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POLB: A new role of DNA polymerase beta in mitochondrial base excision repair

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Abstract

The mitochondrial genome is a matrilineally inherited DNA that encodes numerous essential subunits of the respiratory chain in all metazoans. As such mitochondrial DNA (mtDNA) sequence integrity is vital to organismal survival, but it has a limited cadre of DNA repair activities, primarily base excision repair (BER). We have known that the mtDNA is significantly oxidized by both endogenous and exogenous sources, but this does not lead to the expected preferential formation of transversion mutations, which suggest a robust base excision repair (BER) system. This year, two different groups reported compelling evidence that what was believed to be exclusively nuclear DNA repair polymerase, POLB, is located in the mitochondria and plays a significant role in mitochondrial BER, mtDNA integrity and mitochondrial function. In this commentary, we review the findings and highlight remaining questions for the field.

Keywords

POLB; mtDNA repair; BER; SN-BER; dRP lyase; Mutagenesis

1. Introduction

Mitochondrial function depends on the proteins encoded in the nuclear and mitochondrial genomes. The diploid nuclear genome is inherited in a Mendelian fashion with equal contribution from an individual's mother and father. The mitochondrial genome, however, is transmitted exclusively from the mother. Mitochondrial DNA (mtDNA) originates from an endosymbiotic event in which an aerobic ATP producing α -proteobacterium invaded the progenitor of a eukaryotic cell. During eukaryotic evolution, the vast majority of mitochondrial resident proteins have become nuclear encoded (i.e., expressed in the cytoplasm and imported into the mitochondria). The 13 remaining proteins encoded in the residual mitochondrial genome (16,569 bp) are hydrophobic core subunits essential for mitochondrial respiratory function.

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As with the nuclear genome, mitochondrial genome integrity is essential for health. Many mutations have been described in mtDNA that cause mitochondrial dysfunction and disease [1]. Furthermore, mtDNA damage has been associated with numerous pathological states, and may serve as a mechanism by which mitochondrial dysfunction can arise to exacerbate disease [2]. Given that the nucleus has multiple sophisticated mechanisms to monitor and repair genome adducts, breaks and lesions, it is somewhat surprising that the mitochondrial genome has evolved with only a limited set of repair mechanisms [3,4]. Depending on the tissue, the mitochondrial genome exists from several hundreds to thousands of copies per cell. As such, damage to some genomes can be compensated by others; thus, the full complement of repair pathways in mitochondria might not be necessary. Furthermore, it would appear that some forms of damaged mitochondrial genomes can be removed by mitophagy [5]. Evidence from many studies indicates that the primary DNA repair activity in mammalian mitochondria is base excision repair (BER), which removes oxidative DNA lesions, single strand breaks and alkylated bases. BER proceeds in both the nucleus and mitochondria in several steps [5]. First the damaged base is recognized either a monofunctional or bifunctional glycosylase that remove the base damage and nicks the DNA, respectively, following by removal of the sugar moiety. DNA polymerases fill in the resulting gap and DNA ligase seals the nick. It was widely believed that DNA polymerase gamma (POLG) was the DNA polymerase that did the gap filling with some strand displacement causing a repair tract of several bases [6]. However, two National Institutes of Health (NIH) research teams, headed by Vilhelm Bohr at the National Institutes of Aging [7] and Samuel Wilson at the National Institute of Environmental Health Sciences (see this issue) have now demonstrated that the nuclear DNA polymerase beta (POLB; encoded by *POLB*) is both present in the mitochondria and required for a majority of BER repair activities in the organelle. In this commentary, we review these intriguing new data and discuss some of the remaining outstanding questions.

