 and Polθ and expressing WT Polθ or the Y2387A or Y2391A mutant Polθ protein furnish clear evidence for the lack of any Polζ involvement (Table 1). Thus, even though purified Y2391A mutant Polθ fails to extend synthesis from the nt opposite εdA, this mutation imparts no impairment in TLS through εdA in human cells.
Next, we verified the effects of Y2387A and Y2391A mutations on TLS opposite εdA in Polθ-/- MEFs. In Polθ-/- MEFs harboring the vector or expressing catalytically inactive D570A, E571A mutant Polθ, TLS occurs at ~10% (Table 2). Expression of WT Polθ raises TLS level to ~21%, and expression of the Y2387A or Y2391A mutant Polθ (Figure 5B) also restore WT levels of TLS in Polθ-/- MEFs (Table 2). In Polθ-/- MEFs depleted for Rev1 and expressing either no Polθ or catalytically inactive D570A, E571A Polθ, TLS is almost completely abolished (~1%), whereas expression of Y2387A or Y2391A mutant Polθ raises TLS to the same level (~9%) as expression of WT Polθ (Table 2). Thus, both in HFs and MEFs, Y2387A and Y2391A mutations support TLS through εdA to the same extent as does WT Polθ.

**Y2387 is required for mutagenic TLS by Polθ opposite εdA in human cells**

In human cells, Polθ replicates through εdA by incorporating the correct nt T in over 90% of TLS products, and it also incorporates a C in ~5% or an A in ~3% of TLS products(12). Since Polθ and Rev1 polymerase activity contribute to alternative error-prone TLS pathways(12), in Polθ depleted HFs carrying siRNA sensitive WT Polθ, mutagenic TLS emanating from Rev1 polymerase action occurs at a frequency of ~11% (Table 3). Expression of siRNA resistant WT Polθ raises mutagenic TLS to ~15%, the increase in mutagenic TLS resulting from Polθ contribution (Table 3). Importantly, expression of Y2387A Polθ reduces mutagenic TLS to ~6% (Table 3). Since error-prone TLS by Rev1 polymerase action would remain in these cells, this reduction in mutagenic TLS could have come about if Polθ’s involvement in mutagenic TLS was inhibited by the Y2387A mutation. To confirm this possibility, we examined the frequency of mutagenic TLS in HFs co-depleted for Rev1 and Polθ and expressing Y2387A mutant Polθ (Table 3, last row). Our results that mutagenic TLS is abolished in these HFs concur with a role of Y2387 in encumbering upon Polθ the capacity for mutagenic TLS opposite εdA.

Next, we verified these observations in Polθ-/- MEFs. As shown in Table 4, mutagenic TLS in Polθ-/- MEFs, which would accrue from Rev1 polymerase role, occurs at a frequency of ~10%, and this frequency rises to ~15% in cells expressing WT Polθ; by contrast, expression of Y2387A Polθ in Polθ-/- MEFs reduces mutagenic TLS to ~8%. Our results that in Rev1 depleted Polθ-/- MEFs expressing WT Polθ, mutagenic TLS occurs at ~7% (Table 4, fourth row from bottom) and that mutagenic TLS is abolished in Rev1 depleted Polθ-/- MEFs expressing Y2387A Polθ (Table 4, third row from bottom) add further confirmatory evidence that Y2387 confers upon Polθ the capability for mutagenic TLS in MEFs similar to that in HFs (Table 3).

**Y2391 affects suppression of mutagenic TLS by Polθ opposite εdA in human cells**

In contrast to the effect of Y2387A mutation on the ablation of mutagenic TLS, the frequency of mutagenic TLS is elevated to ~36% in Polθ depleted HFs expressing Y2391A Polθ (Table 3). In Polθ-/- MEFs expressing Y2391A Polθ, mutagenic TLS occurs at ~28% (Table 4). Since mutagenic TLS conferred by both Rev1 polymerase and Y2391A Polθ would operate in these cells, we analyzed the frequency of mutagenic TLS in Polθ-/- MEFs depleted for Rev1 and expressing Y2391A Polθ, since then only the contribution of Y2391A Polθ would remain. We find that mutagenic TLS occurs at ~20% in these MEFs (Table 4, second row from bottom). This observation that Y2391A elevates Polθ-mediated mutagenic TLS implies a role of Y2391 in the suppression of mutagenic TLS.

