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Known Unknowns of Mammalian Mitochondrial DNA Maintenance

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Known unknowns of mammalian mitochondrial DNA maintenance

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Abstract

Mammalian mitochondrial DNA (mtDNA) replication and repair have been studied intensively for the last 50 years. Although recently advances in elucidating the molecular mechanisms of mtDNA maintenance and the proteins involved in these have been made, there are disturbing gaps between the existing theoretical models and experimental observations. Conflicting data and hypotheses exist about the role of RNA and ribonucleotides in mtDNA replication, but also about the priming of replication and the formation of pathological rearrangements. In the presented review we have attempted to match these loose ends and draft consensus where it can be found, while identifying outstanding issues for future research.

Introduction

Mitochondria are cell organelles responsible for oxygen-dependent production of ATP, utilized as energy currency in many cellular processes. Mitochondria have their own genome, mitochondrial DNA (mtDNA), which in mammals is typically a 16.5 kb circular double-stranded molecule existing in thousands of copies per cell. Mitochondrial DNA encodes for

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3 13 subunits of oxidative phosphorylation (OXPHOS) complexes as well as tRNAs and
4 rRNAs required for their synthesis. In mitochondria, mtDNA is packaged in nucleoprotein
5 complexes called nucleoids. Due to its central role in encoding subunits of the OXPHOS
6 complexes, mtDNA is essential for mitochondrial function and any perturbations involving
7 its replication or repair can be detrimental for the host cell. Consequently, disorders involving
8 its replication or repair can be detrimental for the host cell. Consequently, disorders involving
9 its replication or repair can be detrimental for the host cell. Consequently, disorders involving
10 its replication or repair can be detrimental for the host cell. Consequently, disorders involving
11 disturbances of mtDNA replication or gene expression have often dramatic pathological
12 phenotypes, in the worst case failure to thrive [1]. Devastating diseases caused by defects in
13 mtDNA replication include progressive external ophthalmoplegia, ataxia-neuropathy,
14 mitochondrial neurogastrointestinal encephalomyopathy and Alpers-Huttenlocher syndrome
15 [2]. Impaired mitochondrial DNA integrity has been also linked to a range of common
16 diseases and ageing [3], the latter likely connected to stem cell maintenance that affects tissue
17 regeneration [4].
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24 Despite its small size and apparent simplicity, studying mammalian mitochondrial DNA
25 maintenance mechanisms has proven to be challenging. Not only there is disagreement
26 regarding the replication mechanisms of mtDNA, but also our knowledge of the scale and
27 scope of mitochondrial genome repair mechanisms is limited. While mechanisms such as
28 base excision repair of mtDNA are relatively well characterized, other reported mtDNA
29 maintenance mechanisms rely often on rather anecdotal observations [5]. Most of the
30 suspected repair proteins mainly localize to the nucleus, and it is often difficult to
31 unequivocally prove that the protein localizes into mitochondria under normal conditions,
32 instead of being detected there only because of contamination from sample preparation
33 procedures. These issues have been recently exemplified by a critical review regarding the
34 proposed localization of various DNA polymerases in mitochondria [6]. Conversely, the
35 difficulty of finding a rare repair protein in mitochondria does not exclude the protein from
36 having a function in mtDNA maintenance. It might be that some of the mtDNA repair
37 mechanisms operate only under specific circumstances or in a limited number of cell types.
38 Again, demonstrating this can be technically challenging, especially if the protein is essential
39 for nuclear genome maintenance, ruling out knockout studies. However, systematic analysis
40 of the dual localized genes can reveal alternative AUGs [7] or non-conventional
41 mitochondrial targeting signals [8,9], which can be targeted by gene knock-in to interrogate
42 only the mitochondrial function. More focus should be put on the search for enzymes
43 involved in DNA maintenance mechanisms that are necessitated by the nature of nucleic acid
44 metabolism reactions, such as recombination or the repair of protein-DNA crosslinks [10].
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3 The recent discovery of a mitochondrial isoform of tyrosyl-DNA phosphodiesterase 2 [11],
4 capable of removing tyrosine-DNA adducts, could represent the first example of the
5 existence of these DNA repair mechanisms in mitochondria.
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9 Our motivation to write this review was to raise the awareness of still outstanding issues
10 connected to the basic mtDNA maintenance mechanisms and of interesting observations that
11 have not been properly pursued. Despite the apparent simplicity of the mitochondrial
12 genome, its multiple unexplained features demonstrate that our knowledge of mtDNA
13 maintenance is limited at best.
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17 18 **Recursive progress in elucidating mtDNA replication mechanisms**

