

DNA polymerases in the mitochondria: A critical review of the evidence

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1. ABSTRACT

Since 1970, the DNA polymerase gamma (PolG) has been known to be the DNA polymerase responsible for replication and repair of mitochondrial DNA, and until recently it was generally accepted that this was the only polymerase present in mitochondria. However, recent data has challenged that opinion, as several polymerases are now proposed to have activity in mitochondria. To date, their exact role of these other DNA polymerases is unclear and the amount of evidence supporting their

role in mitochondria varies greatly. Further complicating matters, no universally accepted standards have been set for definitive proof of the mitochondrial localization of a protein. To gain an appreciation of these newly proposed DNA polymerases in the mitochondria, we review the evidence and standards needed to establish the role of a polymerase in the mitochondria. Employing PolG as an example, we established a list of criteria necessary to verify the existence and function of new mitochondrial

Table 1. Human DNA polymerases and their function¹

Polymerase	Family	Chromosome	Mol. Wt. (kDa)	Function/comments
alpha (PolA)	B	Xq21.3.-q22.1.	165	Initiates replication
beta (PolB)	X	8p12-p11	39	BER, other functions
gamma (PolG)	A	15q25	140	Mitochondrial replication & repair
delta (PolD)	B	19q13.3.-.4	125	Replication, BER, NER, MMR
epsilon (PolE)	B	12q24.3.	255	Replication, checkpoint control
zeta (PolZ)	B	6q22	344	yREV3 homolog, lesion bypass
eta (PolH)	Y	6p21.1.	78	Lesion bypass, XPV, skin cancer susceptibility
theta (PolQ)	A	3q13.3.1	300	crosslink repair, Dm308, lesion bypass
iota (PolI)	Y	18q21.1.	80	Lesion bypass? BER?
kappa (PolK)	Y	5q13.1.	99	Lesion bypass, mutator when overexpressed
lambda (PolL)	X	10q23	64	TLS, NER
mu (PolM)	X	7p13	55	TdT homolog, NHEJ
nu (PolN)	A	4p16.3.	100	lesion bypass, crosslink repair?
sigma (PolS)	X	5p15	82	TRF4 or PAPD7, sister chromatid adhesion
Rev1	Y	2q11.1.-.2	125	lesion bypass
TdT	X	10q23-24	57	Terminal transferase
PrimPol	AEP	4q35.1.	65	Restart during replication stress, Mitochondrial TLS

¹. Adopted from (125)

proteins. We then apply this criteria towards several other putative mitochondrial polymerases. While there is still a lot left to be done in this exciting new direction, it is clear that PolG is not acting alone in mitochondria, opening new doors for potential replication and repair mechanisms.

2. INTRODUCTION TO MITOCHONDRIA AND BACKGROUND

Mitochondria are essential organelles containing their own DNA that carry out respiration, producing >90% of the cellular ATP. Mitochondria were first isolated in 1948 after the development of zonal centrifugation. In the early 1960s it was determined that these cytoplasmic organelles contain their own DNA. The sequence of human mitochondrial DNA (mtDNA) was determined in 1981 (1) and gene products were assigned by 1985 (2), making mtDNA the first component of the human genome to be fully sequenced. Human mtDNA is composed of a circular 16.5. kb circular genome in very high copy number, coding for 13 essential proteins needed to carry out oxidative phosphorylation, 22 tRNAs and 2 ribosomal RNA genes needed for the synthesis of those 13 polypeptides.

As discussed in detail below, mitochondrial DNA is replicated and repaired by the nuclear encoded DNA polymerase gamma (PolG). In mammals, PolG is comprised of a 140 kDa catalytic subunit containing a

DNA polymerase activity, a 3'-5' exonuclease activity and a 5'-deoxyribose lyase activity, and a dimeric accessory subunit, p55. The catalytic subunit is encoded by the POLG gene on chromosome 15q26 while the accessory subunit is encoded by the POLG2 gene on chromosome 17q23-24. MtDNA integrity depends on the accurate replication and repair of the genome. Repair of mtDNA is limited compared to nuclear DNA, as there is no evidence of nucleotide excision repair (3), and only limited evidence for mismatch repair (MMR) (4). However, due to the highly oxidative environment, mitochondria do have an efficient base excision repair (BER) to remove damaged or oxidized bases (4-9). Most of the machinery needed for BER is shared with the nucleus.

To date, there are 17 DNA polymerases that have been identified from humans (Table 1). Besides PolG, several have been implicated in mitochondria, although their exact role is unclear and the amount of evidence supporting their role in mitochondria varies greatly between the different polymerases. In the past, identification of a protein in the mitochondria was hampered by the inability to obtain highly purified mitochondrial fractions or to determine the genetic or biological function of a putative mitochondrial protein. Furthermore, no universally accepted standards have been set for definitive proof of the mitochondrial localization of a protein. To gain an appreciation of these newly proposed DNA polymerases in the mitochondria, here we review the evidence and

Table 2. Human DNA polymerases proposed in the mitochondria and how they meet the criteria for a mitochondrial protein

Criteria	PolG	PolB	PrimPol	PolZ	PolIN	PolIQ
Mitochondrial targeted sequence identified	Yes	No	No	Suggested*	No	No
Listed in MitoCarta 2.0. or MitoMiner	Yes	No	No	No	No	No
Visualized in mitochondria by Immunofluorescence	Yes	No	Suggested	Suggested	Suggested in yeast	Yes
Identified in highly purified mitochondria ¹	Yes	No	Yes?	No	Not Done	Yes
Genetic mutants or KO demonstrating defect in mtDNA	Yes	No	No	Suggested	Suggested in yeast	Yes
Evidence for biological function in mitochondria	Yes	No	Yes	No	No	Suggested
Interaction with other mitochondrial proteins	Yes	No	Yes	Suggested	Unknown	Unknown

¹The proof to identified proteins in highly purified mitochondria requires purification beyond the typical sucrose gradient

standards needed to establish the role of a polymerase in the mitochondria. Employing PolG as an example, we established a list of criteria necessary to verify the existence and function of new mitochondrial proteins. We then apply this criteria towards several other putative mitochondrial polymerases (Table 2).

