HHS Public Access

Author manuscript

DNA Repair (Amst). Author manuscript; available in PMC 2022 March 01.

Published in final edited form as:

DNA Repair (Amst). 2021 March ; 99: 103050. doi:10.1016/j.dnarep.2021.103050.

DNA Polymerase β Outperforms DNA Polymerase γ in Key Mitochondrial Base Excision Repair Activities

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Abstract

DNA polymerase beta (POLβ), well known for its role in nuclear DNA base excision repair (BER), has been shown to be present in the mitochondria of several different cell types. Here we present a side-by-side comparison of BER activities of POLβ and POLγ, the mitochondrial replicative polymerase, previously thought to be the only mitochondrial polymerase. We find that POLβ is significantly more proficient at single-nucleotide gap filling, both in substrates with ends that require polymerase processing, and those that do not. We also show that POLβ has a helicase-independent functional interaction with the mitochondrial helicase, TWINKLE. This interaction stimulates strand-displacement synthesis, but not single-nucleotide gap filling. Importantly, we find that purified mitochondrial extracts cells lacking POLβ are severely deficient in processing BER intermediates, suggesting that mitochondrially localized DNA POLβ may be critical for cells with high energetic demands that produce greater levels of oxidative stress and therefore depend upon efficient BER for mitochondrial health.

Keywords

POLB; POLG; BER; MITOCHONDRIA

3. INTRODUCTION

3.1 Base Excision Repair

Genome stability depends upon robust DNA repair of the numerous endogenous and exogenous insults experienced by nuclear and mitochondrial genomes. The cell devotes

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Author Statement

Beverly A. Baptiste wrote the paper, did experiments and contributed concept, Stephanie L. Baringer did experiments and edited the paper, Tomasz Kulikowicz did experiments and edited the paper, Joshua A. Sommers did experiments and edited the paper, Deborah L. Croteau provided concepts and edited the paper, Robert M. Brosh, Jr. edited the paper and provided concepts, and Vilhelm A. Bohr oversaw the projects and provided concepts and edited and wrote the paper

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

considerable resources to multiple pathways of DNA repair. BER is an essential, highly conserved DNA repair pathway that corrects non-distorting base lesions including base loss, single-strand breaks, and base damage created by many sources such as irradiation and oxidative stress [1]. These lesions, if left unrepaired, will likely cause mutations or decreased cell viability.

BER consists of four basic steps: discovery and removal of the damaged base, removal of the sugar and processing of DNA ends, incorporation of undamaged nucleotide, and re-ligation. After sugar removal, the repair intermediate consists of a single-nucleotide gap that must be processed to have a 3'-hydroxyl (−OH) and 5'-phosphate ($-PO_4$). Depending on which glycosylase removes the damaged base, either the 3' end will be modified by apurinic/apyrimidinic endonuclease (APE1), or a 5' ribose phosphate (5'-dRP) will be processed by the polymerase that also fills the gap, making it suitable for ligation. BER consists of two sub-pathways: short patch (SP) and long patch (LP). SP BER occurs when a single nucleotide is inserted to fill the gap, most often by DNA POLβ, and then a ligase seals the nick. In LP BER, two or more nucleotides are inserted, filling the gap, and displacing downstream nucleotides, which are subsequently endonucleolytically cleaved. In the nucleus, LP BER is performed by the actions of proliferating cell nuclear antigen, DNA polymerase β, δ or ε, flap endonuclease 1 to remove the flap of displaced nucleotides, and ligase I to seal the nick [2, 3].

3.2 Mitochondrial BER

BER is active in both the nucleus and the mitochondria and is the best characterized DNA repair pathway in the mitochondria [4], with many of the same proteins as in the nucleus, including seven of the eleven known human glycosylases, APE1, POLβ, and LIG3. In the nucleus, DNA polymerases delta and epsilon along with DNA Ligase I participate in LP BER [5]. Those polymerases are not present in the mitochondria and LIG3 is the only mitochondrial ligase. POLβ plays a key role in BER in the nucleus [6] and is also localized to the mitochondria in certain tissues [7, 8]. Loss of POLβ results in severe BER defects and embryonic lethality in mice [6]. There is no known complete loss of function POLβ mutation in humans, but single nucleotide polymorphisms of POLβ are highly associated with cancer susceptibility [9]. Loss of POLβ in HEK293 cells [7] or mouse embryonic fibroblasts (MEFs) [8] leads to mitochondrial dysfunction such as decreased oxidative phosphorylation, mitochondrial networking and repair of oxidative lesions. POLβ is a specialized polymerase with an N-terminal 5'-dRP lyase domain, important for removing sugar-phosphate groups from BER intermediates and a C-terminal polymerase domain for inserting nucleotides within the [10]error prone [11]. Depending on sequence context, the error rate for POLβ is approximately 530×10^{-4} [10]. The role of POLβ in the mitochondria may be similar to its nuclear role, but it has not been directly studied in the mitochondria or in direct comparison to the mitochondrial replicative polymerase, DNA polymerase gamma (POLγ). DNA POLγ is the replicative polymerase in the mitochondria [12] and was previously thought to perform the gap-filling role in mitochondrial BER. POLγ has been shown to have 5'-dRP lyase activity [13], though much weaker than the same activity in POLβ [14]. POLγ is a high-fidelity (error rate just 20×10^{-4} [10]), proof-reading, processive enzyme [15], which are important features for a replicative polymerase. POLβ,

on the other hand, is a mostly distributive enzyme [16], a quality that suits a repair enzyme, whose main polymerase function is to fill single-nucleotide gaps.

The human mitochondrial genome is a covalently closed circular molecule composed of 16,569 DNA base pairs. It encodes some components of the oxidative phosphorylation machinery, as well as the ribosomal RNAs and tRNAs required to translate those proteins [17]. Most proteins required for mitochondrial structure, transport and energy production are encoded in the nucleus, but the mitochondrial genome is essential. Despite multiple copies of mtDNA, somatic mutations in the mitochondrial genome are associated with aging and neurodegeneration [18]. Mice expressing a version of POLγ without proof-reading capacity, which results in higher mutation frequencies, display pronounced aging phenotypes [19], demonstrating the importance of mitochondrial genome maintenance.