2. POLB localizes to mitochondria

These two studies used several approaches to demonstrate that POLB, previously thought to be an exclusively cytoplasmic-nuclear protein, was present in significant amounts in the mitochondria. The Bohr group showed POLB immunoreactivity in highly purified mitochondria from mouse brain and kidney, but not in liver, heart or skeletal muscle. Protease resistance assays demonstrated that POLB was encapsulated in mitochondrial preparations isolated from mouse brain. Additional evidence presented POLB immunoreactivity in mitochondrial preparations from HEK293T cells, which showed an increased signal when POLB was overexpressed. In a separate study, the Wilson group detected POLB immunoreactivity in twice-purified mitochondrial preparations isolated from HeLa, HEK293, and mouse embryonic fibroblast (MEF) cell lines. Both groups demonstrated that mitochondria from *POLB* null cells lacked immunoreactivity, removing the possibility of cross-detection of other polymerases. The Wilson group also showed that POLB in mitochondrial preparations was protease resistant, but soluble recombinant protein added to the preparation was efficiently degraded as a protease sensitivity control. Interestingly, alkaline extraction of mitochondria showed that POLB partitioned primarily into the soluble fractions, while POLG was primarily found in the membrane fraction. This

observation may stem from POLG interacting with Twinkle DNA helicase, an alkaline-insoluble protein that is an essential component of the mitochondrial replisome [8]. These data suggest that POLB does not principally associate with the mitochondrial replication apparatus; however, our understanding of a potential role for POLB in this process is nascent (see below).

These studies used numerous approaches to demonstrate mitochondrial localization of POLB, and provided some hints of the mechanism of targeting. The Wilson study estimated that ~30% of POLB localizes to mitochondria through TOM20 colocalization studies. Additional high-quality immunogold electron microscopy (EM) localization studies were performed on WT and *POLB* null MEFs, demonstrating ~20% mitochondrial matrix localization and 60% nuclear localization, with no signal in *POLB*-ablated cells. Matrix localization is important as it places POLB in the same compartment as mtDNA. The Bohr group studied POLB localization after overexpression, testing the full-length isoform, a mitochondrially-targeted isoform, and a truncated isoform missing the N-terminal 17 amino acids (D17N) after having ruled out alternative splicing as the mechanism for generating a mitochondrial isoform. The mitochondrially-targeted and D17N forms colocalized with Mitotracker, a dye specific for mitochondria, but the full-length isoform was predominantly cytoplasmic. While algorithms do not predict a cleavable mitochondrial matrix leader sequence on POLB, a suggestion of an internal mitochondrial targeting helix was provided but not tested. The D17N mutant, which truncates a verified nuclear localization signal [9], suggests that a dynamic exists between the nuclear localization sequence (NLS) and an internal mitochondrial targeting sequence (MTS). Another potential mitochondrial import mechanism uses the MIA40 system, which has been shown to be responsible for APE1 import into the mitochondria [10]. Thus, the control of mitochondrial targeting for POLB remains unresolved.

3. POLB activity in mitochondria

Base excision repair requires damaged base removal, strand cleavage, new DNA synthesis, and ligation (Fig. 1). For example, lesions such as dUracil are acted on by Uracil glycosylase (UDG) that first removes the base, which is then followed by 5' phosphodiester bond cleavage by APEX1 to expose a 5' deoxysugar moiety (dRP). The 5' dRP must be removed by a dRP lyase activity to undergo single nucleotide (SN)-BER. Although a role for POLG, the mitochondrial replication polymerase, in BER has been suggested based on reconstitution studies, POLG has a dRP lyase activity 20-fold below that of POLB [11]. However, POLG is efficient at strand displacement synthesis, which would result in long patch (LP)-BER (Fig. 1). Furthermore, the high levels of 8-oxoG mtDNA modification [12] do not result in high transversion mutations rates after oxidative stress [13], which demonstrates that such lesions are efficiently repaired. These data also suggest that there should be a more effective source of dRP lyase activity in mitochondria. The studies by the Bohr and Wilson groups identified a robust dRP lyase activity in mitochondria belonging to POLB.

