A fidelity mechanism in DNA polymerase lambda promotes error-free bypass of 8-oxo-dG

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Abstract

8-oxo-7,8-dihydroxy-2'-deoxyguanosine (8-oxo-dG) has high mutagenic potential as it is prone to mispair with deoxyadenine (dA). In order to maintain genomic integrity, post-replicative 8-oxo-dG:dA mispairs are removed through DNA polymerase lambda (Pol λ)-dependent MUTYH-initiated base excision repair (BER). Here, we describe seven novel crystal structures and kinetic data that fully characterize 8-oxo-dG bypass by Pol λ . We demonstrate that Pol λ has a flexible active site that can tolerate 8-oxo-dG in either the *anti*- or *syn*-conformation. Importantly, we show that discrimination against the pro-mutagenic *syn*-conformation occurs at the extension step and identify the residue responsible for this selectivity. This residue acts as a kinetic switch, shunting repair toward long-patch BER upon correct dCMP incorporation, thus enhancing repair efficiency. Moreover, this switch also provides a potential mechanism to increase repair fidelity of MUTYH-initiated BER.

Keywords DNA repair; 8-oxo-deoxyguanosine; MUTYH; polymerase lambda

Subject Categories DNA Replication, Repair & Recombination; Structural Biology

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Introduction

DNA is susceptible to damage by reactive oxygen species (ROS) such as hydroxyl radicals. In particular, hydroxyl radical addition to the C8 position of guanine leads to the formation of 8-oxo-7,8-dihydroxy-2'-deoxyguanosine (8-oxo-dG). 8-oxo-dG is one of the most prevalent types of oxidative DNA damage (Beckman & Ames, 1997; Helbock *et al*, 1998). The damaged base is capable of adopting either an *anti*- or *syn*-conformation and thus can base pair with either deoxycytosine (dC) or deoxyadenosine (dA), respectively (Kouchakdjian *et al*, 1991; Oda *et al*, 1991; Mcauleyhecht *et al*, 1994; Lipscomb *et al*, 1995). As a consequence of this dual coding potential, dAMP is misincorporated opposite 8-oxo-dG by several DNA polymerases (Shibutani *et al*, 1991; Zhang *et al*, 2000; Einolf & Guengerich, 2001; Haracska *et al*, 2002, 2003). Accordingly, failure to remove the DNA lesion prior to replication leads to the

accumulation of guanine to thymine (G–T) transversions (Klungland *et al*, 1999; Hirano *et al*, 2003; Russo *et al*, 2004). The deleterious nature of 8-oxo-dG is further demonstrated by the concerted cellular defense mechanisms employed in all cells to reduce this particular mutagenic burden (Boiteux *et al*, 1987; van der Kemp *et al*, 1996).

The base excision repair (BER) pathway is the main mechanism for purging the genome of oxidative lesions such as 8-oxo-dG (Michaels & Miller, 1992). Two DNA glycosylases initiate repair of 8-oxo-dG lesions. In humans, OGG1 excises 8-oxo-dG adducts from double-stranded DNA, but fails to remove the lesion when mispaired to dA (Aburatani *et al*, 1997; Arai *et al*, 1997; Bjoras *et al*, 1997), as direct removal and subsequent repair would also result in a G–T transversion mutation. Instead, another glycosylase, MUTYH, specifically recognizes the 8-oxo-dG:dA mispair and excises the misincorporated dA (Slupska *et al*, 1996). The MUTYH-initiated BER pathway ultimately results in the formation of a single nucleotide gap that is subsequently filled by a specialized DNA polymerase. While DNA polymerase beta (Pol β) is involved in OGG1-initiated BER (Dianov *et al*, 1998; Fortini *et al*, 1999), DNA polymerase lambda (Pol λ) has been implicated in the MUTYH-dependent BER (Braithwaite *et al*, 2005; Maga *et al*, 2007, 2008; Tano *et al*, 2007; Vermeulen *et al*, 2007; van Loon & Hubscher, 2009; Markkanen *et al*, 2012; Pande *et al*, 2015).

The success of the repair *in vivo* is contingent on the incorporation of dCMP opposite 8-oxo-dG. In agreement with this notion, Pol λ is capable of mediating error-free bypass of 8-oxo-dG (Brown *et al*, 2007; Picher & Blanco, 2007). Interestingly, MUTYH-initiated repair appears to preferentially proceed through the alternative long-patch BER pathway (van Loon & Hubscher, 2009). Long-patch results in the additional incorporation of several nucleotides and thus relies on the ability of a polymerase to extend from the original lesion (Frosina *et al*, 1996). Accordingly, Pol λ extends past an 8-oxo-dG:dC base pair with a higher propensity than an undamaged base pair. Moreover, Pol λ is also capable of discriminating against extension of an 8-oxo-dG:dA mispair at the primer terminus (Picher & Blanco, 2007). Together, these properties make Pol λ well suited to facilitating the error-free bypass of 8-oxo-dG during MUTYH-mediated BER. However, the mechanistic basis for these unique properties has not yet been elucidated.

Here, we describe seven novel crystal structures that fully characterize the 8-oxo-dG bypass reaction in Pol λ . Each structure corresponds to one of the three key steps during long-patch

bypass—initial binding of the DNA (DNA binding), binding of the dNTP (insertion), and polymerization past the lesion site (extension). Curiously, our structures revealed that Pol λ is incapable of discriminating against the pro-mutagenic *syn*-conformation of 8-oxo-dG during initial DNA binding. We have also demonstrated that the high efficiency of incorporation is attributable to the uniquely malleable active site of Pol λ . During insertion, Pol λ can bind 8-oxo-dG in either the *anti*- or *syn*-conformation with minimal structural distortion.

Importantly, we provide structural and kinetic evidence demonstrating that discrimination against the pro-mutagenic *syn*-conformation occurs during the extension step. This bias depends on a conserved active site residue in the thumb subdomain that enhances nucleotide selectivity and fidelity during 8-oxo-dG bypass. Taken together, our results reveal that Pol λ promotes long-patch BER in order to facilitate the error-free bypass of 8-oxo-dG.

Results

In order to elucidate the mechanism of 8-oxo-dG bypass by Pol λ , we structurally characterized several key steps in the context of long-patch BER—initial binding of 8-oxo-dG-containing DNA (DNA binding), subsequent binding of the incoming dNTP (insertion) and polymerization past the lesion site (extension). Each of these steps can function as a fidelity checkpoint and present an opportunity for the polymerase to discriminate against dAMP misincorporation opposite 8-oxo-dG.

Pol λ accommodates 8-oxo-dG in the *syn*-conformation during DNA binding

Solution structures demonstrate that an 8-oxo-dG:dA mispair is more stable than an 8-oxo-dG:dC pair (Kouchakdjian *et al*, 1991; Oda *et al*, 1991). Despite this underlying bias, *in vitro* studies with various polymerases indicate that other factors likely influence polymerization fidelity (Miller *et al*, 2000; Brown *et al*, 2007). Accordingly, the propensity for either dCMP or dAMP synthesis may be determined by the *anti/syn*-conformational equilibrium of 8-oxo-dG in the polymerase active site. We hypothesized that Pol λ may promote dCMP incorporation by preferentially stabilizing the *anti*-conformation of 8-oxo-dG upon initial binding. Therefore, we decided to crystallize Pol λ in complex with a gapped oligonucleotide containing 8-oxo-dG in the templating position (Fig 1A).

