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Polμ tumor variants decrease the efficiency and accuracy of NHEJ

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ABSTRACT

The non homologous end-joining (NHEJ) pathway of double-strand break (DSB) repair often requires DNA synthesis to fill the gaps generated upon alignment of the broken ends, a complex task performed in human cells by two specialized DNA polymerases, Polλ and Polμ. It is now well established that Polμ is the one adapted to repair DSBs with non-complementary ends, the most challenging scenario, although the structural basis and physiological implications of this adaptation are not fully understood. Here, we demonstrate that two human Polμ point mutations, G174S and R175H, previously identified in two different tumor samples and affecting two adjacent residues, limit the efficiency of accurate NHEJ by Polμ *in vitro* and *in vivo*. Moreover, we show that this limitation is the consequence of a decreased template dependency during NHEJ, which renders the error-rate of the mutants higher due to the ability of Polμ to randomly incorporate nucleotides at DSBs. These results highlight the relevance of the 8 kDa domain of Polμ for accurate and efficient NHEJ, but also its contribution to the error-prone behavior of Polμ at 2-nt gaps. This work provides the first demonstration that mutations affecting Polμ identified in tumors can alter the efficiency and fidelity of NHEJ.

INTRODUCTION

Double-strand breaks (DSBs) are one of the major threats to cell viability as they can directly lead to cell death if unrepaired (1). Eukaryotes are endowed with at least two major pathways devoted to DSB repair, homologous recombination (HR) and non-homologous end joining (NHEJ) (2), although recently, the existence of an additional mechanism for DSB repair, termed microhomology-mediated end joining (MMEJ) or alternative NHEJ (a-NHEJ), has become more evident (3,4). Among these three pathways, NHEJ is characterized by operating by directly rejoining the broken ends requiring little or no microhomology and limited resection (5). These characteristics render NHEJ operative throughout the cell cycle (2), and arguably the major pathway for DSB repair in mammalian cells. However, this mechanism of action also implies significant error-proneness; in fact, NHEJ is frequently associated with short deletions (5). Interestingly, the ends of a DSB are usually imperfect for re-connection and their alignment can result in mismatches and gaps that impede direct ligation. For this reason, NHEJ generally requires processing prior to ligation, which may involve DNA polymerases to fill the gaps generated after alignment of the ends (5). NHEJ is arguably the most demanding scenario for DNA synthesis, requiring DNA polymerases to tolerate primer/template discontinuities, mismatches and even damaged bases, which are frequently associated with DSBs (6). Accordingly, conventional DNA polymerases are mostly inactive during NHEJ and only highly specialized polymerases can synthesize DNA using these complex substrates.

DNA polymerases from the X family (PolXs) are small monomeric enzymes specialized in filling small DNA gaps either during NHEJ or base excision repair (BER) (7). PolXs structural organization comprises a catalytic core that consists of a polymerase domain and of an 8 kDa domain. The 8 kDa domain is essential to PolX adaptation to filling small DNA gaps as it interacts specifically with the downstream strand of the gap through one or more helix–hairpin–helix motifs (HhH), and also through a positively charged pocket that binds the 5′-phosphate (5′P) group of

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the downstream strand (7). In addition to the catalytic core, PolXs specialized in NHEJ harbor an additional domain termed BRCT (BRCA1 C-Terminal) that is strictly required for the interaction with NHEJ core factors such as the Ku70/80 heterodimer and the XRCC4/ligase IV complex (8–12). Four PolXs have been identified in mammalian cells: Polβ, which is adapted to BER; Polλ and Polμ, which are specialized in NHEJ; and TdT, which is adapted to generate diversity during V(D)J recombination. Interestingly, although TdT activity was traditionally considered to be strictly template-independent, recent studies demonstrate that, similarly to Polμ, TdT can bridge broken DNA ends and incorporate template-directed nucleotides in this context (13,14).

PolXs are highly conserved through evolution and analysis of single PolXs orthologs suggest that NHEJ is arguably their most ancestral role. Pol4, the only PolX found in both budding and fission yeast, is a relevant example having been shown to be specialized in NHEJ (15,16), and even to impact chromosomal translocations in budding yeast (17). In spite of the clear link between PolXs and NHEJ, the fact that higher eukaryotes harbor two DNA polymerases specialized in NHEJ, Polλ and Polμ, suggested functional redundancy. Nevertheless, multiple studies addressed this by demonstrating that Polλ and Polμ are specialized in repairing DSBs with ends of different complementarity, thus having non-overlapping roles (10,18). Polλ is specialized in the repair of partially complementary broken ends and it is uniquely accurate if the gaps to be filled have two template nucleotides (nt). Polλ accuracy in this context is remarkable because other PolXs, such as Polμ, can be particularly error-prone if the primer and downstream strands of a gap are distant, as in the case of 2-nt gaps (18–22), due to their inherent need to simultaneously interact with both 3′ and 5′ends of the gap. Polλ overcomes this problem by scrunching the template DNA to stabilize the template base that follows the first to be copied in an extrahelical position (23). On the other hand, Polμ is mainly specialized in the repair of DSBs with non-complementary ends, an extremely complicated task given that the primer terminus to be extended and the template DNA are not paired in this context. Different studies have identified some structural features that enable Polμ to synthesize DNA in such a complicated scenario, and among them, Polμ loop1 motif stands out. Loop1, also conserved and first defined in TdT (24), is strictly required by Polμ to operate at non-complementary ends (10,20). In spite of the lack of direct structural information for Polμ loop 1, it was proposed to act as a connector, bridging the unpaired primer-terminus and 3′-protruding template portion of non-complementary ends (10,20). Recent work supports that hypothesis, showing for the first time a crystal structure in which TdT Loop 1 contributes to align and connect the two DNA ends of a DSB (14). Polμ ability to catalyze DNA synthesis using non-complementary broken ends implies higher promiscuity; in fact, Polμ can also catalyse template-independent synthesis, again strictly requiring loop 1 for this task (20). Furthermore, analyses of sugar selectivity at the active site of Polμ also demonstrates a low discrimination (25,26), a relevant feature for Polμ-mediated NHEJ *in vitro* (27), and paralleled by other PolXs such as TdT and Pol4 (28–30), recently shown to be relevant for damage tolerance in the case of the latter (31).

Although previous studies have clearly supported Polμ specialization in the NHEJ of non-complementary DSBs and identified some of the structural elements of Polμ required for this complex task, there are still some aspects of this adaptation that remain unknown. Analyses of Polβ and Polλ tumor variants helped to identify residues relevant for their activity and even suggested that these mutations could contribute to tumorigenesis as they led to increased mutagenesis and genomic instability (32–35). In clear contrast, whether tumor mutations of Polμ could alter the properties of this enzyme has never been reported, and similarly to the Polβ and Polλ analyses, this could be relevant not only to evaluate the potential contribution of Polμ to tumorigeneis but also to identify key structural elements required for NHEJ by Polμ. Herein, we report the characterization of two tumor-associated variants of Polμ, G174S and R175H, affecting two conserved adjacent residues located in the 8 kDa domain. One of these adjacent residues, Arg^{175}, is known to directly bind the 5′P group of the downstream strand of the gap and accordingly, it was shown to be relevant for Polμ-mediated NHEJ (11). Conversely, the role of the other residue, Gly^{174}, remains unknown. Herein, we demonstrate that the tumor mutations G174S and R175H hinder the efficiency of Polμ-mediated NHEJ both *in vitro* and *in vivo*. Remarkably, the mutations specifically hamper the incorporation of template-directed nucleotides but not the insertion of untemplated and incorrect nucleotides at DSBs, thus increasing the error rate of Polμ during NHEJ. Taken together our data highlight the relevance of the 8 kDa domain and more specifically of the Gly^{174} and Arg^{175} residues for efficient and accurate synthesis during NHEJ *in vitro* and *in vivo*.

