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Deployment of DNA polymerases beta and lambda in single-nucleotide and multinucleotide pathways of mammalian base excision DNA repair

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Abstract

There exist two major base excision DNA repair (BER) pathways, namely single-nucleotide or "short-patch" (SP-BER), and "long-patch" BER (LP-BER). Both pathways appear to be involved in the repair of small base lesions such as uracil, abasic sites and oxidized bases. In addition to DNA polymerase β (Polβ) as the main BER enzyme for repair synthesis, there is evidence for a minor role for DNA polymerase lambda (Polλ) in BER. In this study we explore the potential contribution of Polλ to both SP- and LP-BER in cell-free extracts. We measured BER activity in extracts of mouse embryonic fibroblasts using substrates with either a single uracil or the chemically stable abasic site analog tetrahydrofuran residue. The addition of purified Polλ complemented the pronounced BER deficiency of *POLB*-null cell extracts as efficiently as did Polβ itself. We have developed a new approach for determining the relative contributions of SP- and LP-BER pathways, exploiting mass-labeled nucleotides to distinguish single- and multinucleotide repair patches. Using this method, we found that uracil repair in wild-type and in Polβ-deficient cell extracts supplemented with Polλ was ~80% SP-BER. The results show that recombinant Polλ can contribute to both SP- and LP-BER. However, endogenous Polλ, which is present at a level ~50% that of Polβ in mouse embryonic fibroblasts, appears to make little contribution to BER in extracts. Thus Polλ in cells appears to be under some constraint, perhaps sequestered in a complex with other proteins, or post-translationally modified in a way that limits its ability to participate effectively in BER.

Keywords

Abasic sites; UraciI DNA repair; Fen1 nuclease; DNA repair synthesis; 5'dRp and AP lyases

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Conflict of Interest statement

The authors declare that there are no conflicts of interest

1. Introduction

DNA repair is essential for maintaining genome integrity, and thus for the genetic stability and survival of cells. BER is a 'housekeeping' function that deals with frequently generated lesions such as deaminated, alkylated or oxidized bases, abasic sites, and various types of DNA strand breaks [1]. *In vitro* studies have demonstrated the existence of a single-nucleotide repair or "short-patch" pathway (SP-BER) and a multinucleotide "long-patch" pathway (LP-BER) [2–5]. For both, a lesion-specific DNA glycosylase removes the damaged base, following which an AP endonuclease cleaves the apurininc/aprimidinic (AP) site; hydrolytically generated AP sites also enter BER here [6]. The repair of certain oxidative lesions initiated by the bifunctional glycosylases NEIL-1 and −2 may proceed by an Ape1-independent pathway in which these glycosylases cleave the AP sites by a dual β-/δ-elimination reaction that removes the abasic residue, and polynucleotide kinase/phosphatase removes the 3'-phosphate [7,8]. Next, DNA polymerase β (Polβ) replaces the missing nucleotide, and via a separate active site catalyzes the removal of the 5'-deoxyribose-5- phosphate (5'-dRP) moiety to generate a normal 5'-phospho-nucleotide, which can be ligated to the 3'-hydroxyl in the final BER step [6]. In the case of LP-BER, two or more nucleotides are inserted by Polβ and probably other DNA polymerases [9,10], which also displaces the 5' strand. The displaced 5' "flap" is cleaved by flap endonuclease-1 (Fen1) to generate a ligatable substrate [6].

In mammals, Polβ is thought to be the main polymerase involved in BER [11–14]. As noted above, Polβ has two enzymatic activities in BER, namely a DNA repair polymerase and a 5'-dRP lyase. DNA synthesis is carried out by the C-terminal 31-kDa DNA polymerase domain, while the removal of 5'-dRP is catalyzed by the N-terminal 8-kDa dRP lyase domain [15,16]. DNA polymerase λ (Polλ) is another X-family DNA polymerase and the closest homolog of Polβ. Human Polλ is a ~64-kDa protein having 33% sequence identity with Polβ in the region of homology [17,18]. Like Polβ, the catalytic core of Polλ possesses an 8-kDa dRp lyase domain and a 31-kDa DNA polymerase domain [19–21]. In addition, Polλ has a large N-terminal BRCT domain that mediates protein-protein interactions with proteins involved in nonhomologous end joining [22,23]. An adjacent serine-/proline-rich segment undergoes post-translational modifications [24,25]. Consistent with the 5'-dRP lyase and DNA polymerase activities of Polλ, several *in vitro* studies imply a role for it in BER [26,27]. Polλ can substitute for Polβ in a reconstituted SP-BER reaction with purified proteins [19]. S.H. Wilson and coworkers [28–30] have demonstrated that the elimination of Polλ makes cells hypersensitive to H_2O_2, and that Polβ-deficient MEF cells have some residual BER activity that can mostly be ascribed to Polλ. Moreover, eliminating both Polβ and Polλ in MEF and chicken DT40 cells has an additive effect on BER activity and the cytotoxicity of H_2O_2 [29,30]. In addition, Polλ physically interacts with some DNA glycosylases involved in BER [30,31] and with PCNA and Fen1, consistent with a role for the protein in BER [32,33].

In that the known phenotypes of cells devoid of Polβ are more dramatic than those of Polλ-deficient cells, Polλ is generally viewed as a "back-up" enzyme for BER. In the present study, we sought to further understand the contributions of Polλ to both SP- and LP-BER. We analyzed BER activity in extracts from *POLB*-knockout MEF cells supplemented *in*

vitro with either full-length or a truncated version of Polλ that corresponds to the Polβ homology domain. The distribution of SP- and LP-BER in the repair of a uracil residue in these extracts was determined using a mass-labeling approach. Additionally, in an effort toward understanding the roles of 5'-dRP lyase and polymerase activities in influencing the preferred pathway for repair, we measured BER activity in extracts from Polβ-deficient MEF extracts expressing either a lyase-dead or a DNA polymerase-dead variant of Polβ.

2. Materials and Methods

2.1 Materials

All oligonucleotides were synthesized and HPLC purified by Eurofins Genomics [U-top (5'-[Tetramethylrhodamine]-TTGCATGCCTGCAGGTCGA**U**TCTAGAGGATCCCCGGGTACCGAGCTCGA-3'), F-top (5'-GCTTGCATGCCTGCAGGTCGA**F**TCTAGAGGATCCCCGGGTACCGAGCTCGA [Tetramethylrhodamine]-3'), G-template (5'-GTCGAGCTCGGTACCCGGGGATCCTCTAGA**G**TCGACCTGCAGGCATGCAAGC-3') and Biotin-31U-primer (5'-[Phos]TCGG(dT-biotin)ACCCGGGGATCCTCTAGAGTCGACC**U**-3'). Uracil-DNA glycosylase (UDG), T4 DNA polymerase, T4 DNA ligase, EcoRI (HF), PstI (HF) and SalI (HF) were from New England BioLabs. Human Ape1 and Polβ were purified as previously described [34]. pGEM-3Zf(+) plasmid, R408 helper phage and JM109 competent cells were from Promega. The DNA kilobaseBINDER kit was from Thermo Fisher Scientific. Full-length human Polλ protein and rabbit anti-Polλ polyclonal antibody were kind gifts from Drs. Thomas Kunkel and Samuel H. Wilson respectively. Polλ−39 was a generous gift from Dr. Miguel Garcia-Diaz. G418 and hygromycin were from Sigma. 'Heavy' dGTP or 2'-deoxyguanosine 5'-triphosphate ($^{13}C_{10}$, 98%; 96–98% $^{15}N_5$,) was procured from Cambridge Isotope Laboratories, Inc.

2.2 Cell lines

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The wild-type (WT), *POLB*$^{+/+}$ MEF cell line (Mβ 16tsA) and the isogenic *POLB*$^{-/-}$ cell line (Mβ 19tsA) were cultured as described [12]. The cell lines expressing Polβ in a *POLB*$^{-/-}$ background MB38Δ4 (Flag-K35A/K68A/K72A-Polβ, or Flag-D256A-Polβ) and MB36.3 expressing WT Polβ were kindly provided by Dr. Samuel H. Wilson (NIEHS, National Institutes of Health) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. In addition, MEF expressing the Polβ-variants were cultured in G418 (300 μg/ml). All cell lines were grown at 37°C under 5% CO_2.

