DNA polymerase beta participates in mitochondrial DNA repair

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Running title: A novel function for DNA polymerase beta in the mitochondria

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Supplementary Methods:

Cell lines: SV40 large T-antigen transformed MEF cell lines MB16tsA, clone 1B5 (Polβ +/+) and passage matched line MB19tsA, clone 2B2 (Polβ -/-) are derived from Day 10.5 embryos from the same mother and purchased from American Type Tissue Culture (ATCC, Manassas, VA). Recently, the MB19tsa line was also found to be Polymerase iota deficient [3]. The HEK293T cell line is a human embryonic kidney cell line that has SV40 T-antigen purchased from ATCC. All cell lines were cultured and maintained as per documentation provided by ATCC and were regularly checked for mycoplasma contamination. Primary mouse embryonic fibroblasts (detailed in supplementary methods) were taken from embryos at Day 12 and used for experiments at passages 4 to 7. All cell lines were grown with antibiotics protection using penicillin and streptomycin.

Animals: All mice were maintained on a standard NIH diet with a 12 hour light/dark cycle. Animals were group housed where possible and had *ad lib*. access to food and water. All mice were bred at the National Institute of Aging (Baltimore, MD). P*olβ* $^{(+/-)}$ mice on a mixed genetic background were originally obtained from Dr. AR Heydari (Wayne State University, Detroit, MI). Wild type (WT) mice were derived from breeding P*olβ* $^{(+/-)}$ mice. The P*olβ* colony was maintained by breeding WT and P*olβ* $^{(+/-)}$ since homozygous mice are embryonic lethal. Refer to previous publication for further information[5].

Preparation of mouse embryonic fibroblasts: Primary mouse embryonic fibroblasts (MEF) were obtained from embryos produced by mating Polβ (+/-) mice. Pregnant mice were euthanized by cervical dislocation after day 12 of gestation. Each embryo was removed from the mother and the transferred to a plate containing trypsin solution (5 mL trypsin/ EDTA). The embryos were minced and transferred to tubes containing 1ml trypsin solution, vortexed and incubated at 37 °C. The homogenate was transferred to a new tube and centrifuged for 5 minutes at 1000 rpm. 5 mL of trypsin solution was added, the pellet re-suspended and re-incubated at 37 ºC. The trypsinization procedure was repeated 2 to 3 times. Dulbecco's modified Eagle's medium (DMEM) (Gibco®- Thermo Fisher scientific, Waltman, MA) supplemented with 15% fetal bovine serum (Gibco) was then added to the supernatant and the mixture transferred in 100 mm dishes for culture. Each embryo was harvested individually and resulted in a unique cell line. These cell lines were maintained in DMEM+10% FBS and 1% non-essential amino acids with 1% penicillin/streptomycin (Gibco) at 36 °C in a humidified incubator with 5% CO_2. After two days in culture a cell pellet was collected for genotyping. To genotype DNA was extracted using the DNeasy™ Blood and Tissue kit (Qiagen, Valencia, CA) as per manufactures instructions. Extracted DNA was initially quantitated using a spectrophotometer (Nanodrop®, Thermofisher Scientific) with all samples then normalized to

100 ng/µl lambda DNA standard using Quant-iT™ Picogreen® dsDNA quantitation (Thermofisher Scientific). Genotyping was conducted using a custom made PCR probe (Integrated DNA technologies, Coralville, IA) specific to the deleted *polβ* region in the mouse genome forward primers (CCTCAACGGCGGCATCA) and reverse primers (TCCCGGGATGGAGGTGT) with an amplicon of 171 nucleotides. RT-PCR was conducted on 7900HT RT-PCR machine (Applied Biosystems, Thermofisher scientific) with GAPDH used as an endogenous control (TAQMAN probe Mm99999915_g1, Applied Biosystems). Each assay was done with a minimum of four technical replicates and confirmed by western protein analysis.