MATERIALS AND METHODS

DNA and proteins

Human wild-type Polμ and the G174S and R175H mutants were purified as previously described (36). The G174S and R175H mutants were devised by site-directed mutagenesis on the over-expressing construct pRSET-hPolμ (37), using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and the following synthetic oligonucleotides as primers: G174S-forward (5′-GAAGGCAGTGAGAGCC GCCTCCTCACC-3′) and G174S-reverse (5′-GGTGAG GAGGCGGCTCTCACTGCCTTC-3′) in the case of the G174S mutant; R175H-forward (5′-GGCAGTGAGGGC CACCTCCTCACCTTC-3′) and R175H-reverse (5′-GAA GGTGAGGAGGTGGCCCTCACTGCC-3′) in the case of the R175H mutant. DNA oligonucleotides for site-directed mutagenesis were purchased from Sigma-Aldrich. Synthetic DNA oligonucleotides used for DNA polymerization assays were purchased from Isogen and purified by 8 M urea–20% polyacrylamide (w/v) gel electrophoresis. Synthetic oligonucleotides used as primers were radiolabeled using [γ-^{32}P]ATP (3000 Ci/ mmol) (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs) for 45 min at 37°C. Hybridizations were performed in 1× hybridization buffer (50 mM Tris–HCl pH 7.5, 0.3 M NaCl) for 10 min

at 80°C. Unlabeled ultrapure dNTPs were supplied by GE Healthcare.

DNA polymerization assays

For 1 nt gap-filling assays, a 5′[^{32}P]-labeled 15-mer DNA primer (5′- GATCACAGTGAGTAC-3′) was hybridized to a 28-mer template (5′-AGAAGTGTATCTCGTACTCA CTGTGATC-3′) and to a 12-mer downstream oligonucleotide harboring a 5′P (5′P-AGATACACTTCT-3′) to generate a dsDNA molecule with a 1 nt gap. DNA (2.5 nM) was incubated with purified Polμ (25 nM) and the indicated amounts of dGTP for 30 min at 30°C. The reaction mixture (20 μl) contained 2.5 mM $MgCl_2$ and 1X reaction buffer (50 mM Tris-HCl/ pH 7.5, 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA). After incubation, reactions were stopped by addition of loading buffer (95% (v/v) formamide, 10 mM EDTA, 0.1% (w/v) xylene cyanol and 0.1% (w/v) Bromophenol Blue), and primer extension was analyzed by 8 M urea–20% polyacrylamide (w/v) gel electrophoresis. After electrophoresis, extended products were detected using a BAS-1500 phosphorimager (Fujifilm) and band intensities were determined by densitometry. For 2 nt gap-filling assays, a 5′[^{32}P]-labeled 15-mer DNA primer (5′-TCTGTGCAGGTTCTT-3′) was hybridized to a 32-mer template (5′-TGAAGTCCCTCTCGACGAAGAAC CTGCACAGA-3′) and to a 15-mer downstream oligonucleotide harboring a 5′P (5′P-TCGAGAGGGACTTCA-3′) to generate a 2 nt-gapped dsDNA. Reactions were performed as described above for the 1-nt gap-filling experiments but using the indicated concentrations of either dCTP or dGTP. After 30 min of incubation at 30°C, primer extension was analysed as described previously.

To test the ability of Polμ to perform NHEJ of compatible ends *in vitro*, a 5′[^{32}P]-labeled 13-mer DNA (5′-CCC TCCCTCCCGT-3′) was hybridized to an 11-mer oligonucleotide (5′-GGGAGGGAGGG-3′) to generate a labeled dsDNA primer molecule with a 3′-protruding end. Additionally, a 15-mer oligonucleotide (5′-GCACTCACGTC CCCA-3′) was hybridized to a 13-mer oligonucleotide harboring a 5′P (5′P-GGGACGTGAGTGC-3′) to generate an unlabeled dsDNA molecule with a 3′-protruding end that will be recognised mainly a as a template by Polμ due to the presence of a 5′P group flanking the protrusion. Reactions were performed by incubating the labeled DNA (7 nM) with the unlabeled DNA (18 nM), in the presence of 200 nM of purified Polμ. The reaction mixture (20 μl) also contained 2.5 mM of $MgCl_2$, 1× reaction buffer and the indicated amounts of dGTP. After 30 min of incubation at 30°C, primer extension was analysed as described previously.

To assess the effect of the mutations on the NHEJ of non-compatible ends *in vitro*, a 5′[^{32}P]-labeled 12-mer DNA primer (5′-CCCTCCCTCCCN-3′, where N = dA or dC) was hybridized to the 11-mer oligonucleotide (5′-GGG AGGGAGGG-3′), to generate a labeled dsDNA primer with a 3′-protrusion of 1 nt. A 14-mer oligonucleotide (5′-GCACTCACGTCCCN-3′, where N = dA or dC) was hybridized to the 13-mer oligonucleotide harboring a 5′P (5′P-GGGACGTGAGTGC-3′) to generate an unlabeled dsDNA template molecule with a 3′-protrusion of 1 nt, which was used in the assays only when indicated. Reactions were performed as described above for NHEJ of compatible ends, using the indicated amounts of dGTP to test the efficiency of template directed insertions, the indicated amounts of dATP to test the efficiency of misinsertions, or 5 μM of each individual dNTP to test the overall fidelity of the NHEJ reaction. After incubation for 30 min at 30°C, primer extension was analysed as described previously.

Terminal transferase activity of Polμ was assayed by incubating 5 nM of a 5′[^{32}P]-labeled 21-mer polydT with 600 nM of purified Polμ and 100 μM of each individual dNTP. The reaction mixture (20 μl) also contained 1 mM of $MnCl_2$ and 1× reaction buffer. After 30 min of incubation at 37°C, reactions were stopped with loading buffer as described previously. Primer extension was also analysed as described above.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed using a 5′[γ^{32}-P] labeled 13-mer oligonucleotide (5′-CCCTCCC TCCCGT-3′) hybridized to a 11-mer oligonucleotide harboring a 5′P group (5′P- GGGAGGGAGGG-3′). Purified Polμ (either 200, 400 or 600 nM) was incubated with 14 nM of the labeled DNA and 1× reaction buffer in a final volume of 10 μl. After 10 min of incubation at 30°C samples were mixed with 3 μl of 30% glycerol and subsequently resolved by native 4% polyacrylamide (w/v) gel electrophoresis. After electrophoresis the gels were dried and the labeled DNA was detected by either autoradiography or using a BAS-1500 Phosphorimager (Fujifilm). Quantification of the retarded enzyme:DNA complex was performed by ImageJ quant software, and K_d values were determined using specific binding non-linear regression in Prism 6.0 (Graphpahd Software).

Primary sequence alignments and 3D protein structure visualization assays

Multiple alignments of aminoacid sequences were performed using the MULTALIN server (38) (http://multalin.toulouse.inra.fr/multalin/). 3D structural images were created with the PyMol Molecular Graphics System (Version 1.2r2, Schrödinger), using the following PDB IDs: 4LZD for human apoenzyme Polμ, 4M04 for human Polμ ternary pre-catalytic complex, 4M0A for human Polμ post-catalytic complex (39), 1BPY for human Polβ complexed with a gapped DNA and ddCTP (40), 1XSN for Polλ in complex with a 1 nt-gapped DNA and ddTTP (41), 5D46 murine TdT post-catalytic complex with a DSB with partially complementary ends and 5D4B for murine TdT post-catalytic complex with a DSB with native non-complementary ends (14).

Extrachromosomal NHEJ repair assay

The cell lines used for the extrachromosomal repair assays were prepared and maintained as described previously (18). Substrates were assembled by ligation of ∼15 bp double-stranded DNA caps (Table S1) containing the desired overhang sequence to a 280 bp core fragment digested with BsaI-HF (NEB) at a ratio of 3:1 (cap:core). Substrates were then

purified using a Qiaquick PCR purification kit (Qiagen), and resolved on a native acrylamide gel to ensure substrates preparations were free of unappended core and excess cap. The extrachromosomal repair assays were carried out as previously described (18). Briefly, electroporations were performed using the NEON transfection system (Invitrogen) with 20 ng of substrate, 600 ng pMAX-GFP carrier plasmid, and 2×10^5 cells using a 1350 V, 30 ms pulse in a 10 μl chamber. Following transfection, cells were incubated in antibiotic-free media for 1 h at 37°C. Cellular DNA was then harvested using a QiAamp DNA mini kit (Qiagen). Each electroporation was repeated three times on different days. Transfection experiments with protein complementation were carried out as described above with the addition of purified protein (10 or 100 ng) to the substrate transfection solution immediately before electroporation.

Repair junctions were amplified using Cy5-labeled PCR primers and characterized by restriction enzyme digests. The restriction enzymes used for each substrate were as follows: SalI for the +G junctions of the C3′ substrate, Hpy166III for the +X junctions of the C3′ substrate, AfeI for the +C junctions of the GCG3′ substrate, MluI for the +C products of the GACG3′ substrate, or AatII for the +TC products of the GACG3′ substrate. Digestion products were resolved on a 5% non-denaturing polyacrylamide gel, visualized using a Typhoon Imager (GE Healthcare), and quantified using ImageJ software.