2.3 Preparation of linear duplex and plasmid substrate

Linear duplexes containing a uracil or a tetrahydrofuran (F) residue were prepared by annealing the 'U-top' or 'F-top' oligonucleotides with the 'G-template' oligonucleotide in a 1:1.1 ratio in a buffer containing Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol.

Closed circular pGEM-3Zf (+) plasmid DNA containing a site-specific uracil was prepared as previously described [35], but with biotin-31U primer and 'heavy' dGTP. Briefly, single stranded pGEM-3Zf (+) plasmid DNA was prepared by first transforming JM109 bacteria with the pGEM-3Zf (+) plasmid. Transformed bacteria were infected with R408 helper phage to produce pGEM-3Zf (+) phage. The phage ssDNA was purified by the cetyltrimethylammonium bromide precipitation method [36]. The biotin-31U primer (200 pmol) was annealed to the ssDNA template (100 pmol) and primer extension was performed. Each primer extension reaction contained 100 pmol of primed ssDNA template, 20 mM Hepes-KOH (pH 7.8), 2 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 100 μg/ml bovine serum albumin, 450 μM each of dATP, dTTP, dCTP, and 'heavy' dGTP, 200 units of T4 DNA polymerase, and 20,000 units of T4 DNA ligase. Reactions were incubated at 37°C for 4 h and subsequently terminated by the addition of EDTA to a final concentration of 20 mM. Covalently closed circular duplex DNA was purified by ethidium bromide-cesium chloride density ultracentrifugation and extraction with *n*-butanol [35], followed by dialysis against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Purified dsDNA was analyzed on a 1% agarose gel and verified for the presence of uracil by treating an aliquot with a modest excess of UDG and Ape1. Typically, >98% of DNA contained uracil.

2.4 Preparation of cell-free extracts

Cell extracts were prepared as described previously [28]. Briefly, cells were trypsinized to remove them from culture dishes and collected by centrifugation at 500×g. The cells were then washed in phosphate-buffered saline, and centrifuged again before resuspending in buffer containing 10 mM Tris-HCl, pH 7.8 and 200 mM KCl (typically using 40 μL). Next, the suspension was mixed with an equal volume of a second buffer containing 10 mM Tris-HCl, pH 7.8, 600 mM KCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 40% glycerol, 0.2% Nonidet P-40, 2 mM dithiothreitol, and supplemented with protease inhibitor cocktail (Roche) and fresh 0.5 mM phenylmethane sulfonyl fluoride. The suspension was mixed gently for 1.5 h at 4°C and centrifuged at 16,000×g for 10 min, and the supernatant collected. The extracts were typically split into 20-μL or 100-μL aliquots, flash-frozen, and stored at −80°C. The protein concentrations of the extracts were determined using the Bio-Rad Coomassie brilliant blue protein assay, using bovine serum albumin as the standard.

2.5 In vitro BER assay with cell-free extracts

All assays were carried out in reactions containing 45 mM HEPES-KOH, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM dithiothreitol, 2 mM ATP, 1 mM nicotinamide adenine dinucleotide, 100 μg/ml bovine serum albumin and 20 μM each of dTTP, dCTP, dGTP and dATP. Typically, 3.3 pmol of linear duplex DNA and 50 μg of extract were used for each reaction. Reactions were incubated at 37°C, aliquots removed at the indicated times, and the reactions stopped by adding EDTA to a final concentration of 20 mM.

2.6 Analysis of BER reaction products

At each time point, the total amount of repaired DNA was assayed by heat-killing proteins at 65°C for 20 min, followed by treatment with a moderate excess of UDG and Ape1 (for uracil substrate) or just Ape1 (for F substrate) and incubation at 37°C for 15 min to ensure cleavage of any unrepaired DNA. The reaction products were resolved on a 16%

polyacrylamide gel containing 7 M urea and 25% formamide in 90 mM Tris-HCl, 90 mM boric acid and 2 mM EDTA, pH 8. The gel was visualized on a Typhoon FLA laser scanner (GE Healthcare). Densitometric analysis of bands corresponding to the repaired and intermediate(s) DNA species was carried out using ImageJ software. The fraction of DNA repaired was determined by normalizing the densitometric intensity of the repaired DNA band to that for the total DNA in all bands.

2.7 In vitro SP-BER with purified enzymes

The reaction mixtures contained 25 pmol of circular DNA substrate, 10 pmol each of UDG, Ape1 and Polβ, 20 μM dTTP, 1.5 mM ATP and 800 units of T4 DNA ligase in 45 mM HEPES-KOH, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 1mM dithiothreitol and 100 μg/ml bovine serum albumin. Following incubation at 37°C for 60 min, the repair patch size analysis was performed as described in section 2.8.

2.8 Repair patch size analysis

BER reactions containing 25 pmol of plasmid substrate and 850 μg of cell extracts were prepared in the standard buffer described above. After a 60 min incubation at 37°C, samples were subjected to phenol-chloroform extraction followed by ethanol precipitation of DNA. The DNA in each sample was digested with 70 units each of EcoRI and PstI (Supplemental Fig. S1). The digested DNA was captured on streptavidin Dynabeads (Thermo Fisher Scientific) according to the manufacturer's instructions. The DNA-beads suspension was incubated with a modest excess of UDG and Ape1 for 3 h at 37°C, after which the beads were collected on a magnetic stand, and the supernatant was discarded. The DNA-containing beads were resuspended in distilled water and subjected to restriction digestion by 30 units of SalI at 37°C for 3 h with intermittent shaking, which releases a 10-nucleotide DNA fragment Supplemental Fig. S1). After digestion, the supernatant was collected in a fresh tube and used as the sample for matrix-assisted laser desorption ionization/time-of flight mass spectrometry (MALDI-TOF-MS) analysis. The samples were concentrated in an evaporator, and purified using a ZipTip C_{18} resin. The sample was mixed with a 3-hydroxypicolinic acid matrix and applied to a MALDI target plate. The co-crystallized mixture on the plate was subjected to MALDI-TOF-MS analysis using the Voyager-DE *STR* (Applied Biosystems) instrument. The peak intensities generated in this way were used to determine the relative amounts of SP- and LP-BER.

2.9 Transfection

Transfection of the circular DNA uracil substrate was carried out in HEK293 cells. The cells were seeded at a density of 2.2×10^6 cells in 10-cm dishes. When the cells had reached ~80% confluence, transfection was carried out using the Lipofectamine 2000 reagent (Thermofisher) according to the manufacturer's protocol. For each plate, 16 μg of the circular DNA substrate and 70 μL of transfection reagent were used. Plasmid DNA was recovered at 16 h post-transfection in two steps: alkaline lysis of the cells to precipitate out the chromosomal DNA, following which the lysate was loaded on to a Qiagen Genomic-tip 20/G, and the DNA purified according to the manufacturer's instructions.

3. Results

3.1 Defective SP- and LP-BER in extracts of POLB$^{-/-}$ cells

We began by assessing the BER capacity of extracts from WT and *POLB*$^{-/-}$ MEF using linear duplex substrates containing a single uracil or F residue. The latter is a synthetic analog of an AP site that cannot be repaired by SP-BER [37]. Cell-extracts from MEF devoid of Polβ had a pronounced BER defect for both substrates tested, ~4-fold lower for uracil and ~8-fold for F (Fig. 1). This defect in BER activity in Polβ-deficient cells was reversed by adding purified Polβ (Fig. 2A) to the extract in amounts equal to the endogenous Polβ level, as determined by western blotting (Supplemental Fig. S2) and consistent with an earlier study [38]. These results indicate the importance of Polβ in both SP- and LP-BER, consistent with prior studies [9,11,12,39]. Our results specifically corroborate previous reports [13,40] of a critical role for Polβ in LP-BER.

3.2 Restoration of BER in POLB$^{-/-}$extracts by addition of Polλ

In order to explore the possible BER roles of Polλ, we tested whether supplementing Polβ-deficient extracts with the catalytic domain of Polλ (Polλ−39) could alleviate the BER defects of those extracts (Fig. 2B). Interestingly, adding Polλ−39 in an amount equivalent to the endogenous Polβ level fully restored the BER activity for both uracil and F in both rate and extent (Fig. 2A). These data show that Polλ−39 has the potential to support both SP- and LP-BER as effectively as does Polβ.