We obtained crystals that diffracted to a resolution of 2.08 Å and contained four molecules of Pol λ in complex with DNA per asymmetric unit (Table EV1, PDB: 5IIO). This structure represents the initial DNA binding step (Fig 1B). Overall, this structure is very similar to that of the enzyme in a reference structure with unmodified gapped DNA (PDB: 1XSL, RMSD of 0.380 Å over 320 C α atoms) (Garcia-Diaz *et al*, 2005).

Surprisingly, our structure reveals that Pol λ accommodates 8-oxo-dG exclusively in the *syn*-conformation (Fig 1C, left panel). In the *anti*-conformation, the C8-carbonyl would clash with the 5'-phosphate (Fig 1C, right panel, red-dotted line) in the absence of a substantial rearrangement of the DNA backbone. Such a rearrangement is likely prohibited as 8-oxo-dG(*syn*) is stabilized by an intramolecular hydrogen bond between its N2-amino group

and a non-bridging oxygen on its 5'-phosphate (3.2 Å) (Fig 1D). Additionally, the oxidized base also forms multiple interactions with Tyr⁵⁰⁵ and Arg⁵¹⁷. Consistent with what is observed in the structure with unmodified gapped DNA (PDB: 1XSL), our structure shows that Tyr⁵⁰⁵ obstructs the position of the incoming dNTP and forms a hydrogen bond with the C6-carbonyl of 8-oxo-dG (2.8 Å) (Fig 1D). Conversely, Arg⁵¹⁷ provides a van der Waals stacking interaction with the templating base. Importantly, this residue also forms a hydrogen-bonding interaction with another non-bridging oxygen on the phosphate backbone of 8-oxo-dG (2.9 Å) (Fig 1D). Together, these interactions likely stabilize 8-oxo-dG in the *syn*-conformation.

Pol λ indiscriminately accommodates 8-oxo-dG during catalysis

Despite the bias in favor of 8-oxo-dG(*syn*) upon initial binding, Pol λ is capable of incorporating dCMP opposite 8-oxo-dG with an efficiency similar to that of an undamaged base (Brown *et al*, 2007; Picher & Blanco, 2007). In order to identify the structural features that promote error-free incorporation, we crystallized Pol λ in a complex with an 8-oxo-dG:ddCTP base pair in the active site (Fig 2A). Our crystallization attempts yielded crystals of a pre-catalytic insertion complex that diffracted to a resolution of 1.72 Å (Table EV1, PDB: 5IIJ). This structure depicts a polymerase poised for catalysis after undergoing a dNTP-induced conformational change (Fig 2B).

The structure reveals that Pol λ can tolerate an 8-oxo-dG:ddCTP base pair in the active site. The damaged base adopts the *anti*-conformation and forms a Watson–Crick base pair with an incoming ddCTP (Fig 2C). Moreover, the C8-carbonyl of 8-oxo-dG is far removed from the 5'-phosphate (Fig 2C) so as to avoid an electrostatic clash.

Interestingly, the geometry of the pair is similar to other canonical Watson–Crick base pairs. The λ_R angle for the incoming ddCTP is 57.4°, while the λ_Y angle for the templating 8-oxo-dG is 58.1°. Moreover, the C1'–C1' width of the base pair is 10.6 Å which is similar to other base pairs (10.5 Å—dA:ddTTP, 10.6 Å—dA:dUMPNPP) (Garcia-Diaz *et al*, 2005, 2007; Fig 2C).

In Pol λ , formation of a catalytically active complex requires a dNTP-induced conformational change that repositions several residues, including Asn⁵¹³ and Arg⁵¹⁷. These residues form sequence-independent interactions with the minor groove and probe for correct geometry of the nascent base pair in the active site (Garcia-Diaz *et al*, 2005). Asn⁵¹³ establishes a hydrogen bond with the O2 of the incoming ddCTP while Arg⁵¹⁷ interacts with the N3 of the templating 8-oxo-dG. These interactions are mostly preserved (Fig 2C). However, the hydrogen-bonding distance between Arg⁵¹⁷ and 8-oxo-dG is longer than in a normal Watson–Crick base pair (3.2 Å; Garcia-Diaz *et al*, 2005).

Despite this perturbation, an overlay with an undamaged pre-catalytic structure reveals minimal structural distortion around the primer terminus and catalytic active site residues (Fig 2D). This is further evidenced by the small RMSD between our structure and a reference structure containing undamaged DNA (PDB: 2PFO, RMSD of 0.241 Å over 233 C α atoms). Together, these results suggest that Pol λ is capable of accommodating 8-oxo-dG (*anti*) in a catalytically relevant conformation without significant distortion in the active site during catalysis. These results are also consistent with the high

Figure 1. Pol λ exclusively stabilizes 8-oxo-dG in the *syn*-conformation upon initial binding.

- A Substrate used for crystallization of the DNA binding complex. The oligo contains a templating 8-oxo-dG shown in green.
- B The 39-kDa catalytic domain (8-kDa subdomain not shown for clarity) is colored by subdomain: fingers (salmon), palm (yellow), and thumb (purple). The polymerase is in complex with a 1-nt gapped 16-mer oligo (gray) containing a templating 8-oxo-dG (green). Key residues involved in stabilizing the templating base (Y⁵⁰⁵ and R⁵¹⁷) are shown in black.
- C Left panel: 8-oxo-dG (green) preferentially adopts the pro-mutagenic *syn*-conformation. A simulated annealing *Fo*-*Fc* omit electron density map is shown for 8-oxo-dG (contoured at 3 σ). Right panel: 8-oxo-dG(*anti*) (black, modeled using 2PFO) was overlayed with the *Fo*-*Fc* omit electron density map. In the *anti*-conformation, the C8-carbonyl would directly clash with the 5'-phosphate (red-dotted line).
- D 8-oxo-dG(*syn*) (green) is stabilized by multiple interactions, including Tyr⁵⁰⁵ and Arg⁵¹⁷ (gray surfaces).

efficiency of dCMP insertion opposite 8-oxo-dG observed in Pol λ (Brown *et al.*, 2007; Picher & Blanco, 2007).

In addition to being highly efficient at polymerizing opposite 8-oxo-dG, Pol λ is also capable of discriminating against dAMP misincorporation. However, misincorporation is still a relatively frequent event (twofold preference for incorporating dCMP over dAMP) (Brown *et al.*, 2007; Picher & Blanco, 2007). In order to obtain insight into the structural basis of this reaction, we crystallized Pol λ in complex with an 8-oxo-dG:dATP mispair in the active site (Fig 3A and B). Our crystals diffracted to a resolution of 1.80 Å (Table EV1, PDB: 5III).

As expected, 8-oxo-dG adopts the *syn*-conformation and forms a Hoogsteen mispair with the incoming dATP (Fig 3C). In contrast to the 8-oxo-dG(*anti*):dC base pair, which does not substantially alter the DNA conformation, the Watson–Crick base-pairing edge of 8-oxo-dG(*syn*) protrudes into the major groove of the DNA. However, given the absence of protein contacts with the major groove of the DNA in this region (Garcia-Diaz *et al.*, 2005), the Hoogsteen base pair appears to be well tolerated. The λ_R angle for the incoming dATP and the λ_Y angle for the templating 8-oxo-dG are 60.5 and 45.9°, respectively. Interestingly, the C1'–C1' width

of the base pair is 10.6 Å which is also comparable to a Watson–Crick base pair (Garcia-Diaz *et al.*, 2005, 2007). Moreover, an 8-oxo-dG(*syn*):dA mispair mimics the minor groove geometry of a canonical Watson–Crick base pair. Accordingly, Asn⁵¹³ and Arg⁵¹⁷ are capable of interacting with the N3 group on the incoming dATP and C8-carbonyl of the templating 8-oxo-dG(*syn*) (Fig 3C). Similar to what has been observed in other DNA polymerases (Briebe *et al.*, 2004; Batra *et al.*, 2012; Freudenthal *et al.*, 2013a,b; Vyas *et al.*, 2015), 8-oxo-dG(*syn*) is also capable of hijacking these interactions in Pol λ , thus circumventing an important fidelity checkpoint mechanism.