3. HISTORY AND EVIDENCE SUPPORTING POLG AS THE REPLICATIVE MTDNA POLYMERASE

3.1. Identification of PolG as the mitochondrial replicase

The polymerase activity responsible for replication of mtDNA was first identified in 1970 (10, 11). This activity was found to be RNA dependent, though it failed to utilize natural RNA as a substrate, and was distinct from PolA and PolB (10-14). In 1975 the new polymerase was officially designated as Polymerase Gamma (PolG), and two years later it was shown to localize to the mitochondrial compartment (see below) (15, 16). Based on extensive homology alignments (17), PolG has been grouped with *E. coli* pol I in the Family A DNA polymerase class (Table 1). More extensive details on the history, structure, and function of PolG has been previously reviewed (18).

The identification of PolG as a mitochondrial polymerase was done in the reverse order from most proteins. Instead of assigning mitochondrial localization to a known cytosolic or nuclear protein, DNA polymerase activity was detected from isolated mitochondria and was characterized as having the same activity as the gamma polymerase isolated from the cytosol (16). Evidence supporting the functional role of PolG in mitochondria was obtained two years later in a study of isolated brain synaptosomes- resealed membrane derived from pinched off nerve endings that contain residual cytoplasm, mitochondria, and synaptic vesicles (19). PolG is the only polymerase found in these synaptosomes, and it was shown that (³H)TTP was actively incorporated into mtDNA during replication (19). The role of PolG as the

only replicative polymerase was confirmed in subsequent studies which showed that introducing antibodies directed against PolG inhibited mtDNA replication (20). Cloning of the human *POLG* gene in 1996 allowed for the identification of the mitochondrial targeting sequence. The sequence was examined by PSORT and MitoProt, two programs designed to predict mitochondrial targeting sequences (see below), and both programs readily identified a 25 aa mitochondrial targeting sequence (MTS) and cleavage site (21).

Subsequent *in vivo* studies confirmed mitochondrial localization of PolG. Early immunofluorescence staining for PolG utilized rabbit polyclonals against the human protein and found that the PolG was colocalized with mitochondria as stained with MitoTracker Red, even in the absence of mtDNA (22). Fluorescence from GFP-labeled PolG has also been shown to associate with mitochondria co-stained with MitoTracker Red, indicating that PolG is exclusive to mitochondria (23). Sub-localization studies showed myc-tagged PolG primarily localized to the inner mitochondrial membrane fraction, with a small portion found in the outer membrane fraction (similar to mtTFA, and mtSSB) (23). Furthermore, recent visualization of the p55 accessory subunit tagged with GFP revealed distinct punctate staining of the PolG2 and associated with mitochondrial nucleoids (24).

3.2. Genetic evidence supporting PolG in mitochondria

3.2.1. Genetic evidence in model organisms

In 1989 the yeast *mip1* gene was isolated and sequenced and found to be homologous to bacterial Family A DNA polymerases (25, 26). Inactivation of *mip1* lead to a loss of mitochondrial polymerase activity, resulting in a ρ^0 (no mitochondrial DNA) phenotype and proving that Mip1 is responsible for mtDNA replication (27). Inactivation of the exonuclease by site directed mutagenesis provided further genetic evidence for the proofreading function in the fidelity and maintenance of yeast mtDNA (28). Site

directed mutagenesis of essential residues in the three exonuclease domain motifs in *mip1* led to a several hundred fold increase in spontaneous mutations (28).

Genetic evidence beyond yeast is also plentiful demonstrating the role of PolG in mitochondrial DNA maintenance. In the first mouse model that eliminated PolG exonuclease activity, the PolG variant was transgenically targeted to the heart resulting in severe cardiomyopathy accompanied by mtDNA mutations and deletions (29). Several years later, two groups independently created mice homozygous for exonuclease-deficient PolG (30, 31). These mice exhibited premature aging between six and nine months, characterized by graying hair, loss of hair and hearing, curvature of the spine, enlarged hearts, and decreased body weight and bone density (30, 31). Using the “random capture method,” where the frequency of mutations that cause resistance to restriction endonuclease digestion is enriched, Vermulst *et al.* determined the mutation frequency in mtDNA in homozygous exonuclease deficient mice was ~2500-fold higher than wild type (32). Knockout of the *Polg* gene as well as the *Polg2* gene causes embryonic lethality in mice at embryonic day ~8.5 with concomitant loss of mtDNA (33, 34).