The ability of exogenous Polλ−39 to enable wild-type levels of SP- and LP-BER is something of a paradox, as Polλ is already present in the *POLB*$^{-/-}$ cells. We estimated the level of Polλ protein in MEFs by western blotting (Supplemental Fig. S2). This estimate gave a value ~50% of the Polβ level (0.1 pmol Polβ per 10 μg cell extract or ~7×10^4 molecules/cell), which in principle could support considerable BER activity - yet it does not. One possibility is that the separated 39-kDa domain of Polλ is hyperactive in BER compared to the full-length protein. We therefore tested whether addition of recombinant full-length Polλ could restore BER in *POLB*$^{-/-}$ extracts. For both uracil and F repair, the added full-length Polλ complemented the BER defect nearly as efficiently as did exogenous Polλ−39 and Polβ (Fig. 2C). Collectively, these results show that, in MEF extracts, added exogenous Polλ−39 and full-length Polλ can support efficient BER.

It seemed possible that we had misestimated the Polβ and Polλ levels such that we were adding excessive amounts of the proteins to the extracts. We therefore performed a series of titration experiments with Polβ and full-length Polλ, adding the purified proteins in amounts 10–200% of the level used for Fig. 2. The results (Fig. 3) indicate that these enzymes were not in large excess, with a level of 50% already showing reduced SP- and LP-BER, and still further reductions at lower levels. Thus, there appears to be sufficient endogenous Polλ to have considerable potential in BER.

3.3 Validation of method to assess BER patch size

We developed a new approach to determine the contribution of the single-nucleotide and multinucleotide pathways in BER. As described in section 2.3, we constructed substrates

with a single uracil residue with nearby mass-labeled dGMP residues (Fig. 4A). The assay is based on the principle that replacement of each "heavy" dGMP by a normal dGMP during the course of repair would decrease the mass number of the repaired DNA by ≈15 mass units, with a corresponding shift in the m/z peak in the MALDI mass spectrum (Fig. 4B). For illustration purposes, the schematic shows six nucleotides downstream from the lesion. In our experiments, the final DNA fragment generated after digestion with restriction enzymes is a 10-mer with three nucleotides downstream from lesion position (Supplemental Fig. S1, Supplemental Table 1). To test the robustness of this approach and to validate the assay, we reconstituted SP-BER with purified proteins (and only dTTP, to prevent LP-BER; Fig. 4A) acting on the mass-labeled plasmid substrate. The repaired sample was processed for analysis by MALDI-MS (see Section 2.8). The calculated mass of the substrate/unrepaired oligonucleotide is 3070 mass units, while that of the expected SP-BER product is 3084 (Supplemental Table 1), resulting from the replacement of a dUMP residue by a dTMP. MALDI –MS analysis of the repaired sample resulted in a peak at m/z= 3083, while the control (unrepaired) sample generated a peak at m/z= 3071 (Supplemental Fig. S3 A, B), consistent with the expected action of SP-BER. An error of ± 1 mass unit in the observed mass is within the calibration error of the instrument for these samples (analysis in the linear mode results in a broad peak, and Gaussian smoothing of the curve can result in small discrepancies).

In addition to validate the method in a more complete system, we measured repair of the mass-labeled uracil substrate using HEK293 cell extracts. In those experiments, 60–80% of the starting substrate was repaired (data not shown). Nearly two-thirds of the repair events proceeded via SP-BER (Fig. 4C), which was consistent with previous studies demonstrating single-nucleotide repair as the predominant pathway for uracil repair in other mammalian cell extracts [2,11]. Next, in order to determine whether the mass-labeled substrate is repaired effectively *in vivo,* we transfected the plasmid substrate into HEK293 cells (which has relatively high transfection efficiency; [41]). Of the total recovered plasmid DNA (see Fig. 5A), ~45% was repaired (Fig. 5B). Unfortunately, however, the low overall yield of DNA recovered in this procedure was too low for MALDI-MS analysis to determine repair patch size.

3.4 Analysis of repair patch size for uracil in MEF cell extracts

Uracil can be repaired by both SP- and LP-BER, as has been demonstrated both with purified enzymes and in mammalian cell-free extracts [2,4,5,11,42,43]. Here we sought to ascertain the distribution of SP-and LP-BER in uracil repair with WT extracts, or *POLB*$^{-/-}$ MEF extracts supplemented with purified Polλ−39. Similar to the result observed with the linear duplex substrate, we found that, compared to WT extracts, *POLB*$^{-/-}$ extracts were deficient in repairing the mass-labeled circular DNA substrate (Fig. 6A). As seen with the linear DNA substrates, adding recombinant Polβ to Polβ-deficient extracts restored the BER activity for the circular DNA substrate. Again, adding either Polλ−39 or the full-length Polλ increased the total substrate repaired during 60 minutes (Fig. 6A). We then determined the uracil-repair patch size using the mass-label assay. In WT MEF extracts, the majority (80%) of repair occurred by SP-BER (Fig. 6B; Supplemental Fig. S4A); the amount of repaired DNA in the *POLB*$^{-/-}$ extract was too low to allow a repair patch size analysis. In Polβ-

deficient extracts supplemented with Polλ−39, ~8 0% of the repair was via SP-BER and 20% by LP-BER (Fig. 6B; Supplemental Fig. S4B). Together with the observations from Fig. 2, we conclude that Polλ−39 is able to participate effectively in SP- and LP-BER in cell-free extracts.

In preliminary experiments we determined the sensitivity limit in our mass spectrometry analysis to be ~50 fmol oligonucleotide (in the buffer 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and desalted using a C_{18} resin). In some experiments, the peak for LP-BER (which was typically 25% of the SP-BER peak) was not always well resolved (signal: noise ≤3), possibly due to a lower amount of material (<50 fmol) and the presence of low-molecular-weight species that increased the overall noise. The noise from low-molecular-weight species in the range of 1000–2000 Da (Supplemental Fig. S3) might be due to buffer components or cell extract material carried over into the final sample (see Section 2.8).

3.5 5’-dRP lyase and DNA polymerase activities of Polβ in BER

Polβ possesses a 5’-dRP/AP lyase activity contained in its 8 kDa N-terminal domain, and a DNA polymerase activity contained in its 39 kDa C-terminal domain [44]. It has been shown that the 5’-dRP lyase activity of Polβ, rather than the DNA polymerase activity, is required for the resistance of MEF cells to the DNA alkylation damage caused by methyl methanesulfonate [12]. To test the contribution of these activities in SP- and LP-BER in the extracts, we measured the BER activity of *POLB*$^{-/-}$ MEF extracts expressing either a lyase-dead (K35A/K68A/K72A) or a polymerase-dead (D256A) variant of Polβ [39] (see Fig. 7A). For the K35A/K68A/K72A protein, a partial deficiency in uracil repair was observed as expected (Fig. 7B, left). However, we did not expect the observed 2- to 3-fold reduction in repair of an F-residue (Fig. 7B. right), which cannot be excised by the lyase. Extracts from cells expressing the D256A variant exhibited an acute repair defect for both substrates equivalent to that of MEF extracts devoid of Polβ (Fig. 7B). This result indicates a critical role of the polymerase activity of Polβ in both SP- and LP-BER, which contrasts with the *in vivo* result in which the polymerase activity was dispensable [39]. In our scenario, other polymerases in the extract cannot substitute for Polβ, even in LP-BER. However, it was possible that D256A-Polβ binds to the BER substrate tightly enough to prevent access by other DNA polymerases. To rule out this possibility, we tested whether supplementing the D256A-expressing extracts with either Polβ or Polλ affects BER activity. For both uracil and F-substrates the level of BER was restored by the addition of a recombinant polymerase (Fig. 7C).

For the circular DNA substrate, the overall repair (at 60 min) in both the lyase- and DNA polymerase-deficient extracts was ≤50% of that observed in the WT extracts (Figure 7B). The lower amounts of repaired DNA in those samples were not sufficient for the patch size analysis.