To test whether POLB plays a role in mitochondrial DNA repair, the Bohr group evaluated WT and heterozygous *POLB* mouse brain mitochondrial extracts for nucleotide

incorporation and ligation activity using a model damaged substrate. The substrate was a covalently-closed circular plasmid carrying a single abasic DNA analog, tetrahydrofuran (THF) modification, which unlike natural abasic sites, THF analogs are not prone to spontaneous ring opening and phosphate cleavage. WT and *POLB* heterozygous extracts showed modest, but significant differences for repair intermediates but not ligation activity. Similarly, *POLB* null HEK293T cells showed lower levels of radiolabel incorporation. In a reciprocal approach, HEK cells overexpressing *POLB* protein showed strong induction of repair intermediates and ligated products. N-Ethylmaleimide (NEM), an inhibitor of both DNA ligase and *POLG* [14,15], was used to show that full-length product required ligation and was not due to strand displacement (i.e., LP-BER). Presumably the observed polymerase activity in the NEM addition lanes would be from *POLB*, which is not affected by this concentration of NEM [16].

The Wilson group provided a compelling approach using BER repair assays with mouse mitochondrial extracts from *POLB* knockout (KO) cells or *POLB*-immunodepleted cells compared to control cells. The assay used duplex DNA substrate containing a site-specific dU substitution. BER was followed by label incorporation through gap filling activity then ligation to achieve full-length product. From mitochondrial extracts in which *POLB* protein was not depleted, this group observed efficient BER that did not require supplementation with *UDG* and *APEX1*, indicating robust glycosylase, endonuclease, *DRP* lyase, DNA repair synthesis and DNA ligase activities existed in these extracts.

4. Effects of *POLB* deficiency on mitochondria

POLB presence and activity in mitochondria was strongly supported in both studies, but the overall impact of *POLB* activity on mitochondrial function needed to be further established. Both groups provided some initial observations. Starting with effects on mtDNA, the Bohr group showed that *POLB* KO cells had an increase in the steady-state levels of DNA polymerase blocking lesions in mtDNA as shown by a quantitative PCR (qPCR)-based assay of long DNA targets [17]. A direct measure of mtDNA damage, such as strand breaks or 8-oxoG levels, was not provided. The Wilson group used a complementary approach by examining mtDNA repair kinetics after exogenous oxidant exposure using a similar qPCR assay. In MEF cells treated with the exogenous oxidant hydrogen peroxide, mtDNA is extensively damaged, but will begin to repair after media is changed (i.e., oxidant removal) [9]. The Wilson group showed that *POLB*-deficient cells demonstrated slower recovery, which improved when *POLB* was re-expressed. To ascertain how basal mtDNA damage might connect to mutation rates, the Bohr group extended their work to evaluate chloramphenicol (CAP) conversion in WT and *POLB* KO cells. CAP resistance arises from specific mutations in the mitochondrial 12S ribosomal RNA encoded in the mitochondrial genome. The overall mutation rate was inferred by the rate of formation of CAP-resistant colonies. Somewhat surprisingly, the number of CAP resistance colonies were dependent upon *POLB*. Why unresolved mtDNA damage (due to lack of *POLB*) prevents mutation has not been explored. Perhaps the damaged mitochondrial genomes are cleared by mitophagy, or it is possible that *POLB*, which lacks a proof-reading function, introduces mutations during repair.

Both groups noted significant differences in mitochondrial distribution within cells with POLB deficiency. The Bohr group noted that both mitochondrial morphology and function differed between WT and *POLB* KO cells, with *POLB* null cells showing altered mitochondrial content and increased fragmentation. Concomitantly, the Bohr group reported that total cellular superoxide was not increased, but mitochondrial superoxide was untested. The Wilson group showed that low-dose hydrogen peroxide exposure increased the production of superoxide in POLB-deficient cells. The meaning of this result will require a more detailed understanding of cellular antioxidant status, redox homeostasis, and the source of superoxide. EM micrographs by the Wilson group showed that cells lacking POLB have aberrant mitochondrial membrane morphology. Increased lipid droplets were also reported, but were not corroborated by other assays such as fluorescence microscopy, histological staining, or quantitative assays. Accumulation of lipid might suggest a decrease in mitochondrial fatty acid oxidation, but this was not directly assessed. However, the Bohr group noted that *POLB* KO cells exhibited a slight decrease in mitochondrial membrane potential with no change in basal respiration, but a decrease mitochondrial respiratory reserve capacity of ~25% when normalized to baseline, demonstrating a mild overall mitochondrial respiratory dysfunction.