3.2.2. Genetic evidence in human cells

Cells overexpressing PolG mutants that have no polymerase activity (but have active exonuclease activity intact), such as the D1135A and D890N mutants, have decreased levels of mtDNA 96 hours after expression (23). In addition to replication activity, *in vivo* proofreading (3'-5') activity of mtDNA has also been demonstrated for PolG. Alanine substitution of crucial residues leads to accumulation of mtDNA point mutations, demonstrating a direct role for PolG in mtDNA replication and repair (23, 35).

3.3. PolG in health and disease

A wide array of mitochondrial disorders are caused by defects in nuclear proteins that are responsible for the replication and maintenance of mitochondrial DNA. Multiple mtDNA deletions and point mutations can cause disorders such as progressive external ophthalmoplegia (PEO) and ataxia-neuropathy syndromes, and MtDNA depletion can lead to early childhood disorders such as Alpers-Huttenlocher syndrome (AHS), hepatocerebral syndromes, myocerebrohepatopathy spectrum (MCHS), and fatal myopathies (36-38). To date, over 300 point mutations in PolG have been associated with mitochondrial disease, and PolG mutations are the most common cause of inherited mitochondrial disorders (38), reviewed in (4, 39-41) (42).

4. CRITERIA TO SUPPORT THE EVIDENCE FOR MITOCHONDRIAL PROTEINS

We can easily look back on the literature and determine what was used to establish and convince

the scientific community that PolG is mitochondrial localized and is the only replicative DNA polymerase in the mitochondria. Our goal is to use that evidence, as well as new methodologies and techniques, to set up a list of standards for other putative mitochondrial proteins. Therefore, we have created criteria from old and new techniques that focus on two fundamental areas: 1) Localization of the protein in the mitochondria, and 2) Biological function. Subsequent sections evaluate the evidence supporting the presence of other polymerases in the mitochondria based on these criteria. These criteria can also readily be applied to any proposed mitochondrial protein.

4.1. Localization

4.1.1. MitoCarta 2.0. and MitoMiner

Current online tools and databases, such as MitoCarta 2.0. and MitoMiner, list and predict putative mitochondrial proteins. MitoCarta is a dynamic inventory of mitochondrial proteins (<http://www.broadinstitute.org/scientific-community/science/programs/metabolic-disease-program/publications/mitocarta/mitocarta-in-0>). Developed by V. Mootha and coworkers, MitoCarta 2.0. (43) is an update of MitoCarta 1.0. (44) and is a comprehensive inventory of mitochondrial proteins as determined by mass spectrometry of isolated mitochondria, GFP-tagging, and microscopy that is integrated with several other genome datasets of mitochondrial localization. In this inventory, PolG and PolG2 are included, but no other human or mouse DNA polymerases are found in MitoCarta 2.0. (Table 2). However, this does not rule out the possibility of other DNA polymerases transiently existing in the mitochondria. For example, p53 is also not in this inventory, but is generally accepted to have a role in mitochondrial mediated apoptosis (45, 46). MitoMiner (<http://mitominer.mrc-mbu.cam.ac.uk/release-4.0/>) is an integrated web resource of proteins with mitochondrial localization evidence and phenotype data from mammals, zebrafish and yeasts. It is integrated with several other databases and prediction programs (see next section) as well as with MitoCarta 2.0.

4.1.2. Identifying the mitochondrial targeting sequence (MTS)

Mitochondrial proteins are directed to the mitochondria by targeting sequences (MTS), usually located at the N-terminus, that are recognized by the TOM and TIM23 complexes for transport across the outer and inner membranes, respectively, followed by proteolytic cleavage of the MTS. Several computation algorithms can predict both the MTS and in some cases the cleavage site; the most popular programs are Target P (<http://www.cbs.dtu.dk/services/TargetP/>), MitoProt II (<https://ihg.gsf.de/ihg/mitoprot.html>), Predotar (<https://urgi.versailles.inra.fr/predotar/predotar.html>), and PSORT II (<http://psort.hgc.jp/>). Each program uses slightly different criteria for prediction, and thus multiple programs should be used to help identify a putative MTS.

To confirm a predicted MTS, the sequence can be engineered to drive a reporter gene product, such as GFP, into the mitochondria. Further proof can be provided by disruption of this MTS by point mutations followed by failure to drive the reporter protein. While the majority of MTS sequences are found at the N-terminus, a handful of proteins contain a C-terminal or internal MTS, and these MTS's can elude prediction by the programs mentioned above. For example, Dna2 is a protein with an internal MTS that can drive the EGFP fusion protein into mitochondria (6).

4.1.3. Fluorescence microscopy

Visualizing proteins by immunofluorescence has come a long way since its first introduction, and today there are several choices of mitochondrial dyes, protein labels, and much improved high-resolution fluorescence microscopy that allow for much greater confidence in colocalization studies. Some of the original colocalization of PolG in mitochondria relied on polyclonal antibodies co-staining with MitoTracker Red (22). For polymerases or other proteins expected to act on mtDNA, new advances in high-resolution fluorescence microscopy allow for the visualization of discrete puncta within a field of MitoTracker Red staining mitochondria, as has been recently shown using GFP labeled PolG2 (24). Because mtDNA is the substrate for DNA polymerases, a requirement of localization should be to visualize the DNA polymerase coincident with nucleoids. It is worth noting that some polymerases might localize to mtDNA under normal growth conditions, while others might require conditions that obligate mitochondrial DNA replication (growth on glycerol for yeast or growth in galactose media for mammalian cells) or subjected cells to DNA damaging stress. Therefore testing multiple conditions should be required to satisfy this fluorescence requirement.