4. Discussion

In vitro studies with cell-free extracts have established Polβ as a central BER enzyme, with Polλ playing a secondary role or acting on a restricted category of substrates. Fitting a

central BER role for Polβ, this DNA polymerase acts preferentially at nicks or one-nucleotide gaps in repair intermediates [45]. Although Polλ can act processively in small gaps in DNA [20], the enzyme has limited strand-displacement activity on nicked and one-nucleotide gapped structures [32]. These characteristics might be expected to give Polλ a greater role in SP-BER than in LP-BER. However, in the presence of FEN1 and PCNA, the strand displacement activity of Polλ is stimulated [32,33], which would support LP-BER. Here we have shown that the Polβ-like Polλ−39 protein can participate in both SP- and LP-BER when added to MEF extracts lacking Polβ. Full-length Polλ certainly can also participate in LP-BER, as the enzyme effectively supports repair of an F residue, which cannot be handled by SP-BER. Despite its evident potential, endogenous Polλ was unable to compensate for the loss of Polβ in MEF extracts, consistent with previous observations [28].

The 5'-dRP lyase activity of Polλ is ~4-fold lower than that of Polβ [19], while the single-nucleotide gap-filling activity of both enzymes is similar [46], which might again point to a greater proportion of LP-BER with Polλ than with Polβ. The results seen with the titration of recombinant Polλ into *POLB*$^{-/-}$ MEF extracts (Fig. 3) indicate that the 5'-dRP lyase activity is not rate-limiting for the overall repair of a uracil residue *in vitro*. As a corollary, it seems unlikely that the slower 5'-dRP lyase activity of Polλ could be the reason for the observed repair deficiency in Polβ-deficient extracts. These observations suggest that endogenous Polλ is somehow restricted from participating in BER, at least for added DNA substrates.

The N-terminal 250 residues of Polλ can be divided into two major regions, a BRCT domain (residues 36–132), and a serine-/proline-rich region (residues 133–244) [27]. The BRCT domain of Polλ interacts with components of the non-homologous end joining (NHEJ) pathway, namely Ku protein and the XRCC4-DNA ligase IV complex. Consistent with these interactions, the BRCT domain is required for Polλ activity on a NHEJ model substrate *in vitro* [23,47,48]. The serine-/proline-rich region is a target for Cdk2-cyclin A phosphorylation, which affects Polλ's interaction with PCNA, prevents Polλ degradation via the ubiquitin-proteasome pathway, and promotes recruitment of the DNA polymerase to chromatin [49–51]. More recently, threonine-204 was found to be phosphorylated by the ATM and DNA-PKcs kinases, which modulates Polλ activity and the protein's interaction with other NHEJ proteins [24]. It is conceivable that other post-translational modifications influence activity or localization of the protein with regard to BER. Phosphorylation in the serine-proline-rich region does not influence the DNA polymerase activity of Polλ on an open primer/template structure [49]. However, Shimazaki et al. [52] showed that the activity of full-length Polλ on a poly(dA)-oligo(dT) substrate was ~3-fold lower than that of Polλ −39. In one experiment with a SP-BER reaction reconstituted using a linear uracil substrate and purified enzymes, Polλ−39 generated twice as much repaired product as did the full-length protein (Supplemental Fig. S5), which suggests that full-length Polλ may have reduced activity at BER substrates relative to Polλ−39. Nonetheless, the observation that exogenous full-length Polλ effectively complemented the BER defect in *POLB*$^{-/-}$ cell extracts shows that the recombinant protein does not have inherent defects in BER. Thus, endogenous Polλ may be constrained by interactions with other proteins, or modified in a way that limits the protein's activity in BER.

Removal of the 5'-dRP group is critical in BER, and this step can be rate-limiting in some circumstances [53]. The excision of the 5'-dRP moiety may occur either by the 5'-dRP lyase activity of a DNA polymerase or other enzyme, or by its removal as part of the "flap" during LP-BER [54]. Since the 5'-dRP lyase activity of polymerase is not used in LP-BER, we did not expect to see a decrease in BER of the F-substrate in extracts of cells expressing a lyase-deficient form of Polβ. However, we observed a ~2-to 3-fold reduction in repair of the F-substrate (Fig. 7B, right), which may be due inefficient PCNA-dependent repair of linear substrates [55]. This triply-substituted variant of Polβ also has decreased binding of the 5'-phosphate on the downstream strand [15], which could affect the overall repair rate. In the case of uracil repair, extracts with lyase-deficient Polβ showed only a partial reduction in uracil repair activity on both linear and circular substrates (Fig. 6A, 7B), suggesting that other lyases can partially compensate for the loss of Polβ's 5'-dRP lyase function. Another possibility is that the failure to process this 5'-blocking group skews the repair distribution toward LP-BER.

We also explored how a defect in the DNA polymerase activity of Polβ affected the repair of the uracil and F substrates. Although polymerases other than Polβ are implicated in LP-BER [9,10], in our experiments Polβ's polymerase activity was essential for the efficient repair of both the uracil and the F-substrate. This result implies that, at least for a linear DNA substrate, Polβ plays a critical role in LP-BER too, perhaps for initiating DNA repair synthesis, which would be consistent with some previous observations [13,40]. It is also worth noting that, compared to WT cell-free extracts, the repair activity in *POLB*$^{-/-}$ extracts for the circular uracil substrate was only ~2-fold lower after 60 min, while it was ~4-fold lower for the linear substrate (compare Fig. 1A and 6A). The somewhat better Polβ-independent repair for the circular substrate may reflect a higher contribution of PCNA-dependent repair to it than to the repair of linear substrates [55].

Using a mass-label method, we report the measurement of the relative contribution of SP- and LP-BER to uracil repair. In our reaction conditions, SP-BER was the predominant pathway in extracts of both HEK293 and WT MEF cells, consistent with previous observations with other mammalian cell-free extracts [2,11]. The distribution was retained when Polβ-deficient extracts were supplemented with recombinant Polλ−39. This result contrasts somewhat with a study reported by Mosbaugh's group [56] using a different approach, in which >50% of uracil repair in MEF extracts was ascribed to LP-BER. More insightful would be to assess BER patch sizes *in vivo*. To this end, we have been able to recover circular DNA from HEK293 cells following transfection and demonstrated that it was ~50% repaired. Future efforts will focus on improving DNA recovery and optimizing the mass-label method to enable the eventual analysis of repair products formed *in vivo.*

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Krokan HE, Bjoras M (2013) Base excision repair. Cold Spring Harb Perspect Biol 5: a012583. [PubMed: 23545420]
2. Dianov G, Price A, Lindahl T (1992) Generation of single-nucleotide repair patches following excision of uracil residues from DNA. Mol Cell Biol 12: 1605–1612. [PubMed: 1549115]
3. Matsumoto Y, Kim K, Bogenhagen DF (1994) Proliferating cell nuclear antigen-dependent abasic site repair in Xenopus laevis oocytes: an alternative pathway of base excision DNA repair. Mol Cell Biol 14: 6187–6197. [PubMed: 7915006]
4. Frosina G, Fortini P, Rossi O, Carrozzino F, Raspaglio G, et al. (1996) Two pathways for base excision repair in mammalian cells. J Biol Chem 271: 9573–9578. [PubMed: 8621631]
5. Klungland A, Lindahl T (1997) Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). EMBO J 16: 3341–3348. [PubMed: 9214649]
6. Kim YJ, Wilson DM 3rd (2012) Overview of base excision repair biochemistry. Curr Mol Pharmacol 5: 3–13. [PubMed: 22122461]
7. Wiederhold L, Leppard JB, Kedar P, Karimi-Busheri F, Rasouli-Nia A, et al. (2004) AP endonuclease-independent DNA base excision repair in human cells. Mol Cell 15: 209–220. [PubMed: 15260972]
8. Das A, Wiederhold L, Leppard JB, Kedar P, Prasad R, et al. (2006) NEIL2-initiated, APE-independent repair of oxidized bases in DNA: Evidence for a repair complex in human cells. DNA Repair (Amst) 5: 1439–1448. [PubMed: 16982218]
9. Stucki M, Pascucci B, Parlanti E, Fortini P, Wilson SH, et al. (1998) Mammalian base excision repair by DNA polymerases delta and epsilon. Oncogene 17: 835–843. [PubMed: 9780000]
10. Fortini P, Pascucci B, Parlanti E, Sobol RW, Wilson SH, et al. (1998) Different DNA polymerases are involved in the short- and long-patch base excision repair in mammalian cells. Biochemistry 37: 3575–3580. [PubMed: 9530283]
11. Singhal RK, Prasad R, Wilson SH (1995) DNA polymerase beta conducts the gap-filling step in uracilinitiated base excision repair in a bovine testis nuclear extract. J Biol Chem 270: 949–957. [PubMed: 7822335]
12. Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, et al. (1996) Requirement of mammalian DNA polymerase-beta in base-excision repair. Nature 379: 183–186. [PubMed: 8538772]
13. Dianov GL, Prasad R, Wilson SH, Bohr VA (1999) Role of DNA polymerase beta in the excision step of long patch mammalian base excision repair. J Biol Chem 274: 13741–13743. [PubMed: 10318775]
14. Horton JK, Baker A, Berg BJ, Sobol RW, Wilson SH (2002) Involvement of DNA polymerase beta in protection against the cytotoxicity of oxidative DNA damage. DNA Repair (Amst) 1: 317–333. [PubMed: 12509250]
15. Prasad R, Beard WA, Chyan JY, Maciejewski MW, Mullen GP, et al. (1998) Functional analysis of the amino-terminal 8-kDa domain of DNA polymerase beta as revealed by site-directed mutagenesis. DNA binding and 5'-deoxyribose phosphate lyase activities. J Biol Chem 273: 11121–11126. [PubMed: 9556598]
16. Kumar A, Widen SG, Williams KR, Kedar P, Karpel RL, et al. (1990) Studies of the domain structure of mammalian DNA polymerase beta. Identification of a discrete template binding domain. J Biol Chem 265: 2124–2131. [PubMed: 2404980]
17. Nagasawa K, Kitamura K, Yasui A, Nimura Y, Ikeda K, et al. (2000) Identification and characterization of human DNA polymerase beta 2, a DNA polymerase beta -related enzyme. J Biol Chem 275: 31233–31238. [PubMed: 10887191]

