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1 **Mechanisms Ensuring Fidelity of Family X DNA Polymerases in**
2 **Programmed DNA rearrangements in *Paramecium tetraurelia***

3

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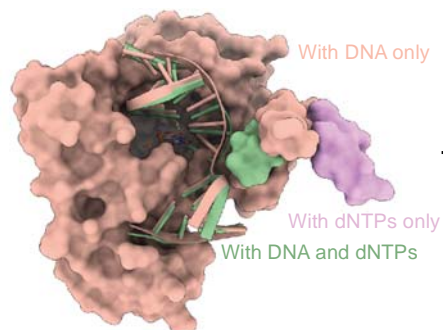
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14 **GRAPHICAL ABSTRACT**

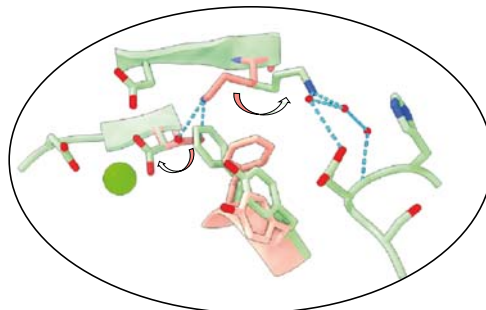
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Mechanisms of fidelity of *Paramecium* PolX

Loop 3 closure movement



Partner exchange in crucial salt bridge



Correct dNTP binding trigger both Loop3 and local rearrangement

16

17

18

19 **ABSTRACT**

20 Repairing programmed DNA double-strand breaks (DSBs) is crucial in the lifecycle of
21 *Paramecium tetraurelia*, especially during its sexual reproduction phase when its somatic
22 highly polyploid macronucleus is lost. The formation of a new macronucleus involves
23 Programmed Genome Rearrangements, introducing DNA DSBs at approximately 45,000 loci.
24 *P. tetraurelia* employs a Non-Homologous End Joining (NHEJ)-related mechanism for the
25 systematic repair of these DSBs. Four genes encoding DNA polymerases of family X are
26 present in the genome, one of which was found recently to colocalize with other proteins of
27 NHEJ. The question arises as to how they make almost no error. Here we show that these
28 enzymes are most similar to metazoan DNA polymerase λ and exhibit high fidelity through
29 two different molecular mechanisms. Using X-ray structure determination of polymerase
30 lambda mutants recapitulating sequence determinants of *P. tetraurelia* PolXs, we find both a
31 local conformational change that involves exchanging partners in a crucial salt bridge in the
32 active site upon binding of correct dNTPs, and a larger conformational change involving the
33 closure of Loop3. This stabilizes the template DNA in the active site, only in the presence of
34 the correct incoming dNTP. Differences with human pol λ and pol β are discussed.

35

36 INTRODUCTION

37 *Paramecium tetraurelia* exhibits nuclear dimorphism, with a single cell containing both a
38 micronucleus (MIC), used for reproduction, and a macronucleus (MAC), utilized for gene
39 expression (1). The MAC harbors up to 1600 copies of the 72 Mb somatic genome, while the
40 diploid MIC genome (108 Mb) contains additional sequences, including Internal Eliminated
41 Sequences (IES) (2). During the sexual reproduction cycle of *P. tetraurelia*, the MAC
42 undergoes fragmentation, and a new MAC is formed from a copy of the MIC through
43 extensive genome replication and rearrangements (3, 4). These large-scale alterations, known
44 as programmed genome rearrangements (PGR), entail the precise removal of approximately
45 45,000 IESs. IESs are excised from the MAC DNA by a domesticated transposase known as
46 PiggyMac (Pgm), which forms active complexes with non-catalytic Pgm-like proteins (5, 6).
47 The DNA elimination complex induces double-strand breaks (DSB) specifically at
48 dinucleotide 5'-TA-3' sites flanking the IES, followed by the elimination of the 5' terminal
49 nucleotide (**Figure 1A**). Recent studies revealed the participation of a specific Ku70/80
50 heterodimer in this complex (7) along with the XRCC4-Ligase IV complex (8–10), in a
51 specialized NHEJ mechanism dealing with programmed DNA DSBs. While the NHEJ system is
52 a priori capable to ensure fidelity in coordinated DNA DSB repair, especially in the presence
53 of cohesive ends, (11, 12), the removal of the 5' nucleotide at each DNA end following DSB
54 induction necessitates an additional gap-filling step before ligation, involving at least one
55 DNA polymerase to make the two DNA ends compatible (**Figure 1A**). These findings prompt
56 the question of the fidelity of the DNA polymerase involved (13, 14).

57 Four DNA PolXs genes, namely *POLXa*, *b*, *c*, and *d*, can be identified in the genome of *P.*
58 *tetraurelia*. In addition, colocalization of one of them, polXa, with the other proteins of NHEJ
59 (Ku and X4L4) has just been described
60 (<https://www.biorxiv.org/content/10.1101/2024.07.18.604053v1>). Here we will focus on these
61 four PolXs.

62 In metazoan (15), the gap-filling step is performed by DNA polymerases of family X (PolX)
63 such as Pol λ (16) or Pol μ (17). Other members of the PolX family participate in short-range
64 base excision repair (BER) (Pol β (18)), or a special version of NHEJ, namely V(D)J
65 recombination (Terminal deoxynucleotidyl transferase (TdT) (19)). Both Pol λ and Pol μ are

66 capable to deal with a wide spectrum of nucleic acids substrates resulting from different
67 scenarii of DNA DSBs (20).

68 Apart from the N-terminal BRCT domain followed by a linker domain, PolX proteins consist
69 of an 8-kDa domain and a DNA polymerase catalytic domain (**Figure 1B**). The 8-kDa domain
70 aids in recognizing the 5' phosphate DNA ends and exhibits a deoxyribose phosphate (dRP)
71 lyase activity in Pol β and Pol λ (21, 22). The DNA polymerase catalytic domain is composed of
72 3 subdomains (fingers, palm and thumb) and contains the catalytic aspartate triad, a steric
73 gate motif (YFTGS, implicated in discriminating NTPs from dNTPs), three loops and two
74 Sequence Determinant (SD1 and SD2) motifs (**Figure 1B**). Loop1 and (SD1) motif are specific
75 to Pol μ and TdT (23) ; their role is to help in the stabilization of a 5' recessing template strand
76 upstream the DSB, followed by a short microhomology (MH) region (24, 25), (26). The SD2
77 motif sequence is unique to each group of PolX. In TdT, the SD2 motif has been proposed to
78 bind an additional divalent Zn²⁺ ion, but its precise role remains unclear (27). For TdT and
79 Pol μ point mutations in this SD2 motif deeply affect the polymerase activity (27, 28). In Pol λ ,
80 the residues equivalent to Loop1 are involved in fidelity by controlling the placement of the
81 primer DNA in the active site (29). The role of the SD2 motif has been shown to participate in
82 the fidelity mechanism of Pol β (30). Loop3 is unique to Pol λ and related homologues; its
83 function is not currently known, but it has been observed by x-ray crystallography to
84 undergo a large conformational change during substrate fixation and catalysis in the active
85 site (31). In the presence of a correct incoming dNTP, Loop3 relocates closely to the DNA
86 template strand and stabilizes the active site (31). Conversely, with incorrect dNTPs, Loop3
87 appears to be flexible, making it challenging to see it in crystal structures. In these instances,
88 it does not interact closely with DNA, which is sub-optimally positioned in the active site for
89 catalysis. Altogether, these recent kinetic crystallography experiments of pol λ show that
90 Loop3 likely plays a role in catalysis and potentially contributes to fidelity in Pol λ , but this
91 requires further experimental functional evidence.

92 Notably, PolXs enzymes involved in NHEJ typically share a permanently closed conformation
93 (32) and display low fidelity, particularly Pol μ and TdT (33). On the other hand, the BER-
94 associated Pol β displays the highest fidelity of metazoan PolX, which is mainly attributed to a
95 large conformational change occurring when the right dNTP binds (34). In the presence of
96 DNA and an incorrect incoming dNTP, the catalytic domain is in an open conformation with

97 its thumb subdomain located away from DNA, and R258 forms a salt bridge with catalytic
98 D192, diverting it from the active site and preventing catalysis (30). However, when DNA and
99 the correct dNTP are present in the active site, the enzyme adopts a closed form, in which
100 the thumb subdomain closes and stabilizes DNA in the active site, and the side chain of F272
101 (involved in the steric gate) now separates R258 and D192. In this active conformation, R258
102 forms a salt bridge with the glutamate residue of the SD2 motif (NEY), and D192 is now
103 available for catalysis. (34). Interestingly, in Pol β the dNTP and DNA binding sites are not
104 preformed, which in contrast with the apo form of Pol μ and Pol λ where there is a preformed
105 DNA binding site, and with the apo form of Pol λ that binds dNTPs in absence of DNA (35).
106 The mutator effect of this prestabilization of dNTPs is counterbalanced by an hydrophobic
107 cluster that regulates the transition to an active state of the catalytic site (35, 36).

108 The four DNA PolXs genes, *POLXa*, *b*, *c*, and *d* stem from two whole genome duplications
109 (WGD) (37). These genes are paralogues and exhibit varying degrees of sequence identity. At
110 the protein sequence level, PolXa and PolXb share 90% sequence identity, while PolXc and
111 PolXd share 84% of sequence identity, and the two subgroups collectively share 71% of
112 sequence identity (**Figure 1B**). Notably, PolXa is overexpressed during PGR in *P. tetraurelia*
113 (38). They share sequence similarities with both human Pol β (37% sequence identity) and
114 Pol λ (39% sequence identity), mainly restricted to the polymerase domain, and they exhibit
115 common features in their domain organization, including a putative N-terminal BRCT domain,
116 a linker domain, an 8-kDa domain containing residues possibly involved in dRP lyase activity,
117 and a catalytic DNA polymerase domain resembling DNA polymerases λ and β (**Figure 1B**).
118 The BRCT domains of *P. tetraurelia* PolXs do not align well with the ones of metazoan PolXs
119 by BlastP searches but AlphaFold confidently predicts the presence of a BRCT domain in all
120 four variants (**Suppl. Figure S1**). However, the linker domain appears to be different from
121 the one in Pol λ , as it is highly SP-rich in Pol λ and predicted by PONDR to be disordered
122 (confirmed by AlphaFold), while it is not in *P. tetraurelia* PolXs and could be partially ordered
123 according to AlphaFold (**Figure S1**). The BRCT domain is responsible of the association of
124 the PolX with the rest of the NHEJ complex, especially the Ku70/80 heterodimer. Here we ask
125 what makes the polymerase domain of these DNA PolX special with respect to human pol μ
126 and pol λ and how they could contribute to the high fidelity of *P. tetraurelia*'s NHEJ process,

127 ensuring fully reliable repair after the excision of IES by Pgm and their recruitment by Ligase
128 IV (8, 12).

129 In the following we present the functional characterization of *Paramecium* PolXs (Ptet-PolX),
130 and show that their enzymatic kinetics parameters are similar to Pol λ 's, but with a better
131 fidelity relying only on their catalytic domain. By structural studies of mutants of human pol
132 lambda mutants carrying sequence determinants of *P. tetraurelia* polX identified by careful
133 sequence analysis, we found two mechanisms that could explain the fidelity of the Ptet PolXs:
134 (i) a local induced fit with an exchange of partner in a salt bridge in the catalytic site, like Pol β
135 (but without its major domain rearrangement and open/closed transition), and (ii) the closing
136 of Loop3, that could be described as a more global substrate-induced conformational
137 change.

