Mitochondrial DNA Replication - a PrimPol Perspective

Laura J. Bailey and Aidan J. Doherty*

Genome Damage and Stability Centre, School of Life Sciences,

University of Sussex, Brighton, BN1 9RQ, UK.

* Corresponding author: Email: ajd21@sussex.ac.uk

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Abstract

PrimPol, (Primase-Polymerase), the most recently identified eukaryotic polymerase, has roles in both nuclear and mitochondrial DNA maintenance. PrimPol is able to act as a DNA polymerase, with the ability to extend primers and also bypass a variety of oxidative and photo-lesions. In addition, PrimPol also functions as a primase, catalysing the preferential formation of DNA primers in a zinc finger-dependent manner. Although PrimPol's catalytic activities have been uncovered *in vitro*, we still know little about how and why it is targeted to the mitochondrion and what its key roles are in the maintenance of this multi-copy DNA molecule. Unlike nuclear DNA, the mammalian mitochondrial genome is circular and the organelle has a number of unique proteins essential for its maintenance, presenting a differing environment within which PrimPol must function. Here, we discuss what is currently known about the mechanisms of DNA replication in the mitochondrion, the proteins that carry out these processes and how PrimPol is likely to be involved in assisting this vital cellular process.

Mitochondrial DNA – Organisation and structure

Mammalian mitochondria contain multiple copies (~1000 per cell) of a circular DNA molecule (mtDNA) that is ~16.5 Kb in length [1]. Unlike nuclear genomic DNA, virtually the entire mtDNA encodes genes that are expressed as 13 proteins, 22 tRNAs and 2 rRNAs with no introns. Only two non-coding regions exist, the non-coding region, (NCR), containing the origin of heavy-strand DNA replication and the transcription initiation start sites HSP and LSP, and $O_{L,}$ the origin of light-strand DNA replication.

Strikingly, significant physical differences exist between the mitochondrial genomes of different eukaryotic organisms (Figure 1). For example, most yeast have linear genomes ranging in size from ~19 to 150 kb, often consisting of circular permutated copies [2]. In plants, the mitochondrial genome varies even more in size, from ~200 kb up to a massive 11 Mb, due to a large number of introns and duplications (reviewed in [3,4]). In contrast, kinetoplast DNA found in the mitochondria of some protists (e.g. trypanosoma) contains two types of DNA circles, large maxi-circles (20-40 kb) containing the majority of the coding DNA that are catenated with smaller mini-circles (0.5–10 kb), which are essential for the production of functional mRNA from the uridylate encrypted maxi-circles [5,6].

In mammalian cells, mtDNA is organised into nucleoid structures containing approximately 1-2 copies of the genome, although this is likely to vary depending on tissue type and energy demand [1,7,8]. Mitochondrial nucleoids are thought to tether mtDNA to the inner mitochondrial membrane to aid in organisation, distribution and segregation. Nucleoids consist of a number of proteins involved the maintenance of mtDNA but the main DNA

packaging protein is TFAM, mitochondrial transcription factor A [9,10]. TFAM is a member of the high mobility group (HMG) of proteins, containing two HMG boxes, and is thought to condense DNA by inducing bending and then wrapping it into compacted nucleoid structures. TFAM forms homodimers with each molecule binding a separate DNA strand in order to generate looped structures or to bring different DNA molecules together leading to condensation (reviewed in [11]).

One striking feature of mammalian mtDNA is a short triple-stranded region found within the NCR, where the addition of a third DNA strand (~0.5 kb), termed 7S DNA, forms a stable displacement D-loop structure [12]. The D-loop was first identified in electron micrograph images of mtDNA and the 7S DNA was later found and named due to its sedimentation rate in caesium chloride gradient studies [12,13]. Despite the length of time since the first identification of 7S DNA and the D-loop, we still know little about its actual function (reviewed in [14]). Its abundance is found to vary greatly between species and tissues, with only 8 % of mtDNA molecules having D-loops in HeLa cells, whilst levels of 7S DNA are as high as 55 % in human placenta [15]. The D-loop is thought to have a number of roles including, acting as a recruitment site for proteins involved in the organisation of mtDNA into nucleoid structures [16,17], maintaining dNTP pools throughout the cell cycle [18] and functioning as a key component of replication (see below). Recent work has shown that this structure may actually be more complex with the identification of an RNA strand on the opposing strand to the 7S DNA forming an R-loop, which may have a role in the organisation and segregation of mtDNA [19].

A wide range of proteins are involved in the organisation, regulation and replication of the mitochondrial genome. All of these proteins are encoded within the nuclear genome and must be transported into the organelle as required. Initially, only a small number of specialised proteins were thought to be required for these processes but, as techniques have improved, we are now identifying numerous additional factors that play roles in the maintenance of mtDNA. Many of these proteins function in both nuclear and mitochondrial compartments, such as DNA2, Fen1, PIF1, Rad51C and XRCC3, some require specialised isoforms, such as Fen1 and PIF1 while others use the same isoform to perform these dual roles [20,21,22,23,24,25,26].