18. Garcia-Diaz M, Dominguez O, Lopez-Fernandez LA, de Lera LT, Saniger ML, et al. (2000) DNA polymerase lambda (Pol lambda), a novel eukaryotic DNA polymerase with a potential role in meiosis. J Mol Biol 301: 851–867. [PubMed: 10966791]
19. Garcia-Diaz M, Bebenek K, Kunkel TA, Blanco L (2001) Identification of an intrinsic 5'-deoxyribose-5-phosphate lyase activity in human DNA polymerase lambda: a possible role in base excision repair. J Biol Chem 276: 34659–34663. [PubMed: 11457865]
20. Garcia-Diaz M, Bebenek K, Sabariegos R, Dominguez O, Rodriguez J, et al. (2002) DNA polymerase lambda, a novel DNA repair enzyme in human cells. J Biol Chem 277: 13184–13191. [PubMed: 11821417]
21. Bebenek K, Pedersen LC, Kunkel TA (2014) Structure-function studies of DNA polymerase lambda. Biochemistry 53: 2781–2792. [PubMed: 24716527]
22. Lee JW, Blanco L, Zhou T, Garcia-Diaz M, Bebenek K, et al. (2004) Implication of DNA polymerase lambda in alignment-based gap filling for nonhomologous DNA end joining in human nuclear extracts. J Biol Chem 279: 805–811. [PubMed: 14561766]
23. Fan W, Wu X (2004) DNA polymerase lambda can elongate on DNA substrates mimicking nonhomologous end joining and interact with XRCC4-ligase IV complex. Biochem Biophys Res Commun 323: 1328–1333. [PubMed: 15451442]
24. Sastre-Moreno G, Pryor JM, Moreno-Onate M, Herrero-Ruiz AM, Cortes-Ledesma F, et al. (2017) Regulation of human pollambda by ATM-mediated phosphorylation during non-homologous end joining. DNA Repair (Amst) 51: 31–45. [PubMed: 28109743]
25. Garcia-Diaz M, Bebenek K, Gao G, Pedersen LC, London RE, et al. (2005) Structure-function studies of DNA polymerase lambda. DNA Repair (Amst) 4: 1358–1367. [PubMed: 16213194]
26. Fiala KA, Abdel-Gawad W, Suo Z (2004) Pre-steady-state kinetic studies of the fidelity and mechanism of polymerization catalyzed by truncated human DNA polymerase lambda. Biochemistry 43: 6751–6762. [PubMed: 15157109]
27. Belousova EA, Lavrik OI (2015) DNA polymerases beta and lambda and their roles in cell. DNA Repair (Amst) 29: 112–126. [PubMed: 25715666]
28. Braithwaite EK, Prasad R, Shock DD, Hou EW, Beard WA, et al. (2005) DNA polymerase lambda mediates a back-up base excision repair activity in extracts of mouse embryonic fibroblasts. J Biol Chem 280: 18469–18475. [PubMed: 15749700]
29. Tano K, Nakamura J, Asagoshi K, Arakawa H, Sonoda E, et al. (2007) Interplay between DNA polymerases beta and lambda in repair of oxidation DNA damage in chicken DT40 cells. DNA Repair (Amst) 6: 869–875. [PubMed: 17363341]
30. Braithwaite EK, Kedar PS, Stumpo DJ, Bertocci B, Freedman JH, et al. (2010) DNA polymerases beta and lambda mediate overlapping and independent roles in base excision repair in mouse embryonic fibroblasts. PLoS One 5: e12229. [PubMed: 20805875]
31. Braithwaite EK, Kedar PS, Lan L, Polosina YY, Asagoshi K, et al. (2005) DNA polymerase lambda protects mouse fibroblasts against oxidative DNA damage and is recruited to sites of DNA damage/repair. J Biol Chem 280: 31641–31647. [PubMed: 16002405]
32. Lebedeva NA, Rechkunova NI, Dezhurov SV, Khodyreva SN, Favre A, et al. (2005) Comparison of functional properties of mammalian DNA polymerase lambda and DNA polymerase beta in reactions of DNA synthesis related to DNA repair. Biochim Biophys Acta 1751: 150–158. [PubMed: 15979954]
33. Maga G, Villani G, Ramadan K, Shevelev I, Tanguy Le Gac N, et al. (2002) Human DNA polymerase lambda functionally and physically interacts with proliferating cell nuclear antigen in normal and translesion DNA synthesis. J Biol Chem 277: 48434–48440. [PubMed: 12368291]
34. Masuda Y, Bennett RA, Demple B (1998) Dynamics of the interaction of human apurinic endonuclease (Ape1) with its substrate and product. J Biol Chem 273: 30352–30359. [PubMed: 9804798]
35. Sung JS, DeMott MS, Demple B (2005) Long-patch base excision DNA repair of 2-deoxyribonolactone prevents the formation of DNA-protein cross-links with DNA polymerase beta. J Biol Chem 280: 39095–39103. [PubMed: 16188889]