4.1.4. Isolating the native protein from highly purified mitochondria

While technical aspects immunofluorescence studies have vastly improved, there are still some potential pitfalls to consider. For instance, fluorescence staining of GFP fusion proteins is usually derived from an overexpression system, and one needs to consider that overexpression of any protein may precipitated in the cytoplasm that causing aberrant immunofluorescence. Therefore it has become important to show direct isolation of the protein(s) in question from purified mitochondria.

Proving a protein is located within mitochondrial can be difficult and highly contentious because of the level of contaminants that can associate with the mitochondrial outer membrane. Mitochondria are highly charged lipophilic organelles, and disruption of the cells can result in many different species of proteins artificially associating with the outer membrane. The conventional purification of mitochondria involves gentle disruption of the cell in hypotonic buffer followed by differential

centrifugation to remove nuclei then followed by pelleting of the mitochondria (47). These enriched mitochondria are then further purified by two-step sucrose gradients (47). As discussed below, after sucrose gradient this enriched mitochondria may still have many other contaminants in the preparation that require further purification or treatment. Further separation of the mitochondria over percoll gradients will usually remove the remaining contaminants, or protease treatment of the mitochondria will remove proteins bound to the outer membrane. Digitonin treatment of purified mitochondria strips away the outer membrane thus more thoroughly accomplishing the same goal as proteolysis treatment, but it is more difficult to use. Trypsin digests can also be used to assign the location of a putative mitochondrial protein to a specific compartment of the mitochondria.

4.1.5. Import assays

The import of mitochondrial proteins can be visualized by an *in vitro* import assay involving the import of radiolabeled *in vitro* translated polypeptides into purified mitochondria. Full length protein is translated *in vitro* by reticulocyte lysate containing ³⁵S-labeled methionine in the presence of isolated mitochondria, followed by protease treatment and re-isolation of the mitochondria (48). The imported protein is then visualized by autoradiography in the re-isolated mitochondria.

4.2. Function in the mitochondria: Biology and genetics

A critical element in determining whether a protein is mitochondrial or not is biological or genetic evidence supporting function. Gene deletions or genetic mutations in the polymerase gene should result in a measurable defect in the mtDNA, either in loss of mtDNA or increase in mutations. Examples of gene knockout and point mutations in the *polG* gene in yeast and mammals were previously discussed. For a repair DNA polymerase, the increase in mtDNA mutations may not be measurable until the cell is challenged with DNA damaging agents that give rise to lesions in mtDNA. It is important to consider the possibility of measurable defects in a wide array of *in vitro* and *in vivo* models. Furthermore, clinical disease alleles can also provide strong evidence for a biological role for a putative mitochondrial protein.

5. PRIMPOL

Human PrimPol (CCDC111) is a member of the AEP superfamily of archaeo-eukaryotic primase/polymerases (49). It had unique DNA and RNA priming activities and has important roles in translesion synthesis (TLS), the ability of a polymerase to insert a nucleotide opposite a lesion or adduct and extend the nucleotide (50-53). We will only briefly discuss key features and activities of PrimPol, as it has recently been reviewed (54), and will primarily focus on PrimPol's role in mitochondria.

PrimPol contains an N-terminal AEP polymerase domain and a C-terminal zinc-finger domain responsible for DNA binding. The C-terminal is responsible for modulating the primase activity, processivity, and fidelity of the enzyme (55). The N-terminal contains 3 catalytic motifs, with the motif I and III responsible for divalent metal ion binding and motif II required for nucleotide binding. PrimPol can utilize either Mn^{2+} or Mg^{2+} *in vitro*, and the choice of divalent cation can affect the activity of PrimPol (see below) (51).

The primary role for PrimPol is to rescue replication forks stalled at bulky lesions by either bypassing the damage through translesion synthesis or adding primers downstream of the damage and restarting replication. Loss of PrimPol results in slower replication forks and increased sensitivity to DNA damaging agents (53, 55). PrimPol's primase activity is unique for eukaryotes in that it is capable of utilizing either dNTPs or NTPs for primer synthesis; Mn^{2+} is a 1,000-fold better activator than Mg^{2+} and results in primers longer than 100 nucleotides in length, whereas the use of Mg^{2+} results in primers of roughly 50 or 20 nucleotides long when dNTPs or NTPs are used, respectively (51). PrimPol is also capable of bypassing lesions that PolG cannot, including pyrimidine 6-4 pyrimidone photoproducts (52, 55), oxidative and UV-induced lesions such as 8-oxo-guanine (51, 55, 56), abasic sites (51), and cyclobutane pyrimidine dimers (52). Translesion synthesis by PrimPol is particularly error prone yet transient, as it has been shown the PrimPol incorporates only 4 bp on average per binding event (55, 57). However, while TLS conducted by PrimPol is highly mutagenic (57), PrimPol repriming events downstream of an AP site, particularly those created an APOBEC/AID family protein, leads to error-free resumption of replication, resulting in an overall anti-mutagenic effect (58). Taken together, these unique roles indicate an important role for PrimPol in maintenance of nuclear and potentially mitochondrial DNA.