PrimPol- a novel eukaryotic primase-polymerase

A recent example of such a player is Primase-Polymerase (PrimPol), a newly discovered member of the archaeo-eukaryotic primase (AEP) superfamily of primases [27,28,29,30] (reviewed in [31,32]). As its name suggests, PrimPol can function both as a primase and polymerase. A number of studies have demonstrated that PrimPol is able to generate both DNA and RNA primers on single-stranded (ss) DNA templates, albeit with a strong preference for dNTPs during synthesis, ~30 fold lower K_d in Mn^{2+} [28,29,33]. In addition, PrimPol is also able to extend a DNA primer in a template-dependent manner and is capable of carrying out trans-lesion synthesis (TLS) across a number of different DNA lesions, such as 8-Oxoguanine (8-oxoG) and 6-4 photoproducts, (6-4 PP) [28,29]. PrimPol contains an N-terminal AEP domain, which contains the key catalytic residues required for all its synthesis activities. Its C-terminal UL52-like zinc finger domain is essential for its priming activity, probably by stabilising the protein's interaction with ssDNA to allow *de novo* dinucleotide synthesis that is subsequently extended to form a primer [34]. PrimPol is

a remarkably unprocessive polymerase, catalysing the insertion of only 1-4 nucleotides before disassociating from DNA and is highly mutagenic, particularly prone to produce insertion / deletion (indels) errors [34,35,36]. The limited processivity of PrimPol may act to limit its mutagenic potential. The elucidation of the structure of the catalytic core of PrimPol has highlighted its relatively small active site cleft, with limited contacts formed between the protein and the incoming primer strand [37].

PrimPol is localised in both the nucleus and the mitochondrion, suggesting that it plays similar roles in maintaining DNA integrity in both compartments [28,29]. Notably, avian cells (DT40) lacking PrimPol exhibit increased sensitivity to a number of DNA damaging agents and exhibit a pronounced G_2 arrest after exposure to UV damage [28,29]. Loss of a PrimPol orthologue (PPL2) in trypanosoma is lethal due to a failure to complete cell division in G_2/M [38]. In contrast, loss of PrimPol alone is not overtly detrimental to mammalian cells, with no obvious signs of damage sensitivity, whilst knockout mice are viable and born at mendelian ratios [28,29]. These differences likely reflect the replication poise of these cells as DT40 cells are mostly in S-phase, whilst mammalian cells sit predominantly in G_1. However, human cells become significantly more sensitive to UV damage when PrimPol and Pol η, a damage tolerance TLS polymerase, are both absent [28,39,40]. Strikingly, loss of PrimPol causes an increase in mtDNA copy number and cells exhibit reduced rates of mtDNA recovery after ethidium bromide-induced mtDNA loss, suggesting that it is important for maintaining “genome” stability within this organelle [29,41]. PrimPol has also been shown to functionally interact with a number of other proteins from both nuclear and mitochondrial compartments. These include mtSSB, RPA,

PolDIP2 and Twinkle, the mitochondrial helicase, which all appear to play roles in regulating PrimPol's cellular activities [35,42,43].

However, much is still to be learned about the function of PrimPol in the maintenance of replication in both mitochondrial and nuclear compartments. Here, we review what is currently know about DNA replication processes in the mitochondrion and discuss how our newfound knowledge of PrimPol's activities informs us about it's possible roles in the duplication of mtDNA.

Priming mtDNA replication – how it all begins

The elusive mitochondrial primase

To begin replication, DNA must first be 'primed' by the generation of short primers, which the replicase is able to extend. In the nucleus, RNA primers are synthesised on ssDNA by the Pol α-associated primase (PriS / Pri1) (reviewed in [44,45]). However, the enzyme responsible for the initiation of mammalian mtDNA replication has taken much longer to be discovered and we are only now beginning to unravel how this process occurs. A mitochondrial primase activity was first identified back in 1985 [46]. This primase activity isolated from mitochondria, was distinct from the replicative polymerase γ (Pol γ) and the mitochondrial RNA polymerase (POLRMT), and was shown to have the ability to catalyse the formation of RNA primers between 9-12 nucleotides long, however the enzyme responsible for this activity was not identified [46]. This activity was further characterised to show that the primase was able to generate a 5' oligoribonucleotide primer with a 3' deoxyribonucleotide termini for polymerase extension and was associated with a structural

RNA molecule, which is essential for its activity [47,48]. Although some further insights were gained in other species and models of mtDNA replication were proposed, little more was learnt about DNA priming or the elusive 'primase' for the next 20 years. The subject of DNA replication initiation in the mitochondrion was reignited with the discovery that the mitochondrial RNA polymerase, POLRMT, was able to initiate lagging strand DNA replication *in vitro* [49].

POLRMT- a mitochondrial primase

Mitochondrial RNA polymerase, like the majority of mitochondrial proteins, is encoded within the nuclear genome and contains an N-terminal mitochondrial targeting sequence, which localises it to this organelle. POLRMT is a single subunit RNA polymerase, which shares significant homology with phage RNA polymerases [50,51]. The C-terminal domain contains the catalytic core and consists of a number of conserved sequence blocks, which form the characteristic thumb and palm structure. Unlike the C-terminus, the N-terminal portion of the protein has no sequence similarity to the phage polymerases, instead this region is structurally related to the T7 RNA polymerase (reviewed in [52]). This structural homology is also mirrored in its functional uses as phage T7 replication employs the RNA polymerase in a similar manner to generate RNA primers [53,54,55]. In addition, POLRMT contains a unique N-terminal extension, containing a novel penta-peptide repeat domain that is essential for promoter-dependent transcription [56]. Unlike T7 RNA polymerase, POLRMT is unable to bind to, bend and melt promoter DNA alone and requires a number of accessory factors to begin transcription. These factors include TFAM, which is thought to alter the DNA structure, allowing initiation, and TFB2M, which interacts directly with the

RNA polymerase to aid its recruitment [57,58,59,60,61]. Similarly, yeast mitochondrial RNA polymerase Rpo41 requires transcription factor mtf1 for tight DNA binding (reviewed in [62]). POLRMT must be targeted to a range of diverse promoter sequences and additional factors are likely to be important in its localisation to different structural DNA elements (e.g. O_L). The generation of POLRMT conditional knockout mice, which have no identifiable 7S DNA, confirmed that POLRMT has an essential role in replication initiation [63].