36. Del Sal G, Manfioletti G, Schneider C (1989) The CTAB-DNA precipitation method: a common miniscale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. Biotechniques 7: 514–520. [PubMed: 2699240]
37. Matsumoto Y, Bogenhagen DF (1989) Repair of a synthetic abasic site in DNA in a Xenopus laevis oocyte extract. Mol Cell Biol 9: 3750–3757. [PubMed: 2779565]
38. Sung JS, Demple B (2006) Analysis of base excision DNA repair of the oxidative lesion 2-deoxyribonolactone and the formation of DNA-protein cross-links. Methods Enzymol 408: 48–64. [PubMed: 16793362]
39. Sobol RW, Prasad R, Evenski A, Baker A, Yang XP, et al. (2000) The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity. Nature 405: 807–810. [PubMed: 10866204]
40. Podlutsky AJ, Dianova II, Podust VN, Bohr VA, Dianov GL (2001) Human DNA polymerase beta initiates DNA synthesis during long-patch repair of reduced AP sites in DNA. EMBO J 20: 1477–1482. [PubMed: 11250913]
41. Thomas P, Smart TG (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharmacol Toxicol Methods 51: 187–200. [PubMed: 15862464]
42. Kubota Y, Nash RA, Klungland A, Schar P, Barnes DE, et al. (1996) Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. EMBO J 15: 6662–6670. [PubMed: 8978692]
43. Nicholl ID, Nealon K, Kenny MK (1997) Reconstitution of human base excision repair with purified proteins. Biochemistry 36: 7557–7566. [PubMed: 9200707]
44. Beard WA, Wilson SH (2014) Structure and mechanism of DNA polymerase beta. Biochemistry 53: 2768–2780. [PubMed: 24717170]
45. Beard WA, Prasad R, Wilson SH (2006) Activities and mechanism of DNA polymerase beta. Methods Enzymol 408: 91–107. [PubMed: 16793365]
46. Fiala KA, Duym WW, Zhang J, Suo Z (2006) Up-regulation of the fidelity of human DNA polymerase lambda by its non-enzymatic proline-rich domain. J Biol Chem 281: 19038–19044. [PubMed: 16675458]
47. Ma Y, Lu H, Tippin B, Goodman MF, Shimazaki N, et al. (2004) A biochemically defined system for mammalian nonhomologous DNA end joining. Mol Cell 16: 701–713. [PubMed: 15574326]
48. Nick McElhinny SA, Havener JM, Garcia-Diaz M, Juarez R, Bebenek K, et al. (2005) A gradient of template dependence defines distinct biological roles for family X polymerases in nonhomologous end joining. Mol Cell 19: 357–366. [PubMed: 16061182]
49. Frouin I, Toueille M, Ferrari E, Shevelev I, Hubscher U (2005) Phosphorylation of human DNA polymerase lambda by the cyclin-dependent kinase Cdk2/cyclin A complex is modulated by its association with proliferating cell nuclear antigen. Nucleic Acids Res 33: 5354–5361. [PubMed: 16174846]
50. Wimmer U, Ferrari E, Hunziker P, Hubscher U (2008) Control of DNA polymerase lambda stability by phosphorylation and ubiquitination during the cell cycle. EMBO Rep 9: 1027–1033. [PubMed: 18688254]
51. Markkanen E, van Loon B, Ferrari E, Parsons JL, Dianov GL, et al. (2012) Regulation of oxidative DNA damage repair by DNA polymerase lambda and MutYH by cross-talk of phosphorylation and ubiquitination. Proc Natl Acad Sci U S A 109: 437–442. [PubMed: 22203964]
52. Shimazaki N, Yoshida K, Kobayashi T, Toji S, Tamai K, et al. (2002) Over-expression of human DNA polymerase lambda in E. coli and characterization of the recombinant enzyme. Genes Cells 7: 639–651. [PubMed: 12081642]
53. Srivastava DK, Berg BJ, Prasad R, Molina JT, Beard WA, et al. (1998) Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps. J Biol Chem 273: 21203–21209. [PubMed: 9694877]
54. Dianov GL, Hubscher U (2013) Mammalian base excision repair: the forgotten archangel. Nucleic Acids Res 41: 3483–3490. [PubMed: 23408852]
55. Biade S, Sobol RW, Wilson SH, Matsumoto Y (1998) Impairment of proliferating cell nuclear antigen-dependent apurinic/apyrimidinic site repair on linear DNA. J Biol Chem 273: 898–902. [PubMed: 9422747]

56. Bennett SE, Sung JS, Mosbaugh DW (2001) Fidelity of uracil-initiated base excision DNA repair in DNA polymerase beta-proficient and -deficient mouse embryonic fibroblast cell extracts. J Biol Chem 276: 42588–42600. [PubMed: 11551933]

Highlights

- Added DNA polymerase λ (Polλ) complemented BER defects in *POLB*$^{-/-}$ MEF extracts
- A new mass-label method determined the distribution of SP- and LP-BER in cells
- Added Polλ supported both the SP- and the LP-BER pathways in amounts Polβ
- Polymerase and lyase defects in Polβ both depressed SP- and LP-BER
- Endogenous Polλ is evidently constrained from acting in BER in cell-free extracts

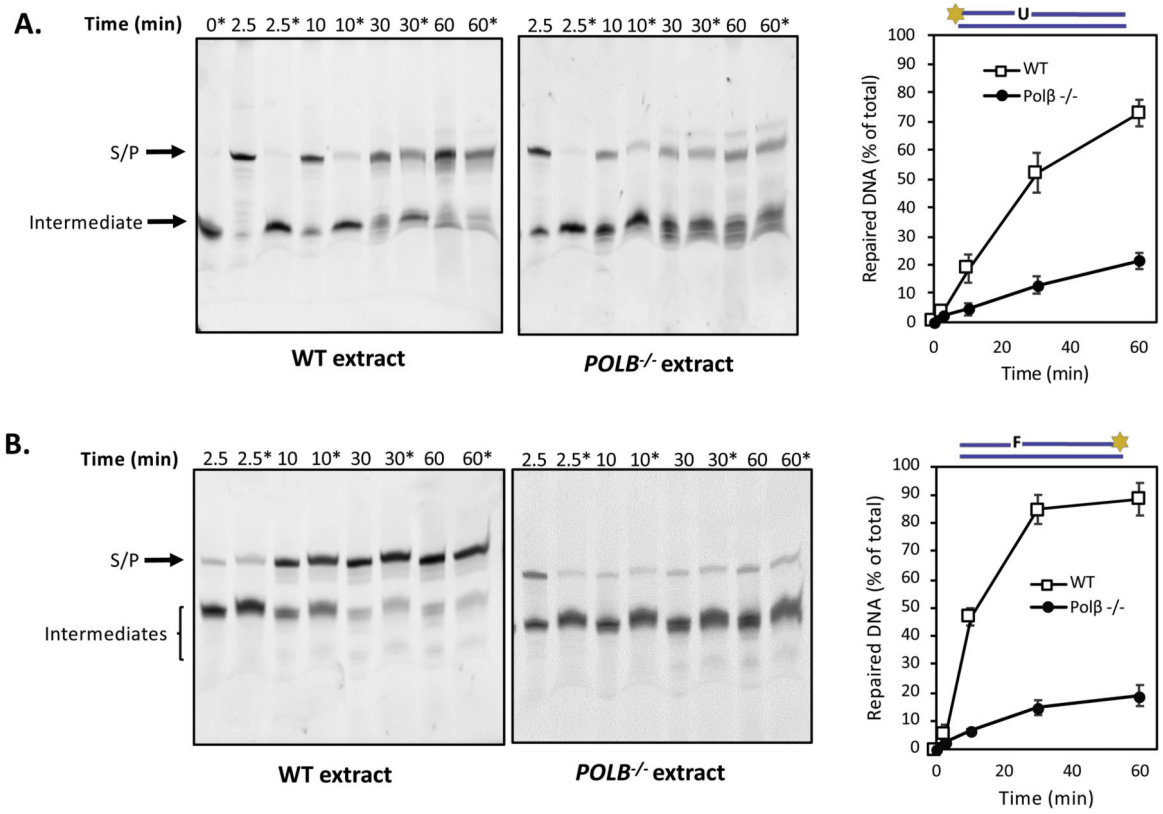

Figure 1. BER activity in MEFs.

Linear oligonucleotide duplexes containing a single uracil (A) or F lesion (B) were used as substrates in repair assays with WT and *POLB*$^{-/-}$ extracts. Reactions were performed and analyzed as described in Section 2.5 and 2.6. Where indicated (* time points), after the reaction an aliquot was treated with UDG and Ape1 (U substrate) or Ape1 alone (F-substrate) to cleave any unrepaired DNA. After electrophoresis, the resolved products from the UDG/Ape1-treated treated samples were quantified to determine the fraction of DNA repaired. Positions of the substrate (S), intermediates, and repaired product (P) bands are indicated. For each experiment, a representative gel image is shown. The means ± standard errors are plotted (right; n=3).