**Epistatic interaction of Y2391 with Y2387 dampens Polθ mutagenicity opposite εdA in human cells**

The abolition of mutagenic TLS by the Y2387A mutation and the enhancement of
mutagenic TLS by the Y2391A mutation suggested that the Y2387 and Y2391 residues interact epistatically such that Y2391 suppresses Y2387 action in mutagenic TLS, and the observed frequency of ~6-8% of mutagenic TLS by Polθ is sustained by that interaction. To explore this possibility, we analyzed the effects of the Y2387A Y2391A double mutation on the frequency of TLS and its mutagenicity in Polθ+/− depleted HFs and in Polθ+/− MEFs. Surprisingly, despite the severe defect in DNA synthesis by the purified enzyme (Figure 3), the Y2387A Y2391A mutant Polθ supports WT Polθ levels of TLS in HFs (Table 1) and in MEFs (Table 2). In Rev1 depleted Polθ+/− MEFs expressing Y2387A Y2391A Polθ, where only the Polθ function in TLS would remain, TLS occurs at WT Polθ rates (Table 2, last row) but mutagenic TLS is abolished (Table 4, last row). The abolition of mutagenic TLS by the Y2387A Y2391A mutation is compatible with an epistatic interaction between Y2387 and Y2391 wherein Y2387 effects mutagenic TLS and Y2391 curtails Y2387 action in mutagenic TLS.

Discussion

Evidence for adoption of a different configuration by the Polθ active site for TLS through εdA in human cells

The observation that similar to that opposite an AP site, purified Polθ predominantly inserts an A opposite εdA has suggested that Polθ replicates through εdA using an ‘AP’ mode wherein εdA becomes extrahelical. In human cells, however, Polθ replicates through εdA by inserting the correct nt T in over 90% of TLS products. Since εdA lacks the W-C edge (Figure 4A), a T could be inserted opposite εdA only if the adduct adopts a syn conformation and forms a Hoogsteen base pair with the incoming T (Figure 6). Hence, Polθ active site must adopt a different configuration for mediating TLS in human cells than that in purified Polθ. Evidence from biochemical and genetic studies with mutations in the highly conserved Y2387 and Y2391 residues in the Polθ active site validates this hypothesis.

In TLS assays with purified Polθ, Y2387A mutant Polθ lacks the capacity to insert a nt opposite εdA whereas Y2391A mutant Polθ primarily inserts a G and to a lesser extent an A (Figure 2B), but it fails to extend synthesis further (Figure 2). Similar to that seen with WT Polθ, the pattern of TLS and of nt incorporation by mutant Polθ proteins opposite εdA resembles that opposite the AP lesion (Figure 2). The complete inhibition of TLS by the Y2387A mutation opposite εdA and the AP lesion indicates that Y2387 is indispensable for TLS opposite both the lesions, and the observation that Y2391A Polθ predominantly inserts a G and less well an A opposite both the lesions suggests that in the absence of functional Y2391, Y2387 promotes the insertion of a G or an A but does not support extension.

In striking contrast to the indispensability of Y2387 and Y2391 for TLS by purified Polθ, mutational inactivation of these residues has no perceptible effect on TLS opposite εdA in HFs or MEFs; these mutations, however, affect the mutagenicity of TLS in HFs and MEFs. Mutational analyses of TLS products in WT HFs and in Rev1+/− MEFs in a previous study(12) and in WT HFs and Polθ+/− MEFs in this study show that whereas TLS mediated by WT Polθ generates ~6-8% of mutational TLS products in which a C (~5%) or an A or G (~1-3%) are incorporated opposite εdA, the Y2387A mutation inhibits mutagenic TLS and the Y2391A mutation increases the misincorporation of C, A, or G to ~20% (Table 4). These results taken together with the observation that mutagenic TLS is also inhibited by the Y2387A Y2391A double mutation (Table 4) suggest that the observed level of mutagenic TLS by WT Polθ (~6-8%) in HFs and MEFs is attained by a mechanism in which Y2387 promotes the misincorporation of nts opposite εdA whereas Y2391 suppresses it.
The indispensability of Y2387 and Y2391 for TLS by purified Polθ (Figure 2) but not for TLS in HFs and MEFs (Tables 1 and 2) implies that for mediating TLS through the εdA adduct, the roles of these highly conserved residues - important for DNA synthesis by the purified enzyme - are minimized in the Polθ active site reconfigured for TLS through εdA in human cells.