5.1. Identification of PrimPol as a mitochondrial protein

The initial characterization of PrimPol was published independently by two groups (50, 51), however it was Garcia *et al.* who first identified PrimPol as a mitochondrial protein. Using HeLa cell fractionation, they found 34% of PrimPol signal was in the mitochondria compartment (compared to 19% in the nuclear compartment). The submitochondrial location was determined by treating mitochondria isolated from HEK 293 cells with either hypotonic buffer or digitonin and trypsin; PrimPol was resistant to trypsin digest in a manner similar to TFAM, indicating it is located in the matrix (51). At the time of this review no mitochondrial targeting sequence has been identified for PrimPol, and no mitochondrial targeting sequence is predicted by MitoProtII using either identified isoform (data not shown). Furthermore, no immunofluorescence studies have been

used to show the colocalization with mitochondria or formation of puncta on mtDNA.

5.2. Biological evidence supporting PrimPol in the mitochondria

In addition to the mitochondria localization, the initial evidence presented for PrimPol in the mitochondria was mainly biological. Fractionation of detergent-solubilized mitochondria showed that PrimPol co-fractionated with mtDNA only after formaldehyde treatment, implying that PrimPol bound to mtDNA, albeit transiently. Furthermore, the mitochondrial fractions that included PrimPol showed robust priming activity. For PrimPol to rescue blocked replication forks in mitochondria, PolG would have to be able to utilize the primers made by PrimPol. This was shown to be the case, as both PolG and PolE can utilize the primers made by PrimPol. The authors also showed that knockdown of PrimPol in human and mouse cells resulted in a decrease in overall mtDNA levels and inhibited the cell's ability to recover after chemical depletion of mtDNA (51).

In addition to the initial study, further evidence has supported the presence of PrimPol in mitochondria. Immunoprecipitation assays using mass spectroscopy to identify binding partners of PrimPol identified mtSSB as a *bona fide* binding partner. Further studies showed that mtSSB inhibits PrimPol primase primer extension activities. The mechanism for this inhibition appears to be due to mtSSB occluding PrimPol binding to DNA, as PrimPol was unable to displace mtSSB bound to single-stranded DNA. This is in direct contrast to PolG, which is stimulated by mtSSB and easily displaces mtSSB when encountered along single-stranded DNA (57).

PrimPol has been shown to interact with PolDIP2 (PDIP38), a polymerase-interacting protein that has been proposed to regulate TLS (59, 60). This interaction has been shown to stimulate PrimPol's polymerase activity, DNA binding, processivity, and bypass of 8-oxoG (61). PolDIP2 has also been shown to localize to the mitochondria. PolDIP2 has a robust N-terminal mitochondria targeting sequence (the probability of mitochondrial localization is 0.9998 as predicted by MitoProt II), and is included in the MitoCarta 2.0. library. Immunocytochemistry staining showed colocalization between mitochondria and a C-terminally HA tagged recombinant PolDIP2. Cellular fractionation showed the majority of PolDIP2 is present in the mitochondrial fraction, and trypsin digests suggest PolDIP2 is present in the matrix (62, 63). While the specific role of the PolDIP2-PrimPol interaction in mitochondria has yet to be elucidated, it is possible that these two proteins interact to increase TLS on mtDNA.

Taken together, these data support a role for PrimPol that is similar to its nuclear role, where it rescues stalled replication forks through repriming events at bulky

DNA adducts. This repriming activity is proposed to be mediated by mtSSB. While PrimPol is proposed to have a nuclear role in TLS, to date the evidence for this role in mitochondria is lacking. In fact, recent *in vitro* data showed that addition of PrimPol to the mitochondrial replisome does not increase replication past an 8-oxo-G lesion or abasic site (64). However, this study was performed without PolDIP2 or any other potential cofactors for PrimPol, which may be required for any mitochondrial TLS.

5.3. PrimPol in health and disease

There is currently only one disease mutation associated with PrimPol. PrimPol^{Y89D} is a mutation that is proposed to be involved in high myopia. On a molecular level, this mutation causes a decrease in RNA primase activity, decreased processivity, reduced DNA binding, slower replication fork progression, and increased cell sensitivity to UV damage (65). At this time it is unclear if the replication defects of PrimPol^{Y89D} directly lead to high myopia, and if these effects are mitochondrial or nuclear in nature.

Mitochondrial DNA replication has also been shown to be a secondary target of nucleoside-reverse transcript inhibitors, a class of antivirals that targets HIV reverse-transcriptase (HIV-RT). The structural similarities between PolG and HIV-RT result in misincorporation of the nucleoside reverse transcriptase inhibitor (NRTI), leading to chain termination and mitochondrial dysfunction. Recently it was shown that PrimPol can also incorporate NRTI's, albeit with different discrimination than PolG. PrimPol readily incorporated AZT-TP and CBV-TP, two commonly administered NRTIs for HIV treatment, while PolG discriminates against these two analogs by a factor of 58 and 270,000. Both of these analogs have showed mitochondrial dysfunction with long-term use, and the lack of discrimination by PrimPol could be a potential mechanism for this toxicity (66).

6. DNA POLYMERASE BETA

DNA polymerase beta (PolB) is a 39 kDa single subunit family X DNA polymerase involved in nuclear base excision repair, specifically single nucleotide base excision repair (67). PolB prefers to incorporate single nucleotides and is distributive on most DNA substrates. High resolution X-ray crystal structures have been solved for Pol b which shows that it is structurally arranged with the polymerase in a stable 31 kDa domain and a cleavable 8 kDa domain (68, 69). Located in the 8 kDa domain is a 5'-deoxyribose-5-phosphate lyase activity that efficiently removes the 5deoxyribose-phosphate moiety from the downstream DNA following nicking by AP endonuclease (70-72). This 5-sugar phosphate moiety blocks ligation and needs to be removed prior to ligation. Because of this lyase activity and the single nucleotide incorporation, this polymerase is ideally suited for base excision repair.