Immunofluorescence: Cells were fixed in 4% paraformaldehyde/PBS, blocked with 10% goat serum at room temperature for 60 min, and incubated with primary antibody at 4 °C overnight. Cells were washed with PBS and PBS-0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO), incubated for 60 min with Alexa-Fluor 488 goat anti-rabbit secondary antibody (Invitrogen, Thermo-fisher Scientific), and mounted in prolong gold anti-fade reagent with DAPI (Invitrogen). For mitochondrial co-localization experiments, a TFAM mouse antibody was used (ab89818; abcam). Z-stacks of images were taken using a Nikon Plan Fluor 60×/0.50–1.25 oil objective mounted on a Nikon Eclipse TE2000 confocal microscope equipped with a Hamamatsu EM-CCD camera at 37 °C using prolong gold anti-fade mounting medium with DAPI and the Volocity-5 software (PerkinElmer, Waltman, MA).Post-acquisition image processing was limited to contrast enhancement. Alternate Polβ antibodies tested that exhibited more significant cross reactivity; ab26343, ab3181 and ab1831 (abcam), SC-5925 (N-19) and SC-48819 (H-147) (Santa Cruz).

Expression of human Polβ transcripts: RT-PCR analysis of *polβ* transcripts was conducted using RNA from human organs (Ambion, Thermo fisher scientific) as previously described[4]). cDNA was made from total RNA using MultiScribe reverse transcriptase (Roche). Commercial probes (Applied Biosystems) targeted to the sequences predicted to transcribe the

N-terminal (Hs01099716_g1), catalytic (Hs01099724_m1) or C-terminal (Hs01099715_m1) domains of polβ were utilized to measure the frequency of these transcript regions. A probe for the house-keeping gene GAPDH was used as the internal loading control with each probe. Assay was conducted with a minimum of four technical replicates.

TWINKLE pulldown: The plasmid expressing human Polβ with Myc/FLAG tags were purchased from Origene (Cat# RC210765) and amplified and purified with Qiagen EndoFree Plasmid Maxi Kit. The plasmids were transfected into 293T cells with JetPRIME DNA transfection reagent (Polyplus, New York, NY) as per manufacturer's instructions. 293T cells transfected with FLAG-tagged Polβ as well as un-transfected 293T cells were harvested and suspended in lysis buffer (50 mM Tris.Cl PH7.4, 200 mM NaCl, 2 mM MgCl2, 0.6% Triton x100, 0.4% NP-40, 1x protease inhibitor cocktail, 1x phosphatase inhibitor cocktail, 20 U/mL benzonase), and disrupted by sonication. After removing cell debris by centrifugation, cell extracts were incubated with anti-FLAG Magnetic beads (M8823, Sigma-Aldrich) overnight at 4^{o}C. The beads were washed with washing buffer (20 mM Tris.Cl 7.4, 150 mM NaCl, 0.2% Triton x100) a minimum of 5 times, then mixed with 1x SDS loading buffer. Proteins were denatured at 95 ^{o}C for 5 min and submitted for western blotting. Polβ-FLAG, TWINKLE and tubulin were detected with FLAG antibody (Sigma, F3165), TWINKLE antibody (GTX116636, GeneTex, Irvine, CA) and tubulin antibody (Sigma, T9026), respectively.

Plasmid-based BER assay: Substrates containing a single defined lesion were prepared as previously described (Frosina, Cappelli et al. 1999; Akbari, Otterlei et al. 2004). Briefly, oligonucleotides were commercially synthesized (Integrated DNA Technologies) containing either a uracil or a cytosine in a defined location. The oligonucleotides were phosphorylated using phosphonucleokinase (New England Biolabs, Ipswich, MA) and annealed to single-stranded DNA prepared from pGEM-3Zf(+). Subsequently, the primed plasmid was replicated

using T4 DNA polymerase (New England Biolabs) and ligated with T4 DNA ligase (New England Biolabs) in the presence of T4 Gene 32 Protein and bovine serum albumin (BSA). To purify covalently closed, circular DNA substrates, reaction products were separated by size and purified (Qiagen). Proteins were extracted from animal brain sections by homogenizing with an equal tissue volume (1x TV) of Buffer I (10 mM Tris-HCl, pH 8.0, 200 mM KCl), followed by addition of another 1x TV 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 Scientific). Extracts were incubated at 4 ºC for 2 hours with rotation. Next, extracts were centrifuged at 14,000 rpm in a microfuge at 4 ºC for 15 min. Supernatants were removed to a new tube and protein concentrations were determined (BCA) followed by aliquoting the extracts into small volumes and storage at -80 ºC.