An overlay with an undamaged precatalytic structure reveals minimal structural distortion around the active site (Fig 3D). This is consistent with the small RMSD between our structure and a reference undamaged structure (PDB: 2PFO, RMSD of 0.243 Å over 234 C α atoms). Together, these results suggest that Pol λ can bind 8-oxo-dG in the *syn*-conformation without significant distortion in the active site. These findings are in agreement with previous kinetic results demonstrating that dCMP and dAMP are incorporated with similar efficiency opposite 8-oxo-dG by Pol λ (Brown *et al.*, 2007; Picher & Blanco, 2007).

Figure 2. Pol λ accommodates 8-oxo-dG in the *anti*-conformation during ddCTP binding.

- A** Substrates used for crystallization of the 8-oxo-dG:dATP insertion complex. The primer terminal base (ddC) is shown in cyan. The incoming dNTP (ddCTP) and corresponding templating 8-oxo-dG are shown in magenta and green, respectively.
- B** Overview of the 8-oxo-dG:dATP insertion complex. The 39-kDa catalytic domain (8-kDa subdomain not shown for clarity) is colored by subdomain: fingers (salmon), palm (yellow), and thumb (purple). The polymerase is in complex with a 1-nt gapped 16-mer oligo (gray) containing a templating 8-oxo-dG (green). The primer terminal ddC and the incoming ddCTP are shown in cyan and magenta, respectively. Key residues that form the nascent base pair binding pocket (N⁵¹³ and R⁵¹⁷) and are involved in catalysis (D⁴²⁷, D⁴²⁹, and D⁴⁹⁰) are shown in black. The catalytic metal (A) and the nucleotide binding metal (B) are shown in red and neon green, respectively.
- C** 8-oxo-dG (green) establishes a Watson–Crick base pair with ddCTP (magenta). Binding of the 8-oxo-dG in the *anti*-conformation is only possible because the C8-carbonyl is far enough away not to clash with the 5'-phosphate. Asn⁵¹³ and Arg⁵¹⁷ (purple) interact with the minor groove of the nascent base pair. The base pair geometry (C1' distance and λ angles) is indicated at the bottom of the figure. A simulated annealing *Fo*-*Fc* omit electron density map for the pair is also shown (contoured at 3 σ).
- D** Overlay of the primer terminus and active site with an undamaged reference structure (2PFO, black). The catalytic aspartic acids are shown in yellow, the primer terminus in cyan, and the incoming dATP in magenta. A water molecule (red) and Mg²⁺ (neon green) are occupying the metal A and metal B sites, respectively.

Active site rearrangements facilitate incorporation opposite 8-oxo-dG

Close inspection of both structures reveal that the C8-carbonyl of 8-oxo-dG(*anti*) and the N2-group of 8-oxo-dG(*syn*) impose local structural changes in the DNA backbone (Fig 4A and B, red-dotted lines). Binding of 8-oxo-dG is only possible because Pol λ can tolerate a drastic repositioning of the 5'-phosphate to avoid these clashes (Fig 4A and B, red arrows). This in turn leads to a notable change in the conformation of active site residue Arg⁵¹⁴, which is displaced by 3.1 Å (Fig 4A and B, black arrows). In an undamaged complex, Arg⁵¹⁴ forms a van der Waals stacking interaction with the templating base and a hydrogen bond with the 5'-phosphate (Garcia-Diaz *et al*, 2005). However, upon repositioning of the 5'-phosphate, this hydrogen bond can no longer be established (Fig 4A and B, blue-dotted lines).

Upon ddCTP binding, the ϵ guanidinium group of Arg⁵¹⁴ is positioned to hydrogen bond with the C6-amino group of 8-oxo-dG (Fig 4A). This interaction may function as a conserved mechanism to stabilize the oxidized lesion. Accordingly, Pol λ exhibits a high efficiency of dNTP incorporation opposite 8-oxo-dG (Miller *et al*, 2000; Brown *et al*, 2007). This rearrangement is also observed in the presence of an 8-oxo-dG:dATP mispair. In this case, Arg⁵¹⁴ forms a hydrogen bond with the C6-amino group of the incoming dATP (Fig 4B).

Interestingly, Arg⁵¹⁴ forms a sequence-dependent interaction with the nascent base pair in both of the 8-oxo-dG-containing structures. We thus hypothesized that these interactions may facilitate oxidative bypass of the lesion. In order to characterize the putative role of Arg⁵¹⁴, we generated an R514L active site substitution. The R514L substitution is expected to exhibit a van der Waals stacking profile similar to the wild-type (WT) Arg⁵¹⁴, while

Figure 3. Pol λ fails to discriminate against dATP misincorporation opposite 8-oxo-dG(syn).

- A** Substrates used for crystallization of the 8-oxo-dG:dATP insertion complex. The primer terminal base (ddC) is shown in cyan. The incoming dNTP (dATP) and corresponding templating 8-oxo-dG are shown in magenta and green, respectively.
- B** Overview of the 8-oxo-dG:dATP insertion complex. The 39-kDa catalytic domain (8-kDa subdomain not shown for clarity) is colored by subdomain: fingers (salmon), palm (yellow), and thumb (purple). The polymerase is in complex with a 1-nt gapped 16-mer oligo (gray) containing a templating 8-oxo-dG (green). The primer terminal ddC and the incoming dATP are shown in cyan and magenta, respectively. Key residues that form the nascent base pair binding pocket (N^{513} and R^{517}) and are involved in catalysis (D^{427} , D^{429} , and D^{490}) are shown in black. The catalytic metal (A) and the nucleotide binding metal (B) are shown in red and neon green, respectively.
- C** 8-oxo-dG (green) establishes a Hoogsteen base pair with dATP (magenta). Asn⁵¹³ and Arg⁵¹⁷ (purple) interact with the minor groove of the nascent base pair. The base pair geometry ($C1'$ distance and λ angles) is indicated at the bottom of the figure. A simulated annealing *Fo-Fc* omit electron density map for the pair is also shown (contoured at 3σ).
- D** Overlay of the primer terminus and active site with an undamaged reference structure (2PFO, black). The catalytic aspartic acids are shown in yellow, the primer terminus in cyan, and the incoming dATP in magenta. A water molecule (red) and Mg^{2+} (neon green) are occupying the metal A and B sites, respectively.

Figure 4. Structural rearrangements facilitate incorporation opposite 8-oxo-dG.

- A, B** Overlay of the nascent base pair in the insertion structures with an undamaged reference structure (2PFO, black). Binding of 8-oxo-dG (green) in both the *anti*- (A) and *syn*- (B) conformation involves a repositioning of the 5'-phosphate (red arrow). Furthermore, the Arg⁵¹⁴ side chain (purple) that normally stacks with the templating base and interacts with the 5'-phosphate (blue-dotted line), also repositions (black arrow). In both structures, Arg⁵¹⁴ interacts with the C6-carbonyl of the templating 8-oxo-dG(*anti*) (A) or the C6-amino group of the incoming dATP (B). The incoming nucleotide is shown in magenta.