3.3 POLβ in the mitochondria

The discovery of POLβ in the mitochondria [7, 8] suggests that it participates in mitochondrial BER. The BER capabilities of POLγ have been demonstrated *in vitro* but it has not been determined if the two enzymes have separate or overlapping functions in the mitochondria or if they cooperate in replication or DNA repair. As a specialized polymerase for DNA repair, POLβ is known to efficiently insert nucleotides in single nucleotide gapped BER intermediates. Detailed here is the first report to our knowledge directly comparing POLβ and POLγ, normalized for activity, on different repair intermediate substrates. We also present data demonstrating interactions with known mitochondrial proteins and consequences for mitochondrial health when POLβ is not present. We find that POLβ is much more active as a BER polymerase than POLγ on every repair intermediate tested and that mitochondria lacking POLβ are dramatically impaired in BER function. To our knowledge this is the first direct demonstration of defective DNA repair in mitochondrial extracts from cells lacking POLβ.

4. MATERIAL AND METHODS

4.1 Cell culture:

Polb$^{-/-}$ and wild-type mouse embryonic fibroblast cells were purchased from ATCC (Gaithersburg, MD) and maintained in DMEM plus 10% fetal bovine serum and 1% Penicillin-Streptomycin. The cells were maintained in a humidified atmosphere of 5% CO2 at 37°C and the medium was replaced every 2–3 days. Cells were tested quarterly for mycoplasma infection.

4.2 Purified Proteins:

DNA POLβ and APE1 were gifts of David Wilson III. DNA POLγ A and B were gifts of Daniel Bogenhagen, Stony Brook University School of Medicine. DNA POLγA is exonuclease deficient. Uracil DNA glycosylase (UNG) was purchased from New England Biolabs (Ipswich, MA). WT and mutant Twinkle protein was purified from *E. coli* as described [20, 21].

4.3 Primer extension, gap-filling and nicked substrate assays:

Assays were performed at 37°C under the following conditions: 10 mM Tris-HCl, pH 8, 50 mM KCl, 8 mM $MgCl_2$, 2 mM DTT, 25 μM dNTPs, 100 μg BSA and 1 nM DNA substrate. All reactions were kept on ice until addition of radiolabeled substrate and incubated for 30 min or times indicated in figure. Reactions were stopped in formamide and heated to 95°C for 10 min. Reaction products were separated via polyacrylamide gel electrophoresis under denaturing conditions. Gels were visualized using Typhoon Trio+ phosphorimager (Molecular Dynamics) and quantified with Image Quant TL software. For all incorporation reactions, quantification of products corresponding to incorporation of one nucleotide or greater was divided by the total radioactivity of the reaction and multiplied by 100 for "% substrate with at least 1 nt added." The results from at least three separate experiments were combined and presented as the mean +/− standard error of the mean.

When uracil-containing DNA substrates were used for reactions, UNG and APE1 were added to reactions. Experiments using mitochondrial extracts were performed in the same conditions, with the following exceptions: DNA substrate was not labeled (described below), 20 μg of mitochondrial extract was added instead of purified proteins, and 1X Halt™ Protease and Phosphatase inhibitor cocktail was added.

Downstream oligonucleotide displacement assay: This assay was performed the same as the gap filling assay, utilizing a substrate in which the downstream oligonucleotide was labeled. Products were separated using non-denaturing polyacrylamide gel electrophoresis. Gels were visualized using Typhoon Trio+ phosphorimager (Molecular Dynamics) and quantified with Image Quant TL software.

4.4 Mitochondrial isolation and extraction:

Cells were harvested, washed with PBS, and then homogenized in MSHE buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EDTA, pH 7). Homogenized cells were centrifuged at 2200 × *g* for 5 minutes at 4°C. Supernatant was removed and centrifuged at 22,000 × *g* for 10 min at 4°C. Pellets were kept at −80°C until needed. To prepare extracts for repair assays, pellets were resuspended in equal volume of Buffer I (10 mM Tris-HCl, pH 8.0, 200 mM KCl), followed by addition of another 1XCV of Buffer II (10 mM Tris-HCl, pH 8.0, 200 mM KCl, 2 mM EDTA, 40% glycerol, 0.5% NP-40, 2 mM DTT) and protease inhibitors (1X total volume, ThermoFisher, Rockville, IL). Extracts were incubated at 4°C for 2 hr with rotation, followed by centrifugation at 17,000 × *g* at 4°C for 15 min. Supernatants were removed to a new tube and protein concentrations were determined (ThermoFisher) followed by storage at −80°C [22].

4.5 Deoxyribose phosphate (dRP) lyase activity assay:

Assay was performed as previously published [23], with the following modifications. Uracil-containing 3'–^{32}P labelled DNA substrate (50 nM) was treated with 0.05 units of Uracil DNA Glycosylase (Trevigen, 0.1 U/μl) in 50 mM HEPES-NaOH, pH 7.5, 0.5 mM EDTA, 0.2 mM DTT for 5 min at 37°C. Next, $MgCl_2$ (5 mM final conc.) and AP endonuclease (10 nM final conc.) were added and incubated for additional 5 min at 37°C to generate AP site-containing substrate. The substrate was added (10 nM final conc.) to the dRP lyase reaction

(10 μl final volume, 50 mM HEPES-NaOH, pH 7.5, 20 mM KCl, 2 mM DTT) containing indicated amount of mitochondrial extract or 20 nM POLβ, and incubated 30 min at 37°C. The reaction was transferred to ice and reduced for 15 min by addition of 1 μl of 100 mM $NaBH_4$. Finally, 0.5 μl of the reaction was mixed with 10 μl loading dye (98% formamide, 20 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol), heated for 2 min at 75°C, and separated on 20% denaturing urea polyacrylamide gel. Gels were visualized using Typhoon Trio+ phosphorimager (Molecular Dynamics) and quantified with Image Quant TL software.

4.6 Substrates:

Oligonucleotide sequences for all substrates are provided in Table 1. For all but one radiolabeled substrate, 10 pmol of the 'A' oligonucleotide was 5' labeled using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and $\gamma-^{32}$P-ATP (Perkin Elmer, Pittsburgh, PA) at 37°C for 1 hr. In the case of Figure 2D, oligonucleotide B was labeled instead of A, but other conditions were the same as described. After labeling reaction, 15 pmol of oligonucleotides B (and C when appropriate) were added in a solution of 40 mM Tris-HCl, pH 8.0 and 50 mM NaCl. Hybridization reactions were placed in a heat block at 85°C and allowed to cool naturally to room temperature. Unlabeled substrates were prepared exactly as above, but without the $\gamma-^{32}$P-ATP and T4 polynucleotide kinase. Radiolabeled probes were applied to G25 sephadex columns (GE Life Sciences, Pittsburgh, PA) to remove unbound nucleotides and then analyzed by non-denaturing polyacrylamide gel electrophoresis for complete hybridization.