RESULTS

Structural analysis and evolutionary conservation of Polμ Gly^{174} and Arg^{175}

Previous studies compiled in the COSMIC database (http://cancer.sanger.ac.uk/cosmic) (42), have identified two human Polμ point mutations, G174S and R175H, described in skin and ovary carcinomas, respectively (43,44). These tumor mutations affect two adjacent residues, Gly^{174} and Arg^{175}, located within the 8 kDa and in close proximity to the template/downstream base pair forming the 5′ side limit of a DNA gap (Figure 1A). Gly^{174} is positioned near the template base that is paired to 5′ end-terminal base of the downstream strand flanking the gap (Figure 1A). Interestingly, Polβ and Polλ harbor a histidine (His^{34}) or a tryptophan (Trp^{274}) respectively at that position (Figure 1B and C), that can perform stacking interactions with the template base ring moiety and that are highly evolutionary conserved in Polβ and Polλ from vertebrates (Supplementary Figure S1A and B). Conversely, the small size of Gly^{174} minimizes interaction with the DNA, which could suggest a minimal interference with sporadic distortions of the template molecule. As previously reported, Arg^{175} directly interacts with the 5′P group of the downstream strand when Polμ is bound to a DNA gap (Figure 1A; first reported for murine Polμ (45)). Analysis of Polμ structure suggests that Arg^{175} may be the most relevant ligand of the 5′P group compared to the other ligand, His^{208}. Accordingly, mutation of Arg^{175} to alanine pronouncedly diminishes Polμ activity during NHEJ (11). The recently solved structures of the catalytic cycle of a gap filling reaction by human Polμ show that Arg^{175} is mobile when Polμ is not bound to the DNA and consequently its side chain is not visible in this context (Figure 1D, left panel); however, interaction with the 5′P of a DNA strand stabilizes its conformation (Figure 1D, right panel), supporting a relevant role for Arg^{175} in DNA binding.

Primary sequence alignment of Polμ orthologs shows that both Gly^{174} and Arg^{175} are highly conserved in vertebrates (Figure 1C), supporting their functional relevance. A more exhaustive alignment showed that Arg^{175} is more strictly conserved than Gly^{174}, that can be changed to other residues such as cysteine, valine and alanine, in a few Polμ orthologs (Supplementary Figure S2), and possibly, mutation of Gly^{174} to serine in Polμ (as in the tumor variant G174S) could have functional implications, as serine is a small but polar residue. The R175H mutation might also affect the optimal function of Polμ given that histidine interaction with a 5′P could be expected to be weaker than that of arginine, given that at a physiological pH, histidines are generally not positively charged, unlike arginines. The possible relevance of Polμ Arg^{175} is also supported by its invariance in Polλ (Arg^{273}) and by the presence of a conservative substitution in Polβ (Lys^{33}) (Supplementary Figure S1A and B) the two other human PolXs that strongly bind the 5′P of the downstream DNA strand flanking a gap (46–48). Interestingly, both Gly^{174} and Arg^{175} are not conserved in human TdT, possibly suggesting that these residues and this area of the 8 kDa domain are especially important for template-dependent reactions. In fact, the important variability observed at these corresponding positions in TdT among vertebrates also supports this idea (Supplementary Figure S1C). Strikingly, a recent study demonstrated that introducing an arginine in murine TdT (replacing Ser^{187}), enhanced TdT template-dependent activity (14).

The G174S and R175H mutations limit the efficiency of accurate NHEJ by Polμ both *in vitro* and *in vivo*

To determine the effect of the G174S and R175H mutations on the activity of human Polμ, we devised the mutants by site-directed mutagenesis and purified the recombinant proteins. First, the ability of the mutants to fill a 1 nt-gap was tested by incubating the purified proteins with a 5′-labeled dsDNA containing a 1 nt-gap flanked by a 5′P (Figure 2A, see scheme), a group known to stimulate the activity of Polμ *in vitro*. The R175H mutation did not alter the efficiency of primer extension by Polμ during gap-filling of a 1 nt-gap compared to its wild-type counterpart (Figure 2A), in clear contrast to what was described for the R175A mutation, which was partially defective in the filling of 1 nt-gaps (11). It should be noted that we could not detect extension of more than ∼50% of the primer molecules, probably due to imperfect hybridization of the DNA molecules. Regardless, this experiment suggests that, although Arg^{175} is necessary for optimal gap-filling activity, a histidine can replace this residue for this task. Conversely, the G174S mutation reduced primer extension by Polμ during gap-filling, but very mildly (∼1.5-fold) (Figure 2A), suggesting that Gly^{174} is also not strictly required for this reaction. Next, after demonstrating that the tumor variants are active proteins and proficient in gap-filling *in vitro*, we sought to test the possible effect of these mutations on NHEJ, the major role of Polμ *in vivo*. For this, we used

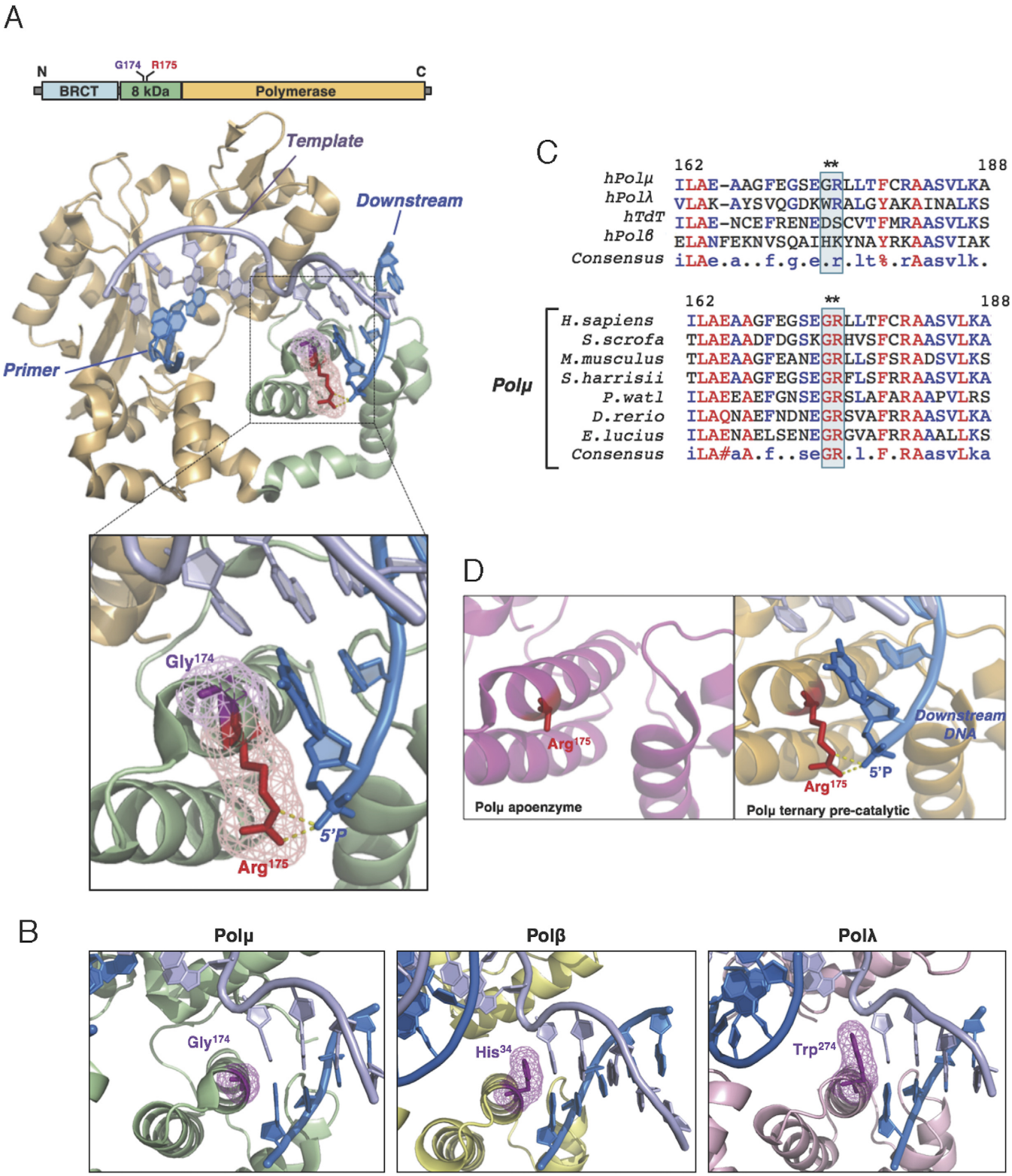

Figure 1. Structure and conservation of human Polμ Gly^{174} and Arg^{175}. (**A**) Structure of the ternary pre-catalytic gap-filling complex of human Polμ (PDB ID: 4M04) showing Gly^{174} and Arg^{175} structure and localization. (**B**) Structural comparison of human Polμ, human Polβ and human Polλ (PDB IDs: 4M04, 1BPY and 1XSN respectively). (**C**) Primary sequence alignments of the human PolX members and of the indicated vertebrate Polμ orthologs. (**D**) Arg^{175} in Polμ apoenzyme (PDB ID: 4LZD), and in the ternary pre-catalytic gap-filling complex of Polμ (PDB ID: 4M04).

