

## BIOCHEMISTRY

# Pol $\theta$ reverse transcribes RNA and promotes RNA-templated DNA repair

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Genome-embedded ribonucleotides arrest replicative DNA polymerases (Pols) and cause DNA breaks. Whether mammalian DNA repair Pols efficiently use template ribonucleotides and promote RNA-templated DNA repair synthesis remains unknown. We find that human Pol $\theta$  reverse transcribes RNA, similar to retroviral reverse transcriptases (RTs). Pol $\theta$  exhibits a significantly higher velocity and fidelity of deoxyribonucleotide incorporation on RNA versus DNA. The 3.2-Å crystal structure of Pol $\theta$  on a DNA/RNA primer-template with bound deoxyribonucleotide reveals that the enzyme undergoes a major structural transformation within the thumb subdomain to accommodate A-form DNA/RNA and forms multiple hydrogen bonds with template ribose 2'-hydroxyl groups like retroviral RTs. Last, we find that Pol $\theta$  promotes RNA-templated DNA repair in mammalian cells. These findings suggest that Pol $\theta$  was selected to accommodate template ribonucleotides during DNA repair.

## INTRODUCTION

Polymerase  $\theta$  (Pol $\theta$ ) is a unique DNA polymerase-helicase fusion protein in higher eukaryotes whose A-family polymerase domain evolved from Pol I enzymes (Fig. 1A) (1, 2). However, contrary to most Pol I enzymes, Pol $\theta$  is highly error-prone and promiscuous (3–6), performs translesion synthesis (TLS) opposite DNA lesions (3, 7, 8), and facilitates microhomology-mediated end-joining (MMEJ) of double-strand breaks (DSBs) by extending partially base-paired 3' single-stranded DNA (ssDNA) overhangs at DSB repair junctions (5, 9–12). Pol $\theta$  is not expressed in most tissues but is highly expressed in many cancer cells, which corresponds to a poor clinical outcome (13, 14). Furthermore, Pol $\theta$  confers resistance to genotoxic cancer therapies and promotes the survival of cells deficient in DNA damage response pathways (11, 13–16). Thus, Pol $\theta$  represents a promising cancer drug target.

Intriguingly, Pol $\theta$  has an inactive proofreading domain due to acquired mutations (Fig. 1A) (2). Inactivating the 3'-5' proofreading function of closely related A-family bacterial Pol I Klenow fragment (KF) enables this polymerase to reverse transcribe RNA like retroviral reverse transcriptases (RTs), which lack proofreading activity (fig. S1A) (17, 18). Because Pol $\theta$  is highly error-prone and promiscuous and contains an inactive proofreading domain, we hypothesized that it has RNA-dependent DNA synthesis activity. Given that ribonucleotides are the most frequently occurring nucleotide lesion in genomic DNA that arrest replicative Pols and cause DNA breaks (19, 20), we also envisaged that Pol $\theta$  would tolerate template ribonucleotides during its DNA repair activities and thus promote RNA-templated DNA repair synthesis (RNA-DNA repair). Although RNA-DNA repair mechanisms have been demonstrated in genetically engineered yeast cells (21, 22), they remain obscure in mammalian cells.

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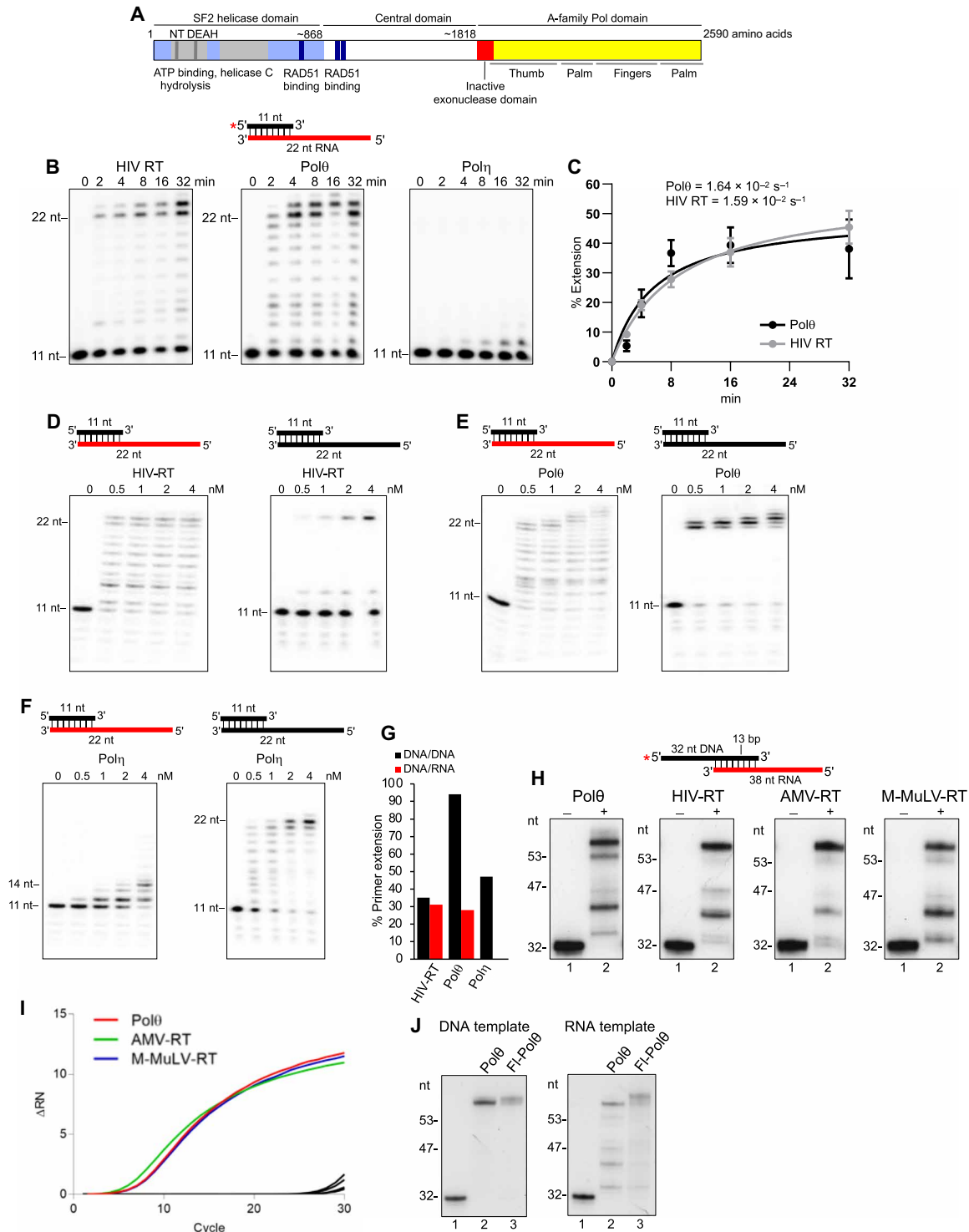
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## RESULTS