Mitochondrial RNA primers (2-10 nucleotides) were first identified at the 5' end of the 7S DNA back in 1979 [64]. More recent work has identified that these primers are generated by POLRMT [49,65,66], originating from the light and heavy strand promoters LSP and HSP, respectively, within the NCR [67,68]. As well as acting as the start site for transcription of the mitochondrial genome, LSP was shown to also be the initiation site for the formation of a persistent RNA-DNA hybrid, believed to act as a primer for leading-strand DNA replication [69]. Transcription initiated from the light strand promoter suffers two fates, it is either terminated within a region containing three conserved sequence blocks (CSBs) and used as a primer for mtDNA replication, or extension continues around the mtDNA molecule to form a poly-cistronic transcript, which is then processed further [70,71]. The ends of this short RNA were shown to map close to previously identified sites of DNA replication initiation and this RNA species was found to be sufficient for the initiation of replication *in vitro* [69,72].

Later work showed that CSB II, a conserved sequence box within the NCR, acted as the termination site for transcription, controlling the formation of a primer rather than extension to complete transcription [66]. CSB II has the potential to form a quadruplex structure, due to its G-rich sequence. However, although this region can form a DNA quadruplex, the majority of these species were actually found to form RNA-DNA hybrid intermolecular quadruplexes [73,74]. These structures act as strong transcription terminators leading to the formation of primers. Thus, the CSB II quadruplex acts as a switch regulating the interplay between transcription and replication of mtDNA. Analysis of variation in the CSB II region of human mtDNA has shown that sequences that cause only weak termination of POLRMT and transcription are avoided. However, all transcription termination events are localised to the same downstream sequences, revealing the importance of the conservation of this region [75]. Yet, it is still important that when required, these RNA molecules are extended into full-length species to provide sufficient mRNA, and therefore protein, as required by the organelle. This switch is also partly controlled by human transcription elongation factor, TEFM, shown to prevent replication primer formation by driving transcription elongation [76,77]. However, the polymerase must then extend the primer beyond this quadruplex structure and it has been shown that Pol γ exhibits a much decreased extension ability on the CSB II sequence, compared with a mutant CSB II sequence that is unable to form a quadruplex structure [73]. In addition, these primer ends fall short of the identified 5' ends of newly replicated DNA molecules [78,79], suggesting things may not be quite so straightforward, therefore we still have much more to learn about the mechanisms that initiate priming of mtDNA replication in the mitochondrion.

MtDNA is double-stranded (ds) and therefore replication must also be initiated on the other strand. The origin of light strand replication, O_L is located approximately two thirds of the way around the genome from the NCR and resides in a much smaller non-coding region of DNA occupying only 30 bp, between the asparagine and cysteine tRNA genes [80]. POLRMT can generate short RNA primers at O_L, allowing complete leading and lagging strand replication (when combined with Pol γ, Twinkle and mtSSB) of a small circular DNA substrate *in vitro* [49]. Further studies *in vivo* confirmed that POLRMT is able to prime specifically at O_L and demonstrated that this specificity is due to a stem loop structure formed when the O_L sequence is exposed after leading strand replication uncovers this region [81]. This stem loop structure is highly evolutionarily conserved and mutations affecting its structure are significantly under represented in the mitochondrial genome, whilst insertions and deletions in the loop region are well tolerated, confirming the importance of such a structure within the mitochondrial genome [82].

PrimPol, the elusive mitochondrial primase?

The discovery of PrimPol within the mitochondria raises a number of important questions regarding its role as a primase within mtDNA replication [28,29]. Although PrimPol has the ability to generate both DNA and RNA primers [28,29], it has a strong preference for utilising dNTPs rather than rNTPs to synthesise primers, and studies using PrimPol isolated by fractionation of purified mitochondria showed that the enzyme generated DNA primers of ~2-12 nt in length [29]. This is in clear contrast to the original activity of the historically unassigned 'mitochondrial primase' thus PrimPol appears not to be this elusive protein and suggests there is still much more to be uncovered. If it is not the replicative

primase then what are PrimPol's primase activities required for within the mitochondrion? Mammalian PrimPol is a non-essential protein as knock-out cell lines and mice strains have been generated, which exhibited no overt phenotypes [28,39]. Thus, it appears that the role of PrimPol can be complemented by other proteins within the organelle or it may itself have a "back-up" role, which may be only required under certain particular circumstances. Therefore, it is clear that PrimPol is not the main enzyme responsible for priming of mtDNA replication and, notably, mtDNA copy number actually increases in the absence of PrimPol [28]. It seems more likely that PrimPol acts to reprime DNA synthesis, or bypass lesions by TLS, when the replicase is stalled or blocked.