138

139 MATERIAL AND METHODS

140 Wild-type and mutant constructs of PolX

141 In this study, we purified different constructs of *Paramecium* PolXs. PolXd (ParameciumDB
142 PTET.51.1.P1010039) was expressed and purified as a full-length construct (PolXdFL), while
143 three constructs lacking residues 1-266 (including the N-terminal BRCT domain and the
144 linker domain) were generated: PolXa Δ Nter (WT sequence: ParameciumDB
145 PTET.51.1.P0210235), PolXb Δ Nter (WT sequence: *Paramecium*DB PTET.51.1.P0360066), and
146 PolXd Δ Nter. Additionally, three mutant versions of PolXa Δ Nter were created: PolXa Δ Nter-
147 K534A (mutation K534A), PolXa Δ Nter-K534R (mutation K534R), and PtetLoop3 β where
148 residues 581 to 588 (Loop3) were replaced by the corresponding sequence of Pol β (GVA).

149 Wild-type versions of human Pol λ (Uniprot Q9HBN3) and Pol β (Uniprot P06746) were also
150 produced. Furthermore, five mutant versions of human Pol λ were generated, all featuring the
151 following mutations originally described in Jamsen et al. (32): Δ 1-241 (Δ Nter), replacement of
152 residues 464-472 (Loop1) with the equivalent sequence in pol β (KGET), and C544A. This
153 construct will be referred to as λ ref in the following. The specific mutations in the other
154 constructs were as follows (**Table 1**): I492R (λ mutR), I492R and residues 528 to 530 (SD2
155 region) replaced by the corresponding –much shorter- pol β sequence NEY (λ SD2 β), a single
156 mutation I492K (λ mutK), and two mutations - I492K and E529D (λ SD2Ptet). Additionally,
157 another construct based on human Pol λ , named λ loop3 β , was produced and purified, with
158 residues 539-547 (Loop3) replaced by the corresponding sequence of Pol β (GVA).

159 All the codon-optimized sequences for WT constructs are indicated in **Supplementary Table**
160 **S1**, and mutated constructs and used primers are described in **Supplementary Table S2**.

161 Cloning, overexpression, and purification of *Paramecium* PolXs (Ptet-PolXs)

162 The synthetic genes encoding Ptet-PolXs were optimized for expression in *E. coli* and
163 synthesized using ThermoFisher's GeneArt service. These genes were then cloned into a
164 modified pRSF1-Duet expression vector with a cleavable N-terminal 14-histidine tag. For the
165 PolXdFL and PolXb Δ Nter constructs, cloning was done using New England Biolabs and Anza
166 (Thermo Fisher Scientific) restriction enzymes, while PCR and NEBuilder HiFi DNA Assembly
167 (New England Biolabs) were utilized for the other constructs. The mutant constructs of

168 PolXa Δ Nter were generated via site-directed mutagenesis performed by PCR (primers are
169 indicated in **Supplementary Table S2**) and using the KLD enzyme kit (New England Biolabs).

170 *E. coli* BL21 Star (DE3) cells (Invitrogen) were transformed with the engineered plasmids and
171 cultured at 37°C in LB medium with kanamycin resistance selection. Induction was carried out
172 at OD = 0.6–1.0 with 1 mM IPTG, followed by overnight incubation at 20°C. After harvesting,
173 cells were homogenized in buffer A (50 mM Tris-HCl pH 8, 600 mM NaCl, 10 mM imidazole),
174 sonicated, and centrifuged to remove bacterial debris. The resulting lysate supernatants were
175 treated with Benzonase (Sigma-Aldrich) and protease inhibitors (Thermo Fisher Scientific)
176 before purification.

177 The proteins of interest were isolated by purifying the clarified cell lysates on a HisTrap
178 column, using buffer A (50 mM Tris-HCl pH 8, 600 mM NaCl, 10 mM imidazole) as the
179 washing buffer and 500 mM imidazole in the elution buffer. For the PolXb Δ Nter construct, a
180 different washing buffer (25 mM Sodium Phosphate pH 8, 1 M NaCl) was utilized to avoid
181 nucleic acid contamination. The collected proteins were then dialyzed to 75 mM NaCl and
182 repurified on a HiTrap Heparin column with an elution at 1 M NaCl. Both purification
183 columns were from Cytiva.

184 Protein purity was analysed using SDS-PAGE 4-15% or 4-12% gels with a molecular weight
185 ladder (Precision Plus Protein, Biorad) as a control. The enzymes were concentrated using
186 Amicon Ultra 30k MWCO centrifugal filters (Merck), flash frozen in liquid nitrogen, and
187 stored directly at –80 °C until further use.

188 **Cloning, overexpression, and purification of human Pol β**

189 The gene encoding human Pol β was commercially synthesized by Genscript and inserted
190 into the modified pRSF1-Duet expression vector with a cleavable N-terminal 14-histidine tag.
191 Production and purification followed the same protocol as described for the previous
192 constructs, with the use of specific buffers: Buffer A (50 mM Tris-HCl pH 8, 500 mM NaCl, 10
193 mM imidazole), Buffer B (50 mM Tris-HCl pH 8, 500 mM NaCl, 500 mM imidazole), Dilution
194 buffer (50 mM Tris-HCl pH 8), Buffer C (50 mM Tris-HCl pH 8, 100 mM NaCl), Buffer D (50
195 mM Tris-HCl pH 8, 1 M NaCl).

196 **Cloning, overexpression, and purification of human Pol λ and mutant constructs**

197 The gene expressing human Pol λ was commercially synthesized by Genscript and inserted
198 into the modified pRSF1-Duet expression vector with a cleavable N-terminal 14-histidine tag.
199 Production and purification followed the same protocol as described for the previous
200 constructs, utilizing the following buffers : Buffer A (50 mM Tris-HCl pH 8, 500 mM NaCl, 10
201 mM imidazole, 1 mM EDTA, 1 mM DTT, 5% glycerol), Buffer B (50 mM Tris-HCl pH 8, 500 mM
202 NaCl, 500 mM imidazole, 1 mM EDTA, 1 mM DTT, 5% glycerol), Dilution buffer (50 mM Tris-
203 HCl pH 8, 1 mM EDTA, 1 mM DTT, 5% glycerol), Buffer C (50 mM Tris-HCl pH 8, 100 mM
204 NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol), Buffer D (50 mM Tris-HCl pH 8, 1 M NaCl, 1 mM
205 EDTA, 1 mM DTT, 5% glycerol).

206 All mutant constructs were obtained by PCR (primers are indicated in **Supplementary Table**
207 **S2**) and using the KLD enzyme mix (New England Biolabs) from the plasmid used for the
208 expression of Hs Pol λ , and purified similarly to the WT protein. An additional step of size-
209 exclusion chromatography was performed on a HiLoad Superdex 200 16/60 PG gel filtration
210 column (Cytiva) in Storage Buffer (50 mM Tris-HCl pH 8, 100 mM NaCl). All purified proteins
211 were concentrated to 16 to 20 mg/ml and stored at -80°C after flash freezing in liquid
212 nitrogen until further use.

213 **Gap-filling and DSB-cis polymerase assays**

214 Primer extension activity assays were conducted in a reaction buffer consisting of 50 mM
215 Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, and 5% glycerol. Reaction solutions
216 comprised 1 μM of templating oligonucleotide, 1 μM of FAM 5'-labelled DNA primer, and
217 either 250 μM dGTP or a mixture of all four dNTPs (250 μM each).

218 For the gap-filling assay, the used oligonucleotides were the following: 5'-FAM-
219 AATCACCAGTACGCCGTTGCGT-3', 5'-p-TATCGCCATGACGCGTTCTGGTCC-3', 5'-
220 GGACCAGAACCGCGTCATGGCGATACACGCAACGGCGTACTGGTGATT-3'. Reactions were
221 incubated for 30 minutes at 27°C with 1 nM of polymerase (Ptet-PolX) or for 5 minutes at
222 37°C with 50 nM of polymerase (HsPol λ). In the DSB cis assay, the oligonucleotides were the
223 following: 5'-FAM-AATCACCAGTACGCCGTTGCGT-3', 5'-p-
224 TATCGCCATGACGCGTTCTGGTCC-3', 5'-TACACGCAACGGCGTACTGGTGATT-3', 5'-
225 GGACCAGAACCGCGTCATGGCG-3'. Reactions were incubated for 30 minutes at 27°C (the
226 optimal growth temperature of *P. tetraurelia*) with various concentrations of PolX ΔNter .

227 Prior to adding the protein, DNA was hybridized by heating up to 90°C and gradually cooled
228 down to room temperature. For the primer extension assay, the used oligonucleotides were
229 the following: 5'-FAM- AATTGTCATAAGCTTATGCG-3', 5'-p-
230 TATCGCCATGACGCGTTCTGGTCC-3', 5'- GGGGTAGCTGCGCATAAGCTTATGACAATT-3'.
231 Reactions were incubated for 30 minutes at 27°C with 1 µM of polymerase (Ptet-PolX) or at
232 37°C with 50 nM of polymerase (HsPolλ).

233 Reactions were terminated by adding two volumes of a buffer containing 10 mM EDTA, 98%
234 formamide, and 1 mg/mL bromophenol blue, and stored at -20°C. Products were preheated
235 at 95°C for 10 minutes, separated using denaturing urea-polyacrylamide (18%) gel
236 electrophoresis, and visualized by FAM fluorescence on a Typhoon FLA 9000 imager. All
237 oligonucleotides were obtained from Eurogentec, Eurofins, or Biomers, dNTPs from
238 Fermentas (Thermo Fisher Scientific), and chemicals from Sigma-Aldrich.

239 **dRP lyase assay**

240 For the dRP lyase assay, DNA was prepared using the following procedure: a 31-mer DNA
241 strand (5'-GTACCCGGGGATCCGTACAGCGCATCAGCTGCAG-3') and its complementary U-
242 containing DNA strand

243 (5'-CTGCAGCTGATGCGCUGTACGGATCCCCGGGTAC-3'), each at a concentration of 50 µM,
244 were hybridized as described above. Next, 4 picomoles of hybridized DNA were mixed with
245 USER3 mix (New England Biolabs) in ThermoPol buffer (20 mM Tris-HCl pH 8.8, 10 mM
246 (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton® X-100), and the mixture was incubated
247 for 2 hours at 65°C.

248 Subsequently, in a reaction volume of 10 µL, 1 µM of USER3-treated DNA was mixed with a
249 250 µM dNTP mix, 400 units of T4 DNA ligase, and 50 nM of each DNA polymerase (HsPolβ
250 or PolXaΔNter), in an activity buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 50 mM KCl, 1 mM
251 DTT, 1 mM ATP). The mixture was then incubated for 30 minutes at 27°C (for Ptet-PolX) or
252 37°C (for HsPolβ) and analyzed by urea-PAGE in denaturing conditions as described
253 previously.

254 **Enzymatic steady state characterization**

255 DNA polymerization assays were conducted between 3 to 12 times using the methodology
256 described above. Each assay utilized 1 μ M of gap-filling DNA and 5 nM of DNA polymerases,
257 with varying concentrations of dNTPs, and proceeded for 10 minutes at 27°C.

258 The resulting gels were subjected to analysis using ImageJ software, and quantification of the
259 product DNA was carried out using Microsoft Excel. Enzyme velocity (measured in nM/min)
260 was plotted against the concentration of dGTP. These plotted data points were then fitted to
261 a nonlinear regression curve utilizing the Michaelis-Menten equation with GraphPad Prism
262 10 software. From the fitted curves, values for k_{obs} and K_M were obtained.

263 **One-point single turnover fidelity assay**

264 The fidelity assays were conducted following the protocol established by Fiala *et al.* (39) in a
265 buffer comprising 50 mM Tris-HCl (pH 8.4), 5 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, 5
266 mM DTT, 10% glycerol, and 0.1 mg/mL BSA. Each reaction mixture contained 30 nM of gap-
267 filling DNA, 120 mM of each dNTP, and 120 nM of DNA polymerases. The reactions were
268 carried out at 37°C (for HsPol λ) or 27°C (for Ptet-PolX) for 50 minutes. Following completion,
269 the reactions were stopped and subsequently analyzed as described previously.