**Mechanism of Polθ for replicating through εdA in human cells**

The indispensability of Y2387 for mutagenic TLS through εdA by purified Polθ and the requirement of this residue for Polθ-dependent mutagenic TLS in HFs and MEFs might suggest that mutagenic TLS in human and mouse cells operates by the same mechanism that the purified enzyme employs for replicating through εdA, wherein εdA adopts an ‘AP’ mode. However, since a C is inserted opposite εdA in mutagenic TLS in WT HFs and MEFs, and since a C could be inserted only if εdA adopts a syn conformation and forms a Hoogsteen base pair with C in anti conformation (Figure 6), Y2387-mediated C insertion opposite εdA would occur via this mechanism. The adoption of syn conformation for C incorporation modulated by the Y2387 residue would suggest that the incorporation of an A or a G opposite εdA by Y2387 also occurs by Hoogsteen pairing between εdA in syn conformation and an A or G in anti conformation (Figure 6). Thus the mechanism of Hoogsteen base pairing via which Y2387 and Y2391 coordinate the incorporation of C, A, or G opposite εdA in HFs and MEFs would differ from the mechanism of adopting an AP-like mode that purified Polθ employs for misincorporating A opposite εdA. And importantly, the predominant incorporation of T opposite εdA (~92%) could only occur by the adoption of syn conformation by εdA in the Polθ active site (Figure 6).

**Possible mechanism for reconfiguration of the Polθ active site for TLS through εdA in human cells**

The lack of requirement of the Y2387 and Y2391 residues for the predominant error free TLS through εdA in human and mouse cells and the proposal that even the mutagenic TLS which depends upon these residues would entail the adoption of a syn conformation by εdA in the Polθ active site can be rationalized if the Polθ active site adopts a different configuration for TLS in human cells than in purified Polθ. To explain the acquisition of a different configuration in the Polθ active site, we posit that Polθ functions in TLS in human cells as a component of a multi-protein ensemble, and that protein-protein interactions and post-translational modifications in the components of this ensemble modulate the Polθ active site such that it promotes rotation of εdA into a syn conformation, allowing for Hoogsteen base pairing with the incoming nt.

In the Polθ active site reconfigured for conducting predominantly error free TLS through εdA in human cells, the roles of Y2387 and Y2391 residues become much less eminent, affecting only the mutagenic TLS. In the structure of purified Polθ, Y2387 participates in H-bonding to the β-phosphate of the incoming dNTP and Y2391 forms part of the active site floor beneath the template residue(18). This explains the requirement of these residues for efficient DNA synthesis on undamaged DNA and for TLS through εdA by the purified enzyme (Figs. 2 and 3). By contrast, the lack of their requirement for predominantly error free TLS through εdA in human cells implies that in the reconfigured Polθ active site, these residues no longer affect the stabilization of the template or the incoming nucleotide for incorporation of the correct dNTP.

TLS Pols such as η, ι, κ, or Rev1 have a preformed active site adapted for replicating through specific types of DNA lesions. In these Pols, the action mechanisms stay the
same for TLS in human cells as those indicated from biochemical and structural studies of the purified Pol; as for example, in the role of Polθ in TLS opposite CPDs and in the role of Polι in TLS opposite εdA. Replicative DNA Pols also utilize similar action mechanisms in vitro and in vivo. Thus, among DNA Pols, Polθ provides the first example where the action mechanism for TLS in human cells differs from the mechanism adopted by the purified enzyme.

**Experimental procedures**

*Polθ expression in yeast*

The human Polθ (1708-2590) protein harboring the catalytically active C-terminal DNA polymerase domain was expressed as a fusion with glutathione S-transferase (GST) from plasmid pPOL507 as described (21). The Y2387A and Y2391A mutations were each generated by PCR using mutagenic oligomers and the Polθ (1708-2590) cDNA in pPOL523 as template. The mutant cDNAs were fully sequenced to confirm the presence of the mutations and were cloned into the expression vector, generating plasmids pJR65, pPOL665, and pBJ2333 which express GST tagged Polθ (1708-2590) Y2387A, Polθ (1708-2590) Y2391A, and Polθ (1708-2590) Y2387A Y2391A, respectively.