Mitochondrial DNA replication – copying the circle

Pol γ, the mitochondrial polymerase

Although a wide range of polymerases are required to replicate and repair nuclear DNA, only one polymerase was identified within the mitochondria and this was believed to be responsible for all of the replication and repair processes that take place within this organelle [83]. Although Pol γ was first identified as early as 1977, it took many more years for it to be isolated from *Drosophila* and confirmed as the key mitochondrial replicase due to the abundance of nuclear polymerases [84,85]. Pol γ is a heterotrimeric complex consisting of two nuclear-encoded components. PolG1 or A is a 140 kDa subunit belonging to the PolA family of polymerases and is thought to share a common ancestry with the T-odd polymerase gp5 [86]. It is essential for mtDNA maintenance and its loss is embryonic lethal [87]. As well as 5′-3′ polymerase activity, POLG1 also contains a highly conserved 3′-5′ exonuclease domain, which confers a key part of its high fidelity, ~100-

fold greater than that observed for nuclear polymerases [88,89]. In addition, POLG1 also has 5′ dRP lyase activity, which allows the enzyme to execute base excision repair, again increasing its fidelity and strengthening its role in DNA repair within mitochondria [90]. The crystal structure of the Pol γ complex has provided further insights into its mechanism and the unique interactions between its subunits. Analysis of a wide number of disease causing mutations within the context of this structure has led to a greater understanding of the molecular changes that these alterations induce [91,92] (reviewed in [93]).

POLG2 or B, is a 55 kDa homodimeric accessory subunit required for tight binding of the holoenzyme on DNA [94]. It is also vital for increasing the processivity of the replication complex and interacts with Twinkle at the replication fork [95,96]. POLG2 evolved from class IIa aminoacyl-tRNA synthases and appears to be less conserved than its catalytic counterpart, with no orthologues identified in fungi [97]. The binding of POLG2 significantly increases the processivity of the enzyme due to a change in the structure of POLG1, which increases its DNA interaction “footprint” from 10 to 25 bp [91]. The addition of POLG2 to the complex decreases the proofreading capacity of the enzyme, which is likely due to a decrease in the ability to switch the template DNA from the polymerase to the exonuclease active site [88,96].

The polymerase is assisted in the completion of replication by a number of other components including Twinkle, the mitochondria helicase, and MtSSB (SSBP1), a single-stranded DNA binding protein. More recently, a much wider range of proteins have been associated with mtDNA replication and repair, many of which have already been identified

based on their roles in nuclear DNA metabolism. These include the helicases DNA2, PIF1 and RecQL4, topoisomerases TopImt, TopIIIα, TopIIβ [21,22,23,24,98,99,100,101], Fen1, DNA ligase III, RNAse H1 [25,102,103,104,105,106].

Mechanisms of mammalian mtDNA replication

The first studies of mtDNA replication were carried out using electron microscopy, which highlighted a number of unique structures within the observed molecules, including double forked structures with one single-stranded branch [107,108,109]. These studies lead to the proposal of a strand-displacement model of mtDNA replication [108,110,111]. This model proposes that replication continues in an asynchronous, unidirectional manner, replication proceeds on the H-strand extending from the primer generated in the NCR region, whilst the unreplicated ssDNA becomes coated with mtSSB. After replication has proceeded two thirds of the way around the molecule then O_L becomes exposed, allowing replication to be primed in the reverse direction, again continuing unidirectionally around the circle to generate a fully duplicated daughter molecule [112].

The use of 2D neutral agarose gel electrophoresis (2D AGE) uncovered a second distinct replication mechanism of mtDNA replication. This technique relies on the fact that DNA can be isolated dependent on its mass and shape by altering separation conditions [113]. Strikingly, when mtDNA replication intermediates were examined by this method, a number of conventional replication bubbles and forks were observed. These structures were resistant to ssDNA nucleases, suggesting a conventional strand-coupled mechanism of replication [114,115,116,117]. Although such dsDNA molecules had been observed

much earlier in EM images of rat liver mtDNA, a strand-coupled mechanism was not pursued [108,109,118,119]. Replication is believed to initiate in a bidirectional manner from a broad zone termed *ori Z,* however, O_H acts as a strong replication barrier therefore DNA replicases are stalled and unable to pass bidirectionally through this region [114,116].

In addition to conventional strand-coupled replication intermediates, 2D AGE also identified replication intermediates with significant regions of RNA-DNA hybridisation [79,117]. This lead to the proposal of the Ribonucleotide Incorporation ThroughOut the Lagging Strand (RITOLS) mechanism of mtDNA replication, where RNA is bound to the displaced strand during asynchronous replication rather than mtSSB [79]. This RNA was identified as mtDNA transcripts, which are thought to be laid down when the L strand is released as ssDNA during replication and *in organello* labelling allowed these RNA species to be followed as replication proceeds [120]. More recent EM studies support this model as it was observed that when conditions were optimised to preserve fragile RNA, no ss mtDNA regions were present [121]. Variations in the types of replication intermediates was observed across different tissues and under different stresses (e.g. damage or mtDNA depletion) suggesting that different mechanisms may be utilised under various circumstances [122].

However, there is still much controversy and disagreement over these different replication mechanisms, with recent work showing that mtSSB occupancy is increased between O_H and O_L on the displaced heavy strand, favouring the strand-displacement model [123].