270 **Clustering of the PolX family**

271 6500 PolXs sequences were obtained by PSI-BLAST (40) of the NCBI (41) non redundant
272 database, using the sequence of Ptet-PolXa as a probe. Sequences were sorted by query
273 coverage, and the last chosen sequence displayed the following parameters: query coverage =
274 52%; e-value = 2.10^{-37} ; % identity = 32,02%. To obtain representative sequences for the bacterial
275 PolXs and yeast PolIV groups, three other PSI-BLAST were performed, using as probes PolX from
276 *Thermus thermophilus* [NCBI ID: WP_096410530.1], PolX from *Deinococcus radiodurans* [NCBI ID:
277 WP_010887112.1] and PolIV from *Saccharomyces cerevisiae* [NCBI ID: AJP37443.1]. From each
278 search, the top 250 sequences were chosen.

279 The FASTA file containing 7250 final sequences was processed by the CLANS web-utility
280 from the MPI Bioinformatics Toolkit (42, 43). The clustering simulation was conducted using
281 the Java version of CLANS (44) with default parameters. The sequences were randomly
282 distributed in 3D space, converging to individual clusters after several hundred steps of the
283 simulation. The simulation was let to run for a total of 20,000 steps, during which no further

284 modification of the positions appeared. The simulation was run without applying a p-value
285 cut-off: additional simulations with cut-offs (10^{-10} and 10^{-20}) resulted in a shrinking and
286 fragmentation of the clusters, leading to the impossibility to observe known relationships
287 between PolX. Clusters were curated manually. Several independent simulations converged
288 to almost identical cluster distributions, with no discrepancy regarding the critical details.
289 Simulations not enriched with bacterial or yeast PolXs sequences resulted in equivalent
290 distributions.

291 **Sequence multialignment**

292 A multialignment of various eukaryotic PolXs sequences was computed using PSI-Coffee (45)
293 and encompass all regions of interest located in the DNA polymerase domain. Graphical
294 representation of the multiple sequence alignments was generated using ESPript 3 (46), with
295 secondary structures from the human Pol λ (PDB 7M43) provided for reference.

296 **Domain prediction**

297 The on-line version of PONDR (47) was used to predict potential disordered regions in *P.*
298 *tetraurelia* and human PolXs. AlphaFold3 (48) was used to predict their structure. The
299 PAGSTscore was calculated over a sliding window of 10 residues and smoothed over a
300 window of 5 residues.

301 **Crystallization**

302 For crystallization of λ Ref and its variants, the DNA substrate was prepared in the following
303 way: an 11-mer template oligonucleotide (5'-CGGCAGTACTG-3') was annealed with a 6-mer
304 upstream primer oligonucleotide (5'-CAGTAC-3') and a 5'-phosphorylated downstream 4-
305 mer primer oligonucleotide (5'-pGCCG-3') in a 1:1:1 ratio. The crystallization plates were
306 prepared according to the procedure outlined by Jamsen *et al.* (31). In brief, the protein (16
307 mg/mL) was combined with DNA in a 1:2 molar ratio and incubated at 4°C for 20h.
308 Following the addition of 2mM dNTP, either dTTP (matched) or dCTP (unmatched), and
309 10mM CaCl₂, the mixture was further incubated for 20h on ice. Crystallization drops were
310 set up at 4°C by mixing in a 1:1 ratio the Pol λ -DNA-dNTP ternary complexes with a
311 crystallization solution consisting of 20 mM Bicine pH 7.5, 300 mM Na-K tartrate, and 14-20%
312 PEG 600/1000/10K/20K/Smear Low/Smear Medium/Smear High. For the crystallization of the

313 mutant λ mutK, an optimization of the best condition (20 mM Bicine pH 7.5, 300 mM Na-K
314 tartrate, 17.5% PEG 20K) was conducted by screening additives (using the Additive Screen HT
315 kit #HR2-428, Hampton Research) and lowering the protein concentration to 10 mg/mL. The
316 resulting crystals were flash-frozen in liquid nitrogen after being cryo-protected with the
317 crystallization solution supplemented with 25% ethylene glycol.

318 **Data collection and refinement**

319 X-ray crystallographic data were collected at the SOLEIL synchrotron (Saint-Aubin, France)
320 utilizing beamlines PX1 and PX2A. The data were processed using XDS (49) with the XDSME
321 pipeline or autoPROC (50). The strong diffraction anisotropy of λ SD2 β dataset was taken into
322 account with StarANISO (Staraniso GlobalPhasing, <https://staraniso.globalphasing.org>).
323 Subsequently, the structures were solved by PHASER (<https://www.globalphasing.com/>)
324 molecular replacement using a HsPol λ model (PDB ID: 7M43) as template and refined in
325 Buster (51), with manual reconstruction performed using Coot (52). Details for all datasets
326 are summarized in Table 2.

327

328 RESULTS

329 Characterization of Ptet-PolXs enzymatic activities, kinetics, and fidelity

330 We tested the activity of several N-terminal truncated versions of *Paramecium* PolXs (PolXa-
331 ,PolXb- and PolXd- Δ Nter) with a relevant DNA substrate, referred to as DSB-cis (**Figure 2A**).
332 This substrate mimics the DNA ends formed after IES elimination by Pgm in *P. tetraurelia*,
333 featuring the conserved TA dinucleotide and presenting a DSB, with the template nucleotide
334 *in cis* relative to the DNA undergoing extension (like in the gap-filling step on **Figure 1A**).
335 Without the 2-bp microhomology between the upstream and downstream DNA, it would
336 resemble a short primer extension scenario, which Ptet-PolXs are indeed capable of doing,
337 although not very efficiently (**Figure S1A**). All DNA polymerases efficiently discriminate
338 dNTPs versus NTPs, as they do not incorporate any ribonucleotide, whether with only GTP or
339 with a mix of NTPs, at any enzyme concentration. This is consistent with the conservation of
340 Pol λ 's steric gate residues YFTGS (**Figure S2**). Moreover, all Ptet PolXs are as efficient with
341 dGTP as with a dNTP mix, leading to a specificity ratio $R = [\text{Intensity of the } +1 \text{ band with } dGTP] / [\text{Intensity of the } +1 \text{ band with dNTP mix}]$ comprised between 0.95 and 1.00.
342

343 To compare Ptet-PolXs activities with human Pol λ , we performed a single nucleotide gap-
344 filling experiment (**Figure 2A**). In this assay four Ptet-PolXs constructs (PolXa Δ Nter,
345 PolXb Δ Nter, PolXd Δ Nter and PolXdFL) demonstrate comparable activity to HsPol λ . They
346 accurately incorporate a single nucleotide on the FAM-labeled primer with dGTP alone or a
347 mix of dNTPs, and efficiently discriminate NTPs. The specificity ratio is 0.90 for human DNA
348 pol λ .

349 The N-terminal BRCT and linker domains do not appear to affect catalysis in this context, as
350 evidenced by identical results obtained with both PolXdFL and PolXd Δ Nter constructs.

351 We evaluated the dRP lyase activity of the PolXa Δ Nter construct, considering that its closest
352 metazoan homologues, Pol β and Pol λ , both exhibit this activity *in vitro*. In an *in vitro* assay
353 designed to reconstitute a short-range base excision repair context, PolXa Δ Nter exhibits
354 lyase activity comparable to HsPol β , indicating a dRP lyase activity (**Figure S1B**), which is
355 probably shared by the all other Ptet-PolXs, as they also carry the needed residues.

356 To further compare their activity to human Pol λ and β , we characterized the kinetics of
357 incorporation of one representative from each subgroup, PolXa Δ Nter and PolXd Δ Nter, under
358 the same conditions as those used previously for human Pol λ (53) and Pol β (54), within a
359 gap-filling context. As depicted in **Figure 2B**, PolXa Δ Nter and PolXd Δ Nter exhibit distinct
360 catalytic characteristics in this assay: the measured k_{obs} of PolXa Δ Nter is three times lower
361 than PolXd Δ Nter but has a three times higher affinity for dGTP, indicated by a higher K_M .
362 Ultimately, they demonstrate similar catalytic efficiencies of approximately $6 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$.
363 Comparing these results with previous data reported for human PolX (53, 54), their kinetic
364 parameters more closely resemble those of Pol λ than Pol β . Specifically, they exhibit a similar
365 affinity for dGTP as HsPol λ Δ Nter, with a K_M in the millimolar range, but also possess a 3 to 10
366 times higher turnover number. Consequently, they appear to be 6 times more efficient than
367 HsPol λ Δ Nter, but they display a low catalytic efficiency compared to HsPol β .

368 To assess the fidelity of Ptet-PolXs in comparison to human Pol λ , we conducted a qualitative
369 one-point single turnover assay inspired by previous experiments (39). The results obtained
370 for HsPol λ and its mutant λ_{ref} lacking both N-terminal BRCT and linker domains are in line
371 with previous results from literature (39) (**Figure 2C**): the full-length enzyme exhibits good
372 fidelity, albeit with a slight dATP incorporation (in line with (55)), while the λ_{ref} construct
373 appears to make more misincorporations, incorporating several dGTP and dTTP, as well as
374 single dATP and dCTP. In the case of *Paramecium* PolXs, PolXa Δ Nter and PolXdFL exhibit a
375 common behavior, both of them effectively incorporating the correct nucleotide (dGTP) and
376 not any incorrect dNTP. Thus, Ptet-PolXs demonstrates good fidelity, at least comparable to
377 full-length HsPol λ . Notably, the fact that the fidelity of Ptet-PolXs remains consistent
378 regardless of the presence or absence of the N-terminal BRCT and linker domains, suggests
379 that this fidelity originates only from the enzyme's polymerase domain.

380 **Comparison of Ptet-PolXs sequences with other DNA PolX**

381 To understand the position of Ptet-PolXs in the PolX family and the origin of their high
382 fidelity, we used the CLANS classification method (44) on 7250 representative PolXs
383 sequences from diverse organisms (*Paramecium*, metazoan, fungi, viridiplantae, bacteria)
384 obtained by PSI-BLAST. As indicated in **Figure 3A**, 12 clusters were identified manually after
385 convergence of the refinement process using 20000 cycles. We confirmed the accuracy of

386 the method by finding the same relationships between the different subgroups of PolXs that
387 were known and analyzed previously using a smaller dataset (56). **Figure 3A** shows that the
388 metazoan clusters Pol μ (#2) and TdT (#1) are close, as expected; the same is true for the Pol λ
389 (#5) and Pol β (#8) clusters. Also, other clusters seem to be close to Pol λ , such as PolXs from
390 plants (cluster #4), that were classified as Pol λ until now (15, 57) or fungal PolXs (cluster #3
391 and #6) (58). The most divergent PolXs clusters are those including yeast PolIV (or Pol4) and
392 bacterial PolXs. Interestingly, bacterial PolXs are separated in two clusters, containing
393 canonical and non-canonical PolX, as described by Prostova *et al.* (59).

394 We then compared representative sequence motifs of PolXs from each cluster (catalytic
395 motifs, SD1/2 motifs, loops 1/2/3, steric gate motifs). The obtained logos (**Figure 3C**) allowed
396 us to compare the 12 groups and define specific motifs that can be used to determine the
397 group of any new PolX. Those results, along with sequence alignments with known
398 eukaryotic PolXs (**Figure S2**), indicated that Ptet-PolXs and the other sequences from their
399 group (cluster #9, associated with the clade Harosa that includes ciliates) share similarities
400 with both Pol λ and Pol β , including in the steric gate and residues involved in dRP lyase
401 activity. Instead, they appear to be distant from TdT and Pol μ as they do not contain Loop1
402 nor the same SD1 motif (60).