Taken together, the data suggest a functional impact of POLB deficiency caused by decreased mtDNA integrity affecting mitochondrial respiration and morphology. We infer the mechanism of deficiency to be through mtDNA, presumably through RNA transcript depletion, but this was not tested in either study. It remains possible that the phenotypes observed in *POLB* null cells are a secondary defect of the absence of nuclear POLB, but the defects were never complemented with an MTS-POLB construct to restore POLB activity specifically to the mitochondria. The Bohr group showed gene expression pathway analysis for *POLB*-deficient cells, which revealed broad mitochondrial down-regulation with overlap for diseases with known involvement of mitochondrial dysfunction. Whether these changes originate with mitochondrial or nuclear POLB deficiency was not resolved, and further work is required to understand the mechanisms of these changes.

5. POLB interacting partners

Both studies used proteomic approaches to help define the interacting partners of POLB in the mitochondria. The POLB protein has two major domains, a N-terminal 8 kDa dRP lyase, followed by a short linker, and a C-terminal 31 kDa polymerase domain. The Bohr lab separated the protein into two parts (the N-terminal dRP lyase + linker and the C-terminal polymerase domains) and fused each to GST. The recombinant protein was added to DNase- and RNase-treated mitochondrial extracts to pull down interacting proteins and exclude contaminants that would be recovered through secondary interactions with nucleic acids. The number of bands recovered from the N-terminal half was significantly higher than the C-terminal half. A large number of DNA repair proteins, mitochondrial RNA-associated proteins, and mtDNA replication enzymes were detected by either mass spectrometry or western blot analysis. This included enzymes required for various steps in BER, including LIG3, TDP1, and PNKP, primarily through the C-terminus. Proteins specific to the classical LP-BER pathway were not detected, although POLB was shown in reciprocal immunoprecipitation experiments to interact with the DNA helicase Twinkle, part of the

mtDNA replisome. Interestingly, the Bohr group also observed a dose-dependent increase in displacement of a 5' flap with the addition of Twinkle to reconstituted POLB repair assays, suggesting the potential for a role of POLB to be involved in LP-BER in conjunction with Twinkle.

To elucidate interacting protein partners, the Wilson group used immunocapture of POLB from mitochondrial lysates isolated from *POLB* null MEF cells expressing vector POLB-FLAG, followed by mass spectrometry. Although this study confirmed the presence of POLB in the mitochondrial preparation, the mass spectrometry did not identify BER proteins or any obvious specific cofactors. This result could be due to recovery of nucleic acids in the preparation that added indirect proteins to the mass spectrometry profile. Nuclease treatment, protein-protein crosslinking and higher stringency washing might provide more specific targets for future study.

6. Key findings and unanswered questions

These two studies provide strong evidence for a robust POLB-dependent mitochondrial SN-BER activity (Fig. 1, right panel), and a working model suggest that perhaps POLG plays a complementary role in LP-BER (Fig. 1, left panel). However, many questions remain unanswered. Sankar Mitra's laboratory has clearly shown an important role for EXOG, a 5' exonuclease that could remove a displaced 5' flap, in the maintenance and integrity of mitochondrial function [18]. It would be expected that this enzyme would be most important for POLG mediated LP-BER (Fig. 1, left panel) and not SN-BER [18]. The majority of the evidence in these two studies was provided in cell culture models, but these studies also touch on the potential that POLB plays a tissue-specific role in SN-BER. Whether the POLB protein is limiting in tissues prone to mitochondrial damage and dysfunction, for example the sub-stantia nigra in Parkinson's disease, will be an important question for the mtDNA repair field. An equally important gap in knowledge is understanding what triggers POLB protein localization to the nucleus or mitochondria. How is POLB trafficked to the mitochondrial matrix? Is mitochondrial localization of POLB stimulated by mitochondrial stress, or is it a static population? Are there genetic deficiencies that prevent POLB mitochondrial localization that contribute to mitochondrial dysfunction and contribute to disease?