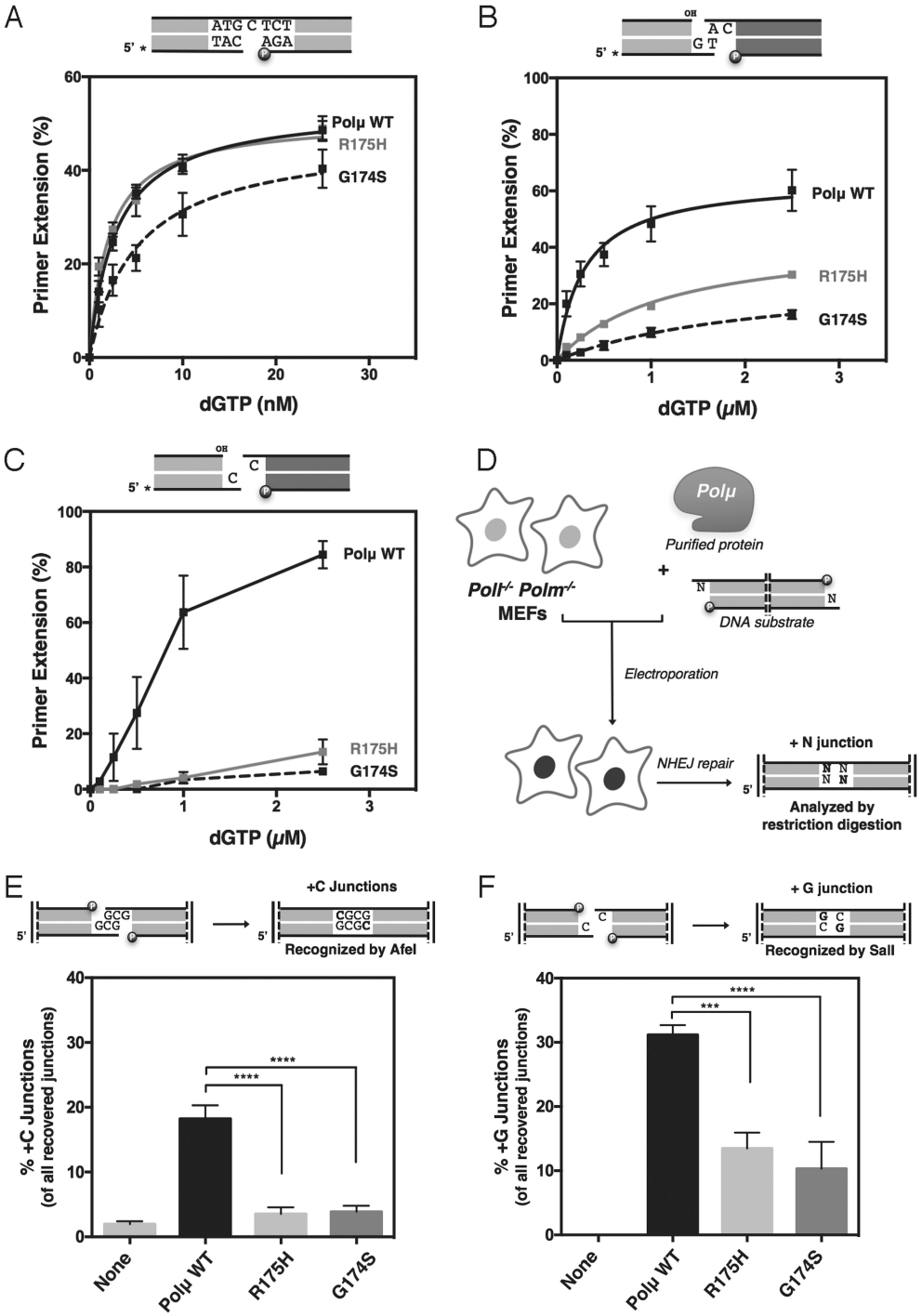

Figure 2. The effect of the G174S and R175H mutations on the efficiency of accurate NHEJ. (**A**) Analysis of the activity of either wild-type Polμ or the G174S and R175H mutants on a 1nt gapped DNA. Data represented are means of at least 3 independent experiments, and the error bars correspond to the SD. (**B**) Kinetic analysis of the effect of the mutations on the efficiency of accurate NHEJ of complementary ends *in vitro*. Data are represented as in (A) and correspond to at least 3 independent experiments. (**C**) *In vitro* kinetic analysis of the efficiency of accurate NHEJ of non-complementary ends by Polμ and the G174S and R175H tumor variants *in vitro*. Data are represented as in (A) and correspond to at least three independent experiments. (**D**) Schematic representation of the extrachromosomal NHEJ cellular system. DNA substrates mimicking different types of DSBs and purified Polμ were electroporated into MEFs deficient for Polλ ($Pol^{-/-}$) and Polμ ($Polm^{-/-}$). Accurate NHEJ could be monitored by reconstitution of a restriction site in the DNA substrates. (**E**) Study of the effect of the G174S and the R175H mutation on accurate NHEJ in the extrachromosomal cellular system using DNA substrate mimicking a DSB with complementary ends. Protein complementation was carried out using 10 ng of the purified proteins and accurate NHEJ of the substrates was analysed by measurement the generation of the +C junction repair product, in which the restriction site of the AfeI enzyme was reconstituted by Polμ activity ($n = 3$, error bars indicate SD, $^{****}P < 0.0001$, one-way ANOVA with P values adjusted by the Bonferroni method). (**F**) Effect of the mutations on the NHEJ of non-complementary ends in the cellular system. Protein complementation was carried out using 10 ng of the purified proteins and accurate NHEJ by Polμ generated the +G junction productions that were detected by digestion of the recovered DNA with the Sal I restriction enzyme ($n = 3$, Error bars indicate SD, $^{***}P < 0.001$, $^{****}P < 0.0001$, one-way ANOVA with P values adjusted by the Bonferroni method).

two dsDNA molecules, one labeled (Figure 2B, depicted in light grey) and the other unlabeled (Figure 2B, depicted in dark grey), and that are endowed with 3′-protruding ends that have a complementarity of 1 nt. After annealing of the complementary ends, which is induced by the polymerase, two 1 nt-gaps are formed, having either dC or dG as template. Importantly, the presence of a gap-flanking 5′P only in the unlabeled molecule, stimulates Polμ to preferably use this molecule as template (dC) and the 3′-protruding end of the labeled molecule as a primer to be extended with dGTP. In this NHEJ-related polymerase assay, that is performed in the absence of other NHEJ factors, both the R175H and G174S mutations decreased Polμ efficiency of incorporation of dGTP, the template directed nucleotide (Figure 2B), approximately 2.5-fold and 5-fold respectively, suggesting that Arg^{175} and Gly^{174} are required for optimal NHEJ of complementary ends *in vitro*. Next, we evaluated the effect of the tumor mutations G174S and R175H on the NHEJ of non-complementary ends, the most specific role of Polμ *in vivo* (18). We performed a similar NHEJ *in vitro* assay but using dsDNA molecules with 3′-protruding non-complementary ends (Figure 2C, see scheme). The experiment showed that both R175H and G174S mutations markedly decreased Polμ NHEJ efficiency when using non-complementary ends *in vitro* (Figure 2C), (approximately 7- and 15-fold respectively), more pronouncedly than during the NHEJ of compatible ends (Figure 2B). Hence, these results demonstrate that the G174S and R175H tumor variants are defective in NHEJ *in vitro*, more markedly when the broken ends are non-complementary, the most specific context of Polμ action. These results also support a relevant role for these two residues of human Polμ in NHEJ, already demonstrated for Arg^{175} (11), but completely unknown in the case of Gly^{174}.