## Pol $\theta$ exhibits RNA-dependent DNA synthesis activity

We tested whether the polymerase domain of Pol $\theta$  (herein referred to as Pol $\theta$ ) reverse transcribes RNA like HIV RT using a DNA primer annealed to a RNA template (DNA/RNA). Pol $\theta$  exhibits a similar rate of RT activity as HIV RT under identical conditions using substoichiometric amounts of enzyme relative to template (Fig. 1, B and C). Previous studies indicated that human Pol $\eta$  has RT activity at high micromolar concentrations (23). We find that Pol $\eta$  fails to perform reverse transcription beyond 3 nucleotides (nt) using conditions identical to those of Pol $\theta$  and HIV RT at multiple concentrations (Fig. 1, B and C; compare Fig. 1F with Fig. 1, D and E; Fig. 1G). However, at significantly higher concentrations, Pol $\eta$  can further extend the DNA/RNA (figs. S1, B to E). Controls show that Pol $\eta$  is active on a DNA/DNA template like Pol $\theta$  and HIV RT (compare Fig. 1F with Fig. 1, D and E; Fig. 1G). Overall, Pol $\eta$  exhibits increased stalling on RNA and requires higher enzyme concentration relative to Pol $\theta$  for reverse transcription (figs. S1, B to E). Pol $\theta$  RNA-dependent DNA synthesis activity is observed under various conditions and on different template constructs (figs. S1, F and G), and sequences (fig. S1H). Despite its robust activity on RNA, Pol $\theta$  strongly discriminates against incorporating ribonucleotides (fig. S1I). Complementary DNA (cDNA) sequencing confirms Pol $\theta$ 's RNA-dependent DNA synthesis activity (fig. S2A) and reveals nucleotide misincorporations and indels, which is consistent with its low-fidelity DNA synthesis activity (fig. S2A) (3). HIV RT and other retrovirus RTs are also highly error-prone, demonstrating a shared characteristic between Pol $\theta$  and retroviral RTs (18, 24–28). In addition to HIV RT, Pol $\theta$  activity on RNA is nearly identical to RTs encoded by Moloney murine leukemia virus (M-MuLV) and avian myeloblastosis virus (AMV) (Fig. 1H). Pol $\theta$  exhibits pausing events nearly identical to those of retrovirus RTs, which is consistent with pausing tendencies for RTs (Fig. 1H) (29, 30). Pol $\theta$  cDNA synthesis on synthetic RNA is similar to M-MuLV and AMV RTs (Fig. 1I and fig. S2B), and Pol $\theta$  also promotes cDNA synthesis of purified *Escherichia coli* 16S ribosomal RNA similar to M-MuLV and AMV RTs (fig. S2C).



**Fig. 1. Polθ exhibits reverse transcriptase activity.** (A) Schematic of full-length Polθ. (B) Denaturing gels showing a time course of DNA/RNA primer-template extension by the indicated polymerases. (C) Plot showing relative rate of DNA/RNA extension by Polθ and HIV RT. Data represent mean ± SD; n = 3. (D to F) Denaturing gels showing DNA/RNA (left) and DNA/DNA (right) extension by the indicated polymerases. (G) Bar plot showing percent extension of DNA/RNA (red) and DNA/DNA (black) by the indicated polymerases (4 nM) [data from (D) to (F)]. (H) Denaturing gels showing DNA/RNA extension by the indicated polymerases. (I) Quantitative PCR chromatogram showing cDNA synthesis by the indicated polymerases. (J) Denaturing gels showing DNA/DNA (left) and DNA/RNA (right) extension by Polθ and FI-Polθ.

The efficient RT activity of Pol $\theta$  appears to be unique among human Pols. Eight other human Pols, representative of at least two enzymes from each polymerase family in humans (A, B, X, and Y), fail to reverse transcribe DNA beyond 2 to 3 nt under conditions identical to those of Pol $\theta$  (Fig. 1F and fig. S3). Y-family polymerase  $\kappa$  (Pol $\kappa$ ) exhibits limited RT activity under various conditions similar to Pol $\eta$  (Fig. S3H). Replicative Pols  $\delta$  and  $\epsilon$  degrade the DNA primer on RNA due to exonuclease activity (fig. S3, F and G). All Pols are active on DNA as expected (Fig. 1F and fig. S3). Full-length Pol $\theta$  (Fl-Pol $\theta$ ) containing an N-terminal superfamily 2 and disordered central domain (Fig. 1A) (1) also has RT activity, suggesting that the endogenous protein performs RNA-DNA repair in cells (Fig. 1J). Because recombinant human Pol $\theta$  and Fl-Pol $\theta$  were purified from different organisms (*E. coli* and *Saccharomyces cerevisiae*, respectively) and by different methods (1, 9), the observed RT activity is not due to a protein contaminant. Consistent with this, human recombinant Pol $\theta$  purified from *S. cerevisiae* also has robust RT activity (fig. S4A) (31).

### Pol $\theta$ exhibits higher velocity and fidelity of deoxyribonucleotide incorporation on RNA

We tested the relative velocity of Pol $\theta$  deoxyribonucleoside monophosphate (dNMP) incorporation on RNA versus DNA. Single deoxyribonucleoside triphosphates (dNTPs) were incubated with Pol $\theta$  during a time course on DNA/RNA versus DNA/DNA with identical sequence (Fig. 2A). Pol $\theta$  exhibits a significantly higher velocity of incorporating deoxycytidine monophosphate (dCMP), deoxythymidine monophosphate (dTMP), and deoxyadenosine monophosphate (dAMP) on RNA (Fig. 2, B to D, and fig. S4B). The velocity of deoxyguanosine monophosphate (dGMP) incorporation was similar on RNA and DNA (Fig. 2E). Fl-Pol $\theta$  also exhibits higher rates of dCMP, dTMP, and dAMP incorporation on RNA (fig. S5). Pol $\theta$  exhibits a twofold higher affinity for DNA/RNA (fig. S4C), which may contribute to the higher velocity of nucleotide incorporation.