5. RESULTS

DNA POLβ is well established as the polymerase most often utilized in nuclear BER. Recent findings that POLβ is also present in the mitochondria [7, 8], in both cultured cells and animal tissues, led to questions about the role of POLβ in mitochondrial BER. Here, we compared POLβ to the mitochondrial replicative polymerase, POLγ, previously thought to be the only polymerase to function in mitochondrial BER in a variety of assays to establish the likely contribution of each polymerase in BER when both are present.

5.1 DNA polymerase beta fills gapped substrates more thoroughly than DNA polymerase gamma

To compare the gap-filling capacity of pols β and γ, we normalized the polymerase activity of purified proteins using a primer extension substrate (Table 1) and titration of proteins. We used a primer extension substrate because both polymerases utilize primed oligonucleotide, with a 3'OH end. POLγ is a multi-subunit protein consisting of a catalytic subunit, POLγ-A and an accessory protein, POLγ-B, used in a 1:2 ratio, respectively, collectively referred to as POLγ [24]. POLγ used in all experiments is exonuclease deficient, preventing removal of newly incorporated nucleotides. The unfilled gap is the fastest migrating band, seen clearly in lanes without any polymerase added. Both POLβ and POLγ readily filled the primer extension substrate. Increasing concentration of POLβ resulted in more fully filled substrate, while even the lowest concentration resulted in completion of at least a single nucleotide addition. In contrast, increasing concentrations of POLγ resulted in more substrate

utilization, as all products were fully filled. This is consistent with POLβ being a distributive enzyme on templates longer than a few nucleotides [25] and POLγ being a processive enzyme [15]. The concentration of enzyme required to fully replicate the primer extension substrate was noted as 1X and used in subsequent comparison assays. For POLγ, 10 nM was required to fill the substrate and for POLβ it was 3 nM (Figure 1A). For POLγ, a slightly lower molecular weight band was considered full activity as POLγ is known to stall close to the ends of linear substrates [26]. Therefore, although the concentration of POLγ required to fill the substrate is 3-fold greater than POLβ, these concentrations represent equal activity.

Next, we compared the activity of the enzymes in filling a single-nucleotide gapped substrate (Table 1), the DNA repair intermediate common in all BER reactions. We utilized the concentration of each enzyme required for full primer extension activity. As expected [13, 14, 27], POLβ performed much better filling the single-nucleotide gap than POLγ. Indeed, at one min, POLβ had already completely filled the gap, but even after 30 min, unfilled gap remained in the POLγ reactions (Figure 1B and 1C). This demonstrates that at equal activity, POLβ can process more single-nucleotide gap than POLγ, suggesting that in conditions of high oxidative stress, cells lacking POLβ may have insufficient BER capacity.

When analyzing labeled single-nucleotide gaps with mitochondrial extracts, we found some substrate degradation (data not shown), with significant banding below 15 nucleotides, the size of an unfilled substrate, suggesting that a single-nucleotide gap may be extended to larger gaps by mitochondrial nucleases. We therefore compared gaps of 1–4 nucleotides (Table 1) in the *in vitro* assay and found that POLβ filled all the gaps robustly, while POLγ performed incrementally better as the gap became larger (Figure 1D). However, even the largest, 4-nt gap, was not filled by POLγ as quickly as by POLβ. These data demonstrate that while POLγ can participate in BER, its activity is severely limited in comparison to POLβ, which may have important biological consequences.

5.2 TWINKLE stimulates POLβ displacement synthesis, but not single nucleotide gap-filling activity, independent of helicase activity.

Previously, we found that POLβ displacement synthesis was stimulated by the nuclear WRN helicase [28], as well as by the mitochondrial helicase, TWINKLE [7]. We reported that WRN stimulation of POLβ was dependent on helicase activity [29]. We wanted to assess if TWINKLE stimulation was also dependent on helicase activity. To that end, we conducted the experiments in the presence or absence of ATP, which is required for helicase activity. We used 0.3 nM POLβ because at this concentration, single nucleotide gap filling is complete, but displacement synthesis is reduced. Consistent with our previous report [7], we found that TWINKLE stimulates POLβ displacement synthesis activity in a concentration-dependent manner (Figures 2A and 2B), although very high concentrations of TWINKLE were inhibitory (data not shown). The purity of the TWINKLE protein used in these assays has been previously published [30]. TWINKLE helicase is a 5’→3’ helicase requiring a 5’ overhang to unwind DNA. Our substrate does not have a 5’ overhang. We supposed that TWINKLE may have another role in stimulating POLβ displacement synthesis. To test this, we employed two strategies. The first was to eliminate ATP, required for helicase activity and the second was to utilize a helicase impaired TWINKLE mutant (K421A) [21]) and

found that POLβ activity was stimulated by 3 nM wild-type TWINKLE in the presence or absence of ATP or by helicase impaired, K421A TWINKLE (Figures 2A and 2B). There were no statistical differences between the two enzymes. Taken together, these data indicate that TWINKLE, in contrast with WRN, stimulates POLβ displacement synthesis in an active helicase-independent manner.

We originally thought that POLβ synthesis by TWINKLE was due to TWINKLE helicase activity displacing the downstream oligo so that POLβ could replicate past the gap. Because we found that stimulation was helicase-activity independent, we wondered how the downstream oligo was displaced in the absence of an active helicase. We reasoned that POLβ was able to separate the downstream double-stranded DNA on its own. To test this, we labelled the 5' end of the downstream oligo in the single-nucleotide gap substrate (Table 1) and replicated the incorporation assay, analyzing the products on a non-denaturing gel. The downstream oligonucleotide was displaced from the hybridized molecule at a POLβ concentration of 0.3 nM (Figure 2C). This confirms that stimulation of POLβ displacement synthesis is not due to TWINKLE unwinding activity, as POLβ activity alone results in displacement of the downstream oligonucleotide. We also considered the possibility that stimulation was due to molecular crowding of the polymerase [31]. However, our reactions contain BSA to provide molecular crowding and doubling the BSA concentration had no effect on POLβ reactions (data not shown).