The pronounced defect caused by these mutations on NHEJ *in vitro*, prompted us to validate these conclusions in a physiological context. For this purpose, we took advantage of a recently described NHEJ extrachromosomal system that operates through the co-electroporation of purified polymerases and specific DNA substrates mimicking DSBs into Polμ- and Polλ-deficient mouse embryonic fibroblasts (MEFs) (Figure 2D; (18)). After electroporation, these DNA substrates are repaired by NHEJ inside the MEFs, as demonstrated by the residual activity detected in a Ku70 deficient background (18), and the accuracy NHEJ-mediated repair is monitored by restriction digestion of the substrate recovered from the cells (Figure 2D). Additionally, the fact that the recipient cells lack endogenous Polμ and Polλ implies that this accurate repair, which is mediated by gap-filling and does not require nuclease processing of the ends, completely relies on the co-electroporated purified DNA polymerases (18). First, we used a DNA substrate mimicking a DSB with 3′-protruding ends with 2nt of complementarity (Figure 2E). Accurate repair of the substrate generates a product, referred to as +C junctions, that is monitored by AfeI restriction. When no protein was co-electroporated with the DNA, +C junctions were barely detectable and only accounted for ∼2% of the total DNA junctions recovered (Figure 2E). Co-electroporation of wild-type Polμ clearly stimulated the level of detectable +C junctions, indicating that accurate NHEJ is enhanced by Polμ-mediated gap-filling (Figure 2E). Conversely, co-electroporation of either R175H or G174S mutants only produced a slight increase in accurate NHEJ efficiency compared to the non-co-electroporated protein condition, and led to 5-fold lower percentage of +C junction recovery compared to the wild-type protein (Figure 2E). According to our *in vitro* data, we expected a less pronounced defect in the case of the R175H mutant compared to the G174S mutant; however, the decrease in efficiency caused by the mutations *in vivo* is overall similar to that observed *in vitro* when using a comparable substrate (Figure 2B), further confirming that these tumor variants are defective in NHEJ. Next, we performed a similar experiment electroporating a DNA molecule that mimicks a non-complementary DSB. Faithful repair by NHEJ of the molecule could be monitored by digestion of the recovered with SalI restriction enzyme (Figure 2E). As previously described, these events of faithful repair, that we named +G junctions, were completely Polμ-dependent, as they are undetectable when this polymerase was not electroporated (Figure 2F) (18). Interestingly, when Polμ wild-type was co-electroporated with the DNA into the MEFs, +G junctions were detectable and accounted for 30% of the total repair events (Figure 2F), however electroporation of either R175H or G174S tumor variants significantly reduced the percentage of +G junctions (∼3-fold in both cases) (Figure 2F), again suggesting that the mutations reduce the ability of Polμ to perform accurate NHEJ efficiently. As this experiment is comparable to others previously performed (18) that completely relied on Ku70, we favor that it constitutes a valid assessment of *bona-fide* classical NHEJ *in vivo*, although we do not formally demonstrate this. Interestingly, it is also worthy to note that the defect caused by the mutations in the cellular system was less pronounced in the case of substrate with non-complementary ends compared to the DNA with complementary ends (Figure 2E), in clear contrast to our *in vitro* data. In fact, this defect on the NHEJ of non-complementary ends was also less pronounced than that detected in the *in vitro* experiment when using a comparable substrate (Figure 2C), which could suggest that the presence of other NHEJ factors could alleviate the defect caused by the mutations specifically during NHEJ of non-complementary ends, which is Polμ's most specific task *in vivo*. In any case, overall, our data unambiguously indicate that the R175H and G174S tumor variants fail to perform accurate NHEJ efficiently *in vivo*.

The G174S and R175H mutations lead to decreased fidelity during NHEJ

Once demonstrated that the G174S and R175H mutations hamper the efficiency of accurate NHEJ, we wanted to evaluate whether this phenotype could impact their overall fidelity. For this purpose, either the tumor variants or the wild-type control were incubated with the dsDNA molecules mimicking a DSB with non-complementary ends and 5 μM of each of the 4 dNTPs, which were added to the mix independently. Wild-type Polμ incorporated dGTP, the template directed nucleotide, much more efficiently than incorrect nucleotides (Figure 3A, right panel); as a control, incorporation of dGTP required the presence of the unla-

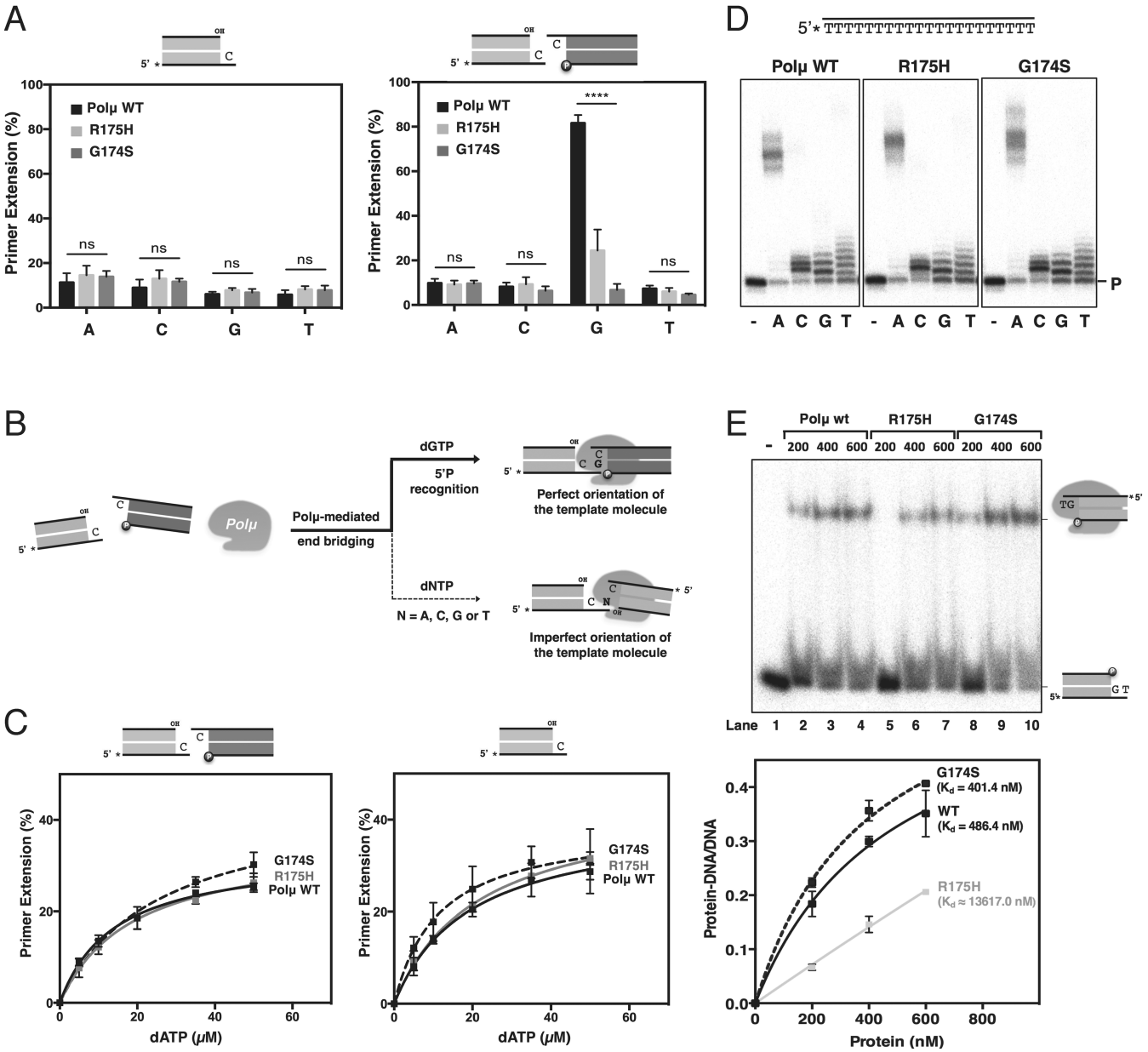

Figure 3. Decreased fidelity caused by the G174S and R175H mutations on NHEJ *in vitro*. (**A**) Purified Polμ and the G174S and R175H mutants (200 nM) were incubated with 5 μM of each individual dNTP and the two DNA molecules mimicking a DSB with non-complementary ends to test the fidelity of these reactions (*right panel*). The effect of the 5′P group flanking the template base on the fidelity of NHEJ was evaluated by repeating the experiment in the absence of the unlabeled 3′-protruding DNA molecule (*left panel*). The data represented in the graphs are of at least three independent experiments and the error bars correspond to the SD. $^{****}P < 0.0001$, ns, not significant, two-way ANOVA with *P* values adjusted by the Bonferroni method. (**B**) Schematic representation of Polμ-mediated end-bridging of the DNA molecules used in (A) explaining template-dependent and template-independent incorporation of nucleotides. (**C**) Kinetic analysis of template-independent nucleotide incorporation by Polμ at DSBs with non-complementary ends, measured by dATP insertion, by Polμ and the G174S and R175H mutants. Data are the means of at least two independent experiments and the SD is represented in the error bars. (**D**) Analysis of the terminal transferase activity of Polμ and the G174S and R175H mutants on homopolymeric DNA. (**E**) Effect of the G174S and R175H mutation on DNA binding monitored by EMSA. The graph represents means of at three different experiments using the indicated concentrations of either Polμ wild-type or the G174S and R175H mutants, and the error bars show the standard error of the mean (SEM). K_d values were determined using specific binding non-linear regression in Prism 6.0 (Graphpad Software).