To assess the relative fidelity of dNMP incorporation by Pol $\theta$  on RNA versus DNA, we measured the relative velocity of nucleotide misincorporation. Remarkably, Pol $\theta$  is significantly more accurate on RNA versus DNA as demonstrated by its severely limited ability to misincorporate nucleotides on RNA versus DNA (Fig. 2, F to J). For example, in Fig. 2 (G and H), Pol $\theta$  fully misincorporates dCMP and dAMP, respectively, on DNA in less than 2 min. Yet, on RNA, Pol $\theta$  is unable to fully misincorporate dCMP or dAMP even after 20 min (Fig. 2, G and H). Pol $\theta$  also fails to effectively misincorporate dTMP on RNA but efficiently misincorporates dTMP on DNA (Fig. 2J). Remarkably, Pol $\theta$  rapidly misincorporates several consecutive dGMPs on DNA yet fails to fully misincorporate a single dGMP on RNA even after 20 min (Fig. 2I). Consecutive dGMP misincorporation events on DNA suggest that Pol $\theta$  more easily misaligns the DNA/DNA primer-template (fig. S6A). The higher fidelity of Pol $\theta$  on RNA versus DNA is also observed in a different sequence context (fig. S6, B to F). Hence, despite the enzyme's overall higher rate of correct dNMP incorporation on RNA, it exhibits a substantially slower rate of misincorporation on RNA. These data suggest that Pol $\theta$  evolved to be more accurate on RNA similar to HIV RT (32).

### Ternary structure of Pol $\theta$ on a DNA/RNA primer-template

The higher fidelity of Pol $\theta$  on RNA suggests that it binds the DNA/RNA and/or active site deoxyribonucleotide:ribonucleotide

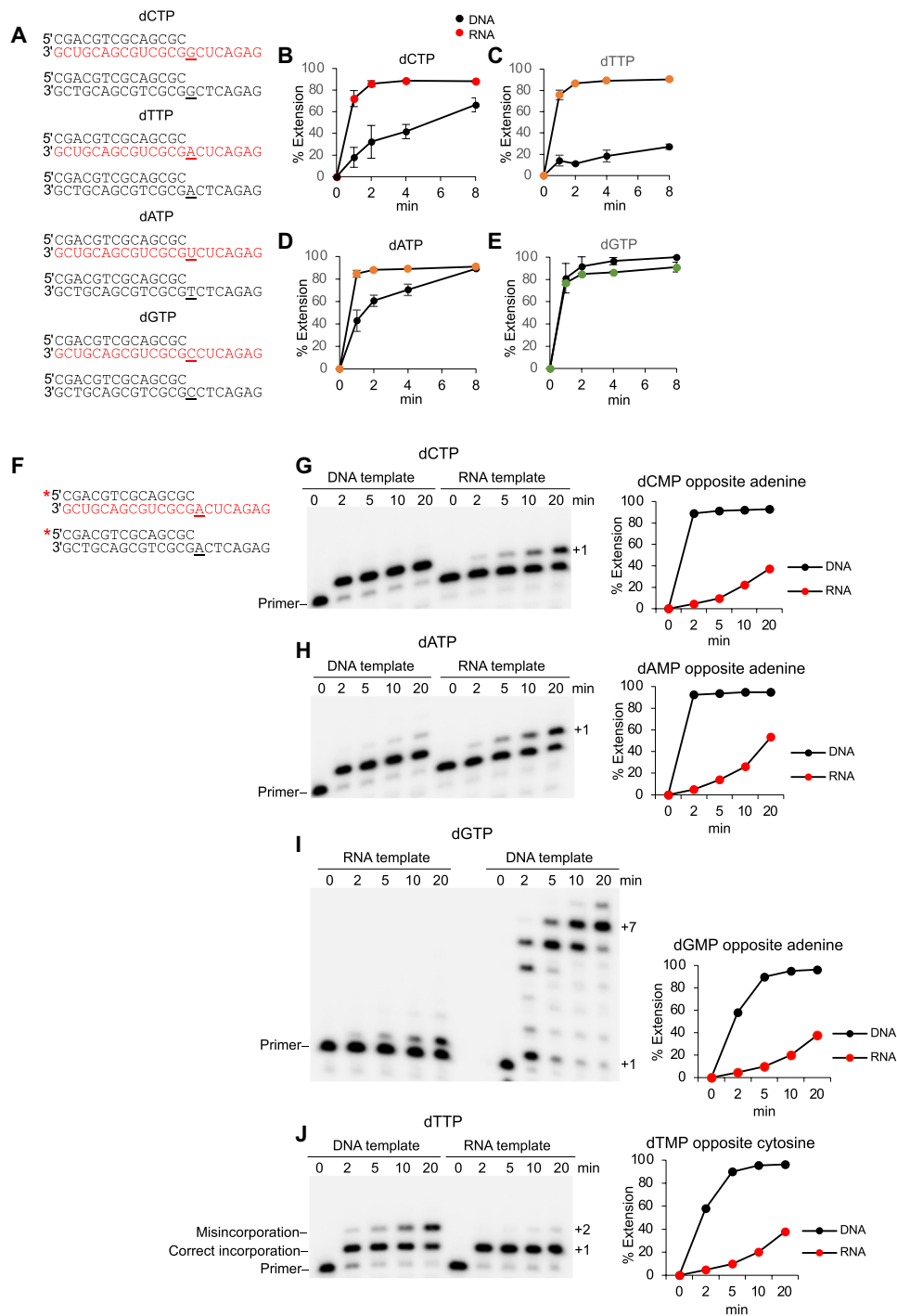
base pair in a distinct conformation. To investigate the molecular basis of Pol $\theta$  RT activity, we solved a 3.2-Å crystal structure of Pol $\theta$  on DNA/RNA with incoming 2',3'-dideoxyguanosine triphosphate (ddGTP) (Fig. 3). The construct used for x-ray crystallography (Pol $\theta$  $\Delta$ L) was engineered to achieve higher *E. coli* expression by replacing five small disordered loops, which were not resolved in previous Pol $\theta$ :DNA/DNA structures (2), with short glycine-serine inserts (Fig. 3A and fig. S7, A to C). The endogenous insert 2 loop promotes Pol $\theta$  MMEJ of DNA/DNA with 3' ssDNA overhangs containing microhomology (9), and inserts 2 and 3 contribute to TLS (2, 7). WT (wild type) and Pol $\theta$  $\Delta$ L exhibit similar DNA/DNA and DNA/RNA primer extension activities, demonstrating that the disordered loops do not substantially contribute to reverse transcription and canonical DNA/DNA extension (Fig. 3B and fig. S7D). In contrast to prior Pol $\theta$ :DNA/DNA:ddNTP structures that were captured in the closed conformation (2), the Pol $\theta$  $\Delta$ L:DNA/RNA:ddGTP complex is in the open form, whereby the O helix of the fingers subdomain is rotated outward by 42° and the bound ddGTP is solvent-exposed (Fig. 3, C and D, and fig. S8a). This demonstrates that Pol $\theta$  shares the induced-fit nucleotide incorporation mechanism with related A-family polymerases (2, 33).