WT and mutant Polθ (1708-2590) expression plasmids were transformed into yeast strain YRP654 and the proteins were expressed and affinity purified using glutathione sepharose as described (22). The GST fusion tag was removed from each Polθ (1708-2590) protein by treatment with prescision protease, leaving a 7 amino acid linker attached to the N-terminus of Polθ. Proteins were quantified by densitometry of Coomassie stained protein samples separated by 11% SDS-PAGE using imagenquant software (GE Biotech).

**DNA polymerase assays**

The standard DNA polymerase assay (5 μl) contained 25 mM Tris HCl pH 7.5, 5 mM MgCl₂, 1 mM DTT, 10% glycerol, 0.1 mg/ml bovine serum albumin and DNA substrate. The DNA substrates consisted of a 32P 5' labeled DNA primer annealed to a 52-mer template with the sequence 5'-TTCGTATA ATGCTCAC ACT[A]GAGT ACCGGAGC ATCGTCGT GACTGGGA AAAC-3', in which [A] at position 20 indicates either an undamaged A, εdA, or a tetrahydrofuran (THF) moiety (AP site analog). The εdA and THF containing templates were synthesized by the Midland Certified Reagent Company (Midland, TX) and were PAGE purified. For running start assays, the 29mer oligo primer 5'-GTTCACGAGATGCCTAC ACT[G]GAGT ACCGGAGC ATCGTCGT GACTGGGA AAAC-3' was annealed to each template. To assay nucleotide incorporation opposite A, εdA, or the AP site, the 23mer primer 5'-GTCACGAGATGCTCCGGTACTC-3' was used. Single dNTPs, dATP, dGTP, dTTP, dCTP or all 4 dNTPs combined were included at concentrations indicated in the figure legends. Reactions were initiated by the addition of 1 μl DNA polymerase in 5x reaction buffer (125 mM Tris HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA) and carried out at 37°C for times indicated in the figure legends before termination by 6 volumes of 95% formamide loading buffer containing 0.06% xylene cyanol/0.06% bromophenol blue. Reaction products were separated by 12% or 20% TBE/8M urea-PAGE. Gels were fixed in 10% methanol:10% acetic acid for 10 min, dried, and products were visualized by phosphorimaging on a Typhoon FLA7000 (GE Biotech).

**Construction of εdA plasmid vectors and TLS assays**

The in-frame target sequence of the lacZ' gene containing the εdA lesion is shown in Figure 4. The detailed methods for construction of lesion-containing SV40 duplex plasmid, for TLS assays, and for mutational analysis of TLS products have been described previously (15,23).

**Stable expression of wild type Polθ and mutant Polθ in HFs or MEFs**
DNAs encoding human wild type Polθ (1708-2590) or the mutant (1708-2590) Y2387A, Y2391A, or Y2387A Y2391A Polθ, respectively, were cloned into vector pCMV7-3xFlag-zeo (Sigma). The resulting vectors were transfected into normal human fibroblast (GM637) cells or Polθ−/− MEF cells by iMFectin transfection reagent (GenDEPOT). After 24h incubation, 0.5 µg of Zeocin (GenDEPOT) were added to the culture media. After 3 days of incubation, cells were washed with PBS buffer and were continuously cultured with the media containing 250 ng of Zeocin for ~ 2 weeks. Protein expression and siRNA knock downs were checked by western blot analysis (Figure 5) as described before (13).

Data availability: All the data are contained within the manuscript.