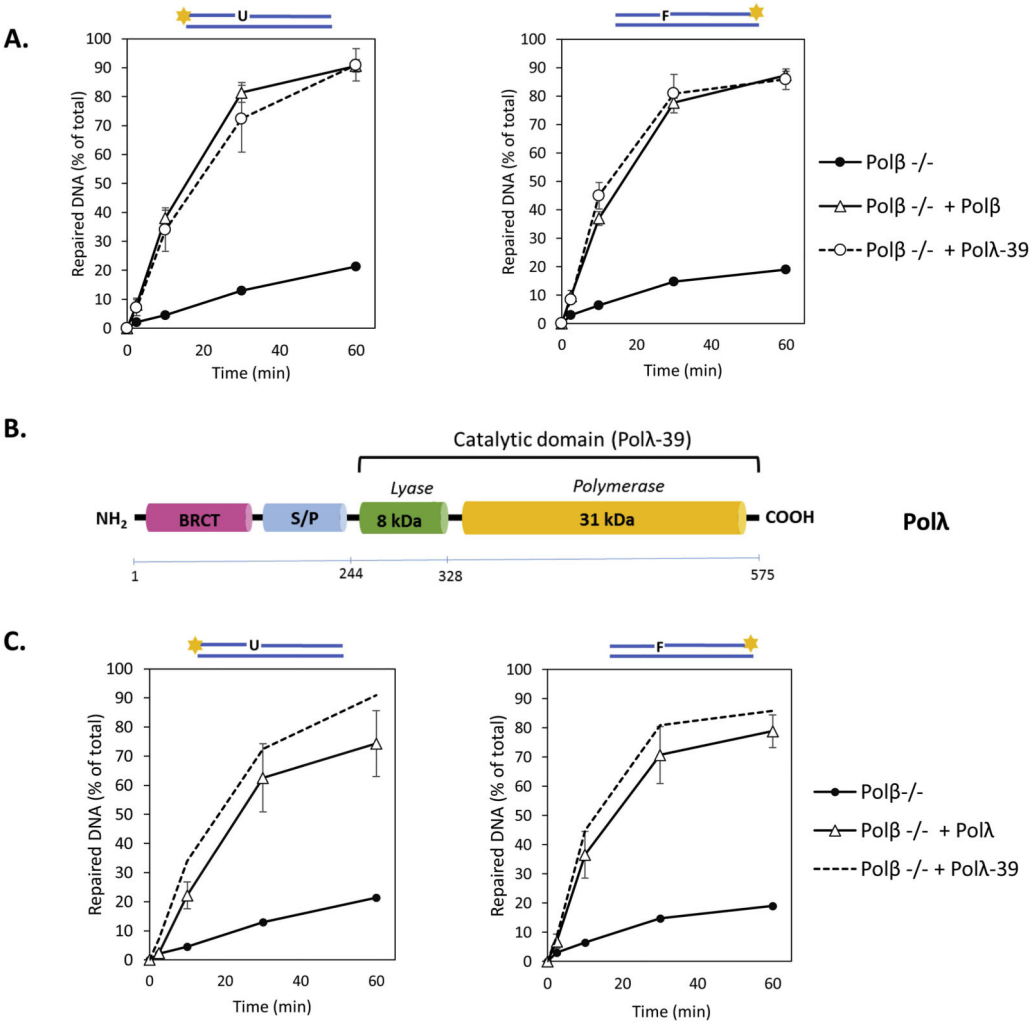

Figure 2. Polλ rescues the in vitro BER deficiency of *POLB*$^{-/-}$ extracts.

(*A*) For each sample, linear DNA substrate containing a single uracil or an F residue was incubated with 50 μg of *POLB*$^{-/-}$ extract, and where indicated, supplemented with 0.5 pmol of Polβ, with 0.5 pmol of Polλ−39, or (C) with 0.5 pmol of full-length Polλ. Reactions were performed and analyzed as described in Section 2. The means ± standard errors are plotted (n=3). Data points from experiment *POLB*$^{-/-}$ extract (Fig.1) are also plotted for reference. (*B*) A schematic showing the domain organization of Polλ.

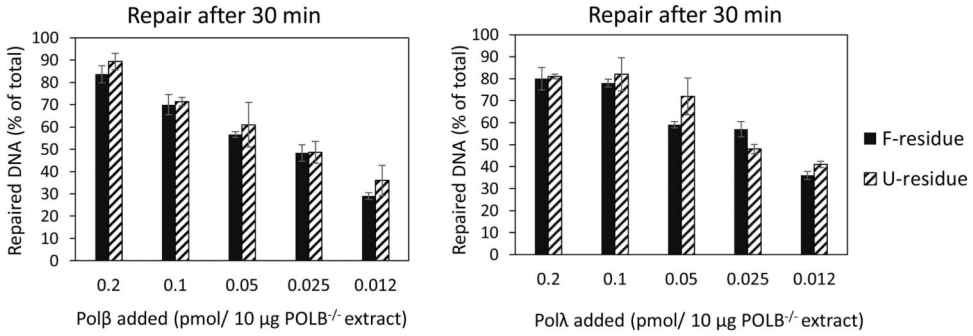

Figure 3. Titration of Polβ and Polλ in *POLB*$^{-/-}$ extracts.

The linear uracil and F substrates were used in a series of experiments where the indicated amounts of purified Polβ (left) or Polλ (right) were added to *POLB*$^{-/-}$ extracts. Reactions were performed with an incubation time of 30 min at 37°C and analyzed as described in Section 2.5 and 2.6. The means ± standard errors are plotted (n=3).

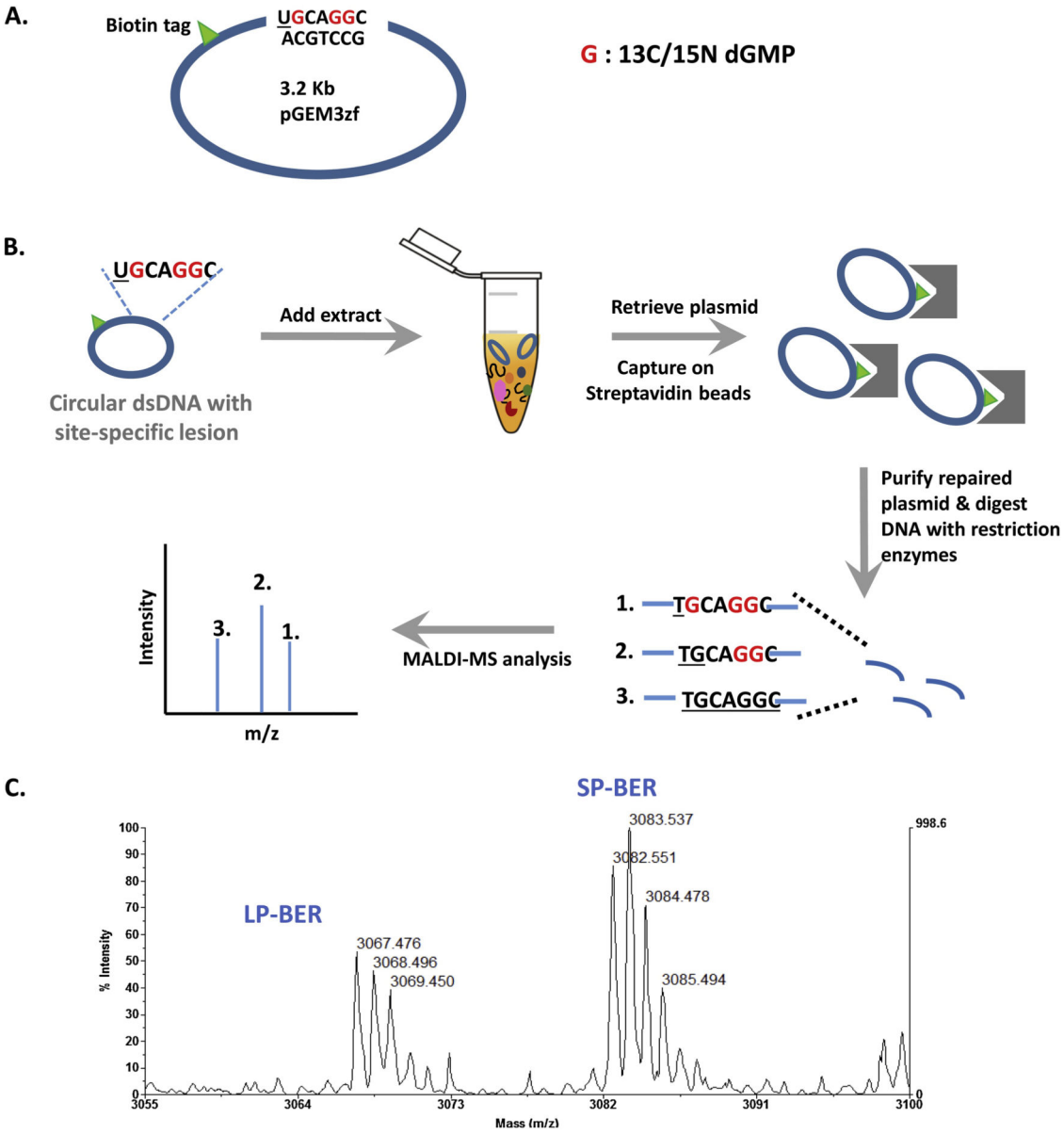

Figure 4. Assay to measure repair patch size.