Table 1. Steady-state kinetic parameters of insertion opposite 8-oxo-dG by Pol λ .

Pol λ	Template	dNTP	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1} \mu\text{M}^{-1}$)
WT	dG	dCTP	0.8 ± 0.2	30.0 ± 4.1	42 ± 5
	8-oxo-dG	dCTP	2.2 ± 0.4	23.2 ± 1.8	11 ± 1
		dATP	1.5 ± 0.3	62.6 ± 1.9	42 ± 5
R514L	dG	dCTP	2.5 ± 0.3	43.7 ± 6.6	18 ± 2
	8-oxo-dG	dCTP	4.3 ± 0.4	26.8 ± 2.6	6.2 ± 0.2
		dATP	4.7 ± 0.1	58.9 ± 4.2	13 ± 1

Each experiment was independently repeated. Reported results are mean $\pm$ s.d. from three independent experiments.

abrogating the hydrogen-bonding potential. Steady-state kinetic parameters for both WT and R514L Pol λ were determined (Table 1). Accordingly, the R514L substitution had a modest, but consistent, effect on all substrates tested (twofold–threefold). Thus, the interactions established in both damaged structures act in a compensatory manner and non-specifically enhance the efficiency of 8-oxo-dG bypass.

Together, these results highlight the importance of the malleability of the Pol λ active site to efficiently polymerize opposite 8-oxo-dG. The high efficiency of incorporation opposite 8-oxo-dG is a direct consequence of the ability of the active site to tolerate substantial distortion in the DNA backbone and the fact that compensatory interactions with active site residues help overcome the structural perturbations induced by 8-oxo-dG.

Pol λ accommodates 8-oxo-dG at the primer terminus

The inability of Pol λ to discriminate against dAMP misincorporation opposite 8-oxo-dG implies that other factors must explain the high fidelity of MUTYH-dependent repair *in vivo*. Since MUTYH-dependent repair tends to proceed through long-patch BER, one possibility is that Pol λ may discriminate against dAMP misincorporation during the incorporation of the next nucleotide (extension). Consistent with this idea, biochemical analysis has shown that Pol λ promotes error-free extension of 8-oxo-dG:dC base pairs (Picher & Blanco, 2007). In order to better understand this mechanism, we crystallized Pol λ in a precatalytic conformation with DNA containing an 8-oxo-dG:dC base pair at the primer terminus (Fig 5A). We obtained crystals that diffracted to 2.15 Å (Table EV1, PDB: 5IIN). This structure represents a complex poised for extension past an 8-oxo-dG:dC base pair (Fig 5B).

Unsurprisingly, the structure reveals that 8-oxo-dG adopts the *anti*-conformation and forms a canonical Watson–Crick base pair with the primer terminal cytosine (Fig 5C). Strikingly, the base pair forms several minor groove interactions with Tyr⁵⁰⁵, Arg⁵¹⁷ and Glu⁵²⁹ (Fig 5C). Here, Tyr⁵⁰⁵ and Arg⁵¹⁷ establish sequence-independent hydrogen bonds with the O2/N3 acceptor groups of the base pair to probe for correct geometry. Interestingly, Glu⁵²⁹ forms a sequence-specific interaction with the N2-amino group of the primer terminal templating 8-oxo-dG in the *anti*-conformation.

Furthermore, an overlay with an undamaged reference structure reveals minimal structural distortion around the primer terminus and the active site (Fig 5D). This is evidenced by the small RMSD between both structures (PDB: 2PFO, RMSD of 0.219 Å over 247 C α

atoms). Thus, Pol λ is capable of tolerating an 8-oxo-dG:dC base pair at the primer terminus without impeding catalysis. Curiously, while Mg²⁺ is occupying the metal B site, metal A is absent, resulting in a non-catalytic position of the 3'-OH (Fig 5D).

The use of non-hydrolyzable analogs in crystallography has been associated with subtle structural deviations (Garcia-Diaz *et al*, 2007). In order to validate whether our precatalytic structure is in a catalytically relevant conformation, we decided to obtain a structure of the post-catalytic complex. Completion of the nucleotidyl transfer reaction and advancement from precatalytic to the post-catalytic complex in Pol λ involves no significant conformational changes, other than stereochemical inversion of the α -phosphate (Garcia-Diaz *et al*, 2005). Therefore, the post-catalytic complex is also representative of a catalytically relevant conformation. Accordingly, we crystallized the post-catalytic complex containing a newly incorporated dTMP in the active site (Table EV1, PDB: 5IJK). An overlay between the pre- and post-catalytic structure reveals minimal structural distortion around the primer terminus and the active site (Fig EV1A) as evidenced by the small RMSD between both structures (RMSD of 0.209 Å over 279 C α atoms). This confirms that our structure is in a catalytically relevant conformation.

We also crystallized Pol λ with DNA containing an 8-oxo-dG:dA mispair at the primer terminus (Fig 6A and B). These crystals diffracted to a resolution of 1.94 Å (Table EV1, PDB: 5IIM). The resulting structure reveals that 8-oxo-dG adopts the *syn*-conformation and forms a Hoogsteen mispair with the primer terminal adenine (Fig 6C). As expected, the mispair mimics the minor groove geometry of a Watson–Crick base pair and is thus capable of interacting with Tyr⁵⁰⁵ and Arg⁵¹⁷ (Fig 6C).

Additionally, an 8-oxo-dG:dA base pair at the primer terminus does not distort the active site of Pol λ (Fig 6D). This is further demonstrated by the small RMSD between our structure and an undamaged structure (PDB: 2PFO, RMSD of 0.295 Å over 247 C α atoms). Similar to the other extension structure, the catalytic metal A was also absent (Fig 6D). We again crystallized a corresponding post-catalytic structure (Table EV1, PDB: 5IIL) further confirming that the presence of an 8-oxo-dG:dA base pair at the primer terminus does not distort the active site (Fig EV1B). This is demonstrated by the small RMSD between the pre- and post-catalytic structures (RMSD of 0.168 Å over 305 C α atoms).

Together, our results suggest that Pol λ is capable of accommodating 8-oxo-dG in both the *syn*- and *anti*-conformations without any significant distortion at the primer terminus, explaining the ability of Pol λ to extend past both pairs.

Figure 5. Pol λ can tolerate an 8-oxo-dG(anti):dC base pair at the primer terminus.

- A** Substrates used for crystallization of the 8-oxo-dG:dC extension complex. The incoming dNTP (dUMP dNTP) is shown in magenta. The primer terminal base (dC) and corresponding templating base (8-oxo-dG) are shown in cyan and orange, respectively.
- B** Overview of the 8-oxo-dG:dC extension complex. The 39-kDa catalytic domain (8-kDa subdomain not shown for clarity) is colored by subdomain: fingers (salmon), palm (yellow), and thumb (purple). The polymerase is in complex with a 1-nt gapped 16-mer oligo (gray) and an incoming dUMP dNTP (magenta). The primer terminal dC and 8-oxo-dG are shown in cyan and magenta, respectively. Key residues that form the primer terminal base pair binding pocket (N⁵¹³, R⁵¹⁷ and Glu⁵²⁹) and are involved in catalysis (D⁴²⁷, D⁴²⁹, and D⁴⁹⁰) are shown in black. The nucleotide binding metal (B) is shown in neon green.
- C** 8-oxo-dG (orange) establishes a Watson–Crick base pair with dC (cyan) at the primer terminus. Tyr⁵⁰⁵, Arg⁵¹⁷, and Glu⁵²⁹ (purple) interact with the minor groove of the base pair. The base pair geometry (C1' distance and λ angles) is indicated at the bottom of the figure. A simulated annealing *Fo*-*Fc* omit electron density map for the pair is also shown (contoured at 3 σ).
- D** Overlay of the primer terminus and active site with an undamaged reference structure (2PFO, black). The catalytic aspartic acids are shown in yellow, incoming dUMP dNTP in magenta, and primer terminal dC in cyan. Expectedly, Mg²⁺ (neon green) is occupying the metal B site. However, the metal A is absent in the structure, resulting in a non-catalytic position of the 3'-OH. This is consistent with other structures containing an incoming non-hydrolyzable analog.