6.1. Proposed role of PolB in the mitochondria

As mentioned above, mitochondria do have an efficient base excision repair system and many of the enzymes in this pathway are shared between the nucleus and mitochondria. For example, uracil DNA glycosylase, OGG1, APE1, FEN1, DNA2 are all involved in base excision repair and all have been found in both the nucleus and mitochondria (4-9). In the mitochondria, as in the nucleus, base excision repair can occur via a short patch (single nucleotide) and long patch repair. The 5'deoxyribose-phosphate moiety that is retained after APE1 cleavage is susceptible to oxidation that would further block lyase action and ligation, if oxidized (5). Long patch BER deals with this 5' block by strand displacement synthesis and cleavage of the flap, and in the mitochondria PolG can perform this long patch repair with displacement of the downstream DNA.

In single nucleotide or short patch BER, the polymerase fills the single nucleotide gap and also cleaves the 5'-sugar moiety with its 5' lyase function. In the nucleus, SN-BER is carried out by PolB that also has a 5' lyase function in its 8-kDa domain. DNA PolG can also carry out SN-BER and does have a weak 5'-lyase function, although it is generally believed that PolG would likely carry out LP-BER because of its robust strand displacement activity. However, when we consider only SN-BER, the rates of 5'-lyase activity by PolG is 40-fold less than PolB (73). Since, all of the other proteins required for BER are shared between the nucleus and the mitochondria, it has been postulated that PolB may be shared between the nucleus and mitochondria to efficiently carry out mitochondrial SN-BER. For this reason, and the historical similarities of PolG and PolB with regards to inhibitors, it is logical to consider PolB as a potential mitochondrial DNA polymerase.

6.2. Conflicting evidence for PolB in mitochondria

In 2000, Nielsen-Preiss and Low reported the identification of a beta polymerase in the mitochondria isolated from bovine heart (74). Using mitochondria isolated by differential centrifugation, Nielsen-Preiss and Low identified and purified a 38 kDa polymerase that was resistant to N-ethylmaleimide and dideoxynucleotides, lacked a 3'-5' exonuclease activity and was distributive in nature. This activity remained associated with the mitochondrial preparation when the mitochondria was further purified over two successive sucrose gradients.

In contrast to the bovine heart preparations, in 2005 Hanson *et al.* presented compelling evidence for the lack of PolB in human mitochondria by obtaining highly purified mitochondrial preparations (75). Using mitochondria purified by differential centrifugation and discontinuous sucrose density gradient centrifugation, they demonstrated that PolB activity was still associated with the mitochondrial preparation and showed by

electron microscopy that this preparation still contained many other cytoplasmic factors such as polysomes, microsomes, and liposomes. Upon further purification of this fraction over a percoll gradient the authors demonstrated that the PolG activity and mitochondrial fraction can be cleanly separated from the polysomes, microsomes, and liposomal fraction as well as PolB (75). PolB activity was found primarily in the polysomes, microsomes, and liposomal fraction, and not the mitochondrial fraction. Furthermore, sequence analysis of mtDNA to detect mutation frequency in mtDNA in *polB* null mouse embryonic fibroblast cells compared to wild type cells did not show any appreciable increase, while PolG exonuclease deficient cells had a >17-fold increase in mtDNA point mutations (75). However, the scenario where cells are under exogenous or increased stress from DNA damaging agents has not been examined as of yet, and it remains possible that PolB is only imported into mitochondria when the mtDNA is under stress.

7. DNA POLYMERASE ZETA

Polymerase zeta is an error-prone TLS polymerase composed of two subunits: the catalytic RevL3 (Rev3) subunit and the structural Rev7 subunit (76). The *rev3* gene produces two isoforms of Rev3 due to the presence of two AUG translation initiation codons. Translation from the first AUG site produces a 3130aa, ~352 kDa protein, and translation from the second produces a 3052 aa, ~343 kDa protein. Rev3 has been shown to be important in the bypass of UV- and chemically-induced DNA damage, although this bypass typically results in mutations (77-79). The regulation of Rev3 expression appears to be crucial for genome stability. Deletion of *rev3* leads to chromosome instability in human and mouse cells and is embryonic lethal in mice (80-83). Conversely, overexpression of *rev3* leads to increased spontaneous mutations, and is associated with multiple types of cancer (84-86). While Rev3 has been long established as a nuclear TLS protein, more recent evidence has suggested that Rev3 is also localized to the mitochondria in humans and yeast to participate in TLS on mtDNA. While Rev7 is also suggested to localize to the mitochondria in yeast, as of the time of this review no such evidence has been reported for Rev7 localizing to the mitochondria in humans.

7.1. Rev3 and Rev7 in yeast

PolZ was initially proposed to be localized to the mitochondria in *S. cerevisiae*. The N-terminal of Rev1 (an accessory protein to PolZ), Rev3, and Rev7 were all predicted to contain mitochondrial targeting sequences by PSORTII, although the exact sequence or probability of localization was not reported. Based on these predictions, the N-terminals of each of the three proteins were fused to GFP and expressed in yeast. Immunofluorescence studies showed that all three were capable of localizing GFP into the mitochondria, and that

this fusion protein was present in mitochondrial extracts (although the presence or absence of contaminating nuclear proteins were not shown) (87).