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Conflict of interest statement. The authors declare that they have no conflict of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References
1. Prakash, S., Johnson, R. E., and Prakash, L. (2005) Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. Ann. Rev. Biochem. 74, 317-353
2. Biertumpfel, C., Zhao, Y., Kondo, Y., et al. (2010) Structure and mechanism of human DNA polymerase η. Nature 465, 1044-1048
3. Johnson, R. E., Kondratick, C. M., Prakash, S., et al. (1999) hRAD30 mutations in the variant form of xeroderma pigmentosum. Science 285, 263-265
4. Johnson, R. E., Prakash, S., and Prakash, L. (1999) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Polη. Science 283, 1001-1004
5. Masutani, C., Kusumoto, R., Yamada, A., et al. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η. Nature 399, 700-704
6. Silverstein, T. D., Johnson, R. E., Jain, R., et al. (2010) Structural basis for the suppression of skin cancers by DNA polymerase eta. Nature 465, 1039-1043
7. Johnson, R. E., Prakash, L., and Prakash, S. (2005) Biochemical evidence for the requirement of Hoogsteen base pairing for replication by human DNA polymerase ι. Proc. Natl. Acad. Sci. U. S. A. 102, 10466-10471
8. Nair, D. T., Johnson, R. E., Prakash, L., et al. (2005) Human DNA polymerase ι incorporates dCTP opposite template G via a G.C+ Hoogsteen base pair. Structure 13, 1569-1577
9. Nair, D. T., Johnson, R. E., Prakash, L., et al. (2006) Hoogsteen base pair formation promotes synthesis opposite the 1,N6-ethenodeoxyadenosine lesion by human DNA polymerase iota. Nat. Struct. Mol. Biol. 13, 619-625
10. Nair, D. T., Johnson, R. E., Prakash, S., et al. (2004) Replication by human DNA polymerase ι occurs via Hoogsteen base-pairing. Nature 430, 377-380
11. Yoon, J. H., Hodge, R. P., Hackfeld, L. C., et al. (2018) Genetic control of predominantly error-free replication through an acrolein-derived minor-groove DNA adduct. J. Biol. Chem. 293, 2949-2958
12. Yoon, J. H., Johnson, R. E., Prakash, L., et al. (2019) DNA polymerase theta accomplishes translesion synthesis opposite 1,N(6)-ethenodeoxyadenosine with a remarkably high fidelity in human cells. Genes Dev. 33, 282-287
13. Yoon, J. H., McArthur, M. J., Park, J., et al. (2019) Error-Prone Replication through UV Lesions by DNA Polymerase theta Protects against Skin Cancers. Cell 176, 1295-1309
14. Yoon, J. H., Roy Choudhury, J., Park, J., et al. (2017) Translesion synthesis DNA polymerases promote error-free replication through the minor-groove DNA adduct 3-deaza-3-methyladenine. J. Biol. Chem. 292, 18682-18688
15. Yoon, J.-H., Prakash, L., and Prakash, S. (2009) Highly error-free role of DNA polymerase η in the replicative bypass of UV induced pyrimidine dimers in mouse and human cells. Proc. Natl. Acad. Sci. U. S. A. USA 106, 18219-18224
16. Chung, F.-L., Zhang, L., Ocando, J. E., et al. (1999) Role of 1,N²-propanodeoxyguanosine adducts as endogenous DNA lesions in rodents and humans. IARC Sci. Publ. 150, 45-53
17. Luczaj, W., and Skrzydlewska, E. (2003) DNA damage caused by lipid peroxidation products. Cell. Mol. Biol. Lett. 8, 391-413
18. Zahn, K. E., Averill, A. M., Aller, P., et al. (2015) Human DNA polymerase theta grasps the primer terminus to mediate DNA repair. Nat. Struct. Mol. Biol. 22, 304-311
19. Haracska, L., Unk, I., Johnson, R. E., et al. (2001) Roles of yeast DNA polymerases δ and ζ and of Rev1 in the bypass of abasic sites. Genes Dev. 15, 945-954
20. Johnson, R. E., Washington, M. T., Haracska, L., et al. (2000) Eukaryotic polymerases ι and ζ act sequentially to bypass DNA lesions. Nature 406, 1015-1019
21. Yoon, J. H., Roy Choudhury, J., Park, J., et al. (2014) A role for DNA polymerase theta in promoting replication through oxidative DNA lesion, thymine glycol, in human cells. J. Biol. Chem. 289, 13177-13185
22. Johnson, R. E., Prakash, L., and Prakash, S. (2006) Yeast and human translesion DNA synthesis polymerases: expression, purification, and biochemical characterization. Methods Enzymol. 408, 390-407
23. Yoon, J.-H., Prakash, L., and Prakash, S. (2010) Error-free replicative bypass of (6-4) photoproducts by DNA polymerase ζ in mouse and human cells. Genes Dev. 24, 123-128