A key conserved residue discriminates against 8-oxo-dG(syn)

While Pol λ appears to tolerate both 8-oxo-dG conformations at the primer terminus, biochemical results (Picher & Blanco, 2007) that we were able to reproduce (Table 2) demonstrate that it strongly discriminates against extension of an 8-oxo-dG:dA mispair. Surprisingly, close inspection of the extension structures revealed a specific interaction with 8-oxo-dG in the *anti*-conformation. A conserved residue in the SD2 region of the thumb subdomain (Romain *et al*, 2009), Glu⁵²⁹, forms a hydrogen bond with the N2-group of the templating 8-oxo-dG in the *anti*-conformation (Fig 7A). Strikingly, this interaction is abolished when 8-oxo-dG adopts the pro-mutagenic *syn*-conformation (Fig 7B, red-dotted line). This is likely due to an electrostatic clash between the negatively charged carboxyl group of the aspartic acid side chain and the C8-carbonyl of 8-oxo-dG (Fig 7B, blue-dotted line). Thus, Glu⁵²⁹ may selectively stabilize correct 8-oxo-dG(*anti*):dC base pairs while destabilizing

8-oxo-dG(*syn*):dA mispairs. Accordingly, this residue may be responsible for the observed extension bias.

To test this hypothesis and characterize the role of Glu⁵²⁹ on 8-oxo-dG discrimination at the extension step, we generated an E529A active site substitution. We determined steady-state kinetic parameters for dGTP incorporation opposite a templating dC containing either an 8-oxo-dG:dC or dA base pair at the primer terminus (Table 2). Interestingly, the E529A substitution had a differential effect on the catalytic efficiency of extension past both base pairs (Fig 7C, Table 2). With respect to the WT protein, the catalytic efficiency for dGMP incorporation decreased by 2.2-fold when an 8-oxo-dG:dC base pair was at the primer terminus. Conversely, the E529A substitution resulted in a threefold increase in extension activity past an 8-oxo-dG:dA base pair. Furthermore, the E529A substitution eliminated the extension bias, as the catalytic efficiencies for extension of both pairs were nearly identical (Fig 7C, Table 2). Thus, our results demonstrate that Glu529 is responsible

Figure 6. Pol λ also appears to tolerate a primer terminal 8-oxo-dG(syn):dA mispair.

- A Substrates used for crystallization of the 8-oxo-dG:dA extension complex. The incoming dNTP (dUMP) is shown in magenta. The primer terminal base (dA) and corresponding templating base (8-oxo-dG) are shown in cyan and orange, respectively.
- B Overview of the 8-oxo-dG:dC extension complex. The 39-kDa catalytic domain (8-kDa subdomain not shown for clarity) is colored by subdomain: fingers (salmon), palm (yellow), and thumb (purple). The polymerase is in complex with a 1-nt gapped 16-mer oligo (gray) and an incoming dUMP (magenta). The primer terminal dA and 8-oxo-dG are shown in cyan and magenta, respectively. Key residues that form the primer terminal base pair binding pocket (N⁵¹³ and R⁵¹⁷) and are involved in catalysis (D⁴²⁷, D⁴²⁹, and D⁴⁹⁰) are shown in black. The nucleotide binding metal (B) is shown in neon green.
- C 8-oxo-dG (orange) establishes a Hoogsteen base pair with dA (cyan) at the primer terminus. Tyr⁵⁰⁵ and Arg⁵¹⁷ (purple) interact with the minor groove of the base pair. The base pair geometry (C1' distance and λ angles) is indicated at the bottom of the figure. A simulated annealing *2Fo-Fc* omit electron density map for the pair is also shown (contoured at 3 σ).
- D Overlay of the primer terminus and active site with an undamaged reference structure (2PFO, black). The catalytic aspartic acids are shown in yellow, incoming dUMP in magenta, and primer terminal dA in cyan. Expectedly, Mg²⁺ (neon green) is occupying the metal B site. However, the metal A is absent in the structure, resulting in a non-catalytic position of the 3'-OH. This is consistent with other structures containing an incoming non-hydrolyzable analog.

for the inherent discrimination against extension of 8-oxo-dG:dA mispairs and is likely a key element that allows Pol λ to participate in MUTYH-dependent repair. Interestingly, Glu⁵²⁹ is conserved among all species in Pol λ (Fig EV2), highlighting its evolutionary importance and suggesting that the ability to selectively extend past an 8-oxo-dG:dC base pair is a unique and essential adaptation to facilitate MUTYH-dependent repair.

Glu⁵²⁹ does not affect polymerization on undamaged substrates

The observation that Glu⁵²⁹ can interact with 8-oxo-dG(*anti*) prompted us to determine whether this residue can also interact with an undamaged guanine. Analysis of an existing Pol λ complex containing a guanine in the templating position at the primer terminus (PDB: 2PFO) revealed an identical interaction with Glu⁵²⁹ (Fig 8A). Conversely, this interaction was absent in all the structures containing an adenine in this position (PDB:1XSN and others)

Table 2. Steady-state kinetic parameters of extension past 8-oxo-dG by Pol λ .

Pol λ	Template: Primer	K_M (μ M)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1} \mu\text{M}^{-1}$)
WT	dG:dC	2.5 $\pm$ 0.2	39.1 $\pm$ 1.5	16 $\pm$ 1
	8-oxo-dG:dC	3.3 $\pm$ 0.4	101.2 $\pm$ 3.5	31 $\pm$ 3
	8-oxo-dG:dA	3.5 $\pm$ 0.7	12.3 $\pm$ 0.5	3.7 $\pm$ 0.5
E529A	dG	3.3 $\pm$ 0.5	43.9 $\pm$ 1.3	14 $\pm$ 2
	8-oxo-dG:dC	6.7 $\pm$ 0.4	93.8 $\pm$ 2.3	14 $\pm$ 1
	8-oxo-dG:dA	4.9 $\pm$ 0.3	51.9 $\pm$ 1.9	11 $\pm$ 1

Each experiment was independently repeated. Reported results are mean $\pm$ s.d. from three independent experiments.

(Fig 8B) (Garcia-Diaz *et al*, 2005). Surprisingly, this observation suggests that Glu⁵²⁹ is able to establish a sequence-specific interaction with the DNA template. This is unexpected for a polymerase,

Figure 7. Pol λ discriminates against extension past an 8-oxo-dG(syn):dA mispair.