Biologically, it has been proposed that PolZ has both error-prone and error-free roles in bypassing lesions in mitochondria. Deletion of *rev3* or *rev7* led to reduced spontaneous and UV-induced frameshift mutations (87, 88). However, point mutations were increased in both deletion strains that had been treated with UV compared to wildtype strains, suggesting that PolZ is capable of error-free lesion bypass and competes with an error prone system, most likely involving PolG (88, 89). This is in direct contrast with the nuclear role for PolZ, as spontaneous point mutations are reduced in knockout strains.

It has been proposed that PolZ and PolG have a complex interaction when maintaining mtDNA. With regard to frameshift mutations, *rev3mip1* double mutants had only a slight increase in both spontaneous and UV-induced frameshift mutations, suggesting that PolG and PolZ belong to the same epistatic pathway (87). However, overexpression of Rev3 can rescue the increased point mutability found in certain *mip1* mutants in a manner that is dependent on Rev7 but not Rev1 (89). This suggests that Rev1 function is not required in mitochondria, although it is potentially present in yeast mitochondria. While the exact mechanistic details remain to be worked out, it appears that PolZ contributes to genome stability in yeast mitochondria.

7.2. Identification of Rev3 as a mitochondrial protein in humans

Currently there is only one report of human Rev3 in mitochondria. Singh and colleagues found a putative MTS in the N-terminus of the short isoform of Rev3, a 107 aa tag with a 76.9% confidence as predicted by MitoProtII (90). The long isoform contains 78aa upstream of the MTS, which makes it less likely that this isoform is localized to the mitochondria. Immunofluorescence of cells expressing a plasmid containing the 107aa putative MTS fused to GFP showed some overlap between GFP and Mitotracker red, suggesting that some of the GFP colocalized with mitochondria. In an earlier work, it was shown that the first 100aa of the long isoform do not contain a MTS, as GFP fused to the first 100 N-terminal aa did not colocalize with mitochondria, supporting the idea that only the short isoform contains an active MTS (87). Western blots on cytoplasmic, nuclear, and mitochondrial protein fractions showed small amounts of the short isoform of Rev3 in the mitochondrial fraction with only trace amounts of the long isoform (90).

7.3. Biological evidence for Rev3 in human mitochondria

Singh *et al.*, also showed there is interplay between *rev3* expression and mitochondria function. Deletion of *rev3* in MEF cells showed decreased levels

of COXII mRNA and protein, and lower Complex IV activity (90). The Rev3^{-/-} cells also showed signs of mitochondria distress with higher glucose consumption rates, decreased mitochondrial membrane potential, and lower ROS levels, all presumably due to the lower Complex IV activity. Conversely, *rev3* expression was higher in cells treated with inhibitors of oxidative-phosphorylation (OXPHOS) and in rho⁰ cells, suggesting that increased Rev3 levels can help compensate for lowered OXPHOS.

The authors also attempted to show that Rev3 directly acts on mtDNA to help protect it from UV damage. MtDNA had increased levels of lesions following UV treatment in cells expressing Rev3 that is lacking the MTS as measured by quantitative PCR. MtDNA copy numbers were also decreased in Rev3^{-/-} cells. ChIP assays using WT cells with and without UV exposure showed Rev3 bound to both the D-loop (a non-coding region of mtDNA responsible for transcription and replication initiation) and CoxII of mtDNA. However, these were the only two regions presented so a comprehensive picture of Rev3 binding sites, particularly on mtDNA, is still lacking (90).

7.4. PolZ in health and disease

There currently are no disease mutations associated with mitochondrial disorders for either Rev3 or Rev7. However, in the study by Singh *et al*, the authors suggested a link between mitochondrial Rev3 and tumorigenesis, and showed that overexpression of Rev3 missing the nuclear localization signal increased cell migration and survival after UV exposure compared to overexpression of WT Rev3. In light of these results they suggest that the mitochondrial functions, and not nuclear, are primarily responsible for the higher tumorigenicity of the cells (90).

8. DNA POLYMERASE ETA

Polymerase eta (PolH) is a Family Y TLS polymerase that lacks 3'-5' proofreading exonuclease activity and can perform both error prone and error free TLS (91, 92). The primary role for PolH is the bypass of UV photoproducts, which performs in a primarily error-free manner. The active site of PolH is very open, allowing for full accommodation of a thymine-thymine cyclobutane pyrimidine dimer and preferentially incorporates two adenines during replication (93-95). PolH also appears to contribute to the error-free bypass of (6-4) pyrimidine-pyrimidone photoproducts (78, 96). In addition to photoproducts, PolH is capable of bypassing cisplatin induced lesions (97), 8-oxo-G (98), and me⁶G (99). In a more mutagenic role, PolH also contributes to somatic hypermutation and DNA replication under conditions of nucleotide deprivation (100, 101). Loss of PolH function leads to UV-sensitivity and skin cancer and causes xeroderma pigmentosum in the XP variant (XPV) complementation group (102).