Unexpectedly, the thumb subdomain undergoes a major reconfiguration. Fifty-seven percent of thumb subdomain residues refold from  $\alpha$  helices to loops (Fig. 3D and fig. S7C, thumb domain), which may be necessary to accommodate the thicker A-form DNA/RNA. A loop shift in the palm subdomain involving E2246 is also observed on the opposite side of the DNA/RNA, suggesting a specific ribose 2'-hydroxyl interaction with its main-chain carbonyl likely mediated through a water molecule (Fig. 3E, gray). Superposition of the DNA/RNA from our structure onto DNA/DNA from the prior Pol $\theta$ :DNA/DNA:ddGTP structure reveals that the DNA/RNA has a shorter distance between neighboring base pairs near the 3' primer terminus (Fig. 3F and fig. S8B). The general features of the DNA/RNA are A-form-like, whereas the DNA/DNA is B-form-like upstream from the active site (Fig. 3F and fig. S8b). Structural irregularities (weakly paired bases and mismatches) are observed near the upstream portion of the DNA/RNA (Fig. 3I). The incoming ddGTP and complementary cytidine on the RNA template also show a significant shift relative to the Pol $\theta$ :DNA/DNA:ddGTP complex (Fig. 3G).

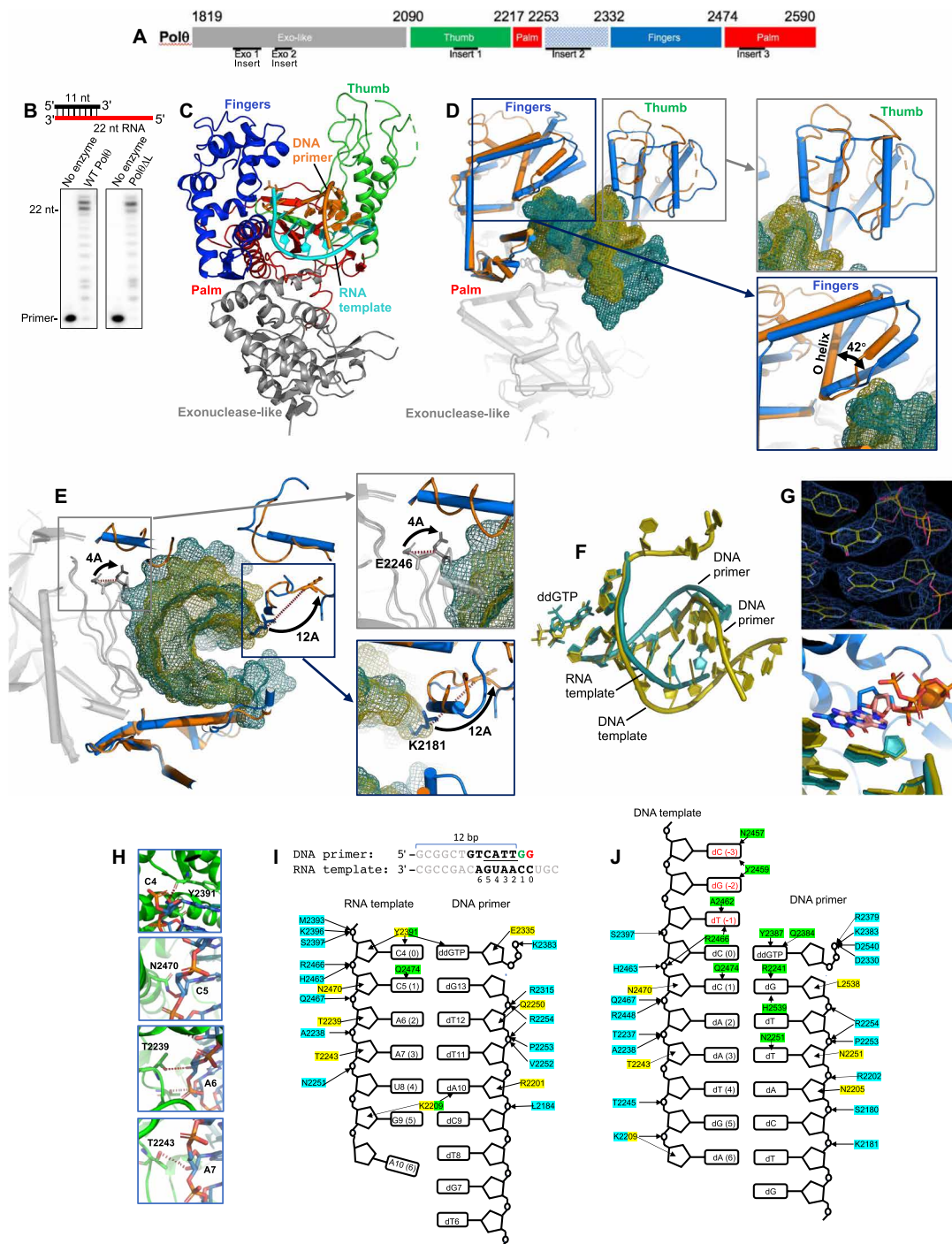
Pol $\theta$  accommodation of DNA/RNA also involves many RNA template interactions (compare Fig. 3, I and J). Multiple hydrogen bonds with ribose 2'-hydroxyl groups along the RNA template are observed (Fig. 3, H and I). Pol $\theta$ , HIV RT, and M-MuLV RT form a similar carbonyl hydrogen bond with the 2'-hydroxyl group of the active site template ribose, suggesting a conserved mechanism of active site RNA template binding (fig. S9, C and D) (34, 35). This interaction and additional Pol $\theta$ :2'-hydroxyl ribose hydrogen bonds along the RNA template may suppress Pol $\theta$  template misalignment errors and thus potentially contribute to its higher fidelity on RNA. Overall, the formation of multiple specific Pol $\theta$ :RNA interactions combined with the major thumb subdomain reconfiguration and palm subdomain loop shift reveal how the polymerase becomes active on a DNA/RNA hybrid.