- A Glu⁵²⁹ (purple) forms a hydrogen bond with the C2-amino group of the primer terminal templating 8-oxo-dG (orange) in the *anti*-conformation.
- B This interaction is abolished when 8-oxo-dG (orange) adopts the *syn*-conformation. An overlay with the 8-oxo-dG(*anti*)-containing structure reveals that Glu⁵²⁹ (black) would likely clash (red-dotted line) with the C8-carbonyl of 8-oxo-dG(*syn*). Repositioning of Glu⁵²⁹ (purple) places the side chain 4.5 Å away from the C8-carbonyl.
- C Comparison of catalytic efficiencies for extension past an 8-oxo-dG(*anti*):dC, 8-oxo-dG(*syn*):dA and dG:dC base pairs at the primer terminus using WT and E529A Pol λ . Each experiment was independently repeated. Plotted data are mean $\pm$ s.d. (error bars) from three independent experiments.

where interactions are typically non-specific, so as to avoid inducing sequence context effects. Therefore, we decided to analyze whether Glu⁵²⁹ influences polymerization on undamaged substrates in a sequence context-dependent manner. Interestingly, our kinetic results indicate that the substitution has no effect on undamaged substrates (Fig 7C, Table 2), which explains why the mutational spectrum of the polymerase does not appear biased toward sites preceded by a guanine residue in the templates (Bebenek *et al*, 2003).

Discussion

Pol λ is an important repair DNA polymerase that has been implicated in MUTYH-dependent BER (Braithwaite *et al*, 2005; Maga *et al*, 2007, 2008; Tano *et al*, 2007; Vermeulen *et al*, 2007; van Loon & Hubscher, 2009; Markkanen *et al*, 2012; Pande *et al*, 2015). The DNA glycosylase, MUTYH, initiates repair by excising the misincorporated adenine. The resulting gap must then be filled with dCMP. The structural and kinetic studies presented here reveal the unique mechanism by which Pol λ contributes to the error-free bypass of 8-oxo-dG.

Figure 8. Glu⁵²⁹ forms a sequence-specific interaction with guanines.

- A, B Undamaged structure containing a templating dG at the primer terminus (2PFO) was overlaid with a structure containing an 8-oxo-dG:dC base pair (black) or an 8-oxo-dG:dA mispair (red) at the primer terminus (A). Undamaged structure containing a templating dA at the primer terminus (1XSN) was overlaid with a structure containing an 8-oxo-dG:dC base pair (black) or an 8-oxo-dG:dA mispair (red) at the primer terminus (B).

Pol λ preferentially stabilizes 8-oxo-dG in the *syn*-conformation prior to dNTP binding

Surprisingly, Pol λ initially binds 8-oxo-dG-containing DNA exclusively in the *syn*-conformation, which is stabilized by Tyr⁵⁰⁵ and Arg⁵¹⁷. The clear preference for binding 8-oxo-dG(*syn*) is particularly striking, as this would predispose Pol λ to misinsert dAMP. Interestingly, the structurally related DNA polymerase, Pol β , also facilitates the error-free bypass of 8-oxo-dG *in vitro* (Miller *et al*, 2000; Brown *et al*, 2007) and has been extensively characterized in the context of this lesion (Krahn *et al*, 2003; Batra *et al*, 2010, 2012; Freudenthal *et al*, 2013a,b, 2015; Vyas *et al*, 2015). However, in contrast to Pol λ , Pol β can initially accommodate 8-oxo-dG in both the *anti*- and *syn*-conformation with no strong preference (Batra *et al*, 2012). The reason for this is largely related to the substantial differences in the dNTP-induced conformational changes that take place in both enzymes. While Pol λ remains in a closed conformation throughout the catalytic cycle, Pol β initially adopts an open conformation where the equivalent residues to Tyr⁵⁰⁵ (Tyr²⁷²) and Arg⁵¹⁷ (Arg²⁸³) are far removed from the templating base (Batra *et al*, 2012). Thus, in Pol β , the binding pocket for the templating base is only partially formed prior to dNTP binding, likely explaining why 8-oxo-dG freely adopts either conformation.

Pol λ struggles to discriminate against 8-oxo-dG(*syn*) during insertion

During dNTP binding, Pol λ is capable of tolerating 8-oxo-dG in either the *anti*- or *syn*-conformation. Correct and incorrect nucleotides are discriminated from each other on the basis of proper base pairing geometry. Interestingly, the minor groove geometry of an 8-oxo-dG(*syn*):dA mispair is similar to a canonical Watson–Crick base pair. Thus, 8-oxo-dG(*syn*) is capable of interacting with the minor groove probing residues in Pol λ , Asn⁵¹³ and Arg⁵¹⁷. This appears to be a common theme among DNA polymerases that rely on this particular type of interaction for fidelity, such as T7 DNA polymerase (Briebe *et al*, 2004) and *Bacillus stearothermophilus*

DNA polymerase I (Hsu *et al*, 2004). Accordingly, an 8-oxo-dG (*syn*):dA mispair is also capable of hijacking these minor groove interactions in Pol β (Batra *et al*, 2010, 2012; Freudenthal *et al*, 2013a,b, 2015; Vyas *et al*, 2015).

Moreover, in Pol β , it has been postulated that a transient third metal ion (metal C) is important for catalysis. The presence of metal C has also been suggested to play a role in proper base pair discrimination (Freudenthal *et al*, 2013a,b). In agreement with the idea that an 8-oxo-dG(*syn*):dA mispair mimics a canonical base pair, metal C binding has been observed following dATP binding (Vyas *et al*, 2015). This further demonstrates the difficulty in distinguishing between the *anti*- and *syn*-conformations of 8-oxo-dG. Evidence for the presence of metal C was not seen in any of our structures. It is possible that this mechanism is not conserved in Pol λ . However, due to the transient nature of metal C binding, this might also be due to the fact that we were not able to adequately trap the reaction intermediate.

The active site of Pol λ is malleable, allowing for the efficient bypass of 8-oxo-dG

Interestingly, the active site of Pol λ is extremely malleable during 8-oxo-dG bypass. Binding of 8-oxo-dG in the *anti*-conformation is only possible because Pol λ can tolerate a drastic repositioning of the 5'-phosphate. This backbone rearrangement alleviates the electrostatic clash between the C8-carbonyl and 5'-phosphate of 8-oxo-dG. Additionally, binding of an 8-oxo-dG(*anti*):dCTP also results in a repositioning of the active site residue Arg⁵¹⁴. Here, the ϵ -amino group of Arg⁵¹⁴ establishes a weak hydrogen bond with the C6-amino group of 8-oxo-dG. This interaction may stabilize the *anti*-conformation and facilitate error-free incorporation of dCMP. Similar rearrangements have also been observed in the active site of Pol β (Krahn *et al*, 2003).

Curiously, unlike in Pol β (Batra *et al*, 2012; Vyas *et al*, 2015), these rearrangements were also observed during dATP binding opposite 8-oxo-dG in Pol λ . Thus, our data imply that Pol λ non-specifically enhances 8-oxo-dG bypass. As a consequence, Pol λ is able to maintain a high efficiency of incorporation opposite 8-oxo-dG. In contrast, DNA polymerase iota (Pol ι) has a restrictive active site that promotes dCMP incorporation opposite 8-oxo-dG(*syn*) (Kirouac & Ling, 2011). Consistently, the restrictive nature of the Pol ι active site likely explains its reduced insertion efficiency opposite the lesion (Zhang *et al*, 2001; Kirouac & Ling, 2011). Furthermore, most eukaryotic polymerases involved in replication incorporate nucleotides opposite 8-oxo-dG with very low efficiency relative to undamaged DNA (Shibutani *et al*, 1991; Zhang *et al*, 2000; Einolf & Guengerich, 2001; Haracska *et al*, 2002, 2003).