Whilst it has been claimed that mtSSB is lost due to proteinase K treatments used to observe RITOLS intermediates and that R-loops are prevalent in mtDNA but not replication intermediates, others claim RNA loss during preparation leads to mtSSB binding [121,123,124]. Although more studies are required to fully unravel the mechanisms of mtDNA replication, it seems likely that this process consists of a combination of the proposed models.

Although it currently seems unlikely that PrimPol plays a direct role in replicating mitochondrial DNA, as it is non-essential in mammalian cells, it probably plays roles in maintaining on-going replication when the replicative complex of Pol γ and Twinkle hits an obstacle. Cells are clearly able to maintain replication in the absence of PrimPol and, notably, the majority of yeast species lack PrimPol orthologues. However, the presence of this additional primase-polymerase is likely to aid in overcoming replication barriers thus ensuring timely completion of replication.

Damage tolerance during mtDNA replication

For many years, it was believed that mitochondria did not have their own DNA repair proteins / pathways as they were unable to remove CPDs after UV damage [125]. Thus, replication must be able to proceed beyond such damage, which is likely to be perpetuated within the DNA. However, these original ideas have now been refuted and many new players in the maintenance and repair of mtDNA are regularly being added to the team of proteins maintaining the “genome” within this organelle. Although a wide range of repair pathways are now known to be able to remove DNA lesions within mitochondria,

this can be a relatively slow process and therefore Pol γ probably requires additional damage tolerance / bypass mechanisms to allow timely progression of replication.

Bypass of lesions- overcoming obstacles on DNA

Along with its ability to generate primers, PrimPol is also a template-dependent polymerase with the ability to bypass some lesions. For example, it has the ability to perform TLS bypass of 8-oxoG lesions, a prevalent product of oxidative damage but is unable to bypass abasic sites or thymidine glycol lesions unless supplemented with manganese [28,29]. In addition, PrimPol has the ability to bypass damage caused by UV exposure and, unlike known mammalian polymerases, PrimPol can bypass distorting 6-4 PPs lesions that induce DNA bending. Although it is not able to directly bypass CPDs in the presence of magnesium, it can extend from this lesion when templated with two dA residues, unlike many other polymerases [28]. In addition, PrimPol has been reported to scrunch the template, realigning the priming strand in order to bypass intolerable DNA lesions, which seems the more likely mode of bypass given the proteins small active site cleft [36,37]. Thus, it seems likely that PrimPol may also play a role in the TLS bypass of these types of damage within the mitochondrial genome (Figure 2).

UV damage has been shown to accumulate in mitochondrial DNA, particularly in skin cells that are regularly exposed to low levels of UV irradiation (reviewed in [126]). These lesions are commonly repaired by the nucleotide excision repair (NER) pathway [127]. However, this pathway has not been identified within the mitochondria therefore such lesions must be repaired by other pathways such as BER, MMR, NHEJ or homologous recombination

and may even persist within the DNA. To prevent the catastrophic formation of double-strand breaks during DNA replication, the organelle must have a method to bypass such lesions. Studies have shown that Pol γ has the ability to bypass CPD lesions *in vitro*, albeit at a level much lower than that of undamaged DNA [128]. Although it often incorporates the incorrect base at such sites, it is still able to excise these mutations using its exonuclease domain. However, such methods of bypass either by PrimPol or Pol γ are likely to significantly delay the completion of replication. Although the mitochondria has multiple DNA copies, unperturbed replication of the full genome is thought to take up to one hour [110], therefore only a small amount of damage is likely to have a drastic effect on the organelles functionality as a whole. Indeed, although low levels of UV or oxidative damage do not affect the mitochondrial DNA copy number, they do cause changes in the mechanism of DNA replication [129]. It has been shown that after such UV damage, replication switches from a RITOLS mechanism to a strand-coupled mechanism, likely due to its ability to complete replication more rapidly and allow mtDNA copy number to be maintained in the presence of damage [129]. It is believed that the RITOLS mechanism may represent a high fidelity method of genome replication, whilst the strand-coupled mechanism may be more error-prone but allow replication to be completed. Notably, tissues with a higher oxidative stress load use a higher degree of strand-coupled replication[122][129]. However, for strand-coupled replication to be successful, particularly in the presence of DNA damage, the replication machinery must be able to generate multiple new primers to allow the continuation of the replication fork.

PrimPol can also reprime DNA synthesis after lesions and it is this function that is likely to be vital in allowing replication to proceed after DNA stalling. Allowing the replication fork to

continue beyond such lesions would significantly decrease the completion time and thus allow processes that take a longer time, such as repair of the lesion itself, to take place in a post-replicative fashion. PrimPol is a poorly processive polymerase, only capable of incorporating 1-4 bases before falling off the DNA, suggesting it may be required to produce short primers for the more accurate and exonuclease-containing polymerase, Pol γ to take over and complete mtDNA replication. PrimPol interacts with RPA, mtSSB and PolDIP2, which modulate its synthesis activities *in vitro* [35,42]. Although PrimPol's polymerase activity is stimulated by Twinkle [43], mtDNA replication studies showed that PrimPol is unable to enhance replication after Pol γ stalling at oxidative lesions [43]. Therefore, although it has the capability to perform TLS *in vitro*, the jury is still out on whether or not PrimPol actually uses such a mechanism to restart replication *in vivo*.