### Pol $\theta$ promotes RNA-templated DNA repair

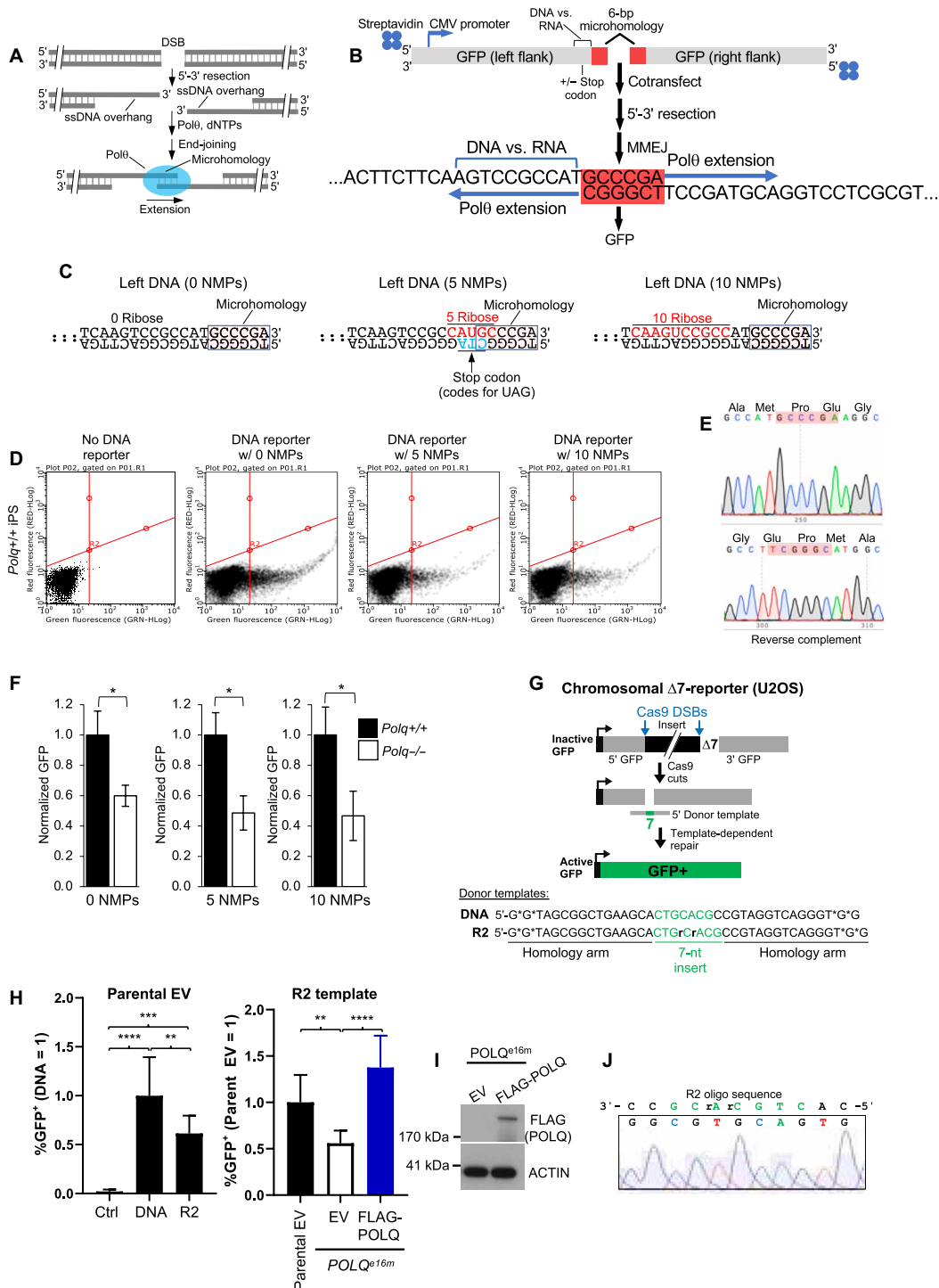
To test whether Pol $\theta$  promotes RNA-templated DNA repair synthesis (RNA-DNA repair) in a biological setting, we developed a cellular green fluorescent protein (GFP) reporter assay that can simultaneously quantitate Pol $\theta$  MMEJ and RNA-DNA repair



**Fig. 2. Polθ exhibits a higher fidelity and velocity of deoxyribonucleotide incorporation on RNA.** (A) Schematic of DNA/DNA and DNA/RNA templates used for the indicated dNTPs. Underlined base codes for the incoming nucleotide. Red, RNA; black, DNA. (B to E) Plots showing relative velocity of the indicated deoxyribonucleotide incorporation by Polθ on DNA/RNA and DNA/DNA primer-templates. Red, DNA/RNA; black, DNA/DNA. Data represent mean ± SD;  $n = 3$ . (F) Schematic of DNA/RNA and DNA/DNA primer-templates. Underlined base codes for the correct incoming nucleotide. Red, RNA; black, DNA. (G to J) Denaturing gels showing a time course of Polθ primer extension on the indicated DNA/RNA and DNA/DNA primer-templates in the presence of the indicated deoxyribonucleotide (left). Plots showing percent extension over time (right). Red, DNA/RNA; black, DNA/DNA. dTMP, thymidine monophosphate.