Although Pol λ incorporates dCMP opposite 8-oxo-dG more frequently than many other polymerases (Hashimoto *et al*, 2004; Brown *et al*, 2007; Picher & Blanco, 2007), the fidelity of this reaction is modest at best (2:1 preference—dCMP/dAMP incorporation opposite 8-oxo-dG). This suggests that the high efficiency of incorporation is achieved at the expense of replication fidelity. Thus, during the insertion step, efficiency may be more important than fidelity.

Failure to complete BER can result in accumulation of BER intermediates that are cytotoxic to cells (Sobol *et al*, 2000, 2003). Thus, the low fidelity of incorporation opposite 8-oxo-dG might be an

acceptable price to pay to avoid the cytotoxic consequences of stalling BER. Moreover, the high efficiency of 8-oxo-dG bypass by Pol λ is consistent with its role during NHEJ (Fan & Wu, 2004; Lee *et al*, 2004; Nick McElhinny *et al*, 2005; Capp *et al*, 2006), where the polymerase would frequently encounter oxidatively damaged ends.

Pol λ is uniquely adapted to discriminate against 8-oxo-dG during extension

A very interesting and yet unexplained observation is that Pol λ is endowed with the extraordinary ability to extend past 8-oxo-dG with both high efficiency and fidelity (Picher & Blanco, 2007). Here, we have identified a residue in the SD2 region of the thumb subdomain that forms a unique sequence-specific hydrogen bond with 8-oxo-dG (*anti*). In fact, owing to this interaction, extension of an 8-oxo-dG:dC pair is even more efficient than extension of an undamaged dG:dC pair (Picher & Blanco, 2007). Conversely, the same residue results in an unfavorable interaction with an 8-oxo-dG(*syn*):dA mispair, which reduces the catalytic efficiency of extension. Together, these two effects result in a clear bias toward extension of correct 8-oxo-dG:dC pairs.

Interestingly, the analogous residue in Pol β is also a glutamate (Glu²⁹⁵) (Fig EV2). However, Glu²⁹⁵ does not interact with the primer terminus (Sawaya *et al*, 1997). Instead, this residue plays a role in the catalytic transition from the inactive to active conformation upon dNTP binding (Sawaya *et al*, 1997). Prior to dNTP binding, one of the active site aspartic acids (Asp¹⁹²) forms a salt bridge with Arg²⁵⁸, sequestering it in an inactive conformation. Binding of the correct nucleotide results in a conformational change that ultimately disrupts this salt bridge. As a result, Asp¹⁹² moves in position to anchor the catalytic metal B, while Arg²⁵⁸ instead establishes an interaction with Glu²⁹⁵.

Taken together, this is striking example of a residue that is conserved in two related enzymes that play completely different functional roles. This functional divergence stresses how subtle differences in the active site can drastically affect both the functional properties and perhaps the cellular role of a DNA polymerase.

Pol λ -dependent MUTYH-initiated BER pathway

The structural and kinetic results that we describe here provide a framework for understanding error-free bypass of 8-oxo-dG by Pol λ in the context of MUTYH-initiated BER. Interestingly, MUTYH-initiated BER appears to predominantly proceed through long-patch BER (van Loon & Hubscher, 2009). Consistent with this observation, the short-patch protein, DNA ligase III, is inefficient at ligating the 3'-terminus of a correct 8-oxo-dG:dC base pair (Hashimoto *et al*, 2004). As a result, short-patch BER is futile and repair must instead proceed through the alternative long-patch BER route.

Our results lead to a model for MUTYH-dependent repair (Fig 9) where Pol λ plays a central role acting as a kinetic switch that drives the reaction toward long-patch BER. Pol λ and Pol β exhibit a similar efficiency and fidelity with respect to nucleotide insertion opposite 8-oxo-dG (Brown *et al*, 2007; Picher & Blanco, 2007). Thus, the DNA polymerase involved during the insertion step may be interchangeable. However, Pol λ appears to have the unique ability to promote error-free bypass during the extension

Figure 9. Proposed mechanism for the MUTYH-initiated BER pathway.

MUTYH initiates the excision of the misincorporated adenine opposite 8-oxo-dG. The resulting 1-nt gap is then filled by Pol λ in an error-free (C) or error-prone (A) manner. Completion of the canonical short-patch (SP) pathway is futile as DNA ligase III cannot seal an 8-oxo-dG:dC base pair at the primer terminus. Thus, Pol λ promotes the long-patch (LP) extension past a correct base pair to complete error-free BER. Conversely, Pol λ is capable of stalling ligation (SP) or extension (LP) of an 8-oxo-dG:dA mispair, thus promoting its excision by proofreading. The resulting intermediate can then proceed through the error-free pathway.

step. Strikingly, Pol λ bypasses an 8-oxo-dG(*anti*):dC pair at the primer terminus with a higher propensity than an undamaged pair, thus promoting long-patch BER extension (Picher & Blanco, 2007). Moreover, error-free bypass of 8-oxo-dG by Pol λ is promoted by the long-patch BER auxiliary factors PCNA and RPA (Maga *et al*, 2007). Together, these properties make Pol λ well suited in facilitating the error-free bypass of 8-oxo-dG during MUTYH-mediated long-patch BER.

Pol λ is also capable of discriminating against error-prone extension, thereby preventing error-prone long-patch BER. While it is possible that DNA ligase III can seal the 3'-terminus of an 8-oxo-dG:dA mispair (Hashimoto *et al*, 2004), this would result in a futile cycle of short-patch repair. Instead, failure to extend the mispair may promote removal of the misincorporated dA in an exonuclease-dependent manner. Accordingly, this increased fidelity may be attributed to the high pyrophosphorolytic activity of

Pol λ and its ability to preferentially operate on 8-oxo-dG:dA mispairs (Crespan *et al*, 2012). Another possibility is that an extrinsic exonuclease is instead recruited. Interestingly, the primary abasic endonuclease involved in BER, APE1, possesses 3'-5'-exonuclease activity. Moreover, the exonuclease activity of APE1 is enhanced at 3'-termini of mismatches (Wong *et al*, 2003), which suggests a possible proofreading role during BER. Together, our results indicate that Pol λ may stall extension past the 8-oxo-dG:dA mispair to promote excision, thus acting as form of proofreading.

In summary, we have shown here how a family X polymerase utilizes subtle active site adaptations to carry out a critical repair reaction. Our results highlight the importance of Pol λ as a key enzyme during MUTYH-initiated BER, provide a mechanistic explanation for its unique behavior during repair, and uncover a potential mechanism to enhance the fidelity of repair.

Materials and Methods

Protein purification

The sequence corresponding to residues 242–575 of human DNA polymerase lambda (Pol λ) was previously cloned into the bacterial expression vector pET-22b (Garcia-Diaz *et al*, 2004). Site-directed mutagenesis was performed on the Pol λ expression vector to generate the R514L and E529A mutants. WT, R514L, and E529A Pol λ were subsequently expressed in the *E. coli* strain BL21-CodonPlus(DE3)-RIL and purified as described (Garcia-Diaz *et al*, 2004).

Oligonucleotides for crystallography

Oligonucleotides used for crystallography (Fig EV3A–D) were synthesized by solid-state synthesis methods using an automated DNA synthesizer and were subsequently purified by HPLC, ethanol precipitated, and quantified by UV absorbance (A_{260}).