beled molecule with the 5′P to be efficient (Figure 3A; compare dGTP incorporation in both left and right panels). Importantly, when the molecule harboring the 5′P was not present in the assay, dGTP incorporation, although much less efficient, was still detectable; and it could be due to the 'alignment' of two labeled molecules to use of one of the 3′ protruding dC as a template (Figure 3B). Interestingly, wild-type Polμ could also catalyse the incorporation of the 3 incorrect nucleotides, and with comparable efficiency irrespectively of the presence of the molecule with the 5′P group (Figure 3A). Furthermore, in the absence of the DNA ends with the 5′P, incorrect nucleotides were incorporated as efficiently as dGTP, suggesting that when the phosphate group that flanks the template base is absent, the templating base dC cannot be 'read' correctly by Polμ, probably due to defective binding and/or orientation of the two DNA ends (Figure 3B). In fact, we speculate that these insertion events are completed by virtue of the terminal transferase activity of Polμ (20), known to be limited by the presence of 5′P groups (49), and therefore, they may not necessarily involve simultaneous binding of two DNA molecules. In agreement with our previous results, the G174S and R175H variants incorporated dGTP (the accurate insertion when the dC protruding end had a neighbor 5′P) less efficiently than wild-type Polμ (Figure 3A, right panel). The G174S variant incorporated dGTP very inefficiently, being unaffected by the presence/absence of the 5′P, and comparably to the insertion of incorrect nucleotides (Figure 3A, compare left and right panels). Strikingly, wild-type, R175H and G174S gave very similar values of misincorporated nucleotides in these NHEJ assays. The fact that dGTP incorporation by the Polμ variants was slightly (R175H) or not (G174S) stimulated by presence of a 5′P group flanking the template base suggests that they cannot correctly bind or orientate the 3′protrusion to serve as a template (Figure 3B).

Furthermore, kinetic analyses of dATP incorporation further confirmed that the G174S and R175H mutations did not affect incorporation of non-templated nucleotides during NHEJ of non-complementary ends. Again, dATP incorporation was as in the wild-type, and took place similarly either in the absence or presence of a 5′P flanking the dC template base (Figure 3C), further confirming that this reaction does not occur via *bona-fide* NHEJ. Interestingly, and irrespective of the 5′P group, certain sequence contexts have been shown to impact Polμ activity during NHEJ; for instance, some bases, such as A, are poor templates during Polμ-mediated NHEJ of non-complementary ends *in vitro*, as they seem to limit the efficiency of template directed additions, rendering them comparable to non-templated insertions (27). The tumor variants showed a similar rate of incorporation of dNTPs compared to the wild-type Pol μ in this sequence context (template A) (Supplementary Figure S3). This behavior is consistent with the variants' defect in template-directed synthesis in NHEJ *in vitro*. As mentioned earlier, we speculate that the incorporation of template-independent nucleotides during NHEJ is due to the terminal transferase activity of Polμ. Also in line with our previous results, the G174S and R175H mutations did not affect the terminal transferase activity of Polμ measured by incorporation of dNTPs on a homopolymeric ssDNA molecule (Figure 3D).

Taken together, our results demonstrate that the G174S and R175H mutations specifically decrease Polμ ability to incorporate template-directed nucleotides during NHEJ of non-complementary ends. The mutations on the other hand, do not impact whatsoever the incorporation of incorrect dNTPs at DSBs, which likely occurs through terminal transferase-like reactions. Hence, these results indicate that these tumor mutations could impact the fidelity of Polμ-mediated NHEJ via interfering with template-dependent selection of nucleotides *in vivo*. Given that the residues affected by the mutations localize in the 8 kDa domain, which is involved in binding the template/downstream junction at a gap, the low efficiency of *bona-fide* NHEJ caused by the mutations could be consequence of defective binding/orientation of the templating base, either directly or indirectly caused by a defective 5′P recognition. Electrophoretic mobility shift assays indicated that the R175H variant, but not the G174S, bound defectively to a 3′-protruding DNA substrate harboring a 5′P next to the protrusion (Figure 3E). This suggests that defective NHEJ by R175H is thus a consequence of impaired DNA binding in agreement with the fact that Arg^{175} is the main ligand of the 5′P group. However, this experiment could also indicate that although R175H and the G174S variants lead to a similar phenotype, they might affect NHEJ differently, as the G174S mutation apparently does not impair binding of the DNA. We speculate that this mutation might impair proper orientation of the templating base that is likely facilitated by the small and flexible Gly^{174} *in vivo*.

The G174S and R175H mutations differentially impact 2nt gap-filling by Polμ

The adaptation of the members of the PolX family to filling small DNA gaps -a common DNA transaction in both BER and NHEJ- relies on their 8 kDa domain, which allows these polymerases to interact not only with the 3′ end of the primer, like a conventional polymerase, but also with the 5′ end of the downstream strand of the gap. Consequently, PolXs can be particularly ineffective if these two flanks of the gap are too distant. In fact, a recent *in vivo* study demonstrated that in mammalian NHEJ, PolX activity is mainly restricted to the filling of 1nt and 2nt gaps (18). However, 2nt gaps are already a challenging scenario for NHEJ PolXs: whereas Polλ can accurately fill 2nt gaps due to its unique ability to scrunch the template (23), Polμ lacks this capacity and is consequently more error-prone in this context (18–22). In fact, when dealing with 2nt gaps, Polμ was shown to preferably copy the template base closer to 5′ end of the downstream strand, rather than the first template base, a reaction termed skipping-ahead that potentially leads to frameshift mutations (18–22). Due to the specific role of the 8 kDa in interacting with the 5′ end of the downstream DNA strand, this domain has already been anticipated to possibly influence Polμ's error-proneness during the filling of 2nt gaps (22). Moreover, it could also be anticipated that the lack of stacking interactions with the base pair flanking the gap, a stark difference between Polμ and Polλ or Polβ (Figure 1B), could also have an influ-

ence on how differently these enzymes behave on 2nt gaps. Hence, we decided to address the role of the Gly^{174} and Arg^{175} residues on the filling of 2nt gaps by Polμ by analyzing the effect of the G174S and R175H mutations. For this, we used a 5′-labeled dsDNA molecule harboring a 2nt gap in which incorporation opposite the second template base (skipping ahead) and opposite the first template base (regular primer extension) could be monitored by incorporation of dGTP and dCTP respectively (Figure 4A). As expected, under these conditions, Polμ was clearly more efficient (roughly 7-fold) using the second template base to direct nucleotide incorporation (dGTP in this case) rather than the first, although it could use either of them for catalysis (Figure 4B, note the different scale of nucleotide concentration). Strikingly, the R175H mutation pronouncedly decreased the overall efficiency of Polμ in both reactions, so markedly that incorporation of the nucleotide complementary to the first template base (dCTP) was not detected in the experiment (Figure 4B). Consequently, these data indicate that Arg^{175} is indispensable for the filling of 2nt gaps by Polμ, and that a histidine cannot replace it for this task, similarly to what was observed in the NHEJ experiments (Figure 2). Conversely, this result contrasts with that obtained in a 1nt gap-filling assay (Figure 2A), where the R175H had no detectable defect, suggesting that the 2nt gap is a more challenging scenario, likely more dependent on an optimal interaction with the 5′P of the downstream strand. On the other hand, the G174S mutation led only to a moderate decrease in efficiency (∼1.5-fold) in the skipping-ahead reaction compared to wild-type Polμ, but an improved (∼2.5-fold) conventional incorporation of the nucleotide complementary to the first template base (Figure 4B). Hence, although the G174S mutants still showed a clear preference for the skipping-ahead reaction, it performed this reaction only 2.5-fold more efficiently than the incorporation of nucleotides opposite the first template base, in contrast to wild-type Polμ that skipped-ahead 7.1-fold higher efficiency (Figure 4C). Overall, these data suggest that Gly^{174} and Arg^{175} have different impact on 2nt gap-filling by Polμ; while Arg^{175} is required for any reaction mediated by Polμ involving a 2nt gap, Gly^{174} limits nucleotide incorporation opposite the first template base. Further analyses using the cellular NHEJ system indicated that both G174S and R175H mutants decreased similarly (2-fold) the ability of Polμ to perform skipping-ahead reactions on 2nt gaps during NHEJ, consequent with their effect on these reactions and their overall limitation during NHEJ (Supplementary Figure S4). However, likewise wild-type Polμ, the mutants could not fill the 2nt gap by incorporating 2 nucleotides and extending the primer normally, suggesting that, although Gly^{174} limits normal primer extension at 2nt-gaps *in vitro*, its mutation to serine may not be sufficient to perform accurate 2nt gap-filling *in vivo*.