In addition, POLRMT may also play a key role in repriming DNA replication within the mitochondrion. Although POLRMT has been demonstrated to initiate replication at three defined sites in the genome, it is also capable of generating RNA primers on ssDNA [49,130,131]. In contrast to PrimPol, it generates RNA primers of ~ 25-75 nucleotides long [49,130], although these have been found to be as short as 9-18 nt in yeast in the presence of the transcription factor mtf1 [131]. These differences suggest both may play key roles in repriming but under different circumstances.

More than one polymerase on the block

Unlike mammalian mitochondria, which for many years were thought to contain only a single DNA polymerase, a number of additional polymerases have been found to also reside within yeast mitochondria. Like mammalian mtDNA replication, the main replicative

polymerase is a Pol γ homologue, Mip1, that consists only of the large catalytic subunit, with no PolG2 homologue identified [132,133]. In addition, Pol α has also been identified within the mitochondria in *S. cerevisiae* [134]. Its dual localisation was confirmed by immunofluorescent labelling and, although its function within the organelle is yet to be elucidated, it is likely to have a role in repair and gap-filling as it is unable to rescue cells lacking Mip1 [134]. In another *S. cerevisiae* study, Pol ζ, consisting of Rev3, Rev7 and Rev1, was also found to localise within the mitochondria as well as in the nucleus. [135]. Whilst depletion of Rev3 or Rev7 had no effect on mitochondrial mutation levels, loss of Rev1 lead to a decrease in the frequency of spontaneous mutations within the mtDNA. [135] Further studies have shown that, although loss of Pol ζ causes a decrease in spontaneous and UV-C induced frame-shift mutations, it also causes a large increase in point mutations after UV-C damage, suggesting an alternative, more mutagenic, pathway is used in its absence [136]. Interestingly, over-expression of Pol ζ is able to rescue pathological Mip1 mutants, which cause an increase in mutations [137], whilst over-expression of the protein in the nucleus causes an increase in mutations [138]. In addition, a proportion of Pol η has also been identified within the mitochondria of budding yeast [139]. Like Pol ζ, it was also found that loss of Pol η causes an increase in the number of mutations produced after UV-C damage within the mitochondrial DNA, confirming an important role in the maintenance of DNA integrity. Other organisms have also been found to have a much wider range of mtDNA polymerases (Figure 1), with trypanosomes having a large number of Pol β and Pol κ-like polymerases [6,140].

New players join the mammalian mitochondria polymerase team

A recent study using a mtDNA-specific damaging agent has provided clear evidence for additional polymerases within mammalian mitochondria. Pol θ is a member of the A family of DNA polymerases but, unlike many of the other members, it is a highly promiscuous polymerase that exhibits a wide range of activities on a broad variety of substrates. Indeed, it appears to have roles in inter-strand crosslink repair, BER, microhomology-mediated end-joining (MMEJ), translesion synthesis, as well as having lyase activity [141,142,143](reviewed in [144]). Although it has no mitochondrial targeting sequence, it is recruited to the organelle after oxidative damage [145]. Strikingly, loss of Pol θ leads to a decrease in cellular oxygen consumption and mitochondrial membrane potential, indicative of decreased oxidative phosphorylation, whilst mtDNA actually shows a decrease in mutations, suggesting that other types of damage may abound [145]. This is not unique, as loss of Pol ζ in the nucleus has a similar effect on mutation levels [146,147]. Although these TLS polymerases are useful for maintaining replication across lesions, thus preventing the formation of possible DSBs as replication and the cell cycle progresses, their ability to perform TLS comes with a high mutagenic cost. Therefore, the cell must balance such consequences to ensure that a functional copy of the genome is passed on to daughter cells. In the mitochondrion, mutations represent less of a risk than in the nucleus, as each gene is available in multiple copies, thus any mutated copy represents a much smaller fraction of the available product pool. Although mutations have been shown to cause a range of mitochondrial diseases, some with catastrophic consequences for cellular survival, these mutations must cross a threshold level before their effects are observed. Mitochondrial DNA copy number appears to be relatively flexible and it has been reported, in a number of model organisms, that copy number varies widely across tissues and with age [148,149]. Interestingly, mtDNA copy number

has been found to vary widely across different cancers, likely due to mutations in a number of regulatory genes and, in a number of cases, mtDNA copy number has been linked to prognostic outcomes [150,151,152]. In addition, it has been reported that high levels of random mutations are tolerated by cells. For example, studies on mice lacking the exonuclease domain of Pol γ showed premature ageing. However, when mutation levels were analysed in the heterozygotes, they were shown to be almost as high as homozygote litter mates but showed no premature ageing phenotypes [153].

In addition, extension of work initially carried out in yeast has shown that a specific isoform of Rev3 is also found within mammalian mitochondria, yet unlike in yeast, the other components that form Pol ζ have not been identified [154]. Loss of this protein causes mitochondrial dysfunction and an increase in DNA damage after UV-C irradiation [154].

It is now becoming clear that, like nuclear DNA maintenance, mitochondria also utilise a broad range of repair and tolerance mechanisms to overcome DNA damage, which may occur spontaneously or arise due to many different environmental issues. The presence of several TLS polymerases within the organelle suggests that mitochondria also utilise such specialised polymerases to overcome DNA lesions. As well as direct extension of the replication fork, such polymerases are also likely to be vital for post-replicative repair across lesions. In many cases where the fork is stalled, replication may be reprimed beyond a lesion by PrimPol or POLRMT allowing replication to proceed. However, a polymerase able to bypass the lesion is then required to fill in the gap left behind, thus allowing repair of the lesion that must be done on dsDNA to prevent the formation of double-strand breaks, which are highly deleterious for the cell.