403 Ptet-PolXs also exhibit a similarity with Pol β in their second catalytic motif (RIDLK). In
404 metazoan Pol β , this motif comprises five conserved residues: RIDIR. The C-terminal arginine
405 residue (R258) plays a crucial role in the induced-fit mechanism of Pol β (30). Unusually
406 among PolXs, *Paramecium* PolXs also display a positively charged residue at this position
407 (K534). They also have an equivalent for all the other residues involved in this induced-fit
408 mechanism: the three catalytic aspartates, the steric gate F506, and a negatively charged
409 residue at the second position of the SD2 motif (NEY in Pol β , SDH in Ptet-PolX). On the basis
410 of this conservation of equivalent residues (especially the positively charged side chain in the
411 second catalytic motif) we formed the hypothesis that Ptet-PolXs could also benefit from an
412 induced-fit mechanism to achieve their high fidelity. In the following, we will test this
413 hypothesis experimentally.

414 Additionally, Ptet-PolXs display a Loop3 sequence, like metazoan Pol λ s and related
415 sequences (Plants PolXs, Pol λ -like PolXs of Fungi) that is more positively charged than in

416 metazoan Pol λ 's (pI of 10.3 vs 8.23 in HsPol λ). Since Loop3 is involved in DNA stabilization
417 during correct catalysis in human Pol λ (31), it could have the same role in Ptet-PolX. This
418 hypothesis will also be tested here by site-directed mutagenesis.

419 **Mutants of Ptet-PolXs and human Pol λ designed to probe a Pol β -like induced-fit** 420 **mechanism**

421 Considering the observed accuracy and fidelity of Ptet-PolXs compared to HsPol λ , we
422 explored the first hypothesis suggested by sequence analysis, involving a pol β -like induced-
423 fit mechanism. First we conducted a fidelity assay to compare the fidelity of WT and mutant
424 versions of PolXa Δ Nter (**Figure 4A**) involving K534. The PolXa Δ Nter K534A construct displays
425 strong misincorporations, as it incorporates any dNTP. The mutation at this position also has
426 an effect on the kinetics of the enzyme, since this mutant is the only tested construct to have
427 transformed all of the initial substrates with the correct incoming dNTP. On the other hand,
428 the K534R mutant seems to have a lower effect on fidelity in this assay, and no effect on
429 correct catalysis.

430 To further understand this mechanism, we engineered and purified five other mutants of
431 human DNA polymerase λ , the closest relative of Ptet-PolX, at positions believed to confer
432 partly or totally the putative induced-fit mechanism of Pol β . The λ mutR construct I492R
433 involves the position equivalent to Pol β 's R258 residue, and the λ SD2 β mutant has an
434 additional mutation giving it the SD2 motif of Pol β (NEY).

435 Two other mutants were prepared to give Pol λ the equivalent residues of Ptet-PolXs : λ mutK
436 has the mutation I492K, and the λ SD2Ptet mutant has a second mutation E529D, giving it the
437 SD2 motif of *Paramecium* PolXs (SDH). See Table 1 for a full description of these mutants
438 and their rationale.

439 After purification, all mutants underwent a steady-state characterization in gap-filling context
440 (**Figure 4B and 4C**) and x-ray crystallography analysis (**Figure 5**) in presence of Ca²⁺ ions to
441 prevent catalysis (61). The λ mutR mutant exhibits diminished enzymatic activity compared to
442 the WT enzyme (λ ref), indicated by a 2-fold lower k_{obs} along with an increased affinity for
443 incoming nucleotides, denoted by a 19-fold lower K_M . The structural analysis of this mutant
444 (see **Figure 5**, top left panel) revealed that the side chain of the introduced arginine residue

445 is positioned in proximity to the catalytic residue D490 (~ 3 Å), which is diverted away from
446 the active site. When this mutant is further engineered to include the SD2 motif of Polβ (NEY),
447 it triggers significant differences in enzymatic behavior and local structural changes, but no
448 large movements of domains. This λSD2β mutant displays near-complete inactivity, as
449 evidenced by a 10-fold reduced k_{obs} , despite maintaining an almost normal K_M in the
450 millimolar range. Crystallographic data for this mutant produced different results compared
451 to other mutants. While all other mutants crystallized in the P2₁2₁2₁ space group with one
452 molecule per asymmetric unit, this specific mutant crystallized in the H32 space group with
453 two molecules in the asymmetric unit, displaying highly anisotropic diffraction data (**Table 2**),
454 resulting in two slightly different structures (see **Figure 5**, middle and bottom left panels).
455 However, both molecules exhibit the same structural characteristics: closed conformation,
456 absence of an incoming nucleotide in the active site, with residue R492 positioned between
457 the catalytic D490 residue and the NEY SD2 motif, at approximately 3.5-4 Å from both. This
458 proximity suggests potential stabilization through salt bridges, either with catalytic D490 or
459 with both glutamate and tyrosine of the SD2 motif. Additionally, in this instance, Loop3 does
460 not engage with an interaction with the DNA template strand, which is improperly placed for
461 catalysis in the active site (**Figure 6B**).

462 The λmutK mutant was designed to emulate in Polλ the putative polβ-like induced-fit
463 mechanism of *Paramecium* PolX. Remarkably, its kinetics closely resemble that of the λref
464 construct, particularly in terms of k_{obs} , despite exhibiting a 3.8-fold lower K_M , indicative of a
465 higher affinity for the incoming nucleotide. However, the most notable feature of this mutant
466 is its reduced fidelity: under the same steady-state conditions as those used for testing the
467 correct incorporation of dGTP, it is the only mutant that demonstrated clear
468 misincorporations and double incorporations of the correct nucleotide in the 1-nt gap filling
469 substrate (**Figure 4B**). Structurally, the mutant closely resembles the WT enzyme with a
470 closed conformation, but the side chain of K492 is participating in a salt bridge with E529
471 (WT SD2 motif) (see **Figure 5**, top right panel). A significant departure from the WT enzyme
472 is observed in the orientation of the side chains of Y271 and F272. In this mutant (as well as
473 in λSD2β and λSD2Ptet mutants), these two residues adopt a perpendicular conformation
474 resembling that seen in the inactive binary complex of HsPolλ or Polβ with DNA, which is
475 unexpected given the presence of the correct incoming nucleotide.

476 The λ SD2Ptet mutant encompasses all residues potentially implicated in an induced-fit
477 mechanism in *Paramecium* PolX. Its sole sequence difference with the λ mutK mutant is the
478 E529D mutation, which mimics the SD2 motif of *Paramecium* PolX. Unlike the λ mutK mutant,
479 this variant exhibits no misincorporation behavior and demonstrates kinetics closely
480 resembling λ mutK, with slightly improved k_{obs} and marginally lower K_M , resulting in enhanced
481 catalytic efficiency ($1.64 \mu\text{M}^{-1}\cdot\text{min}^{-1}$ for λ_{ref} vs. $9.94 \mu\text{M}^{-1}\cdot\text{min}^{-1}$ for the λ SD2Ptet mutant and
482 $6.45 \mu\text{M}^{-1}\cdot\text{min}^{-1}$ for λ mutK). Structural analysis shows two different local conformations within
483 the active site (**Figure S3**), both present in the structure obtained in presence of the correct
484 incoming nucleotide (dTTP). However, no major conformational changes are observed for
485 this mutant, whose polymerase domain conserves a closed form. In a so called "active"
486 conformation (see **Figure 5**, middle right panel), all catalytic aspartates are correctly oriented
487 in the catalytic site, with K492 engaged in water-mediated bonds with D529 of the SD2 motif.
488 This aspartate participates in bonds not only with its side chain but also with its main chain
489 carboxyl group. In contrast, in an "inactive" conformation (see **Figure 5**, bottom right panel)
490 obtained with an incorrect incoming nucleotide (and within the same structure with the
491 correct nucleotide, see **Figure S3**), K492 is located 3.7 \AA from catalytic D429, which is
492 directed toward this lysine, suggesting the presence of a salt bridge between the two,
493 diverting D429 from the catalytic site. In this scenario, the incorrect nucleotide (dCTP)
494 occupies the active site with an incorrect placement.

495 In conclusion, these structural and functional studies support a mechanism by which K492
496 can locally switch between two conformations, an inactive one forming a salt bridge with the
497 catalytic aspartate D429, and a catalytically active one where it is engaged in a completely
498 opposed salt bridge with D529 from SD2 region. This occurs without a big domain
499 rearrangement as seen in $\text{pol}\beta$.

500 **Determination of the role of Loop3 in fidelity**

501 To investigate the impact of the conserved Loop3 in the fidelity of Ptet-PolXs and HsPol λ , we
502 generated and purified mutant versions of PolXa Δ Nter and HsPol λ , lacking their respective
503 Loop3 sequences, namely PtetLoop3 β and λ Loop3 β . We conducted a qualitative fidelity
504 assay to compare the fidelity of the mutant and WT versions (**Figure 6A**) and the results
505 clearly demonstrate that the constructs lacking the Loop3 display a stronger

506 misincorporative behavior. Specifically, the PolXa Δ Nter construct demonstrates minimal
507 errors, while the PtetLoop3 β construct erroneously incorporates dATP, dTTP, and dCTP. A
508 similar trend is observed for HsPol λ , with the mutant construct incorporating dATP and dTTP
509 but seemingly no dCTP. Notably, the correct incorporation of dGTP remains unaffected by
510 the introduced mutations. The predominant misincorporation involves double dTTP
511 incorporation.

512 Structural analysis allowed us to put forward a tentative explanation for these enzymatic
513 results. For the λ SD2 β mutant, Loop3 appears to be positioned at a distance from the DNA
514 and exhibits increased flexibility with high B-factors (130 to 260 \AA^2), which contrasts with
515 other structures where it interacts with DNA both upstream and downstream of the
516 templating nucleotide (**Figure 6B**). Upon Loop3 closure, specific interactions are made with
517 DNA through salt bridges involving its positively charged residues (K544, R538, H541) and
518 DNA phosphates, as well as G542 which interacts with K521 to stabilize a phosphate group
519 (**Figure 6B and 6D**). Additionally, in those structures, H530 of the SD2 motif also interacts
520 with the phosphate group of the -3 nucleotide (**Figure 6D**). In the λ SD2 β structures, which
521 lack this interaction, DNA is displaced, and the templating nucleotide is not properly
522 positioned for catalysis, likely leading to its inability to stabilize an incoming nucleotide.

523 **DISCUSSION**

524 ***Paramecium* PolXs activity resembles that of human Pol λ**

525 We assessed the activity of Ptet-PolXs with their physiological substrate encountered during
526 the programmed genome rearrangement in *P. tetraurelia*. Our results indicate an enzymatic
527 resemblance between these PolXs and both human DNA polymerases β and λ , as they all
528 exhibit gap-filling and dRP lyase activities, and do not incorporate NTPs. Their steady-state
529 characterization, compared with published results obtained for human Pol λ (53) and Pol β
530 (54), provide further insights into their enzymatic mechanism and points to a higher similarity
531 to Pol λ 's. Thus, it is possible that Ptet-PolXs employ a structural mechanism akin to that of
532 Pol λ and other NHEJ-related PolX, maintaining a closed form of their active site throughout
533 catalysis, contrary to what is seen in pol β .

534 Ptet-PolXs appear to be more accurate than HsPol λ , independently of their BRCT and linker
535 domains.

536 To understand the origin of this high fidelity, we compared Ptet-PolXs sequences with more
537 than 7000 PolXs sequences from various organisms. The definition of 12 subgroups of PolXs
538 allowed us to study the distinctive features of each group, and the similarities between them.
539 Close examination of the conserved sequences and crucial motifs allowed to make two
540 distinct hypotheses that could explain Ptet-PolXs fidelity: a local induced-fit mechanism, and
541 a conformational change involving Loop3.