DISCUSSION

The genetic alterations leading to the transformation of a normal cell into a cancer cell are commonly point mutations (e.g. insertion, deletions, single base modifications), and due to the role of DNA polymerases as DNA synthesizers, alterations in the function and fidelity of these enzymes could contribute somehow to the accumulation of these mutations, and in turn to the tumorigenesis process (nicely reviewed in (50)). Several evidence argue in favor of this idea as for instance Polδ and Polε exonuclease-deficient mice have shorter lifespans and highly increased frequency of carcinogenesis (51–53), but in the case of most DNA polymerases, whether they may contribute to tumorigenesis is not fully understood. Among the members of the PolX family, Polβ is the best studied in this context as some tumor-associated point mutants of Polβ were shown to induce mutations (32,33), and their expression to cause cellular transformation (34). Polλ could also impact tumorigenesis, given that a single point mutation associated to colorectal cancer was shown to decrease Polλ fidelity and to lead to increased genomic instability (35), a common characteristic of cancer cells. Remarkably, these studies are relevant not only because they shed light on whether the altered function of these polymerases could contribute to tumorigenesis but, from a structural and functional standpoint, because they identified critical residues for function of these polymerases.

In this work, we have characterized two tumor-associated Polμ point mutantions, G174S and R175H, previously identified in two different tumor samples (43,44). We have demonstrated that these mutations impact similarly the activity of Polμ during NHEJ decreasing both its efficiency and fidelity, due to the fact that they lead to impaired incorporation of template-directed nucleotides (Figure 2). As shown, *bona-fide* NHEJ reactions by Polμ require the presence of a 5′P group flanking the template base, which enhances both the fidelity and efficiency of these reactions (Figure 3). In fact, the absence of this group leads to the random incorporation of nucleotides at DSBs by Polμ, a natural consequence of its inherent terminal transferase activity (Figure 3). Remarkably, the G174S and R175H mutants were as proficient as wild-type Polμ in these terminal-transferase events, but in contrast to their wild-type counterpart, their activity did not respond to the presence of the 5′P flanking the template base (Figure 3A), suggesting that their defective activity is somehow related to an impaired recognition of the 5′P during NHEJ. Arg^{175} is a ligand of the 5′P group itself, hence the effect of the R175H mutation is not surprising, especially taking into account the previously reported effect of Arg^{175} mutation to alanine, shown to hamper binding to the DNA, bridging of broken ends, and overall, the activity of Polμ on any DNA substrate harboring that group (11). Arg^{175} mutation to histidine, although detrimental, was not as dramatic as mutation to alanine since the R175H mutant retained normal activity 1n-gapped substrates (Figure 2A). Interaction with 5′P group has already been reported to be essential for the activity of most of the studied template-directed polymerases from the X-family (e.g., (14,30,46–48)) and the study of R175H further supports the idea. In line with this, it is interesting to note that other template-directed PolXs, such as Polβ and Polλ, harbor similar or equivalent residues at the corresponding position of Arg^{175}, and also that this residue is not conserved in TdT, a polymerase with strong template-independent activity that displays no positively charged residues interacting with the 5′P within the 8 kDa domain. Moreover, this 5′P biding pocket at the 8 kDa domain, absent in TdT and that includes Arg^{175} in Polμ, is less

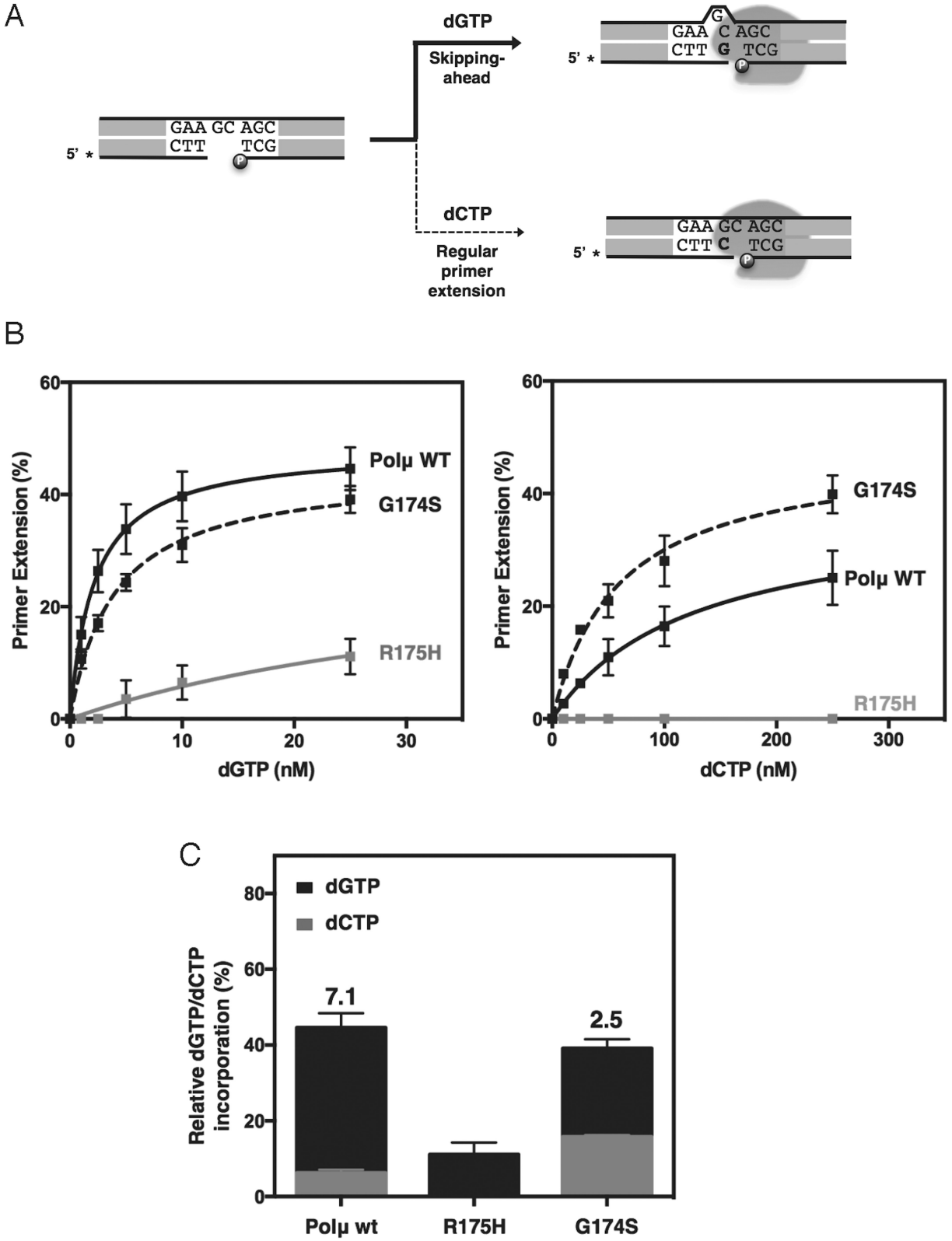

Figure 4. Analysis of the effect of the G174S and R175H mutations on 2nt gap-filling by Polμ *in vitro*. (**A**) Schematic representation of two alternatives for 2nt gap filling by Polμ: copying the second template base (skipping-ahead) or copying the first template base (regular primer extension). (**B**) Effect of the G174S and R175H mutations on the efficiency of both reactions measured by dGTP and dCTP incorporation, respectively. Data represent means of triplicate experiments and the error bars the SD. (**C**) Relative incorporation of 25 nM dGTP and 25 nM dCTP (taken from B) by wild-type or mutant Polμ. Bars correspond to the superimposition of the percentage of primer extension with dGTP (black) and dCTP (gray). The number above the Polμ wild-type and the G174S bar was calculated by dividing primer extension with dGTP by primer extension with dCTP.