8.1. PolH in yeast mitochondria

While there have been no reports of PolH in the mitochondria in humans, one study has reported evidence of Rad30 (the yeast PolH homolog) in mitochondria in *S. cerevisiae*. Chatterjee *et al*. noted colocalization of Rad30-GFP with DAPI signals using fluorescence microscopy, and found presence of Rad30-Myc in mitochondria purified using gradient centrifugation from a strain expressing Rad30-Myc from its native promoter (103). In support of a biological role for Rad30 on mtDNA, the authors showed that deletion of Rad30 leads to an increase in mutations following UV treatment as detected by erythromycin selection, and a small increase in frameshift reversions in an *arg8* reversion assay. This suggests that Rad30 decreases the amount of mutations during UV damage in a similar manner as Rev3. In fact, the authors found Rad30 worked epistatically with Rev3, and proposed that these two proteins function together to promote lesion bypass in a manner that is less mutagenic than if PolG attempted to carry out the bypass (103). However, several key factors in the role of these proteins, the potential interplay between the various repair polymerases, and the relevance in human cells, remains to be elucidated.

9. DNA POLYMERASE THETA

The full-length cDNA for polymerase theta (PolQ) has been shown to encode a 2592 amino acid polypeptide with an amino-terminal helicase domain, a carboxy-terminal polymerase domain, and an intervening spacer region (104, 105). Like PolG, PolQ belongs to the Family A polymerases (104, 106). Biochemical studies have shown that purified full-length human PolQ exhibits template-directed DNA polymerase activity on nicked double-stranded DNA and on a single-primed DNA template. PolQ is capable of conducting translesion DNA synthesis by inserting bases opposite an AP site or thymine glycol residue in the template strand and extending an unpaired primer base opposite these lesions (107). In contrast, PolQ cannot insert bases opposite a cyclobutane pyrimidine dimer or a (6-4) photoproduct (108). More recently, PolQ has been implicated in DSB repair, NHEJ and replication timing (109-113). Fidelity measurements of human PolQ revealed that the polymerase generates single base pair substitutions at a rate 10- to 100-fold higher than other characterized Family A DNA polymerases, making it one of the least faithful members of the Family A DNA polymerases (114).

9.1. Polymerase theta in human mitochondria

Recently, Wisnovsky *et al*. conducted a siRNA screen of known nuclear DNA repair factors in combination with cellular treatment by mt-Ox, a DNA damaging agent that exclusively elicits oxidative damage in mtDNA due to it being tethered to a mitochondria-specific peptide, to measure cell viability when grown in

low glucose. Surprisingly, several known nuclear (but not previously known to be mitochondrial) DNA repair genes were identified that cause a loss of cell viability under these conditions (115). Of these genes, siRNA knock down of PolQ and PolZ induced high levels of intrinsic toxicity when treated with mt-Ox, implicating a role in mitochondrial DNA maintenance. PolQ was further shown to be mitochondrial by identification in enriched mitochondrial fractions and protection from degradation by proteases in these fractions. Immunofluorescence showed that PolQ co-localized with MitoTracker Red and, in fact, demonstrated punctate staining and co-localization with nucleoids when stained by anti-dsDNA antibodies. Finally, *polQ* KO cells showed a reduction in basal and maximal oxygen consumption as measured by the Seahorse XF analyzer and showed a reduction in mtDNA copy number when challenged with the mt-Ox. Finally, mtDNA mutation rates as measured by deep sequencing were reduced in *polQ* KO cells. These results implicate a role in error-prone mitochondrial DNA replication when DNA adducts and damage is encountered (115).

10. TRYPANOSOMATID MITOCHONDRIAL POLYMERASES

Having multiple polymerases acting in mitochondria is not an entirely new concept; it has been known for a few decades that trypanosomes have multiple mitochondrial DNA polymerases. In trypanosomes, the mitochondrial DNA is wrapped up in kinetoplasts (kDNA), where it is replicated and/or repaired by a number of beta-like DNA polymerases and Family A type DNA polymerases (116-121). Early studies showed localization of beta polymerases from *Crithidia fasciculata* mitochondria (119), and two distinct DNA beta polymerases were identified in *Trypanosoma brucei* (122). PolIB is essential for growth and kinetoplast replication in *T. brucei* (123). Four different mitochondrial DNA polymerases belonging to family A DNA polymerases, TbPolIA, IB, IC, and ID have also been identified in *T. brucei* (121). All four polymerases are related to bacterial PolII. Interference with TbPolIB and TbPolIC activities leads to shrinkage of kDNA. Silencing TbPolIC caused depletion of kDNA minicircles and maxicircles, and concomitant accumulation of minicircle replication intermediates (121). PolIB, IC and ID are essential for parasite growth while PolIA was found to be non-essential under normal growth conditions (121). Finally, a kappa DNA polymerase from *Trypanosoma cruzi* has also been found to localize to the mitochondria (124), bringing the total number of DNA polymerase involved in kinetoplast DNA replication to seven. Interestingly, a gamma-like DNA polymerase has never been isolated from or identified in trypanosome mitochondria.

11. SUMMARY

Current literature is clearly moving away from the initial belief that PolG is the only DNA polymerase acting in the mitochondria, opening new doors for potential replication and repair mechanisms. In the past ten years we have witnessed several reports implicating other DNA polymerases in the mitochondria besides DNA PolG, namely, PrimPol, PolB, PolZ, PolH and PolQ. As can be seen from Table 2, there are many gaps in our knowledge about the roles of these other polymerases in the mitochondria and additional research needs to be performed to galvanize and unequivocally determine the roles of these other polymerases in the mitochondria.

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