Because the essential polymerase function in BER is gap filling and not displacement synthesis, we also examined the ability of TWINKLE to stimulate the single-nucleotide gap-filling activity of POLβ or POLγ. To determine stimulation of single-nucleotide gap filling, it was necessary to find a concentration of POLβ at which less than half of the gap was filled in a 30 min reaction. We found that 1000-fold less POLβ (3 pM *vs.* 3 nM) was required for more than 50% of the substrate to remain (Figure 2D) than was required for full synthesis of the primer extension substrate. The full primer extension activity of POLγ results in greater than 50% of the substrate to be unfilled, so no adjustments were made to the POLγ concentration for these experiments. TWINKLE was titrated in the reactions up to 30 nM with or without ATP and even at the highest concentrations did not stimulate either POLβ or POLγ single-nucleotide gap filling (Figure 2E). This suggests that TWINKLE helicase may not participate in SP BER in mitochondria, based on *in vitro* evidence with the two mitochondrial polymerases most likely to function in BER.

5.3 DNA pols β and γ do not cooperate to repair gapped substrates.

To examine the possibility of cooperation between POLγ and POLβ in mitochondrial BER, we measured the effect on product formation in reactions containing both enzymes added together or in sequence. Using the lower POLβ concentration that filled less than 50% of the gapped substrate and did not result in any displacement synthesis (Figure 2D), we titrated POLγ levels from 100 pM up to 20 nM. POLγ and POLβ had a simple additive effect, indicating that they neither cooperated with each other nor inhibited the function of the other (Figures 3A and 3B). Similarly, we performed reactions with a constant 10 nM POLγ and titrated in POLβ from 0.01 nM to 3 nM. Again, we found that the enzymes worked independently with no cooperative or inhibitory effect (Figures 3A and 3B.)

In these reactions, POLγ and POLβ were added together. In certain contexts, polymerases have been shown to hand off substrates, both for insertion in translesion synthesis [32] and extension past those lesions [33, 34]. We wondered if the order of addition of the enzymes would play any role in reaction completion. To address this, we compared each enzyme alone with reactions in which POLγ was added for 5 min before POLβ, in the absence or presence of dNTPs. We also performed similar reactions in which POLβ was added first. All these reactions resulted in the same additive effect seen in Figure 3A (Figure 3C). That is, order of addition of POLγ and POLβ has no effect on BER product formation. Taken together, these data indicate that POLγ and POLβ do not cooperate nor inhibit the activity of each other in utilizing a single-nucleotide gapped substrate.

5.4 DNA POLβ can utilize a wider variety of DNA repair intermediates than POLγ.

To verify that fully filled substrate seen at the highest levels of POLγ titration in Figure 3A were due to use of unfilled gap and not utilization of a nicked substrate resulting from POLβ insertion of a single nucleotide, we artificially created a substrate with three oligos that had no nucleotide gaps but did have a nick, similar to what would be created by insertion of a single nucleotide into a gap without subsequent ligation (Table 1). POLγ was unable to utilize this substrate at any detectable level for up to 30 min (Figure 3D). However, POLβ utilized approximately half of the substrate within 10 min and all of it within 30 min, at 0.3 nM concentration (Figure 3D), equivalent to 10% of the enzyme activity of POLγ. This result indicates that the fully filled products we observed in Figure 3A were due to POLγ using the unfilled gap, and not extending after POLβ filled the gap. It also suggests that in the absence of ligation, POLβ can re-bind the substrate and add additional nucleotides, a potential mutational risk given the error-prone nature of POLβ. These results are consistent with previous reports of POLβ strand-displacement activity on nicked substrates [35–37].

We next examined the ability of the polymerases to utilize a substrate more similar to a natural repair intermediate. We created a double-stranded substrate containing a single uracil and no nicks or gaps (Table 1). This substrate was processed with purified UNG glycosylase and APE1 enzymes, creating a single nucleotide gap flanked by a 3’OH and a 5’dRP (deoxyribose phosphate). This results in a substrate more like the BER intermediates the polymerases would encounter *in vivo*. Next, we titrated in increasing concentrations of POLβ or POLγ. POLβ, but not POLγ, was able to completely fill the gap (Figure 3E). POLγ did not produce any observable products even at the highest concentrations. These data are consistent with published reports that although POLγ has dRP lyase activity, that activity is slow. A direct comparison of POLβ and POLγ dRP lyase activities revealed that POLγ is markedly slower than POLβ [14]. Together these results illustrate that POLβ can effectively utilize a wider variety of DNA substrates, particularly relevant for mitochondrial BER. The lack of activity of POLγ on a more biologically relevant substrate suggests that in the absence of POLβ, under conditions of high oxidative stress, mitochondrial BER may be stalled at the incorporation step.

5.6 Impact of POLβ deficiency for mitochondria

In the previous experiments, we utilized purified proteins to determine the capabilities of POLγ and POLβ to incorporate nucleotides in various substrates. In Figure 2 we

demonstrated that at least one mitochondrial protein, TWINKLE, interacts with and affects the activity of POLβ. Based on this and our previous mass spectrometry findings that showed that POLβ interacts with a number of mitochondrial proteins [7], we reasoned that other mitochondrial resident proteins may assist or inhibit the ability of these polymerases to function in BER. We took advantage of MEFs lacking POLβ to examine BER activities in the context of mitochondrial extracts. These cells lack POLβ in both the mitochondria and nucleus. Because they express POLγ in the mitochondria and the wild-type MEFs express both POLγ and POLβ in the mitochondria, we were able to estimate the contribution of POLβ to mitochondrial BER by comparing purified mitochondrial extracts from wild-type and *polb*$^{-/-}$ MEFs.

First, we compared the extracts in a single-nucleotide gap incorporation assay. In this experiment we utilized an unlabeled substrate and α−^{32}P-dCTP instead of the 5'-end labeled substrate used in previous experiments because of degradation by mitochondrial nucleases in both wild-type and polβ$^{-/-}$ mitochondrial extracts. Wild-type mitochondrial extracts filled significantly more gaps than extracts lacking POLβ (Figure 4A and 4B). These results suggest that mitochondrial POLβ has an important *in vivo* role in filling BER intermediates, and are consistent with a previous report demonstrating dramatically decreased gap-filling in the absence of POLβ [8]. While it is possible that mitochondrial nucleases resulted in a mixture of single nucleotide gapped substrates and primer extension substrates, we believe that those are a minority of the substrate because of the size of the products. Both the purified POLβ and wild-type extracts had clear bands representing single nucleotide insertion.