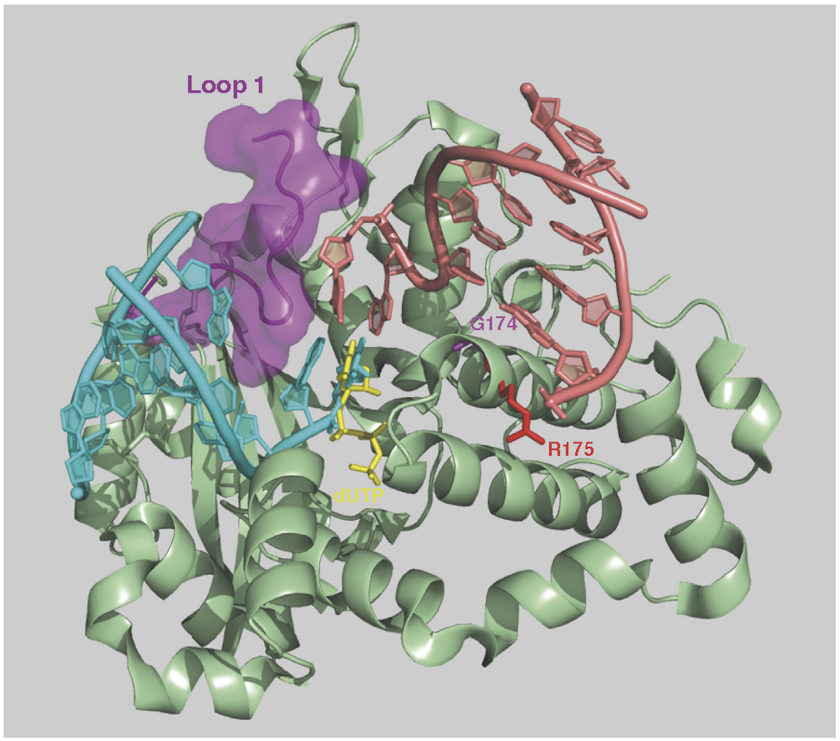

Figure 5. Modeling of human Polμ in a NHEJ reaction. The human Polμ polypeptide (in green) and the incoming nucleotide (dUTP; in yellow) were taken from PDB ID: 4M04, and modeled with a DSB with 2 bp-complementary ends (coral and blue) and Loop1 (magenta) derived from a co-crystal of mouse TdT (PDB ID: 5D46). The neighbor residues Gly^{174} and Arg^{175}, studied in this paper, are indicated.

positively charged in Polμ than in Polβ and Polλ, the latter two lacking template-independent activity. This could suggest that interaction with the 5′P limits terminal transferase activity, as was previously shown for Polμ (49). Moreover, this could also suggest that mutation of the interactors of the 5′P in Polμ, as in the case of the R175H mutant, can limit template-directed nucleotide insertions at DSBs.

However, it has been recently demonstrated that TdT can perform template-directed nucleotide insertions at DSBs, and suggested that these reactions may occur only after addition of non-templated nucleotides (14). Interestingly, introduction of an arginine in mouse TdT enhanced the incorporation of template-directed nucleotides (14), in full accordance with the role of this residue as a DNA ligand for NHEJ in Polμ. Interestingly, the overimposition of the structure of human Polμ:DNA-gap post-catalytic complex to the structures of mouse TdT-DSB post-catalytic complexes with a DSB with partially complementary or non-complementary ends (14; the first of this kind in the case of PolX members), provides an excellent fit at the area of Arg^{175} and Gly^{174} (Supplementary Figure S6). This allowed us to make a structural extrapolation of Polμ in a NHEJ context (Figure 5), which emphasizes the direct role of Loop 1 in connecting the two ends in both TdT and Polμ, and further supports that Arg^{175} in particular plays a direct role as DNA ligand during NHEJ, as shown in this paper. However, additional studies aimed to determining the structure of Polμ in complex with a DSB would be needed to further validate these conclusions.

Analysis of the R175H mutant also lead to another relevant conclusion: interaction with the 5′P flanking the gap is essential for the efficiency of Polμ on 2nt gaps. Several studies have demonstrated that when Polμ faces a 2 nt gap it preferably copies the second template base, which is the one closer to the 5′ end of the downstream DNA, at the cost of skipping the first available template base (18–22). This error-prone behavior is a stark difference between Polμ and Polλ, since the latter was shown to correctly fill 2nt gaps *in vivo* (18), thanks to its unique template scrunching ability (23). Interestingly, our work also indicates that, although the G174S and R175H mutations caused a similar defect in NHEJ, this was not the case in 2nt gap-filling reactions, indicating that they are defective NHEJ for different reasons. In fact, the G174S mutation was not deleterious for 2nt gap-filling, as it stimulated nucleotide incorporation opposite the first template base, decreasing –but not reverting- the preference of Polμ for the skipping ahead reaction. As mentioned earlier, Polλ harbors at the corresponding position of Gly^{174} a tryptophan (Trp^{274}), a bulky residue that performs stacking interaction with the template base paired to the 5′P-containing nucleotide that limits the gap. This stark difference was noticed when the structure of Polμ was first solved (45), although this has never been understood. Given the stimulating effect of Gly^{174} on skipping ahead during 2nt gap-filling by Polμ, it is temping to speculate that stacking interactions with the template DNA proximal to the gap may limit skipping ahead at 2nt gaps in the case of Polλ. Comparison of the structures of Polλ bound to a 1nt gap and to a 2nt gap reveals that Trp^{274} is

placed in close proximity to where the DNA is scrunched by Polλ in a 2nt gap (Supplementary Figure S5), and that in fact, it moves accordingly to the template DNA when it is scrunched (Supplementary Figure S5), suggesting that it may contribute to this process. In any case, the dramatic effect that the G174S mutation had on NHEJ by Polμ, both *in vivo* and *in vitro*, suggests that, in contrast to Polλ, Polμ requires a small, flexible and non-polar residue at that position to perform NHEJ efficiently, although at the cost of enhanced error-proneness at 2nt gaps. The fact that R175H and G174S showed similar defectiveness during NHEJ, but that only the latter was more effective in 2nt gaps indicates that the defect caused by the G174S mutation in NHEJ proceeds via inappropriate orientation of the DNA, but not by defective DNA binding. Different positioning of the DNA by the G174S mutant could explain why it incorporates nucleotides opposite the first template base of a DNA 2nt-gap more efficiently than wild-type Polμ. Moreover, unaffected binding of this mutant is in agreement with the fact that Gly^{174} is not a ligand of the 5′P group, unlike Arg^{175}. Our EMSA experiments support this idea, as only the R175H mutant showed a DNA binding defect. Hence, we speculate that Gly^{174}, being such a small and flexible residue, could allow maximal movement of the templating base so that Polμ can correctly orientate it for catalysis. This could be particularly relevant for NHEJ, especially of non-complementary ends where the primer and primer and template are not physically connected.

Overall, our characterization of the G174S and R175H mutants highlights the relevance of the characterization of tumor-associated variants of DNA polymerases for structure–function analyses and to anticipate to certain extent whether they could contribute to tumorigenesis. However, we must emphasize that our work does not establish a direct link between Polμ and the tumorigenesis process itself. Given that some consider that an early tumorigenesis step may be the mutation of genes that modulate the fidelity of DNA repair to increase the frequency of mutagenesis (54), it is tempting to speculate that the G174S and R175H could contribute to the process, due to their increased error-proneness during NHEJ *in vitro*. Although we have no explicit measurement of their fidelity of synthesis *in vivo*, the *in vitro* data suggests that they could lead to increased mutagenesis *in vivo* in response to DSBs. Furthermore, even if these mutants do not display increased error-proneness *in vivo*, defective Polμ activity *in vivo* could lead to remodeling of the DNA ends, implying end resection and loss of genetic information, so that they become compatible with repair by Polλ-mediated NHEJ. Regardless, further studies are required to determine the real impact of the G174S and R175H on tumor development, if any.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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