542 ***Paramecium* PolXs fidelity can be explained by an induced-fit mechanism like in Pol β ,
543 but without closing the whole polymerase domain**

544 By testing the effects of point mutations located in the second catalytic motif of Ptet-PolX,
545 we showed that the presence of a positively charged residue at position 5 of the second
546 catalytic motif is necessary for the fidelity of the enzyme. This fidelity mechanism was
547 analyzed by enzyme kinetics and crystallographic structures of mutant constructs of Pol λ .
548 Indirect structural evidences, combined with direct enzymatic characterization experiments,
549 allow to suggest that PolXs involved in NHEJ have a local induced fit mechanism, which relies
550 on a lysine residue in the second catalytic mechanism exchanging salt-bridge partner.
551 Importantly, the structural analyses indicated that the steric gate residues were displaced

552 when the residues involved in this mechanism were present : they display an unusual
553 conformation in presence of DNA and of a correct incoming nucleotide, similar to the
554 conformation seen in Pol λ in absence of an incoming nucleotide, which allows the fixation of
555 any nucleotide in the active site. In Pol λ , such a conformation is associated with a lowering of
556 fidelity, but here it seems that the local induced-fit mechanism can counterbalance this
557 lowering effect.

558 **Human Pol λ and *Paramecium* PolIXs may share a crucial conformational change** 559 **involving Loop3**

560 Fidelity assays on mutants of HsPol λ and PolIXa Δ Nter lacking Loop3 revealed strong error-
561 prone behaviors, indicating a fundamental role of Loop3 in fidelity (**Figure 6**). We suggest
562 that the mechanism by which this loop stabilizes template DNA could be also dependent on
563 the interaction of DNA with H530, the third residue of the SD2 motif in Pol λ and *Paramecium*
564 PolIX. Indeed, the interaction of H530 with DNA will be facilitated by the hybridization of the
565 correct incoming nucleotide with the templating nucleotide in the active site. In Pol λ It has
566 been shown that the hybridization of the correct incoming dNTP triggers repositioning of
567 the template DNA (62). This suggests a mechanism whereby, upon the entry of a correct
568 incoming nucleotide into the active site, its hybridization with the templating nucleotide is
569 further stabilized by R556 and R559 (R514 and R517 in Pol λ). This repositions DNA and
570 brings it closer to H530, possibly allowing their interaction and triggering a cascading
571 interaction of Loop3 residues with DNA, "closing it" around DNA. Loop3 provides further
572 interactions with DNA, facilitating the catalysis. Previous studies have shown that the role of
573 Pol λ 's R517 is to stabilize and control the nascent base pair during dNTP-induced template
574 strand repositioning (62, 63) : when R517 is mutated, DNA does not reposition, neither do
575 the Loop3. This proposed mechanism is in line with the model proposed by Showalter and
576 Tsai (64) : they proposed that the nucleotide binding in the active site of polymerases
577 triggers conformational changes that allow for closing of the active site around substrates,
578 and that fidelity is dictated by the approach of the chemistry step, at which the energy
579 difference between Watson-Crick and mismatched nucleotide incorporations are at the
580 maximum. In Pol λ , there is no closing of the polymerase domain; however, the movement of
581 Loop3 mimicks the closing of the tip of the thumb subdomain as in Pol β and has the same
582 consequence: the stabilization of the DNA in the active site (**Figure 7**).

583 **A local induced-fit and a conformational change of Loop3 in a single DNA polymerase**

584 The absence of enzymatic activity of the λ SD2 β construct suggests that the fidelity
585 mechanisms of Pol λ (based on Loop3) and Pol β (due to global induced fit and a remodeling
586 of the active site) cannot co-exist in the sequence context of one another, likely because Pol λ
587 has evolved towards a permanently closed form and a mechanism relying only on a smaller
588 conformational change. The λ SD2 β mutant demonstrated an inability to transition to an
589 open state, likely impeded by the permanently closed conformation of Pol λ 's catalytic
590 domain (32). As previously discussed, in Ptet-PolXs the induced-fit mechanism is limited to a
591 local side chain movement exchanging salt-bridge partners in the active site, and does not
592 require the opening of the active site to be operative. Instead, the hybridization of a correct
593 nucleotide with DNA can trigger its optimal positioning in the active site, allowing the
594 release of the catalytic aspartate from its interaction with K534. This mechanism, combined
595 with the stabilization of DNA with the Loop 3 in the presence of a correct nucleotide, could
596 explain their high fidelity.

597 **Conclusion and Perspectives**

598 In summary, our study elucidates that PolXs of *Paramecium tetraurelia* share similarities with
599 both Pol λ and Pol β . Notably, they exhibit a fidelity level surpassing that of HsPol λ , a
600 distinctive attribute within the spectrum of NHEJ-associated PolXs. Our studies are
601 compatible with a fidelity enhancement underpinned by two distinct mechanisms, both
602 triggered by the entrance of the correct dNTP and DNA in the active site: a local induced-fit
603 activation of the catalytic site; and a conformational change of Loop 3 originally
604 demonstrated with Pol λ by Jansen *et al.* (31). Even though those conclusions are supported
605 by indirect structural evidence, functional results clearly indicate roles for Loop 3 and
606 residues involved in the local induced fit mechanism in fidelity in Ptet-PolXs. Surprisingly,
607 there are other DNA polymerases of family X that share the residues at the origin of those
608 two fidelity mechanisms: Plants PolXs (cluster #4 in **Figure 3**) display an equivalent of Pol β 's
609 R258 as a lysine and also contain a Loop 3, like HsPol λ . Their steric gate (AWTGN) and SD2
610 (DDT) motifs also depart from known sequences, so that further studies could reveal a similar
611 function in highly accurate DNA repair. Some fungal "Pol λ / μ -like" (clusters #3 and #6 in
612 **Figure 3**) polymerases also seem to share equivalents of Loop3, but with distant SD2 motifs.

613 On the evolutionary level and based on the fidelity mechanisms in this family of DNA
614 polymerases. *P. tetraurelia* PolXs could represent a missing link in the evolution between
615 Pol β and Pol λ .

616 The CLANS analysis of the whole PolX family could be used to refine the evolutionary
617 scenario proposed by Bienstock *et al.* (56) who proposed that the PolX family originates from
618 bacterial PolX, followed by yeast PolIV, which then separated in eukaryotes in two groups :
619 Pol μ /TdT and Pol λ /Pol β . We propose that the Pol μ /TdT group could originate from the Pol μ -
620 like group of PolX found in Fungi; and that eukaryotic Pol λ and Pol β could originate from
621 PolX found in Plants and ciliates like *P. tetraurelia*, as those groups display similarities with
622 both Pol λ and Pol β (**Supplementary Figure S3B**).

623 In addition to specific features in the polymerase domain of *Paramecium* DNA polXs the role
624 and structure of their conserved linker domain is particularly intriguing. This domain could
625 play a significant part in the intricate dynamics of the complete NHEJ complex in
626 *Paramecium*, and it strongly differs from that of human Pol λ . Disorder predictions and
627 AlphaFold structure predictions suggest that this domain could contain secondary structure
628 elements like α -helices (**Supplementary Figure S1**). The presence of a structured linker
629 domain between the polymerase and BRCT domains could significantly aid in maintaining
630 the precise orientation of the NHEJ complex and the DNA, thereby enhancing the efficiency
631 of the repair process in the NHEJ complex. Such a structured linker domain could be
632 essential in *Paramecium*'s specialized NHEJ system to position uniquely and efficiently the
633 DNA substrate and the attached DNA polymerase during DNA ends processing, which is
634 possible because there is only a single type of DNA substrate to deal with. This is in contrast
635 to metazoan Pol λ and Pol μ , which must handle a wide variety of DNA ends (e.g., varying
636 microhomology lengths, different orientations of microhomologies, ...) resulting from various
637 DNA DSB. For *Paramecium* PolX, the ability to stabilize their unique physiological substrate
638 effectively in conjunction with the NHEJ machinery would be highly advantageous. Further
639 structural characterization of the full NHEJ short-range complex in the presence of polX
640 would be needed to answer this question.

641 **DATA AVAILABILITY**

642 The crystallographic data have been deposited to the PDB under the codes 9EWB, 9EWC,
643 9EWD, 9EWE and 9EWG.

644

645 **SUPPLEMENTARY DATA**

646 Supplementary data are available online.

647

648 **AUTHOR CONTRIBUTIONS**

649 Antonin Nourisson: Conceptualization, Data collection and processing, Analysis, Validation,
650 Visualization, Writing—original draft, review & editing. Ahmed Haouz: Data collection and
651 processing. Sophia Missouri: Conceptualization, Methodology, Writing—Review & Editing.
652 Marc Delarue: Conceptualization, Supervision, Writing – Review & Editing.

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666 **CONFLICT OF INTEREST**

667 None declared.

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- 843

844 **Tables**

845

846 **Table 1.** Mutant constructs of human Pol λ used for enzymatic and structural studies of the
847 fidelity mechanisms of P λ -PolXs.

<i>Mutant</i>	Common mutations	Specific mutations	Expected role of mutations
<i>λmut</i>		None	Facilitate crystallization (Δ 1-241 and [464-472] KGET) and diffraction (C544A)
<i>λmutR</i>	Δ 1-241 [464-472] KGET C544A	I493R	Partly confer to Pol λ the Pol β 's induced fit mechanism (R258 of Pol β)
<i>λSD2β</i>		I493R, [528-530] NEY	Confer to Pol λ the Pol β 's induced fit mechanism (R258 and SD2 motif of Pol β)
<i>λmutK</i>		I493K	Partly confer to Pol λ the P λ -PolXs putative induced fit mechanism (K534 of P λ -PolXs)
<i>λSD2Pλ</i>		I493K, E529D	Confer to Pol λ the P λ -PolXs putative induced fit mechanism (K534 and SD2 of P λ -PolXs)
<i>λloop3β</i>	None	[539-547] GVA	Deletion of Loop3, replacement by the equivalent residues of human Pol β

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849

850 **Table 2.** Crystallographic statistics for datasets of crystals of pol lambda mutants with dNTP
 851 insertion site occupied opposite A in the presence of Ca²⁺. Data in the highest resolution
 852 shell is shown in the parenthesis.
 853 *Values calculated after truncation by STARANISO. Estimated resolution limits along the
 854 three crystallographic directions of the reciprocal lattice a*, b*, c*.
 855 ** Values obtained with MolProbity.