Abbreviations: TLS, translesion synthesis; Pol, DNA polymerase; εdA, 1,N⁶ -ethenodeoxyadenosine; W-C, Watson-Crick; CPD, cyclobutane pyrimidine dimer; AP, abasic
Table 1. Effects of Y2387A, Y2391A, or Y2387A Y2391A Polθ mutations on TLS through the εdA adduct carried on the leading strand DNA template in HFs (GM637)

| siRNA          | Vector expressing | Number of Kan⁺ colonies | Number of blue colonies among Kan⁺ | TLS (%) |
|----------------|-------------------|-------------------------|-----------------------------------|---------|
| NC             | Vector control    | 416                     | 104                               | 25.0    |
| Polθ           | Vector control    | 424                     | 60                                | 14.2    |
| Polθ           | Flag-WT Polθ      | 388                     | 54                                | 13.9    |
| Polθ           | Flag-siR⁻-WT Polθ | 375                     | 90                                | 24.0    |
| Polθ           | Flag-siR-Y2387A Polθ | 405                   | 103                               | 25.4    |
| Polθ           | Flag-siR-Y2391A Polθ | 397                   | 102                               | 25.7    |
| Polθ           | Flag-siR-Y2387A Y2391A Polθ | 364   | 100                               | 27.5    |
| Rev1+ Polθ     | Flag-siR-WT Polθ  | 378                     | 40                                | 10.6    |
| Rev1+ Polθ     | Flag-siR-Y2387A Polθ | 342                   | 38                                | 11.1    |
| Rev1+ Polθ     | Flag-siR-Y2391A Polθ | 366                   | 36                                | 9.8     |
| Rev3+ Polθ     | Flag-siR-WT Polθ  | 286                     | 35                                | 12.2    |
| Rev3+ Polθ     | Flag-siR-Y2387A Polθ | 304                   | 40                                | 13.2    |
| Rev3+ Polθ     | Flag-siR-Y2391A Polθ | 312                   | 42                                | 13.5    |

<sup>a</sup>siR, siRNA resistant
Table 2. Effects of Y2387A, Y2391A, or Y2387A Y2391A Polθ mutations on TLS through the εdA adduct carried on the leading strand DNA template in Polθ−/− MEFs

| siRNA       | Vector expressing          | Number of Kan⁺ colonies | Number of blue colonies among Kan⁺ colonies | TLS (%) |
|-------------|----------------------------|-------------------------|--------------------------------------------|---------|
| NC          | No Polθ (control)          | 403                     | 44                                         | 10.9    |
| "           | D570A, E571A mutant Polθ   | 308                     | 31                                         | 10.1    |
| "           | WT Polθ                    | 342                     | 72                                         | 21.1    |
| "           | Y2387A Polθ                | 412                     | 84                                         | 20.4    |
| "           | Y2391A Polθ                | 431                     | 90                                         | 20.9    |
| "           | Y2387A Y2391A Polθ         | 408                     | 86                                         | 21.1    |
| mRev1a      | No Polθ (control)          | 422                     | 5                                          | 1.2     |
| "           | D570A, E571A mutant Polθ   | 416                     | 6                                          | 1.4     |
| "           | WT Polθ                    | 367                     | 32                                         | 8.7     |
| "           | Y2387A Polθ                | 411                     | 38                                         | 9.2     |
| "           | Y2391A Polθ                | 428                     | 40                                         | 9.3     |
| "           | Y2387A Y2391A Polθ         | 402                     | 40                                         | 10.0    |

a mouse Rev1 siRNA
Table 3. Effects of Y2387A, Y2391A, or Y2387A Y2391A mutations in Polθ on mutation frequencies and nucleotides inserted opposite the εdA adduct carried on the leading strand DNA template in HFs (GM637)