**Fig. 3. Ternary structure of Polθ on a DNA/RNA primer-template.** (A) Polθ polymerase. (B) DNA/RNA extension by Polθ and PolθΔL. (C) Structure of Polθ:DNA/RNA:ddGTP. (D) Superposition of Polθ:DNA/RNA (marine) and Polθ:DNA/DNA (orange, 4x0q). The fingers and thumb subdomains undergo reconfiguration. (E) Superposition of Polθ:DNA/RNA (marine) and Polθ:DNA/DNA (orange, 4x0q) highlighting a 12-Å shift of K2181 (blue box; palm) and a 4.4-Å shift of E2246 (grey box; palm). (F) Superposition of nucleic acids and ddGTP from Polθ:DNA/RNA:ddGTP and Polθ:DNA/DNA:ddGTP structures. (G) Top: Electron density of ddGTP and 3' primer terminus in Polθ:DNA/RNA structure. Bottom: Zoomed-in image of the superposition of active sites, illustrating a different conformation of ddGTP in the Polθ:DNA/RNA (blue) and Polθ:DNA/DNA (salmon) complexes. (H) Interactions between ribose 2'-hydroxyl groups of the RNA template and residues in the Polθ:DNA/RNA structure. Red dashed lines, hydrogen bonds. (I) DNA/RNA used for cocrystallization with Polθ and ddGTP (top). Strong electron density is present for four base pairs [nucleotides located at positions 2 to 5 (underlined) of the DNA/RNA] and two base pairs resulting from an incorporated ddGMP (2';3' dideoxyguanosine monophosphate) (green; position 1) and a bound unincorporated ddGTP (red; position 0) in the active site (top). Interactions between Polθ and nucleic acids in Polθ:DNA/RNA:ddGTP (bottom). Interactions between residues and phosphate backbone, sugar oxygen, or nucleobase are shown in blue, yellow, and green, respectively. Hydrogen bonds between Polθ and ribose 2'-hydroxyl groups are indicated (boxed residues). (J) Interactions between Polθ and nucleic acids in Polθ:DNA/DNA:ddGTP (4x0q). Color scheme identical to (I).



**Fig. 4. Polθ promotes RNA-templated DNA repair synthesis in cells.** (A) Schematic of MMEJ. (B) Schematic of GFP MMEJ reporter assay. (C) Sequences of downstream end of left DNA GFP constructs with and without NMPs. (D) GFP FACS plots following no transfection (left) and cotransfection of the indicated right DNA constructs in *Polθ*<sup>+/+</sup> iPSCs. (E) Sequencing chromatograms showing MMEJ of the left and right constructs. Red, microhomology. (F) Bar plots showing normalized GFP cells following cotransfection of the indicated reporter constructs. Data pooled from three independent experiments performed at least in duplicate. ± SEM. \*Statistical significance from paired t test: *P* = 0.04, 0 NMPs; *P* = 0.025, 5 NMPs; *P* = 0.04, 10 NMPs. (G) Δ7-GFP reporter integrated in U2OS cells for measuring CRISPR-Cas9 donor template [DNA (\*phosphorothioate linkages) or DNA with two RNA bases (R2)]-mediated genome engineering. Ribonucleotides, rCrA; Green text, knock-in donor sequence. (H) Shown are frequencies of GFP<sup>+</sup> cells (±SD) for the oligonucleotides shown, normalized to transfection efficiency and to the DNA template (=1, left) and the R2 template in the parental cell line (=1, right). \*\**P* ≤ 0.0043, \*\*\**P* = 0.0001, and \*\*\*\**P* < 0.0001, one-way analysis of variance with Tukey's (left) and Dunnett's (right) posttests. (I) Immunoblot analysis of FLAG-POLQ and actin control. (J) Sequence of an amplification product from GFP<sup>+</sup> cells isolated from *POLQ*<sup>e16m</sup> cells with the Δ7 reporter assay using the R2 donor template and POLQ expression vector.

(Fig. 4B). Polθ is essential for MMEJ of DSBs, such as those caused by ionizing radiation (1, 9–11), and performs TLS (7, 31). Polθ therefore regularly uses aberrant template bases during its DNA repair activities. Polθ promotes MMEJ by using microhomology [ $\geq 2$  base pairs (bp)] between 3' ssDNA overhangs generated by 5'-3' resection of DSBs and then extends the partially base-paired overhangs (Fig. 4A). The MMEJ GFP reporter assay is described as follows. Left and right DNA constructs respectively encoding the upstream and downstream portion of a GFP expression vector with 6 bp of overlapping sequence (microhomology) were conjugated with streptavidin at 5' DNA termini opposite from the microhomology tract to suppress 5'-3' exonuclease activity at these ends (Fig. 4B). Introduction of 5 or 10 ribonucleoside monophosphates (NMPs) into the nontemplate transcription strand immediately upstream from the microhomology tract within the left construct enables analysis of RNA-templated DNA repair synthesis during MMEJ (Fig. 4, B and C). In the case of the left construct with five NMPs, base mutations within the transcription template strand directly opposite the RNA tract were added to engineer a stop codon in the GFP coding sequence (Fig. 4C, middle). Cotransfection of the right construct and left construct with or without NMP tracts into mouse induced pluripotent stem cells (iPSCs) activates GFP, demonstrating the capacity of MMEJ to use deoxyribose and ribose nucleobases as templates for DNA repair synthesis (Fig. 4D). Sequencing confirms MMEJ of the left and right DNA constructs in cells as well as error-prone repair (Fig. 4E and fig. S10). Cotransfection of the right construct and left construct without NMPs results in significantly higher %GFP in *Polq*<sup>+/+</sup> versus *Polq*<sup>-/-</sup> iPSCs that were previously characterized (Fig. 4F, left) (10). This demonstrates that Polθ promotes MMEJ as expected. Cotransfection of the right construct with the left construct containing 5 or 10 NMPs also results in significantly higher %GFP in *Polq*<sup>+/+</sup> iPSCs, demonstrating that Polθ promotes RNA-DNA repair (Fig. 4F, middle and right). In the case of the left construct containing the stop codon opposite the 5-NMP tract (Fig. 4C, middle), RT activity is essential for generating a transcription template strand lacking the stop codon. Hence, these data confirm that Polθ promotes RNA-dependent DNA synthesis in cells.