(*A*) Plasmid substrate with a site-specific uracil and flanking mass-labeled guanine nucleotides. (*B*) Schematic of the assay workflow. For details see Section 2.3 and 2.8. *(C)* Mass spectrum of repaired DNA using MALDI in the reflector mode. The sample was incubated with a HEK293 extract (see Section 2.8). Carbon isotope peak pattern is shown (C) for both SP-BER (calculated molecular mass= 3084) and LP-BER (calculated molecular mass= 3068).

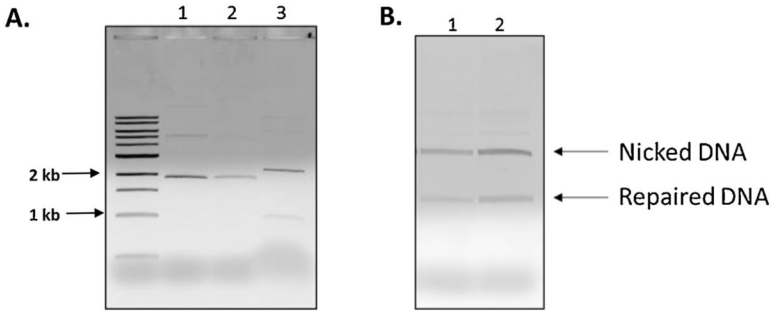

Figure 5. Analysis of repaired plasmid DNA recovered after transfection.

Recovered DNA *(A, lane 2)* was confirmed by restriction digestion with AatII and BamHI *(A, lane 3)*. The pGEM3Zf (+) plasmid was loaded as a marker *(A, lane1)*. Leftmost lane: a 1-kb ladder. The amount of DNA repaired was estimated by treating the samples with UDG and Ape1 (using amounts sufficient to cleave all unrepaired DNA, as determined by experiments with the unrepaired substrate), and the products were resolved on a 0.8% agarose gel containing ethidium bromide. Analysis of the DNA recovered from two experiments is shown *(B, lane 1 and 2)*.

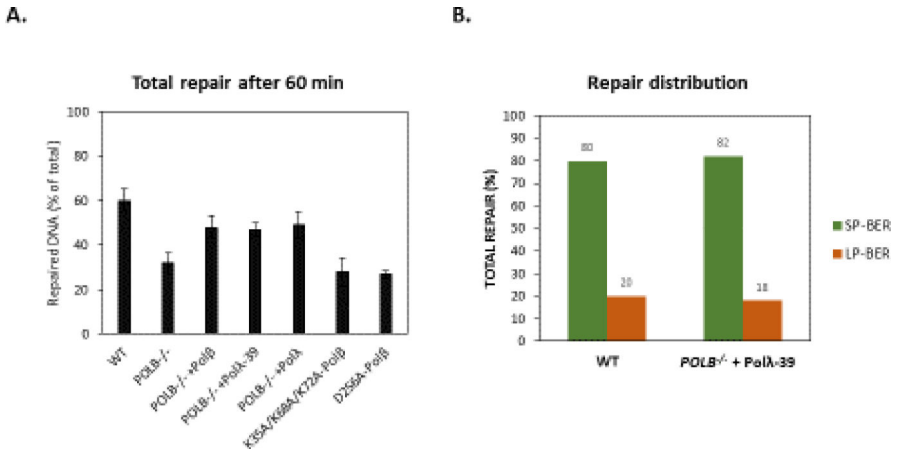

Figure 6. Uracil-repair patch size distribution.

(*A*) Quantitative analysis of total repair of the circular substrate using MEF extracts. Reaction mixtures containing 0.1 pmol of circular substrate in the standard *in vitro* assay reaction buffer (see Section 2.5) and 3.5 μg of cell extract were incubated at 37°C for 60 min, followed by incubation at 65°C for 20 min to inactivate enzymes. The total repaired DNA in each sample was determined by adding UDG and Ape1 to ensure cleavage of any unrepaired DNA. Reaction products were resolved on a 0.8% agarose gel and quantified using ImageJ. (*B*) Uracil-repair patch size distribution determined after MALDI-mass spectrometry. Analysis was performed as described in Section 2.8.

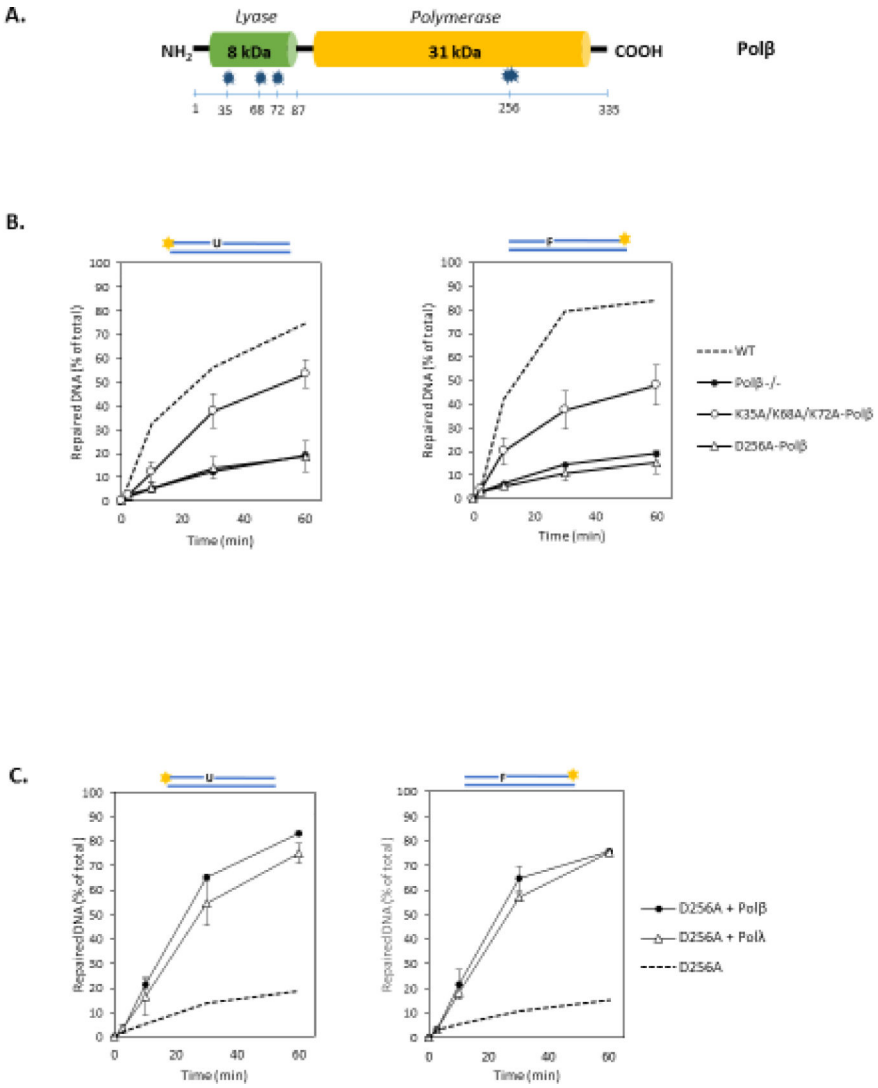

Figure 7. The 5'-dRP lyase and DNA polymerase activities of Polβ in BER.

(*A*) Domain organization of Polβ showing the position of residues changed in the Polβ variants. (*B*) Repair of uracil and F-containing linear substrates in WT, *POLB*$^{-/-}$ MEF extracts and extracts from *POLB*$^{-/-}$ cells expressing lyase-dead (K35A/K68A/K72A) or polymerase-dead (D256A) variants of Polβ, with Polλ, or with Polλ−39. Data points from experiment with WT and *POLB*$^{-/-}$ extract (Fig.1) are also plotted for reference. (*C*) Repair of uracil and F-containing linear substrates in *POLB*$^{-/-}$ MEF extracts expressing D256A variant of Polβ and supplemented with 0.5 pmol of recombinant Polβ or Polλ. Data points from experiment D256A-Polβ expressing extract are also plotted for reference. The means ± standard errors are plotted (n=3).