BER assays were performed under described conditions (Akbari, Otterlei et al. 2004), with few exceptions. Protein was diluted as described in text in 35 µL of purified water. A master mix of 5X reaction buffer (25 mM $MgCl_2$, 200 mM Hepes, pH 7.8, 2.5 mM DTT, 10 mM ATP, 100 µM dNTPs*, 1.8 mg/ml BSA), plasmid substrate (250 ng/reaction), protease and phosphatase inhibitors (Thermo Fisher) and either γ-^{32}P-dCTP or γ-^{32}P-dGTP (2.0 µCu/reaction) as specified in text was added to the diluted proteins for a total of 50 µL reactions. Reactions were incubated at 30 ºC for 1 hour and DNA was purified (Qiagen) before restriction digest. Restriction digests were performed at 37 ºC for 1 hour. An equal volume of loading buffer (90% formamide, 10% Bromophenol Blue (w/v)) was added to each digest and half of the total volume was electrophoresed on a 20% denaturing polyacrylamide gel, at 15W for 1 hour. Labelled fragments were visualized by phosphoimaging (Typhoon, GE Healthcare Lifesciences).

Metabolic studies: A comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments) was used to evaluate the general metabolic status. On the test day, all animals

were allowed to habituate to the testing environment for 30 min in their home cages. Additionally, once placed in the metabolic chambers, animals were given 2 hours prior to formal data collection to acclimatize to the metabolic chamber environment. The Respiratory Exchange Ratio (RER) is the ratio of VCO_2 to VO_2. VO_2 and VCO_2 represent the volume of O_2 consumption and the volume of CO_2 production, respectively. Activity was measured on the x and z axes by using infrared beams to count the beam breaks at consecutive intervals during a specified measurement period (48 hours). Feeding is measured by recording the difference in the scale measurement of the center feeder from one time point to another. The accumulated drink volume is measured by a volumetric drinking monitor which detects when the subject licks a specially designed sipper tube and delivers a measured volume of liquid to the subject, as per [5].

Microarray Analysis: Total RNA was used to generate biotin-labeled cRNA using the Illumina TotalPrep RNA Amplification Kit. In short, 0.5 µg of total RNA was first converted into single-stranded cDNA with reverse transcriptase using an oligo-dT primer containing the T7 RNA polymerase promoter site and then copied to produce double-stranded cDNA molecules. The double stranded cDNA was cleaned and concentrated with the supplied columns and used in an overnight in-vitro transcription reaction where single-stranded RNA (cRNA) was generated incorporating biotin-16-UTP. A total of 0.75 µg of biotin-labeled cRNA was hybridized at 58 °C for 16 hours to Illumina's Sentrix Mouse Ref-8 Expression BeadChips (Illumina, San Diego, CA). Each BeadChip has ~25,600 well-annotated RefSeq transcripts with approximately 30-fold redundancy. The arrays were washed, blocked and the labeled cRNA was detected by staining with streptavidin-Cy3. Hybridized arrays were scanned using an Illumina BeadStation 500X Genetic Analysis Systems scanner and the image data extracted using the Illumina GenomeStudio software, version 1.1.1.1. Raw microarray data were log transformed to yield z-scores. The z-ratio was calculated as the difference between the observed gene z-scores for the experimental and the control comparisons, and dividing by the standard deviation

associated with the distribution of these differences. Z-ratio values of ± 1.5 were chosen as cut-off values and calculated using a 5% false discovery rate (FDR) threshold. A complete set of 522 cellular pathways was obtained from the Molecular Signatures Database (MSigDB, Broad Institute, Massachusetts Institute of Technology, Massachusetts, USA). The complete set was tested for Geneset enrichment using parametric analysis of gene set enrichment [2]. For each pathway z-score, a p-value was computed using JMP 6.0 software to test for the significance of the z-score obtained.