	λ mutR (dTTP)	λ SD2 β	λ mutK d(TTP)	λ SD2Ptet (dTTP)	λ SD2Ptet (dCTP)
<i>Data collection</i>					
<i>Space group</i>	P 21 21 21	H32	P 21 21 21	P 21 21 21	P 21 21 21
<i>a, b, c (Å)</i>	56.28 62.51 140.22	149.904 149.904 272.154	56.03 62.49 141.37	56.4 62.47 139.57	56.35 62.76 139.72
<i>α, β, γ (°)</i>	90 90 90	90 90 120	90 90 90	90 90 90	90 90 90
<i>Wavelength (Å)</i>	0.9801	0.9801	0.9801	0.9801	0.9801
<i>Resolution (Å)</i>	46.74 - 2.32 (2.38 - 2.32)	117.173 - 3.543 (4.018 - 3.543)	24.04 - 1.916 (2.118 - 1.916)	46.55 - 2.12 (2.18 - 2.12)	46.69 - 2.737 (2.987 - 2.737)
<i>Estimated resolution limit (Å)*</i>		5.491 5.491 3.344			
<i>R-pim</i>	0.040 (0.629)	1.514 (3.179)	0.052 (0.807)	0.038 (0.69)	0.204 (0.845)
<i>Completeness (%)</i>	99.9 (98.8)	100 (100)	93.3 (63.9)	99.8 (97.5)	86.4 (48.5)
<i>Multiplicity</i>	13.3 (13.7)	18.2 (19.2)	11.9 (11.7)	13.4 (13.7)	9.3 (9.2)
<i>I/σ(I)</i>	17.85 (1.99)	5.00 (0.70)	11.0 (1.70)	16.6 (1.6)	4.6 (1.40)
<i>CC1/2</i>	99.9% (82.0%)	65.7 % (63.9%)	99.6% (50.4%)	99.9% (79.4%)	97.5% (54.8%)
<i>R-pim*</i>		0.658 (1.192)			
<i>Completeness (%)*</i>		91.7 (74.2)			
<i>Multiplicity*</i>		17.7 (15.2)			
<i>I/σ(I)*</i>		7.80 (1.80)			
<i>CC1/2*</i>		80.5% (74.7%)			
<i>Refinement</i>					
<i>No. of reflections</i>	22111	5991	25444	28561	8572
<i>R_{work} / R_{free}</i>	0.208 / 0.256	0.263 / 0.292	0.204 / 0.245	0.193 / 0.224	0.209 / 0.259
<i>No. non H atoms</i>					
<i>Macromolecules</i>	2459	5036	2312	2514	2497
<i>Ligands</i>	47	0	31	47	28
<i>Solvent</i>	303	21	278	306	76
<i>Protein geometry</i>					
<i>RMSD - bonds (Å)</i>	0.008	0.007	0.009	0.009	0.007
<i>RMSD - angles (°)</i>	0.89	0.81	0.87	0.95	0.83
<i>Ramachandran favored (%)**</i>	97.47	96.54	96.04	94.10	96.54
<i>Ramachandran outliers (%)**</i>	0.00	0.31	0.33	0.00	0.31
<i>Poor rotamers (%)**</i>	1.52	5.63	0.44	1.85	5.64
<i>Clashscore</i>	4	10	4	4	8
<i>B-factors(Å²)</i>					
<i>Mean B-factor</i>	57.19	79.25	73.50	57.10	52.90
<i>Macromolecules</i>	60.92	79.81	49.67	60.89	43.27
<i>Ligands</i>	49.62	0	117.7	49.50	115.40
<i>Solvent</i>	61.03	78.68	53.14	60.92	20.66

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862 **FIGURE LEGENDS**

863

864 **Figures**

865

866 **Figure 1.** Role of PolXs in IES elimination during Programmed Genome Rearrangements in *P.*
867 *tetraurelia*.

868 A: After IES elimination by PiggyMac (PGM), the introduced Double Strand Breaks (DSB) are
869 repaired by the NHEJ repair pathway, involving Ku70a/80c, DNA-PKcs, PolX and XRCC4-
870 Ligase IV. *Paramecium* PolXs (Ptet-PolXs) are involved in the gap-filling step, during which
871 their role is to accurately synthesize the missing base on each strand.

872 B: *Paramecium* PolXs display a domain composition and sequence motifs similar to
873 metazoan PolXs, especially the NHEJ-related Pol λ and Pol μ as well as V(D)J-related TdT. They
874 share high sequence identity to one another and are divided in two groups (PolXab and
875 PolXcd). Their closest homolog among metazoan PolXs is Pol λ , but they are also homologs
876 of Pol β , despite their additional BRCT domain. The domains are described under the figure,
877 and the most important sequence motifs are indicated.

878

879 **Figure 2.** Characterization of Ptet-PolXs enzymatic activities.

880 A: *Paramecium* PolXs display a gap-filling activity similar to human Pol λ , independently of
881 their N-terminal BRCT and linker domains. Like HsPol λ , they efficiently discriminate NTPs.
882 Gap-filling assays were conducted using human Pol λ and *Paramecium* PolXa Δ Nter,
883 PolXb Δ Nter, PolXd Δ Nter, and PolXdFL, with either dNTPs, dGTP only, NTPs or GTP only.

884 B: Steady-state kinetics characterization of *Paramecium* PolX. PolXa \square Nter and PolXd \square Nter
885 have different kinetics profiles in gap-filling, but display a similar catalytic efficiency, which
886 surpass HsPol λ 's. Their behavior in gap-filling is more similar to HsPol λ than to HsPol β .
887 Activities of PolXa Δ Nter (n=12) and PolXd Δ Nter (n=3) were assessed with increasing
888 concentrations of dGTP, employing a gap-filling substrate. Left panel: Velocity curves for
889 PolXa and PolXd, as a function of dGTP concentration. The error bars correspond to the
890 standard deviation on the measurements. Right panel: Kinetic values derived from the plots

891 for each DNA polymerase. The optimal fit values and the 95% confidence intervals (CI) are
892 provided. Literature data for HsPol λ and HsPol β under analogous conditions are included.

893 C: Fidelity of Ptet-PolXs is higher than HsPol λ 's, independently of its N-terminal BRCT and
894 linker domains. Single time-point, single-turnover fidelity assays were conducted with human
895 Pol λ (FL or lacking its N-terminal BRCT and linker domains) and *Paramecium* PolXa Δ Nter and
896 PolXdFL. The substrate is the gap-filling oligonucleotide duplex indicated in B.

897

898 **Figure 3.** Cluster analysis of PolXs sequences along with the sequence determinants of each
899 subgroup.

900 A: Non-hierarchical clustering of PolXs sequences. A 3D distribution of 7250 PolXs sequences
901 was generated with CLANS (47) using the pairwise Blastp scores of sequence similarity
902 between individual sequences. Three planar projections in different directions of this
903 distribution are shown. The 12 clusters of PolXs sequences are colored and numbered. Their
904 names are given on the bottom right. Ptet-PolXs are in the Harosa group (#9).

905 B: A life tree of the 12 groups of PolXs as determined by the of CLANS.

906 C: Sequence motifs of the 12 PolXs clusters. Each cluster is presented with a sequence logo,
907 where the height of each residue type is proportional to its frequency

908

909 **Figure 4.** A possible induced-fit mechanism for the fidelity of Ptet-PolXs carried out in part
910 by K534.

911 A: Role of K534 in fidelity for Ptet-PolX. Single time-point, single-turnover fidelity assay
912 conducted with WT or mutant versions of *Paramecium* PolXa Δ BRCT. The K534A construct
913 displays a strong error prone behavior, but the K534R mutation has a lower effect on fidelity.

914 B: Gap-filling assays were performed with wild-type (λ mut) and different mutant versions of
915 Pol λ , with increasing concentrations of correct and incorrect nucleotides.

916 C: Kinetic constants of dGTP insertion by the different mutant versions of Pol λ . The value in
917 parentheses stands for standard deviation estimated from n=3.

918

919 **Figure 5.** Comparison of the structures of the active site of four mutant versions of Pol λ and
920 comparison with λ ref (PDB 7m43, in green). The residues involved in the activation
921 mechanism of the active site are highlighted, including catalytic residues (D490, D427 and
922 D429), steric gate residues (Y271 and F272), SD2 motif residues (529 and 530), and residue
923 R492 in catalytic motif 2. Distances between residues are indicated in red, while hydrogen
924 bonds are shown in cyan. In the λ mutR mutant (top left panel), R492 is oriented towards the
925 catalytic residues at ~ 3 Å from D490, which is diverted from the active site. The λ SD2 β
926 mutant (middle and bottom right panels) displays two molecules in the asymmetric unit. In
927 those structures, D490 is oriented towards R492, which is located between this catalytic
928 aspartate and the SD2 motif, at a distance of ~ 3.5 -4 Å. In the λ mutK construct (top right
929 panel), the inserted lysine (K492) is located at 3 Å from the SD2 residue E529, forming a salt-
930 bridge with it. In the matched (with dTTP) structure of the λ SD2Ptet mutant (middle right
931 panel, in blue) K492 is stabilized by the SD2 residue D529 through water-mediated bonds,
932 whereas in the unmatched structure (with dCTP, bottom right panel, in orange) K492 is at 3.7
933 Å from the catalytic D429, diverting it from the active site.

934
935 **Figure 6.** Role of Loop3 in catalysis and fidelity in Ptet-PolXs and human Pol λ , and its
936 interaction with the template DNA strand.

937 A: Role of Loop3 in fidelity for Pol λ and for *Paramecium* PolX.

938 Top: Single time-point, single-turnover fidelity assays were conducted with human Pol λ and
939 *Paramecium* PolX Δ BRCT lacking their respective Loop3 or not. For both enzymes, the
940 truncated constructs display a strong error-prone behavior.

941 Bottom: Sequence of the DNA duplex.

942 B: Comparison of the interaction of Loop3 with the template DNA strand in the six obtained
943 x-ray structures. In λ ref (PDB 7m43, in green), λ mutR (light blue), λ mutK (red) and λ SD2Ptet
944 (matched in blue, unmatched in orange) structures, Loop3 interacts with DNA. In the λ SD2 β
945 structure (light purple or yellow), Loop3 is flexible, away from DNA (indicated by bolds
946 arrows) and does not interact with it. Consequently, DNA is displaced and improperly
947 positioned for catalysis (indicated by bolds arrows).

948 C: Example of a 2Fo-Fc electron density map contoured at 1 sigma around the active site,
949 here for the λ SD2Ptet mutant.

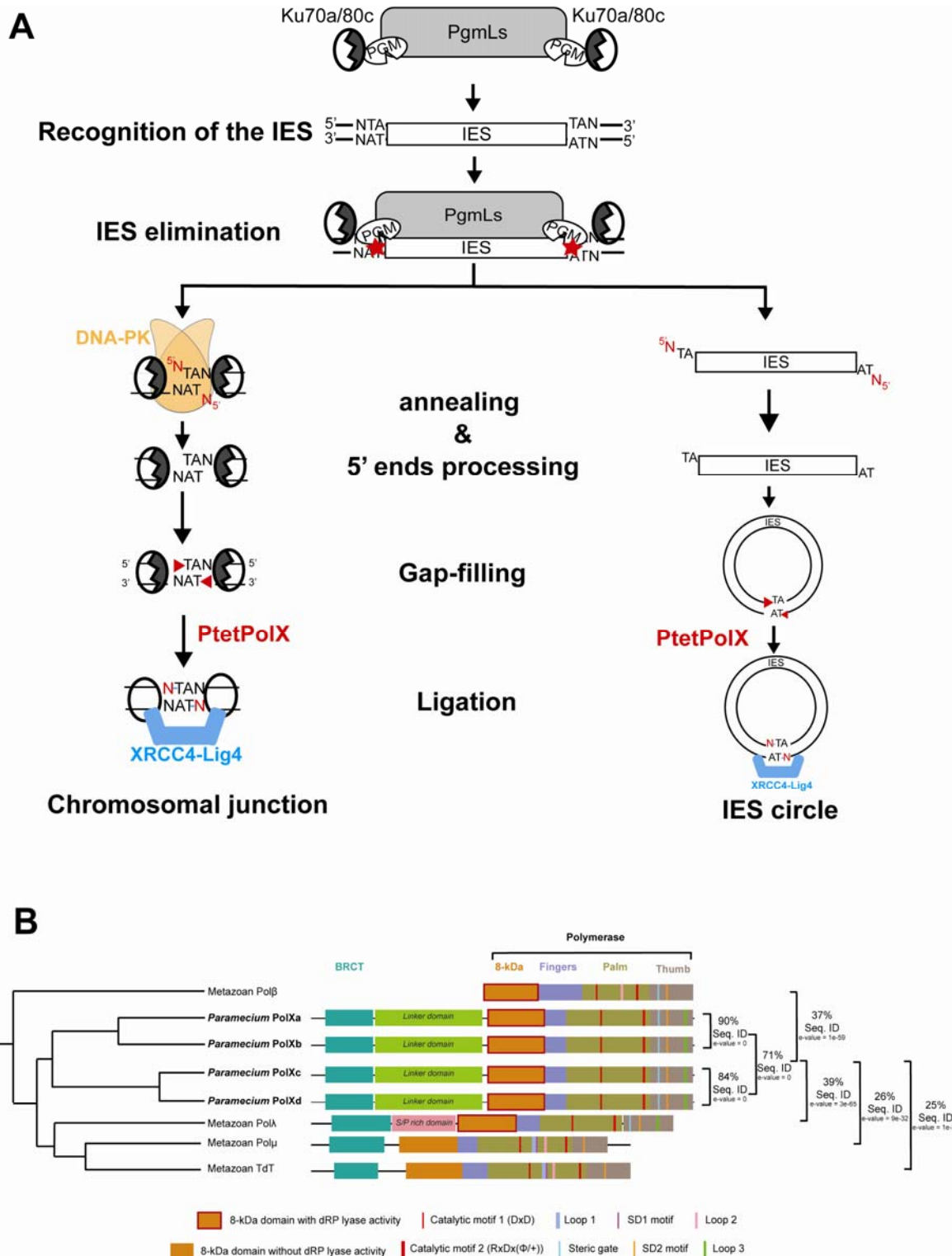
950 D: The λ ref, λ mutR, λ mutK and λ SD2 β constructs interact with DNA through residues mostly
951 located in the SD2 motif and Loop3. Residues in red circles are located in α helices, those in
952 green triangles are found in β strands, and those in blue squares are found in loops.
953 Interaction of the residues with the DNA double helix are indicated with a color code:
954 interactions in cyan are in the minor groove, those in pink are in the major groove, those in
955 yellow involve the sugar moieties, interactions with bases are in grey, and interactions with
956 phosphate groups are indicated in orange