We further investigated Polθ RNA-DNA repair in cells using a previously published GFP knock-in reporter assay ( $\Delta 7$ -reporter) that is chromosomally integrated in human U2OS cells (Fig. 4G) (36). This assay has a GFP expression cassette disrupted by an insert sequence, along with a deletion of 7 bp of GFP sequence ( $\Delta 7$ ). An oligonucleotide donor with these 7 nt flanked by 16-nt homology arms on each side can template restoration of active GFP<sup>+</sup> (36). This event is induced via CRISPR-Cas9 to generate two DSBs that excise the insert. We compared the frequency of such repair from the positive control DNA donor-template versus a variant (R2) donor-template that contains two ribonucleotides within the 7 nt missing from the  $\Delta 7$ -reporter (Fig. 4G, bottom), finding only a modest decrease with the R2 template versus DNA (Fig. 4H, left). To examine the influence of Polθ on the R2-templated event, we used a previously described POLQ-polymerase deficient cell line (*POLQ* <sup>$\epsilon 16m$</sup> ) with this reporter (36), which we compared both to the parental cell line and to the mutant cells with expression of FLAG-POLQ (Fig. 4I). From either comparison, we found that Polθ promotes a significant increase in the frequency of GFP<sup>+</sup> cells using the R2 donor template, which further confirms its ability to reverse transcribe template ribonucleotides during DNA repair in a biological setting (Fig. 4H, right).

## DISCUSSION

Our study unexpectedly reveals that Polθ reverse transcribes RNA and undergoes a significant structural transformation to accommodate a DNA/RNA template. The structural transformation of Polθ's thumb subdomain is likely needed to maintain productive interactions on DNA/RNA, which adopts a significantly different conformation relative to DNA/DNA in the Polθ complex (Fig. 3F and fig. S11B). In contrast, structurally characterized retroviral RTs, such as HIV-RT, do not exhibit structural refolding of their thumb subdomain when acting on DNA/RNA (fig. S11A). The marked structural-functional switch within the thumb subdomain observed in Polθ has not been previously observed in other DNA polymerases or retroviral RTs and therefore may be unique to Polθ, which is an unusually promiscuous enzyme that is capable of acting on a variety of different templates including DNA/DNA, DNA/RNA, ssDNA, partial ssDNA, and single-stranded RNA (1, 5, 9, 37). Together, these structural studies reveal that Polθ has an extraordinary degree of structural plasticity that enables it to efficiently transcribe template ribonucleotides and accommodate a full RNA-DNA hybrid within its active site. Although future studies will be required to fully elucidate the physiological relevance of Polθ RT activity, our findings demonstrate that Polθ accommodates template ribonucleotides in an active configuration and promotes RNA-DNA repair, which may contribute to cellular tolerance of genome-embedded ribonucleotides.

## MATERIALS AND METHODS

### Primer-template extension assays

#### Relative velocity of RT activity (Fig. 1B)

Polθ (0.5 nM), HIV RT, and Polη (catalytic core, residues 1 to 514) were incubated with 10 nM radiolabeled DNA/RNA template (RP559/RP493R) for the indicated times in buffer A [25 mM tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 0.01% (v/v) NP-40, 1 mM dithiothreitol (DTT), bovine serum albumin (BSA; 0.1 mg/ml), and 10% (v/v) glycerol] with 100 μM dNTPs at 37°C. Percent extension was determined by dividing the intensity of the extended product by the intensity of the sum of extended and unextended products for each lane. All primer-template reactions were terminated with 25 mM EDTA and 45% (v/v) formamide then resolved in urea denaturing polyacrylamide gels and visualized by PhosphorImager. The rate of RT activity was determined from the slope of the linear portion of the plot representing steady-state conditions.

#### Comparison of polymerase activities on DNA/DNA and DNA/RNA (Fig. 1, D to G)

The primer extension assays on DNA/RNA (RP559/RP493R) and DNA/DNA (RP559/RP493D) templates were performed using the conditions in Fig. 1B with the following changes. The indicated concentrations of the indicated polymerases were used, and the reactions were performed for 32 min.

#### Relative RT activity (Fig. 1H)

The indicated polymerases were incubated with 10 nM radiolabeled DNA/RNA template (SM98/SM44R) for 20 min in the presence of 10 μM dNTPs at 37°C. Polθ and HIV RT reactions were performed in 25 mM tris-HCl (pH 8.0), 10 mM KCl, 10 mM MgCl<sub>2</sub>, 0.01% (v/v) NP-40, 1 mM DTT, BSA (0.1 mg/ml), and 10% (v/v) glycerol. AMV RT (20 units, New England Biolabs)-containing reactions were performed in buffer [50 mM tris-acetate (pH 8.3), 75 mM potassium acetate, 8 mM magnesium acetate, and 10 mM DTT] and

contained BSA (0.1 mg/ml). M-MuLV RT (400 units, New England Biolabs)—containing reactions were performed in buffer [50 mM tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, and 10 mM DTT] and contained BSA (0.01 mg/ml).

#### **Comparison of RT and DNA-dependent DNA synthesis activities by truncated and full-length Polθ (Fig. 1J)**

One hundred nanomolar of the indicated polymerases were incubated with 10 nM radiolabeled DNA/RNA (SM98/SM44R) or DNA/DNA (SM98/SM44) for 45 min with 50 μM dNTPs and 25 mM tris-HCl (pH 7.8), 2 mM MgCl<sub>2</sub>, 4 mM KCl, 6 mM NaCl, 0.01% (v/v) NP-40, 1 mM DTT, BSA (0.1 mg/ml), 10% (v/v) glycerol, and 750 μM adenosine triphosphate (ATP) at 37°C.

#### **Relative velocity of single dNMP incorporation on radiolabeled DNA/RNA (Fig. 2, A to E)**

Polθ (2 nM) was incubated with 100 nM of the indicated radiolabeled DNA/RNA and DNA/DNA templates for the indicated times with 300 μM of the indicated dNTP in buffer [25 mM tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 4 mM KCl, 6 mM NaCl, 0.01% NP-40, 1 mM DTT, BSA (0.01 mg/ml), and 10% (v/v) glycerol] at 37°C. Percent extension was determined as described above.