Crystallization

All crystallization complexes were formed using an upstream primer, downstream primer containing a 5'-phosphate group, and template. Oligonucleotides were mixed (1:1:1) and heated to 80°C for 10 min before slowly cooling to room temperature in 25 mM Tris-HCl pH 7.5 and 50 mM MgCl₂. Oligonucleotides were subsequently mixed with Pol λ . However, due to the low melting temperature of the downstream primer (DP4, 16°C), a second annealing step (starting at 16°C) in the presence of protein was used to ensure the proper formation of the final substrate. The resulting mixture (100 μ l) contained 15 mM Tris-HCl pH 7.5, 75 mM NaCl, 10 mM MgCl₂, 1 mM DTT, DNA (0.5 mM), and Pol λ (0.4 mM).

DNA binding complex

Binary complexes were formed with the oligonucleotides shown in Fig EV3A. Crystals were grown using the hanging drop method at 4°C by mixing 1 μ l of a protein-DNA solution with 1 μ l of a reservoir solution containing 0.1 M KCl, 0.01 M CaCl₂, 0.05 M HEPES pH 7.0, and 10% PEG 400. The crystals were then transferred to a solution containing 0.1 M KCl, 0.01 M CaCl₂, 0.05 M HEPES pH 7.0, and 30% PEG 400 and 25% w/v glycerol cryo-cooled in liquid nitrogen prior to data collection.

Insertion complex

Precatalytic ternary complexes were formed with the oligonucleotides shown in Fig EV3B. ddCTP (1 mM) and dATP (4 mM) were sequentially added to a prehybridized protein-DNA solution to allow for the proper formation of a dideoxy-terminated oligonucleotide and binding of the incoming dATP. Crystals were then grown as previously described above. Complexes containing a nascent 8-oxo-dG:ddCTP base pair were formed at 4°C in 0.1 M sodium cacodylate pH 7.0 and 1.0 M sodium acetate trihydrate. The resulting crystals were then transferred to a solution containing 0.1 M sodium cacodylate pH 7.0 and 1.1 M sodium acetate trihydrate and 25% w/v glycerol for cryo-storage in liquid nitrogen prior to data collection. A subset of these crystals were also

soaked overnight in a cryo-solution containing 0.1 M sodium cacodylate pH 7.0, 1.5 M sodium acetate trihydrate, 25% w/v glycerol, 100 mM MgCl₂, and 15 mM dATP in order to fully exchange the residual ddCTP with dATP. All crystals were mounted in Mitegen MicroLoops prior to cryo-cooling in liquid nitrogen.

Extension complex

Precatalytic ternary complexes were formed with the oligonucleotides shown in Fig EV3C and D. dUMPdNPP (4 mM) was added to a prehybridized protein-DNA solution. Crystals were then grown as previously described above. Complexes containing either an 8-oxo-dG:dC or dA base pair at the primer terminus were formed at 4°C in 0.2 M ammonium acetate, 0.1 M sodium citrate pH 4.8, and 2–7% PEG 4000, and then transferred to a solution containing 0.2 M ammonium acetate, 100 mM sodium citrate pH 4.8, 2–7% PEG 4000, and 25% w/v glycerol for cryo-storage in liquid nitrogen prior to data collection.

Post-catalytic nick complexes were formed with the oligonucleotides shown in Fig EV3E and F. Crystals were grown using a prehybridized protein-DNA solution as previously described above. Complexes containing either 8-oxo-dG:dC or dA base pair at the -1 position were formed at 4°C in 0.2 M ammonium acetate, 0.1 M sodium citrate pH 4.8, and 3–5% PEG 4000 and then transferred to a solution containing 0.2 M ammonium acetate, 0.1 M sodium citrate pH 4.8, 3–5% PEG 4000, and 25% w/v glycerol for cryo-storage in liquid nitrogen prior to data collection.

X-ray data collection and structure determination

Diffraction data were collected on beamlines X6A, X12C, and X29 of the National Synchrotron Light Source at Brookhaven National Laboratory (BNL) and on beamline BL14-1 at the Stanford Synchrotron Radiation Lightsource (SSRL). All datasets were collected at 100 K using a wavelength of 0.9795, 1.0, or 1.075 Å. All diffraction data were processed using XDS (Kabsch, 2010) and Aimless (Evans & Murshudov, 2013) as implemented in the autoPROC pipeline (Vonnrhein *et al*, 2011). Phases were obtained by molecular replacement using Phaser (McCoy *et al*, 2007); search models for the binary, pre- and post-catalytic complexes were created from 1XSL (Garcia-Diaz *et al*, 2005), 1XSN (Garcia-Diaz *et al*, 2005), and 1XSP (Garcia-Diaz *et al*, 2005), respectively. Model building was carried out in Coot (Emsley *et al*, 2010), followed by refinement in Phenix (Zwart *et al*, 2008), Refmac (Murshudov *et al*, 2011), and BUSTER (Smart *et al*, 2012). The geometric quality of the refined models was assessed with MolProbity (Chen *et al*, 2010) and the structure validation tools in the Phenix suite. Data collection and refinement statistics are shown in Table EV1.

Oligonucleotides for steady-state primer extension assays

Oligonucleotides used for steady-state primer extension assays were obtained from Invitrogen (Fig EV4A–E). Oligonucleotides were purified by HPLC and polyacrylamide gel electrophoresis (PAGE), quantified by UV absorbance (A_{260}), and heated to 80°C for 10 min before slowly cooling to room temperature overnight in 20 mM Tris-HCl pH 7.5 and 150 mM MgCl₂.

Steady-state primer extension assays

Oligonucleotides used to evaluate the biochemical role of Arg⁵¹⁴ and Glu⁵²⁹ in Pol λ are shown in Fig EV4A and B and EV4C–E, respectively. Upstream primers were 5' labeled with a Cy3 fluorophore and downstream primers contained a 5'-phosphate group. Prehybridized gapped DNA (1:1.2:1.2) or an open duplex (1:1.2) were mixed with either WT, R514L, or E529A Pol λ . The resulting mixture (18 μ l) contained 50 mM Tris–HCl pH 8.5, 10 mM MgCl₂, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, P/T (200 nM) and either WT, R514L, or E529A Pol λ . The protein/DNA mixture was directly added to varying amounts of the appropriate dNTP to start the polymerization reaction. Reaction mixtures (20 μ l) were quenched by the addition of 95% v/v formamide, 10 mM EDTA, 0.001% xylene cyanol, 0.001% bromophenol blue (10 μ l). Extended primers were separated by denaturing (8 M urea) 18% w/v PAGE. The fluorescence intensity of the bands was quantified using a Typhoon FLA 9000 imager and ImageQuant software.

Kinetic analysis of the primer extension assays

The observed rate of nucleotide incorporation (extended primer) was plotted as a function of nucleotide concentration. Steady-state kinetic parameters, $V_{\max}$ and K_M , were determined by fitting the data to the Michaelis–Menten equation: $V = V_{\max}[S]/(K_M + [S])$. k_{cat} was determined with the equation: $k_{\text{cat}} = V_{\max}/[E]$.

Accession codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, under accession codes: 5II0, 5IIJ, 5III, 5IIN, 5IIM, 5IIK, and 5IIL.

Expanded View for this article is available online.

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Author contributions

MJB and MGD conceived the project. MJB, EH, and BD performed experiments. MJB, KEG, and MGD analyzed data. MJB, KEG, and MGD wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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