957

958 **Figure 7.** Comparison of conformational changes in the thumb subdomain in Pol β and Pol λ .
959 In Pol β (left panel), when the correct incoming nucleotide enters the active site, a global
960 conformational change stabilizes the DNA by a large movement of whole thumb subdomain
961 that closes the polymerase domain. In Pol λ (right panel), when the correct incoming
962 nucleotide enters in the active site, only Loop 3 (at the tip of the thumb subdomain) moves
963 in to stabilize the DNA. In this case, the thumb subdomain stays in a closed form.

964

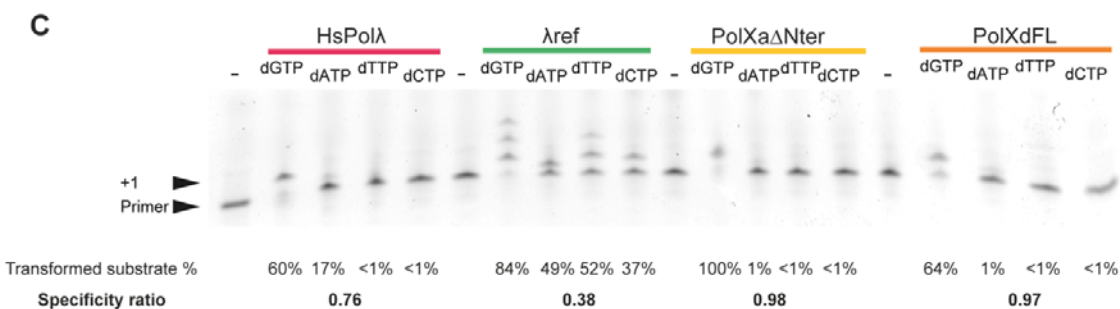
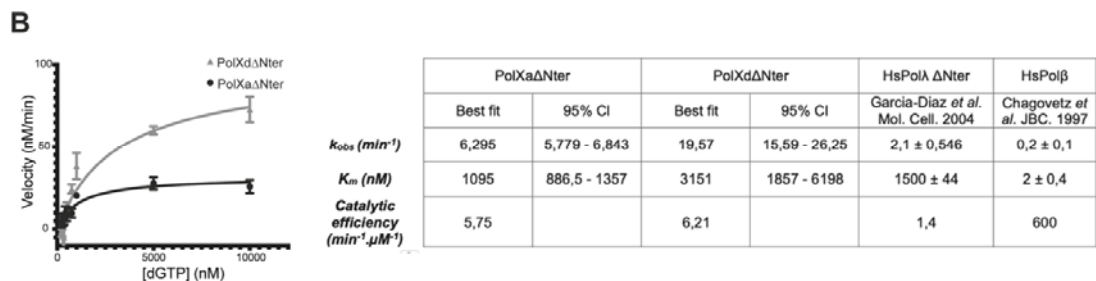
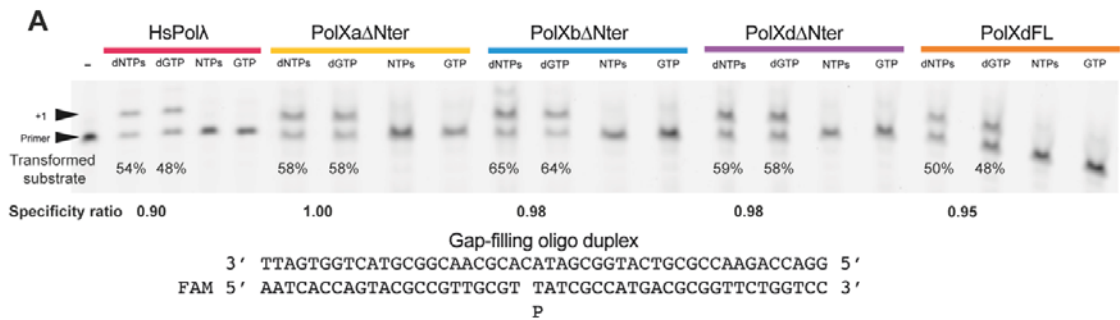
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967

968 Figure 1

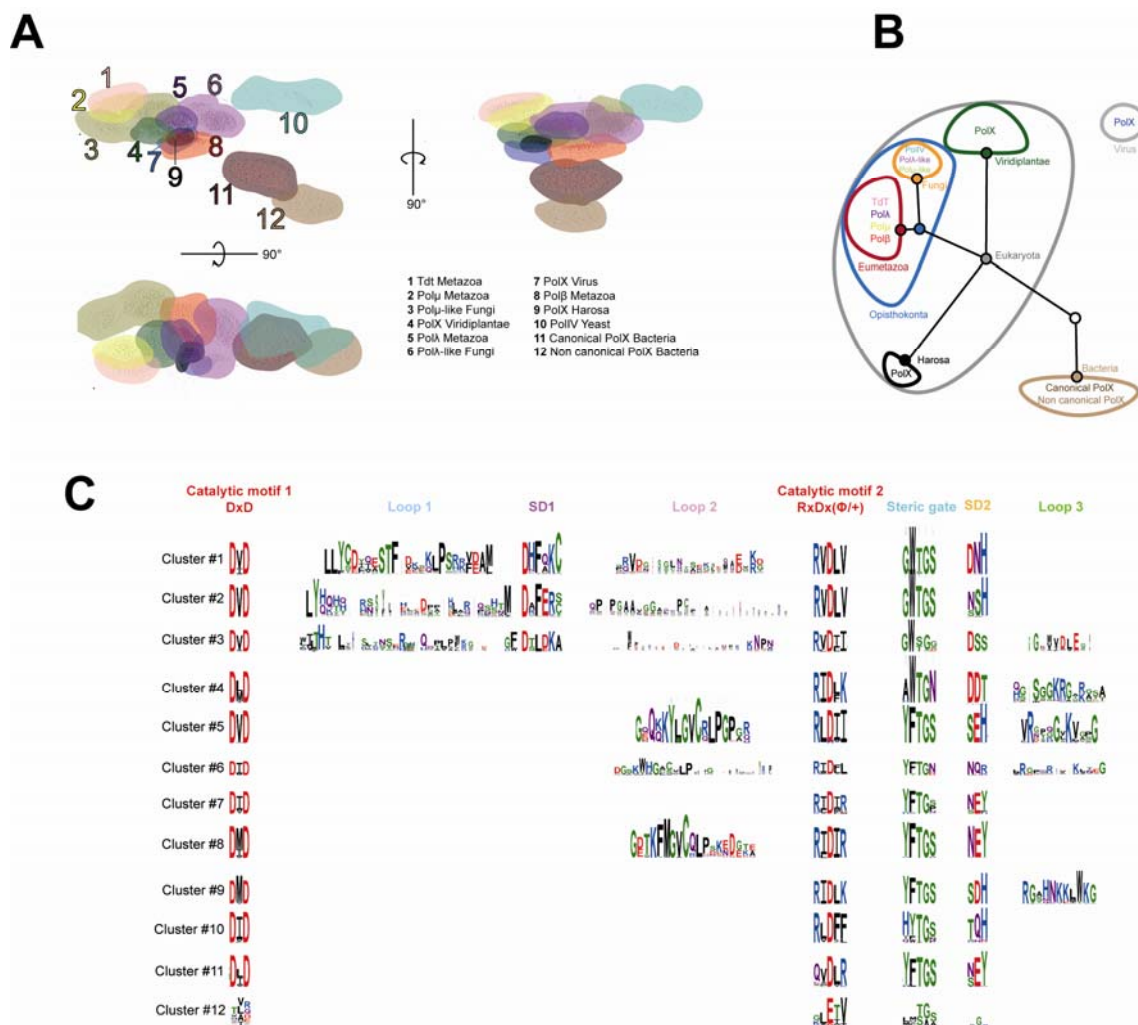
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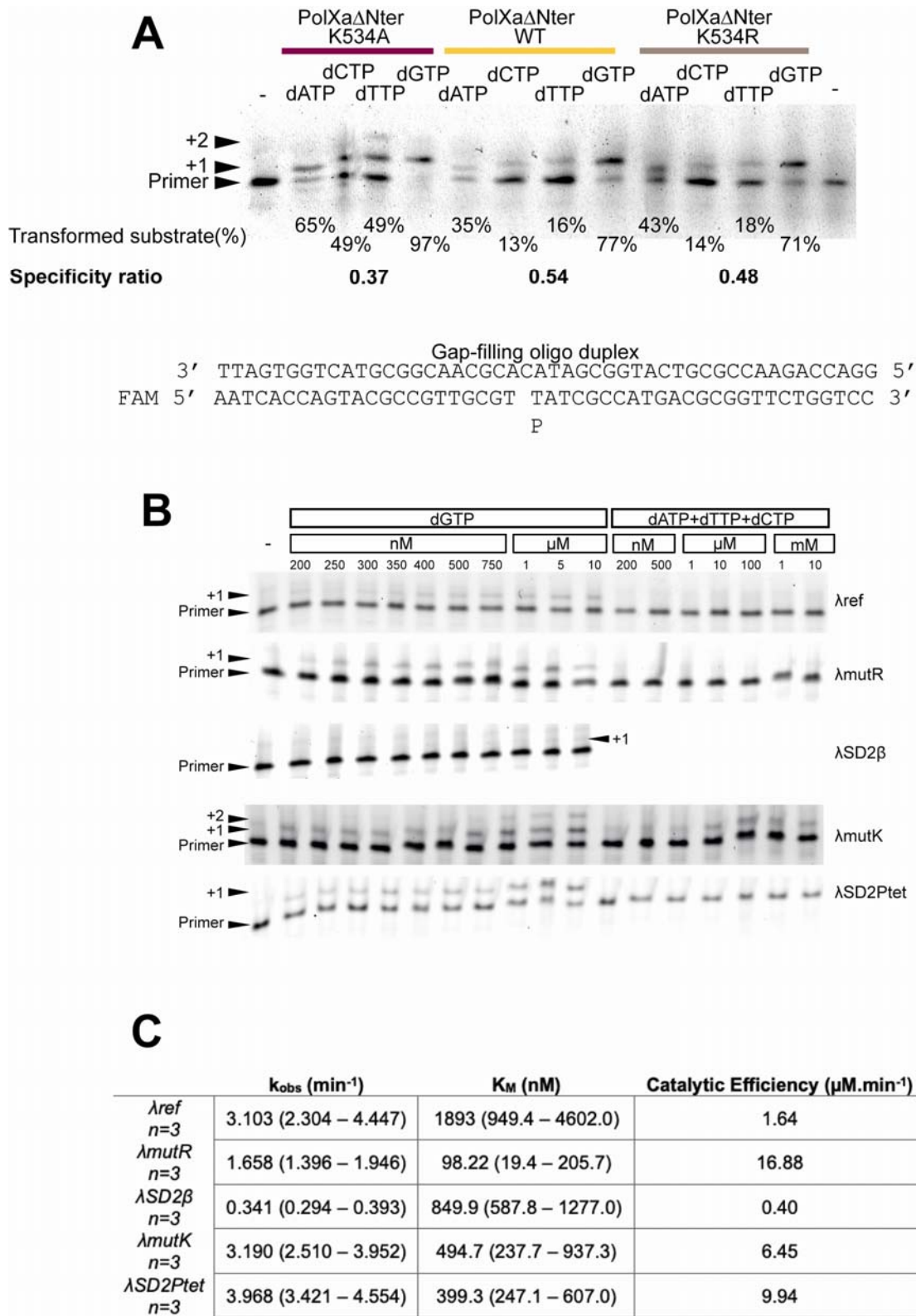
971 Figure 2