Based on our results in Figure 3E that purified POLγ was unable to utilize a substrate containing a dRP group created by APE1 endonuclease activity, we compared the dRP lyase capacity of mitochondrial extracts from wild-type and POLβ-deficient MEFs. We again find that extracts lacking POLβ remove modestly but significantly less dRP substrate than wild-type extracts (Figure 4C and 4D). These data demonstrate that in addition to the results demonstrated with purified POLγ and POLβ, at a cellular level, loss of mitochondrial POLβ creates deficiency in incorporation of nucleotides within a BER intermediate.

Together, the data obtained from mitochondrial extracts confirms that although POLγ has shown activity *in vitro* on BER-relevant substrates, the biological contribution of POLγ to mitochondrial BER is limited in comparison to POLβ.

6. DISCUSSION

Mitochondria are the source of the bulk of cellular oxidative stress to DNA, mostly repaired via BER. The efficiency of mitochondrial BER is highlighted dramatically by the recent discovery that the majority of age-related mitochondrial mutations do not fit the mutational signature expected for unrepaired oxidative lesions [38]. This suggests that either the mitochondrial oxidative environment does not lead to DNA lesions, or more likely, that those lesions are rapidly repaired by the mitochondrial BER pathway. The discovery of DNA POLβ in the mitochondria in several cell and tissue types, by two separate labs [7, 8] raised new questions into the relative roles of pols β and γ in mitochondrial BER [39]. Before this,

it was assumed that POLγ performed the role of gap filling, supported by *in vitro* evidence that it was able to fill a gapped substrate [27] and could function as a dRP lyase [13].

We report the first direct comparison of pols γ and β as gap-filling polymerases with a variety of BER-relevant substrates. In all cases, POLβ is far more effective as a BER polymerase than POLγ. One reason for this may be that POLγ has weak dRP lyase activity, which is slow and may result in delayed removal of the enzyme from the DNA [14]. Our results demonstrate that although POLγ has biochemical activity utilizing BER substrates, when compared in identical conditions to POLβ, it is weak.

Our previous report demonstrated a functional interaction between POLβ and TWINKLE [7]. Here we further explore that interaction and conclude that the stimulation of POLβ by TWINKLE is not dependent upon TWINKLE helicase activity. This is not surprising, as TWINKLE helicase has specific substrate requirements including 10 bases of single-strand DNA 5' to the duplex and a single-stranded tail 3' of the duplex [40], which are not present in our BER substrates. Nonetheless, TWINKLE does stimulate POLβ displacement synthesis activity *in vitro*. Further, we determined that TWINKLE was unable to stimulate the single-nucleotide gap-filling activity of either POLβ or POLγ. It remains to be seen if TWINKLE is involved in *in vivo* mitochondrial BER. TWINKLE has been shown to have helicase activity on some DNA repair structures [21], but a non-helicase role has not been previously demonstrated for any type of mitochondrial DNA repair. The mechanism for this functional interaction is an intriguing area for future research, potentially revealing a more comprehensive role for TWINKLE in mitochondrial DNA metabolism.

Importantly, we found that mitochondrial extracts from cells lacking POLβ have deficient incorporation within BER substrates. Our lab and others reported that there were more polymerase-blocking lesions in the mitochondrial DNA of cells lacking POLβ, as well as decreased respiration and increased mitochondrial ROS in response to oxidative stress [7, 8]. This work further demonstrates that mitochondrial extracts from cells lacking POLβ are deficient in gap filling and dRP lyase activity. This dramatic deficit in gap filling may lead to increased strand breaks, consistent with the lesions described previously. We have previously shown that DNA ligase is the rate-limiting step in mitochondrial BER [41]. In the absence of POLβ in the mitochondria, it may be that the polymerase step is rate limiting in the mitochondria as it is in the nucleus.

The data presented here demonstrate that mitochondrial extracts lacking POLβ have a dramatically decreased capacity for BER incorporation. This is especially important in highly metabolically active tissues with increased mitochondrial ROS production such as the brain. Because the polymerase step of BER occurs after a strand break has occurred in the DNA repair process, it is essential that the gap be filled, and subsequent ligation occur. In the case of a deficient glycosylase, the oxidative lesion would remain, but without strand breakage. For example, we previously reported that mitochondria from OGG1-deficient mice harbor 20 times more 8-oxo-dG than wild-type mice [42].

However, those mice are metabolically similar to their wild-type counterparts [43]. Because wild-type mice do not accumulate mutations over time with oxidative signatures, we know

that glycosylase activity is not limiting in mitochondria [38], making efficient gap-filling and ligation essential. If metabolically active cells lack POLβ, we would expect single-nucleotide gaps to accumulate and result in strand breaks. This is consistent previous reports demonstrating increased polymerase-blocking lesions in the mitochondria of null cells [7, 8]. The mechanism for how the BER deficiency in POLβ-deficient cells leads to altered mitochondrial morphology and dysfunctional oxygen consumption is an area of active investigation.

ACKNOWLEDGEMENT

The authors thank Dr. Daniel Bogenhagen for the generous gift of DNA POLγ, both A and B subunits. We also thank Dr. David Wilson III for the gift of DNA POLβ. This work was supported entirely by funds from the intramural research program of the National Institute on Aging.

FUNDING SOURCES

This research was supported entirely by the Intramural research Program of the NIH, National institute on Aging.

ABBREVIATIONS

2.

BER	Base excision repair
POLβ	DNA polymerase beta
POLγ	DNA polymerase gamma
APE1	Apurinic/apyrimidinic endonuclease
SP	Short patch
LP	Long patch
MEF	Mouse Embryonic Fibroblast
dRP	Deoxyribose phosphate
mtDNA	Mitochondrial DNA
ROS	Reactive Oxygen Species

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Highlights

- POLβ is significantly more efficient in filling BER gapped intermediates than POLγ
- POLβ is stimulated by TWINKLE, independent of helicase activity
- POLβ efficiently utilizes more BER intermediate substrates than POLγ
- Mitochondrial extracts lacking POLβ are deficient at BER incorporation