Assessment of mitochondrial networks: To investigate mitochondria network, 3 x 10^4 cells were plated in 4-well chamber slides and grown overnight. The next day, the media was changed and 10 µl of MitoTracker Green (Thermo Fisher Scientific) was added. Cells were treated for 20 min before being fixed for 15 min in 4% paraformaldehyde in PBS. Cells were then washed in PBS before being mounted in prolong anti-fade gold with DAPI (Invitrogen). Images were acquired at x60 on a confocal microscope coupled with Volocity software as per immunofluorescence methods above.

mtDNA copy number and LA PCR repair assay: DNA was extracted from HEK293T cells and quantitated with the Nanodrop® 2000 spectrophotometer (Thermo Fisher Scientific) to determine total DNA content. Mitochondrial copy number used to normalize the LP-PCR results as per previous [4]. In brief, mitochondrial copy number was measured using the TAQMAN primer combination MTCO1 (Hs02596868_g1) and 18S (Hs99999901_s1) using TAQMAN fast universal PCR master mix and run on a Applied Biosystems StepOne plus RT-PCR machine. The Input DNA amount was 25 and 50ng of total DNA for each sample, which gave a linear amplification. Endogenous mitochondrial DNA damage was quantified using long amplification PCR. The assay was conducted as per [4] with the following changes. Phusion high fidelity DNA polymerase (Thermo Fisher polymerase) was used instead of the now defunct rTth DNA polymerase XL (Thermo Fisher polymerase) with more consistent results achieved. PCR

product quantitation was initially performed using the pico green standard curve approach this was compared to direct band intensity quantification using sybr green (Thermo Fisher Scientific) gel staining and imaged and analyzed using the Chemi-Doc imaging system (BioRad) running ImageLab 5.2.1 with very similar results achieved using both methods. As seen in Figure S4B each gel quantitated had an internal controls to verify that the signal was indeed linear. Any sample that had smearing or inconsistent band formation was omitted from downstream analysis.

Chloramphenicol mitochondrial mutation assay: We used the bacterial ribosome inhibitor chloramphenicol (CAP) to measure the mtDNA mutation rate of HEK293T cells after CRISPR/cas9 knockout of Polβ. Partial CAP resistance can be conferred by a single point mutation in the ribosomal RNA genes of the mitochondria, blocking the ability of CAP to bind to the region. Cells were continuously exposed to CAP at 50 µg /ml in full media for ten days. Media was changed every 2 days containing fresh CAP. Initial experiments used 10^2 to 10^4 cells per 100mm dish to establish colony forming ability of the Polβ KO cells after CAP treatment. CAP assay was then conducted in a 6-well plate plating 500 and 1000 cells in 6 wells. Cells were left to adhere for 4 hours before treatment with CAP. Upon completion of the treatment regime, the cells we fixed and stained as per previous[4]. Plates were left to dry before being scanned and counted by a Celigo cytometer (Nexcelom, San Mateo, CA) and also blinded by two investigators. . An event was deemed to be a colony size of greater than 3x10^4 μm^2 with defined borders.

CRISPR/ CAS9 Knock-out of HEK293T cells: HEK293T cells cells are a human kidney cell line purchased from ATCC and were cultured as per ATCC guidelines in DMEM media with

10% fetal bovine serum and supplemented with 1% penicillin-streptomycin in 5% CO_2 at 37°C. A dual plasmid (addgene #52963, #52962) lentiviral vector[1] system was used to facilitate CRISPR-cas9 mediated mutation of the *polb* gene. Two alternate guideRNA (gRNA) pairs were used; Polβ 1.3 – 5`GAGCAAACGGAAGGCGCCGC predicted to cut at genomic location chr8:42338643 in exon 1 of the gene, Polβ 1.5- 5`CGCCGCAGGAGACT CTCAAC predicted to cut at chr8:42338657 in exon1 of polβ. Cells were maintained in media containing the puromycin selection agent (2.5µg/ml) for 4 days before immunoblot protein validation of the knockout was performed

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