DNA structural barriers in mitochondria - bypass of G4 quadruplex structures

G4 quadruplexes (G4s) are stable DNA secondary structures formed through the planer stacking of quartets of Hoogsteen-bonded guanine bases. They are thought to have a wide range of roles within cells including, replication initiation, telomere maintenance, epigenetic instability, regulated recombination in the immune system and transcription regulation [155]. G4s form in both DNA and RNA and can be either intra-molecular, within one DNA strand, or inter-molecular, between two or more DNA/RNA strands [156]. Along with the well-studied RNA/DNA quadruplex found in the NCR, *in silico* studies have identified a number of other potential G4 forming sequences within mtDNA [157,158]. 80-90 potential quadruplex forming sequences have been found throughout the human mitochondrial genome, strongly biased towards the heavy strand of DNA, which has a significantly high content of guanine nucleotides. Yeast mtDNA contains ~0.373 potential quadruplex-forming sequences per 1000 base pairs, which is an order of magnitude larger than in nuclear DNA (~0.034-0.067) [159,160]. Interestingly, some studies suggest that, along with other helix distorting and intrinsically curved regions of DNA, G4s may be a significant cause of DNA instability within the mitochondrial genome [161]. Dong *et al*. have identified a number of quadruplexes with 2 or 3 consecutive guanines within the mitochondrial genome that are associated with sites of common mitochondrial deletions [158]. They proposed that such structures may induce genome deletions in a number of possible ways including the stalling of replication, causing DNA breaks or extensive tracts of vulnerable ssDNA or through aberrant DNA repair mechanisms [158]. In addition, potential quadruplex forming sequences are found close to sites of common pathological deletions and such sequences are inefficiently unwound by the mitochondrial helicase, Twinkle, suggesting they may cause a significant impediment to progressing replication

forks [162]. In addition, a number of other DNA structures may also persist within mtDNA, which may slow or stall the replicative polymerase, such as triplex or Z DNA, intrinsically bent structures or hairpin loops, such as the loop shown to form at O_L [161,163].

It has recently been demonstrated that PrimPol is crucial for the bypass of G4s in the nucleus of vertebrate cells [164]. Although PrimPol is unable to replicate directly through these structures, it is capable of repriming directly after G4s in a process termed “close-coupled repriming” that enables replication to be restarted almost immediately downstream of G4s (Figure 2). In the absence of PrimPol, a significant amount of uncoupling of the replication fork was observed in cells as large tracts of late replicated DNA in the region of the quadruplex on the leading strand, which was associated with the loss of epigenetic histone marks [164]. This recent discovery has led to speculate that PrimPol is likely to play a similar role in the replication of such structures within the mitochondrial genome. Although Twinkle shows poor activity when confronted with G4 structures [162], a number of helicases have been found to be involved in the bypass of such structures in the nucleus, one of which (Pif1) is also localised to the mitochondria [21,165].

Tolerance of chain terminating Nucleoside Analogues

Another situation where PrimPol has recently been shown to play a vital role is in the maintenance of replication in the presence of chain terminating nucleoside analogues (CTNAs), such as acyclovir and abacavir. Cells lacking PrimPol showed increased sensitivity to such drugs, whilst PrimPol was found to be able to incorporate a number of

these nucleotide analogues and also perform close-coupled repriming downstream of such lesions *in vitro* [40,166]. CTNAs typically lack the essential 3' hydroxyl moiety required for phosphodiester bond formation and therefore prevent on-going replication when incorporated into DNA by terminating strand extension. They are commonly used to control viral infections, (e.g HIV), as they are readily incorporated into DNA by reverse transcriptase, significantly slowing viral replication processes. However, they do have a range of toxic side effects caused by mitochondrial toxicity (reviewed in [167,168,169]), with long-term use of AZT, (azidothymidine) causing a decrease in mitochondrial DNA in skeletal muscle and cumulative mitochondrial myopathy [170,171]. More precisely, these affects have been attributed to the incorporation of these nucleoside analogues by Pol γ (reviewed in [172]). Pol γ inserts nucleoside analogues with varying ease, some incorporated at concentrations similar to standard nucleotides, while others require ~2-5 fold higher concentrations to be efficiently incorporated [173,174,175]. All analogues share the ability to inhibit Pol γ-mediated replication *in vitro* by preventing any further synthesis and therefore they must be rapidly removed otherwise on-going replication is inhibited [174]. The attempted removal of CTNAs from the elongating DNA strand is likely to be first tackled by the exonuclease domain of Pol γ, whose role it is to check newly replicated DNA for accuracy and quickly remove any incorrectly incorporated bases to prevent mutations from being generated [88]. The exonuclease domain of Pol γ increases its fidelity by improving nucleotide selection by a factor of ~200 [88]. Notably, removal of nucleoside analogues by this exonuclease is a slow process. For example, the ddC analogue Zalcitidine has a half-life in DNA of ~2.4 hours due to its stronger binding affinity, compared to standard nucleotides, for the polymerisation domain [176]. In addition, other

analogues (e.g. AZT) have also been shown to inhibit the exonucleolytic activity of Pol γ, causing a decrease in fidelity [174].