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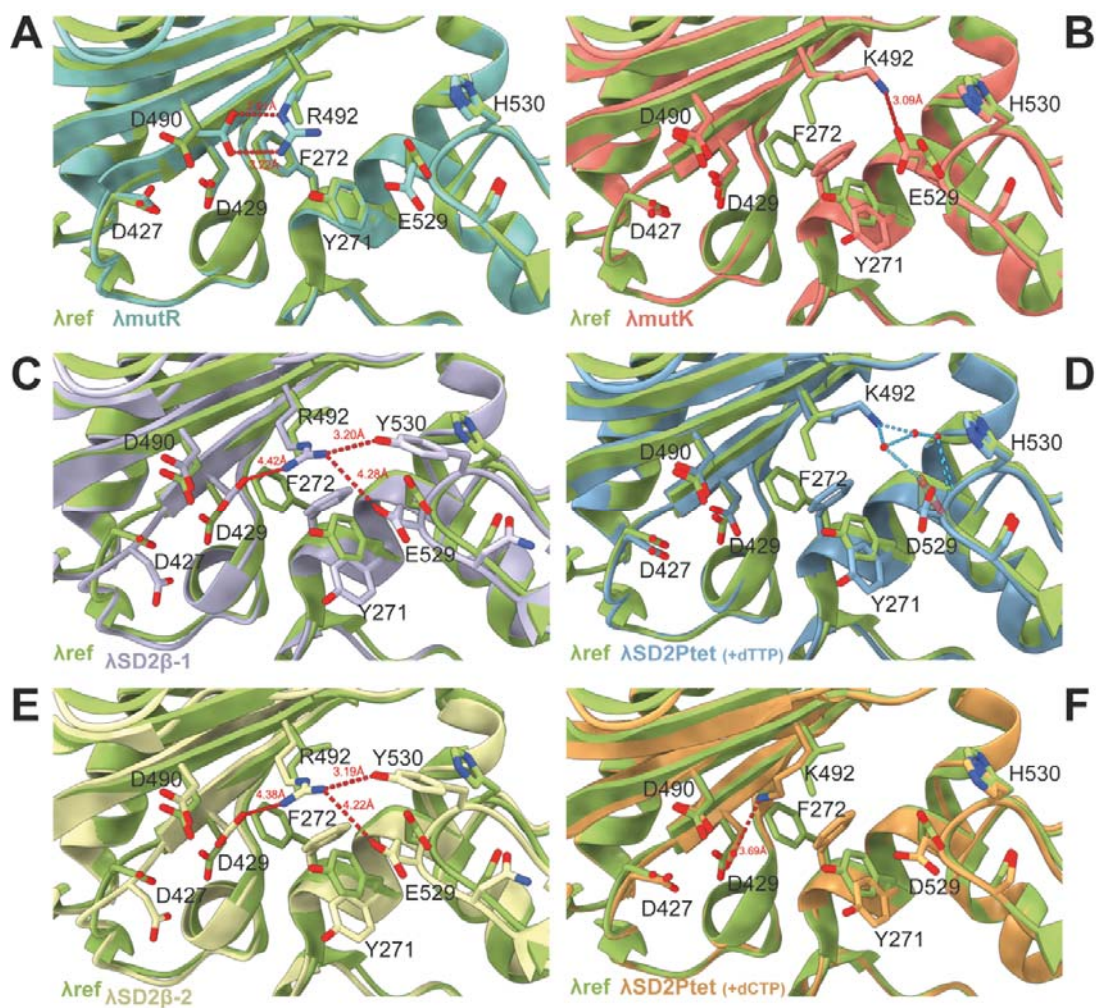
974 Figure 3



975

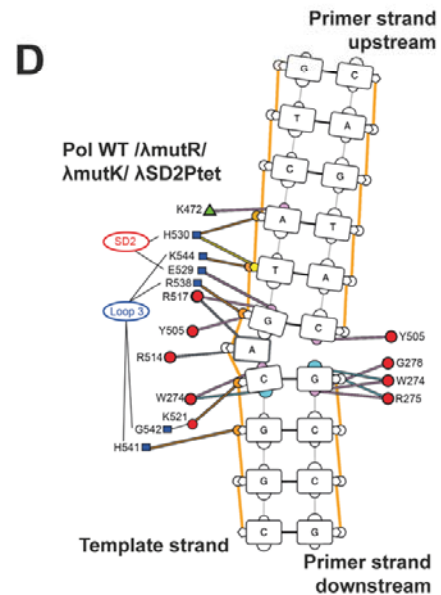
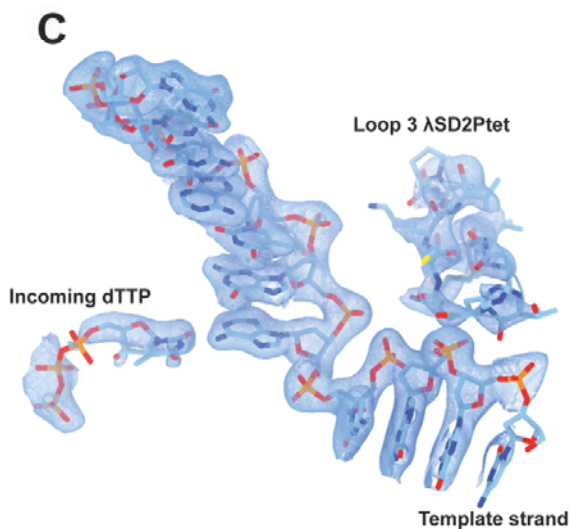
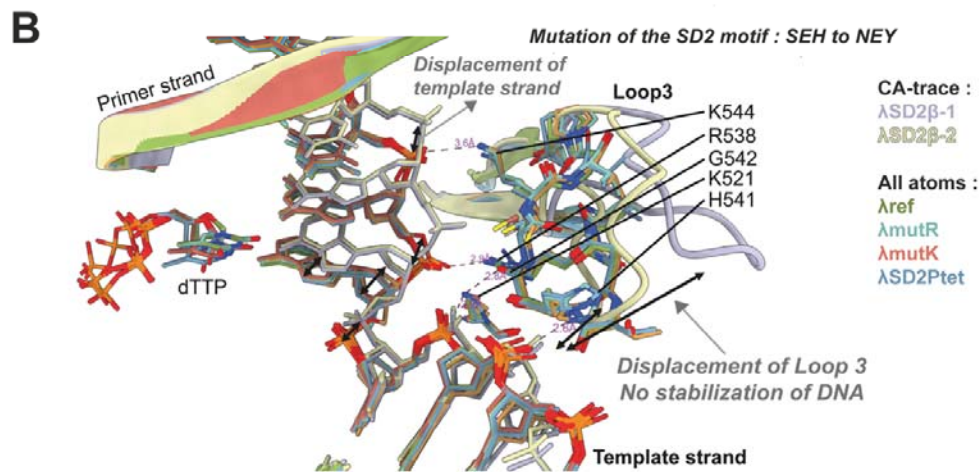
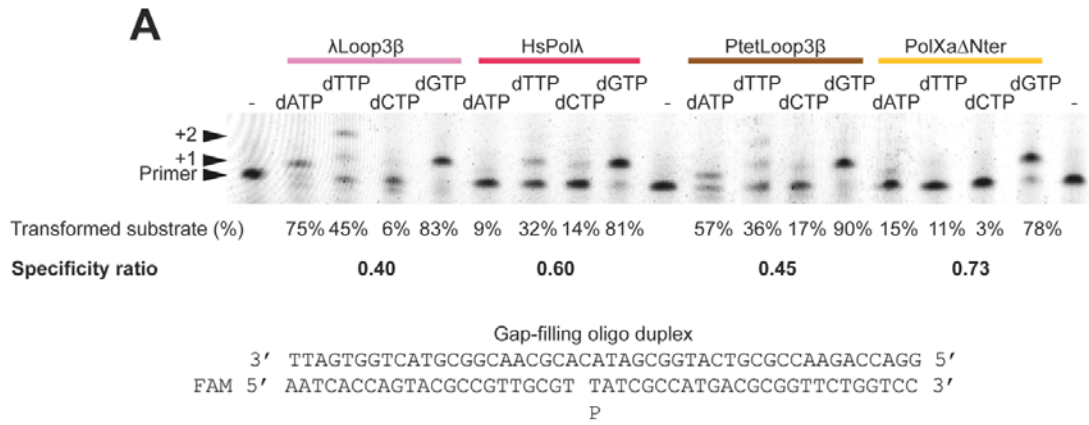
976 Figure 4

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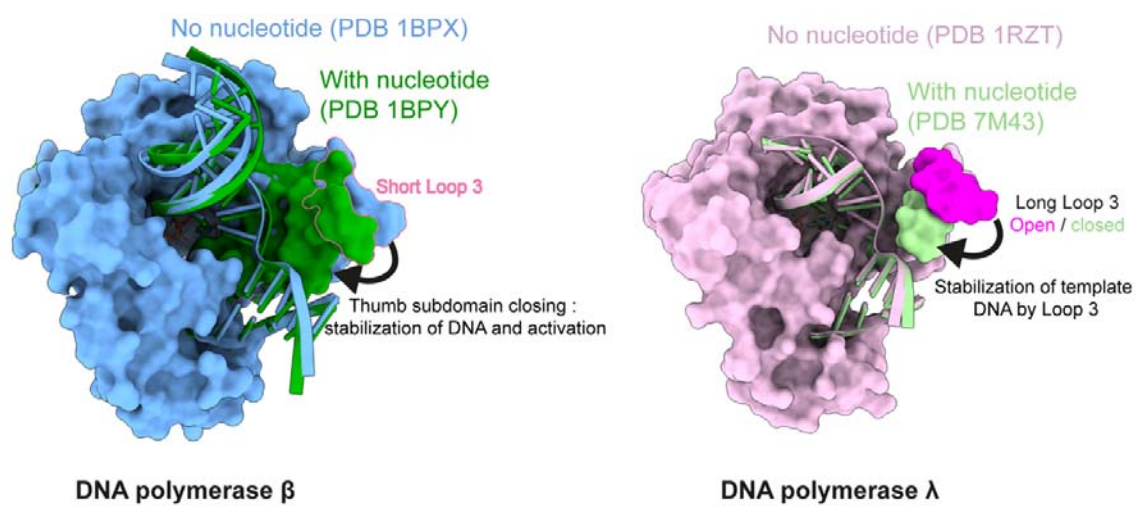
979 Figure 5



980

981 Figure 6

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984

985 Figure 7

986