| siRNA  | Vector expressing          | No. of Kan+ blue colonies sequenced | Nucleotide inserted | Mutation frequency (%) | Error prone pathway that remains active |
|--------|----------------------------|------------------------------------|---------------------|------------------------|-----------------------------------------|
| Polθ   | Flag-WT Polθ               | 146 (16)a                          | A: 3, G: 2, C: 11, T: 130 | 11.0                   | Rev1 Polc                               |
| Polθ   | Flag-siRb-WT Polθ          | 138 (21)                           | A: 5, G: 1, C: 15, T: 117 | 15.2                   | Rev1 Pol, Polθ                          |
| Polθ   | Flag-siR-Y2387A Polθ       | 144 (9)                            | A: 1, G: 0, C: 8, T: 135 | 6.3                    | Rev1 Polc                               |
| Polθ   | Flag-siR-Y2391A Polθ       | 156 (57)                           | A: 20, G: 10, C: 27, T: 99 | 36.5                   | Rev1 Pol, Y2391A Polθ                   |
| Polθ   | Flag-siR-Y2387A Y2391A Polθ| 136 (9)                            | A: 1, G: 0, C: 8, T: 127 | 6.6                    | Rev1 Polc                               |
| Rev1+  | Flag-siR-Y2387A Polθ       | 108 (0)                            | A: 0, G: 0, C: 0, T: 108 | 0                      | None                                    |

aNumber of colonies in which TLS occurred by insertion of a nucleotide other than T are shown in parenthesis

bsiR, siRNA resistant

cThe greater reduction in mutation frequency in rows 3 and 5 than in row 1 is because of the increase in TLS frequency that occurs in cells expressing the Y2387A or the Y2387A Y2391A Polθ (see Table 1) and because of the ablation of Polθ-mediated mutagenic TLS by the Y2387A mutation.
Table 4. Effects of Y2387A, Y2391A, or Y2387A Y2391A mutations in Polθ on mutation frequency and nucleotides inserted opposite εdA carried on the leading strand DNA template in Polθ<sup>-/-</sup> MEFs.

| MEFs (siRNA) | Vector expressing | No. of Kan<sup>+</sup> blue colonies sequenced | Nucleotide inserted | Mutation frequency (%) | Error prone pathway that remains active |
|---------------|-------------------|-----------------------------------------------|--------------------|-----------------------|----------------------------------------|
| Polθ<sup>-/-</sup> | Vector control | 144 (15)<sup>a</sup> | A 3, G 1, C 11, T 129 | 10.4 | Rev1 Pol |
| Polθ<sup>-/-</sup> | Flag-WT Polθ | 138 (20) | A 5, G 1, C 14, T 118 | 14.5 | Rev1 Pol, Polθ |
| Polθ<sup>-/-</sup> | Flag-Y2387A Polθ | 140 (11) | A 2, G 0, C 9, T 129 | 7.9 | Rev1 Pol |
| Polθ<sup>-/-</sup> | Flag-Y2391A Polθ | 144 (40) | A 13, G 5, C 22, T 104 | 27.8 | Rev1 Pol, Y2391A Polθ |
| Polθ<sup>-/-</sup> | Flag-Y2387A Y2391A Polθ | 152 (11) | A 2, G 0, C 9, T 141 | 7.2 | Rev1 Pol |
| Polθ<sup>-/-</sup> | Flag-WT Polθ | 96 (7) | A 4, G 0, C 3, T 89 | 7.3 | Polθ |
| Polθ<sup>-/-</sup> | Flag-Y2387A Polθ | 96 (0) | A 0, G 0, C 0, T 96 | 0.0 | None |
| Polθ<sup>-/-</sup> | Flag-Y2391A Polθ | 90 (18) | A 8, G 4, C 6, T 72 | 20.0 | Y2391A Polθ |
| Polθ<sup>-/-</sup> | Flag-Y2387A Y2391A Polθ | 104 (0) | A 0, G 0, C 0, T 104 | 0.0 | None |

<sup>a</sup>Number of colonies in which TLS occurred by insertion of a nucleotide other than T are shown in parenthesis.

<sup>b</sup>Mouse Rev1 siRNA
Reconfiguration of DNA polymerase θ active site

Figure Legends

Figure 1. Homology among human Polθ and other A-family DNA polymerases. The region encompassing the Polθ O-helix within the finger domain is shown. The arrows above the alignment indicate helical elements as determined from crystallography (PDB: 4X0Q). The amino acid positions in each protein are indicated on the right and left. Mutations in the Y2387 and Y2391 residues (in bold) of human Polθ were examined in this study. Homologous residues are indicated underneath, where an asterisk indicates identical residues, and periods and colons indicate moderately and highly homologous residues, respectively.

Hs = Human Polθ, Mm = Mouse Polθ, Bt = Bovine Polθ, Dm = Drosophila melanogaster Mus308, Ec = E coli Pol1, Taq = Thermus aquaticus Pol1.