Given this propensity for incorporation of CTNAs into replicating mtDNA, it seems likely that PrimPol also plays a key role in repriming replication after the incorporation of these nucleoside analogues by Pol γ (Figure 2). PrimPol-deficient cells are more sensitive to the presence of these analogues and this sensitivity can be complimented by the addition of PrimPol, but not by a primase-deficient mutant of this enzyme [40], indicating that repriming is essential for this process. In addition, PrimPol is able to reprime downstream of incorporated nucleoside analogues *in vitro,* further supporting this proposed role in replication restart. Interestingly, PrimPol is itself able to incorporate a number of the FDA approved CTNAs into DNA, albeit less efficiently than natural nucleotides, with a distinct discrimination profile compared to Pol γ [166]. Thus, PrimPol may also create its own problems, but its low processivity [34] is likely to limit such potential toxicity *in vivo*.

Conclusions

Despite its small size, mitochondrial DNA can represent ~1 % of the total DNA in some cells due to its polyploid nature [17]. However, our understanding of DNA replication processes within this organelle still trails well behind that of nuclear genome duplication. Mitochondrial DNA has its own specialised replicative polymerase and mechanisms that vary from those observed in the nucleus, although some controversy remains within the field over the abundance and validity of the different proposed replication models. However, we are beginning to uncover that mtDNA replication and repair mechanisms may in fact be more similar to those in the nucleus than was initially thought, as many

proteins identified within the nucleus are now also being found to have additional roles in mtDNA replication. Clearly, there is much more to understand about the replication of this small, but far from insignificant, molecule of DNA.

PrimPol is likely to play a similar role within the mitochondrion as it does in the nucleus, maintaining the progression of the replication fork after replisome stalling. Emerging data suggests that PrimPols key role is likely to be in repriming replication restart after a fork stalling lesion or DNA structure [40,164]. In this review, we propose that it is highly likely that PrimPol plays the same roles in mitochondria by repriming DNA replication to allow replication to be completed in an efficient and timely manner (Fig. 2).

However, mitochondrial DNA organisation varies significantly from the compaction of nuclear DNA and therefore the replication mechanisms and proteins required for its duplication are adapted for these different environments suggesting that PrimPol must be a highly flexible protein, which is able to adapt its functions depending on its partners and the problems it encounters. It seems likely that its main role is to reprime the initiation of DNA synthesis after Pol γ is stalled by DNA damage, secondary structures or chain terminating events. This allows replication to proceed, leaving behind the cause of the stalling event to be processed and corrected in a post-replicative fashion. However, its ability to perform TLS opposite template lesions, such as 8-oxoG, may also be significant [28,29]. Complete replication of a mitochondrial DNA molecule is thought to take ~1 hr [110] thus, although each cell contains multiple copies of the mitochondrial genome, it is essential that each round of replication is completed in a timely manner to prevent the accumulation of multiple stalled and / or collapsed replication forks. Indeed, in the absence of PrimPol, the mtDNA copy number is considerably increased suggesting that more copies are required to fully maintain organelle functionality.

Although we have primarily discussed the possible roles of PrimPol during mitochondrial genome replication, it is possible that it may also play additional roles in the repair of damaged DNA in the absence of on-going replication. For example, almost all DNA repair pathways utilise polymerases to fill in gaps generated by nucleolytic repair processes, such as base / nucleotide excision and resection. Notably in this regard, distinct primase-polymerases have evolved to play such bespoke roles in various DNA repair processes in prokaryotes, such as NHEJ [31,32]. We still have much more to learn about the roles and regulation of PrimPol in both the mitochondria and the nucleus and further research is required to better understand the functions this fascinating enzyme fulfils within the cell. [177] A range of PrimPol mutations have been found in cancer cells and other conditions suggesting possible connections to human disease, including mitopathies, although these pathological associations remain to be established. [178].

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Figure legends

Figure 1. The diversity of mitochondrial genomes. This table shows the wide variety of mtDNA sizes found across different kingdoms and how their organisation and replication mechanisms differ. Also highlighted are primases and polymerases shown to be, or speculated to be (?), involved in these processes. Black strands represent parental DNA, with newly synthesised DNA shown in blue and RNA in red.

Figure 2. Potential functions of PrimPol during mtDNA replication. Highlighted here are ways in which PrimPol may play key roles in allowing the maintenance of mtDNA replication in a number of fork stalling situations. **A.** After Pol γ is stalled by a lesion (yellow star), PrimPol is able to reprime synthesis downstream to allow replication to proceed while the slower process of replication across the lesion is dealt with by Pol γ itself or another specialised TLS polymerase. Alternatively, PrimPol may act as a TLS polymerase that directly bypasses the lesion. **B**. PrimPol may play a role in repriming synthesis when the replication fork is stalled by DNA secondary structures, e.g. G4s. By priming after the structure, it allows replication to continue downstream, while specialised helicases are recruited to facilitate synthesis through the structure. **C.** Nucleoside analogues (cyan star), incorporated into the newly synthesised strand, prevent further elongation and must be removed by the exonuclease of Pol γ, which is a slow process. PrimPol may reprime downstream of CTNAs to allow replication to continue in a timely fashion, whilst the process of removing these is completed. In each case, the second DNA strand is shown as dsDNA for simplicity, however, this could be coated with mtSSB or RNA transcripts, depending on the mode of replication.