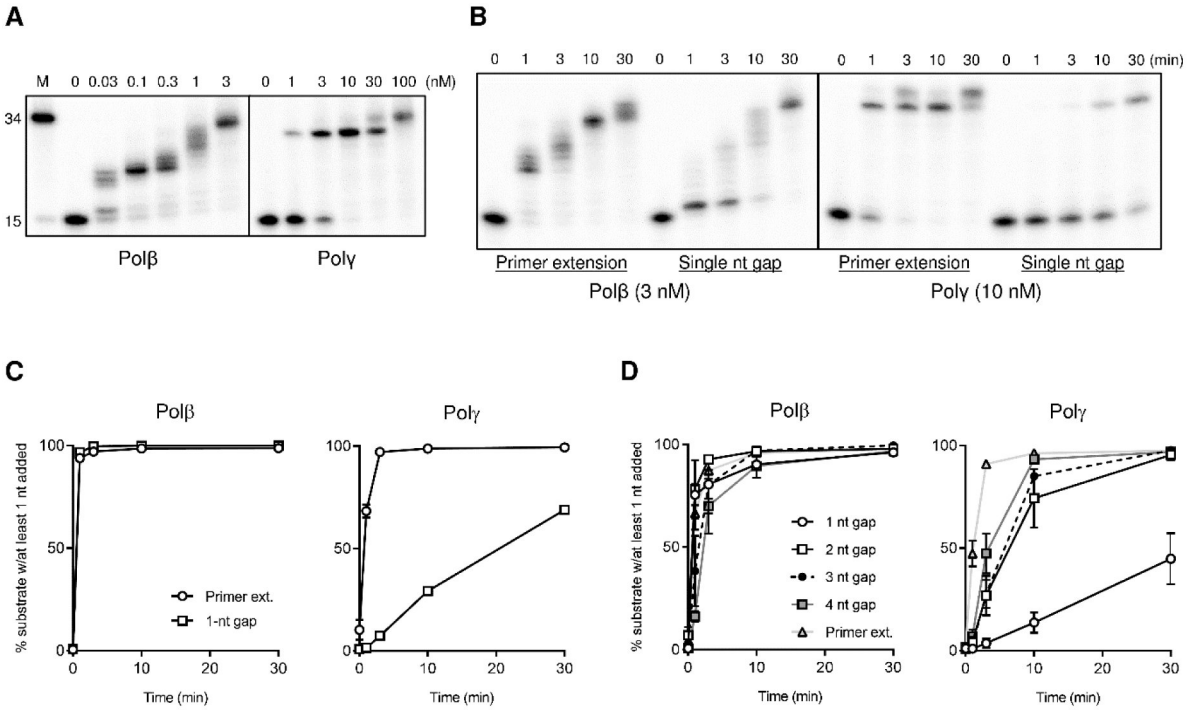


Figure 1.

DNA POLβ fills gapped substrates more thoroughly than POLγ. A. Primer extension assay used to normalize activity of purified pols β and γ. M labels known 34 and 15 base oligonucleotides. Asterisks label the lane representing full activity of each enzyme. B. Comparison of activity of pols β and γ utilizing primer extension or single-nucleotide gapped substrates at full activity concentrations of polymerases over time. C. Graphical representation of B, separated by polymerase. D. Quantitation of incorporation assays utilizing 1-, 2, 3-, and 4-nt gapped substrates. Polymerases were used at concentrations equal to full activity on primer extension substrates and measured incrementally over 30 min.

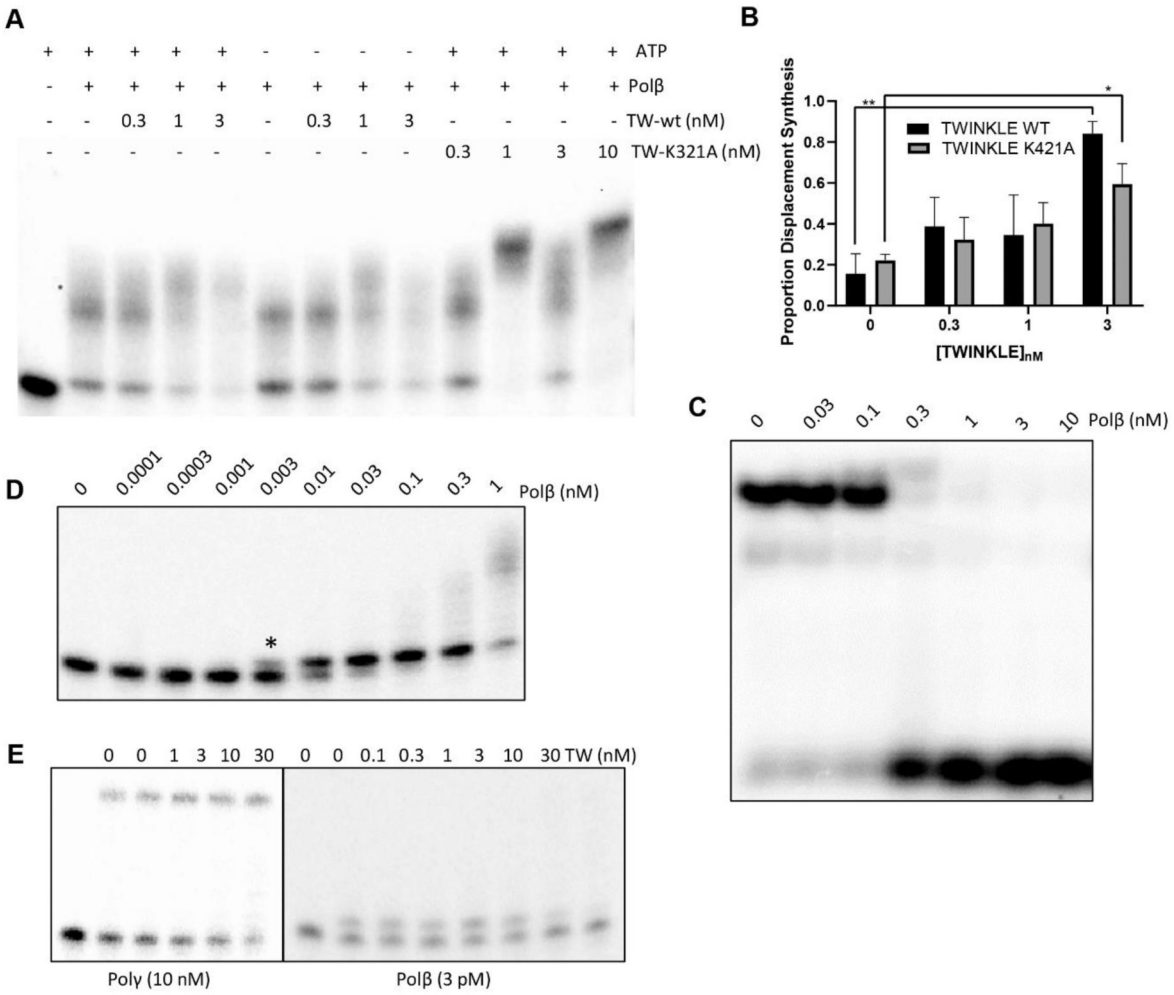


Figure 2:

DNA POLβ displacement synthesis, but not single-nucleotide-gap filling is stimulated by mitochondrial helicase TWINKLE, independent of helicase activity. A. Incorporation assays were performed using POLβ at 0.3 nM in the presence or absence of ATP and TWINKLE wild-type or K421A mutant helicase. Any synthesis greater than a single nucleotide was considered displacement synthesis. B. Quantitation of displacement synthesis by WT or mutant TWINKLE. Statistics performed using 2-way ANOVA with multiple comparisons by Tukey post-hoc tests. Asterisk indicates $p < 0.05$. C. Using the downstream oligo displacement substrate (Table 1), POLβ was titrated from 0.03 nM to 10 nM. The products were separated on a non-denaturing gel. Slower migrating products indicate hybridized molecules, while the faster moving product indicates separation of the labeled band from the other oligonucleotides. D. Incorporation assay was performed as above titrating POLβ from 3 nM down to 0.1 pM. Asterisk marks the concentration of POLβ where less than 50% of gap-filling can be observed. E. TWINKLE helicase was titrated into incorporation assays using single-nucleotide gap substrate and POLγ (10 nM) or POLβ (3 pM).

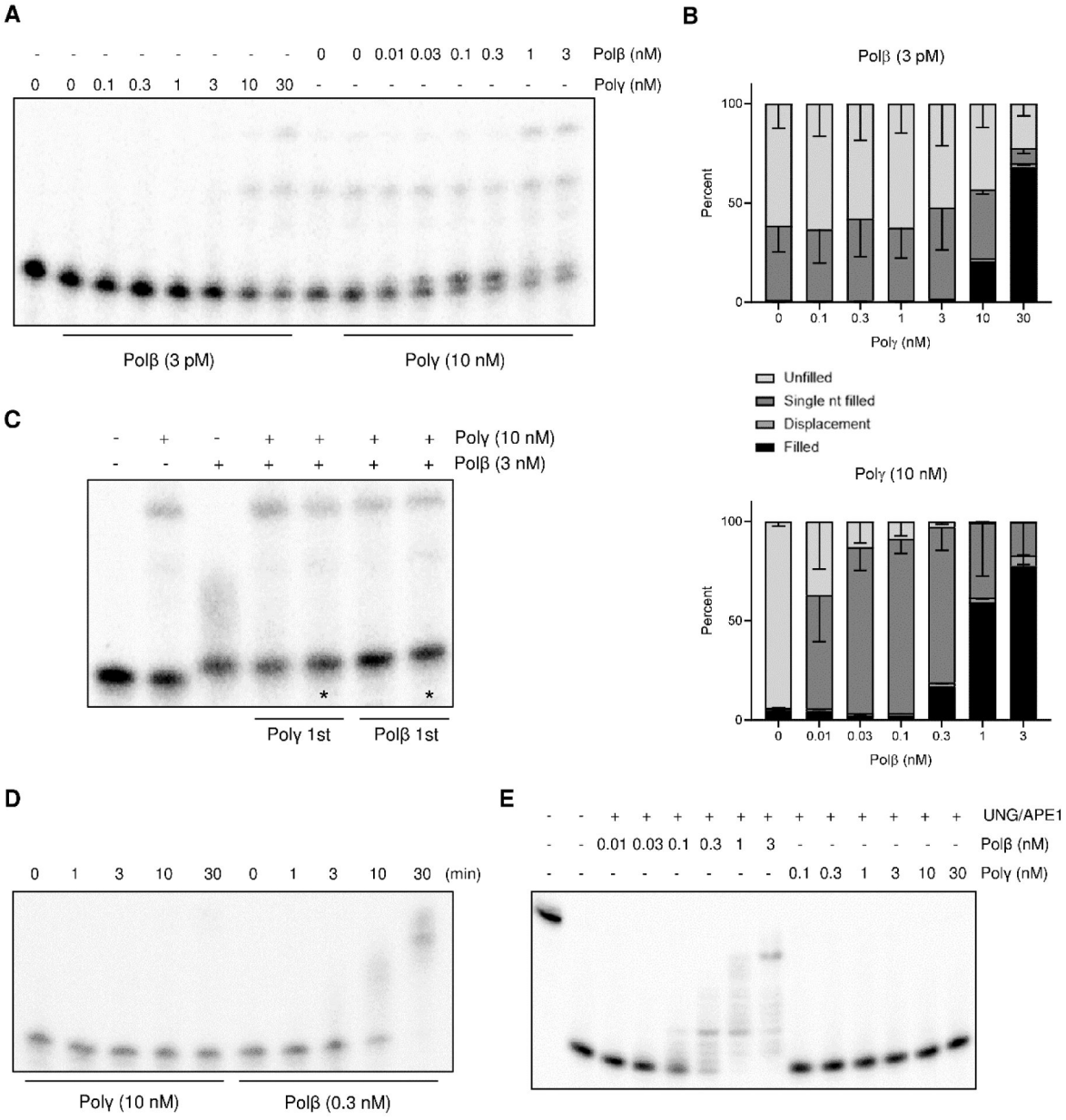


Figure 3.

Pols β and γ do not cooperate or inhibit each other in gap filling or displacement synthesis. A. Keeping the concentration of POLβ constant (3 pM) and titrating increasing concentrations of POLγ (0.1 to 30 nM), or constant POLγ (10 nM) with increasing POLβ (0.01 to 3 nM), incorporation assays were performed with single-nucleotide gap substrate. B. Quantitation of A graphed by percentage of reaction totally unfilled, single nucleotide filled, intermediate displacement synthesis and fully filled. C. Incorporation assay using single-nucleotide gap substrate. Polymerases were added alone or incubated for five minutes before the addition of the other polymerase as indicated. In lanes marked with asterisks, dNTPs were not added until the second polymerase was added to reaction. D. Nicked substrate was used in incorporation assays with POLγ (10 nM) or POLβ (0.3 nM) for times

indicated. E. Labelled, double stranded substrate containing a single uracil lesion was treated with UNG and APE1. POLβ or POLγ was titrated into reactions for 30 min at 37°C.