Figure 2. DNA polymerase activity of (1708-2590) WT Polθ, Y2387A Polθ, or Y2391A Polθ on undamaged, εdA, or AP site-containing DNAs. A, Increasing amounts of each protein were assayed with 10 nM template in the presence of 25 µM each of dGTP, dATP, dTTP and dCTP for 5 min using the standard DNA polymerase assay conditions given in the methods. A diagrammatic representation of the DNA substrate is shown on top, wherein the asterisk indicates the presence of an undamaged A, an εdA, or an AP lesion. Increasing protein amounts are indicted by triangles, and the concentrations for each set were 0.2 nM, 1.0 nM and 10.0 nM. The position of the 29mer primer and the 52 nt full extension products are shown on the right. The position of the template A, εdA, or AP site, 4 nt 3’ to the primer terminus is indicated by the asterisk on the right. B, Nucleotide incorporation by WT Polθ, Y2387A Polθ, or Y2391A Polθ opposite an undamaged A, ε-dA or an AP site. Assays were performed using the standard DNA polymerase conditions and contained 25 µM of either dCTP, dTTP, dGTP, dATP, indicated by C, T, G, or A, or all 4 dNTPs combined, indicated by N. The protein concentration for each assay is given in parentheses, and all assays were carried out for 5min, except those containing the Polθ Y2387A mutant protein, which were carried out for 10 min. A diagrammatic representation of the DNA substrate is shown on top, wherein the asterisk indicates the presence of an undamaged A, an ε-dA, or an AP site. Positions of the primer and full length product are shown on the right. The asterisk on the right indicates the position of the undamaged A, the εdA or the AP site.

Figure 3. DNA polymerase activity of (1708-2590) WT Polθ or Y2387A Y2391A Polθ on undamaged, εdA, or AP site-containing DNAs. Increasing amounts of each protein were assayed with 10 nM template in the presence of 25 µM each of dGTP, dATP, dTTP and dCTP for 5 min using the standard DNA polymerase assay conditions given in the methods. A diagrammatic representation of the DNA substrate is shown on top, wherein the asterisk indicates the presence of an undamaged A, an εdA, or an AP site. Increasing protein amounts are indicted by triangles. The concentrations for each set were 0.2 nM, 1 nM and 10 nM for the WT protein, and 0.2, 1, 10 and 50 nM for the Y2387A Y2391A mutant derivative. The position of the primer and the 52 nt full extension products are shown on the right. The position of the template A, εdA, or AP site, 4 nt 3’ to the primer terminus is indicated by the asterisk on the right.

Figure 4. TLS assay opposite εdA. A, Chemical structure of εdA. B, In the SV40 based plasmid, a 16mer target sequence (shown on top) containing an εdA at A* is inserted between the BamHI and SbfI restriction sites in the lacZ’ gene. The εdA-containing DNA strand is in-frame and therefore functional; it also carries the kan’ gene. TLS through the adduct generates Kan’ blue colonies.

Figure 5. Western blot analysis of Polθ expression. A, GM637 HFs or B, Polθ-/- MEFs. expressing 3xFlag-wild type Polθ (1708-2590), or the (1708-2590), Y2387A, Y2391A, Y2387A
Y2391A mutant Polθ or harboring the vector control. GM637 HFs were treated with Polθ siRNA for 48h. Protein expression was determined by western blot with Flag ab (Sigma). β-tubulin (Santa Cruz Biotechnology) was used as the loading control.

**Figure 6.** Hoogsteen base pairing of εdA in syn with T, C, G, or an A in anti conformation. Dots represent hydrogen bonds and R denotes the sugar moiety.
Figure 1
Figure 2

A

Template  A  idA  AP site

Polθ  WT  Y2387A  Y2391A  WT  Y2387A  Y2391A  WT  Y2387A  Y2391A

B

nucleotide  Polθ  CTGn  CTGn  CTGn  CTGn  CTGn  CTGn  CTGn  CTGn  CTGn

(0.5)  (5.0)  (5.0)  (5.0)  (5.0)  (5.0)  (5.0)  (5.0)  (5.0)  (5.0)

template  A  idA  AP site

A primer
Figure 3
Figure 4
Figure 5
Figure 6
Genetic evidence for reconfiguration of DNA polymerase θ active site for error-free translesion synthesis in human cells
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