Although we know that POLB deficiency increases mtDNA damage or repair intermediates, we do not know whether specific sequences are particularly vulnerable to POLB deficiency, or whether POLB interacts with only damaged mtDNA. POLB deficiency manifests as an overall decline in spare respiration capacity, but is this through gene expression deficits, or is there a cellular signal that reduces mitochondrial biogenesis or increases autophagy? Depending on the bioenergetic demands of the tissue, this decrease in spare respiratory capacity could have important consequences for energy demanding tissue like brain. Another question is whether altered mitochondrial morphology in *POLB* null cells secondary to the respiratory defect, or does POLB play additional roles in mitochondria? Certainly, it is essential that we establish which defects are due to loss of mitochondrial POLB vs nuclear POLB.

While these studies fill an important gap in our understanding of SN-BER in mitochondria, they raise and reiterate more questions. POLB protein contains a strong dRP lyase activity, but does POLB or POLG play the primary role in LP-BER? Moreover, now that more of the players have been defined, which BER pathway is most active under specific stresses, and in which tissues? While both groups identify proteins that interact with POLB, neither provided insights into specific complexes that contain POLB. Do these complexes assemble at the site of damage, or are they pre-formed? Do they slide along the DNA or is there a damage recruitment signal?

Perhaps it is not surprising in that POLB is found in mitochondria, as nearly all proteins in LP- and SN-BER have been found in both the nucleus and mitochondria. These include the majority of glycosylases, endonucleases, ligases, and now polymerases. However, BER proteins join ever-growing list of dual localized proteins involved in DNA metabolism. These include BRCA1, p53, CtIP, MRE11, PARP, KU80, and telomerase [19,20]. This roster could be expanded to transcription factors such as ERR, PGC1 α , and likely others [21,22]. There is also a collection of proteins predicted (with several validated) to localize to mitochondria through alternative translation start sites [23]. Whether splice site switching, translation initiation site switching, or trafficking to mitochondria after post-translation modification, a gap exists in our understanding of the mechanisms of localization and the triggers that activate them. The disentangling of dual localization remains a prominent challenge in the study of mitochondrial genome stability.

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Fig 1.

Working model of POLB activities in mitochondrial base excision repair (BER). Top: Cartoon of removal of modified base (yellow star) from double stranded (ds) DNA. Excision occurs through either monofunctional or bifunctional glycosylases. Monofunctional glycosylase can remove a damaged residue (UNG1, MUTYH, and MPG) followed by 5' phosphodiester cleavage by APEX1 to produce a 5'-deoxyribosephosphate (dRP). Bifunctional glycosylases (OGG1, NTHL1, NEIL1, and NEIL2) along with endonuclease (APEX1) or kinase (PNKP and TDP1) create a single nucleotide gap intermediate. The single nucleotide gap intermediate can also be formed by conversion of the dRP-intermediate by a dRP lyase activity found in POLB or POLG. For long patch (LP)-BER (blue box), strand displacement occurs via POLG, or potentially POLB and TWNK helicase. The displaced sequence is cleaved by EXOG and FEN1 nucleases to reach an unligated repair intermediate (bottom). Single nucleotide (SN)-BER (green box) occurs when the nucleotide gap is filled in by POLB or POLG DNA synthesis to generate an unligated

intermediate (bottom). In both LP-BER and SN-BER, LIG3 joins the nascent DNA to existing sequence to complete the repair (Bottom). Abbreviations: UNG1, Uracil-DNA glycosylase; MUTYH, Adenine DNA glycosylase; MPG, DNA-3-methyladenine glycosylase; APEX1, DNA-(apurinic or apyrimidinic site) lyase; OGG1, 8-oxoG N-glycosylase/DNA lyase; NTHL1, Endonuclease III-like protein 1; NEIL1, Endonuclease 8-like 1; NEIL2, Endonuclease 8-like 2; PNKP, Bifunctional polynucleotide phosphatase/kinase; TDP1, Tyrosyl-DNA phosphodiesterase 1; POLB, DNA polymerase beta; POLG, DNA polymerase subunit gamma-1; TWNK, Twinkle protein, mitochondrial; FEN1, Flap endonuclease 1; EXOG, Nuclease EXOG, mitochondrial; LIG3, DNA ligase 3; dRP lyase (5' deoxyribose-5-phosphate lyase).