#### **Relative velocity of nucleotide (dNMP) misincorporation (Fig. 2, F to J)**

Polθ (20 nM) was incubated with 100 nM of the indicated radiolabeled DNA/RNA and DNA/DNA templates and 300 μM of the indicated dNTP for the indicated times in buffer [25 mM tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 10 mM KCl, 0.01% (v/v) NP-40, 1 mM DTT, BSA (0.1 mg/ml), and 10% (v/v) glycerol]. Percent extension was determined as described above. All oligonucleotides were radiolabeled using T4 polynucleotide kinase (New England Biolabs) and <sup>32</sup>P-γ-ATP (PerkinElmer) in recommended buffer for 37°C for at least 1 hour.

#### **MMEJ cellular assay**

Some of the methods used here are similar to those in a previously published article (38). iPSCs ( $2 \times 10^5$ ) were transfected in suspension with 0.25 μg each of the indicated left- and right-flanking DNA GFP constructs using Lipofectamine 2000 (Invitrogen). As a negative control, similar volume of buffer that was used in experimental wells was used for transfection in control wells. As a positive control to measure transfection efficiency, a wild-type linear DNA GFP expression construct was transfected simultaneously. GFP-positive cell frequencies were measured 3 days after transfection by flow cytometry using GUAVA easyCyte 5-HT (Luminex Corp.) in independent replicates and corrected for transfection efficiency and background events. Data are represented as the mean and SEM of three independent experiments, with at least duplicates per experiment. Statistical analysis was carried out by paired *t* test.

#### **Preparation of GFP MMEJ reporter constructs**

Some of the methods used here are similar to those in a previously published article (38). Polymerase chain reaction (PCR) preparation followed recommended conditions for the Phusion High-Fidelity DNA Polymerase (New England Biolabs M0530) using 10 ng of pCMV-GFP plasmid as template in 1× Phusion HF Buffer. PCR for the left-flank DNA was performed with primers RP500B and RP501. PCR for the right-flank DNA was performed with primers RP502 and RP503B. Following PCR, left- or right-flank DNA products were pooled together and digested with Dpn I (New England Biolabs) in 1× CutSmart buffer and then purified via Qiagen QIAquick PCR Purification Kit. PCR was then conjugated to

streptavidin using PCR (110 ng/μl) and streptavidin (0.8 μg/μl) in 10 mM tris-HCl (pH 7.5) and 100 mM NaCl at 37°C for 1 hour. PCR for the left-flank DNA with five consecutive ribonucleotides was performed with primers RP500B and RP501. PCR was purified and then digested with Dpn I (New England Biolabs) and Sap I in 1× CutSmart buffer. PCR was purified again and then ligated to double-stranded DNA composed of annealed oligonucleotides RP550R-P and RP530a [oligos were annealed in the presence of a ribonuclease (RNase) inhibitor] for 16 hours at 16°C with T4 DNA Ligase (New England Biolabs) in 1× T4 DNA Ligase Buffer. Ligated PCR was purified and then conjugated to streptavidin as described above. PCR for the left-flank DNA with 10 consecutive ribonucleotides was prepared by the same methodology with ligation to the double-stranded DNA composed of oligos RP546P and RP530. Streptavidin conjugation and DNA amplification steps were confirmed in agarose gels stained with ethidium bromide.

#### **CRISPR-Cas9 knock-in GFP reporter assay**

The U2OS parental and *POLQ*<sup>e16m</sup> cell lines with the Δ7-reporter and CAS9/single guide RNA (sgRNA) plasmids to target the DSBs in this reporter, DNA oligonucleotide template, and control oligonucleotide (LUC) were previously described (36). The R2 oligonucleotide has the same sequence as the DNA oligonucleotides, but with two RNA bases in the 7 nt missing from the Δ7-reporter (IDT). The cell lines were seeded at  $1 \times 10^5$  on a 12-well dish and transfected the following day, and % GFP was analyzed 3 days after transfection using a CyAN-ADP (DAKO) cytometer and normalized to transfection efficiency, as previously described (36). Transfections for the reporter assay contained 400 ng each CAS9/sgRNA plasmid, 10 pmol oligonucleotide, and 100 ng of either pCAGGS-BSKX (empty vector) or FLAG-POLQ expression vector (38). Transfections for transfection efficiency contained 400 ng of pCAGGS-NZE-GFP (GFP expression vector), 500 ng of empty vector (EV), and 10 pmol control oligonucleotide (36). Transfections were performed with 4 μl of Lipofectamine 2000 (Thermo Fisher Scientific) in 0.2 ml of Optimem (Thermo Fisher Scientific) and incubated with cells in 1 ml of antibiotic-free medium for 4 hours. Immunoblotting analysis for FLAG-POLQ involved extraction with ELB [250 mM NaCl, 5 mM EDTA, 50 mM Hepes, 0.1% (v/v) Ipegal, and Roche protease inhibitor] with sonication (Qsonica, Q800R) and using antibodies for FLAG (Sigma-Aldrich, A8592) or ACTIN (Sigma-Aldrich, A2066). Sequence analysis of reporter assay with R2 oligo: Cells were transfected as for the reporter assay with the R2 oligonucleotide template in the *POLQ*<sup>e16m</sup> cells with the POLQ expression vector, GFP<sup>+</sup> cells were isolated by fluorescence-activated cell sorting (FACS) (Becton Dickinson Aria Sorter), and the GFP repair product was amplified with CMVFWDFRT5 5' CGCAAATGGGCG-GTAGGCGTG and BGHREVFRT5 5' TAGAAGGCACAGTC-GAGG and sequenced with the CMVFWDFRT5 primer.

#### **cDNA synthesis**

cDNA synthesis reactions were performed by the indicated polymerase in the presence of 100 μM dNTPs and optimal buffer for each enzyme: Polθ [25 mM tris-HCl (pH 8.0), 10 mM KCl, 10 mM MgCl<sub>2</sub>, 0.01% (v/v) NP-40, 1 mM DTT, BSA (0.1 mg/ml), and 10% (v/v) glycerol]; AMV RT [50 mM tris-acetate (pH 8.3), 75 mM potassium acetate, 8 mM magnesium acetate, 10 mM DTT, and BSA (0.1 mg/ml)]; and M-MuLV RT [50 mM tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, and 10 mM DTT]. Reactions with





## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/7/24/eabf1771/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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