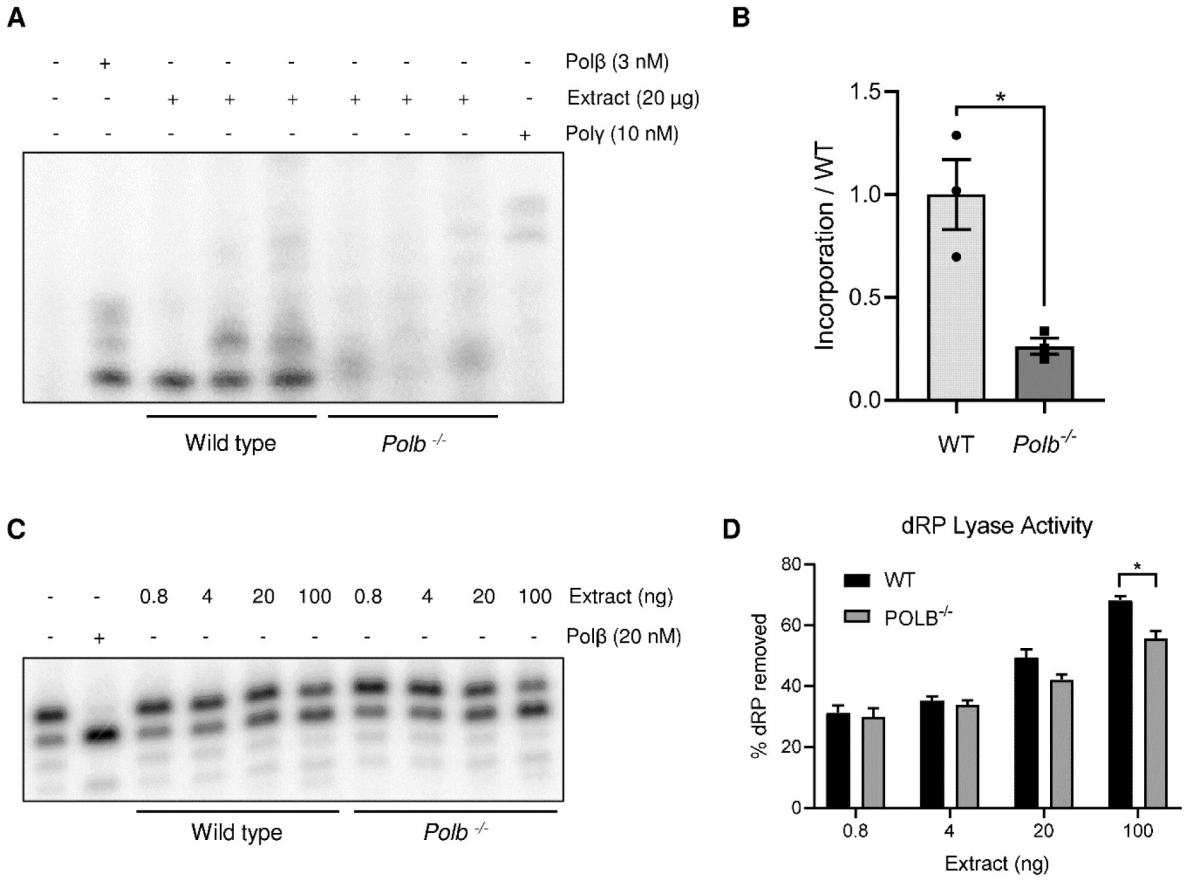
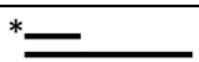
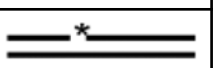
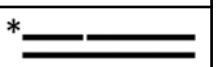
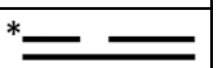
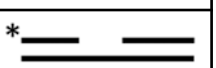
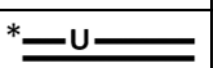


Figure 4.

Extracts lacking POLβ are deficient in key BER activities. A. Mitochondrial extracts from wild-type and POLβ-deficient MEFs were incubated with unlabeled single-nucleotide gap substrate and α–^{32}P-dCTP. B. Quantitation of A graphed with total incorporation normalized to extracts from wild-type cells. Statistics performed using 2-way ANOVA with multiple comparisons by Tukey post-hoc tests. Asterisk indicates $p < 0.05$. C. Titrations of mitochondrial extracts from wild-type and POLβ-deficient MEFs were incubated with labeled dRP substrate and quantitated. D. Quantitation of C, percentage of total substrate present in the faster moving, dRP-removed band. Statistics performed using 2-way ANOVA with multiple comparisons by Tukey post-hoc tests. Asterisk indicates $p < 0.05$.

TABLE 1:

Oligonucleotide substrate names, sequences and visual configurations used in this manuscript.

Substrate	Oligonucleotide Sequence	Configuration
Primer Extension	A: 5’ CTG ATG CTG ATG CGC B: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
Single-nucleotide gap	A: 5’ CTG ATG CTG ATG CGC B: 5’ GTA CGG ATC CCC GGG TAC C: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
Downstream oligo displacement	A: 5’ CTG ATG CTG ATG CGC B: 5’ GTA CGG ATC CCC GGG TAC C: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
Nicked	A: 5’ CTG ATG CTG ATG CGC C B: 5’ GTA CGG ATC CCC GGG TAC C: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
2-nt gap	A: 5’ CTG ATG CTG ATG CGC B: 5’ TAC GGA TCC CCG GGT AC C: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
3-nt gap	A: 5’ CTG ATG CTG ATG CGC B: 5’ ACG GAT CCC CGG GTA C C: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
4-nt gap	A: 5’ CTG ATG CTG ATG CGC B: 5’ CGG ATC CCC GGG TAC C: 5’ GTA CCC GGG GAT CCG TAC GGC GCA TCA GCA TCA G	
Uracil	A: 5’ ATA TAC CGC GUC CGG CCG ATC AAG CTT ATT B: 5’ AAT AAG CTT GAT CGG CCG GGC GCG GTA TAT	
dRP Lyase	A: 5’-CTG CAG CTG ATG CGC UGT ACG GAT CCC CGG GTA C B: 5’-GTA CCC GGG GAT CCG TAC GGC GCA TCA GCT GCA G