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21 Mitochondria are constantly replicating their genome and this replication is thought to be
22 independent of the cell cycle [12,13]. Cells need to replace the mtDNA lost due to
23 mitochondrial turnover or produce additional mtDNA when mitochondrial biogenesis is
24 upregulated. Additionally, mitotic cells have to replicate their mtDNA to maintain a constant
25 copy number when the cytoplasmic compartment is partitioned between the daughter cells.
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31 Due to the evolutionary history of mitochondria, the mtDNA replication machinery in
32 eukaryotes is essentially prokaryotic in its constituents. For example, the mitochondrial
33 single-strand binding protein mtSSB is a homologue of bacterial SSB [14], whereas the
34 replicative helicase TWNK and the catalytic subunit of the mitochondria DNA polymerase
35 Pol γ have resemblance to bacteriophage T7 gp4 primase-helicase and gp5 DNA polymerase
36 [15-17]. The minimal mammalian mtDNA replisome can be reconstituted *in vitro* using these
37 three proteins, with Pol γ being present as a heterotrimeric holoenzyme (one catalytic subunit
38 Pol γ A plus two accessory subunits, Pol γ B) [18]. Intriguingly the mtDNA replication
39 mechanisms seem to have evolved differently in different eukaryotic lineages, representing
40 almost all possible replication modes including recombination-dependent replication [19],
41 rolling-circle replication [20], strand-coupled replication [21] and strand-displacement
42 mechanisms [22]. Even more complicated, more than one mechanism might co-exist in the
43 same group of organisms. For instance at least three different mtDNA replication
44 mechanisms have been proposed to exist in mammals [23]. Despite the ongoing
45 controversies [24,25], there is a general agreement that the mammalian mtDNA replicates
46 mostly in a highly asymmetric manner, with one strand (light, or the L-strand) replicated with
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3 considerable delay compared to the other strand (heavy, or the H-strand) (**Figure 1**). In this
4 mechanism, the earlier initiated replication of the H-strand results in the displacement of the
5 L-strand template as single-stranded DNA (ssDNA), hence initially dubbed as the strand-
6 displacement model (SDM) [12].
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10 11 12 **Unbraiding the displaced strand identity** 13 14

15 Mitochondrial DNA replication was initially studied using transmission electron microscopy
16 (TEM) [26,27] and in fact, fully double-stranded replication intermediates were observed in
17 the first TEM analyses [26,28]. Later studies revealed partially single-stranded replication
18 intermediates [27,29], resulting in the establishment of the strand-displacement model (SDM)
19 of mtDNA replication [12]. In this model, replication is unidirectional and asymmetric, with
20 leading-strand synthesis displacing one strand, which is replicated with delay and resulting in
21 persisting single-stranded intermediates. Decades later the mtDNA strand-displacement
22 model was revisited by a series of studies from Ian Holt's group [30-33] using two-
23 dimensional Brewer-Fangman agarose electrophoresis (2D-AGE) that separate nucleic acids
24 according to their size and shape [34]. Although initially confusing the mitochondrial DNA
25 replication field by introducing the concept of strand-coupled [30] and bi-directional [32,33]
26 mtDNA replication, being opposite to the original model, the studies also provided evidence
27 that the SDM intermediates can be generated from double-stranded replication intermediates
28 by RNase digestion, indicating that the L-strand template is covered by RNA prior its
29 replication [31,35,36]. Random hybridization of RNA on ssDNA during isolation procedures
30 was excluded by experiments showing that RNA could be cross-linked on the displaced L-
31 strand *in vivo* using psoralen [36]. On the other hand, chromatin immune precipitation
32 experiments showed that the occupancy profile of mtSSB displays a distinct pattern that can
33 be expected from replication intermediates that are replicated by the SDM, mtSSB fully
34 covering the exposed single-stranded lagging-strand [24]. However, in the heat of the debate
35 it is often missed that there is in fact much consensus on the mode of mtDNA replication,
36 apart from the identity of L-strand replication intermediates. H-strand replication initiates
37 first at the origin of H-strand replication (O_H) and proceeds unidirectional through the
38 genome. The L-strand replication initiates at one major origin, the O_L , only when H-strand
39 replication has reached it (**Figure 1B**) and the replication of both strands terminates when it
40 they have reached the non-coding region (**Figure 1C**) [25,35,37,38]. The only practical
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3 difference is that in the so-called bootlace model the displaced H-strand is covered by
4 preformed RNA (**Figure 1D**) [25] instead of mtSSB (**Figure 1E**), as in the classical model
5 [12]. Due to the similarity of DNA synthesis in the SDM and bootlace models, methods
6 visualizing newly replicated DNA cannot differentiate between the two [39]. As far as
7 known, the bootlace mechanism of replication would be unique to mitochondrial DNA. It has
8 been proposed that covering the displaced strand with RNA could have a number of
9 advantages: protection of the replication fork against breakage, elimination of mutations and
10 prevention of secondary structure formation [40], but currently there is no experimental
11 support or precedence for these ideas.
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19 The main weakness of the bootlace model is that the recruitment and annealing of preformed
20 RNA at the replication fork should be an active mechanism with a dedicated enzymatic
21 machinery. Until its components are discovered, the issue can be considered unsettled.
22 However, an interesting candidate for the RNA annealing could be the mitochondrial
23 replicative helicase TWNK, which has been shown to have DNA annealing activity *in vitro*
24 [41]. If the annealing activity is not limited to DNA, TWNK's central location at the
25 replication fork would offer a simple mechanistic explanation for the rapid covering of the
26 displaced strand with RNA. Furthermore, TWNK has 5' to 3' unwinding activity [42] and
27 thus moves on the L-strand during DNA replication, making the annealing of RNA
28 mechanistically plausible. The mechanism would probably have to exclude the usage of
29 modified or structurally constrained RNAs, such as ribosomal RNA and tRNAs.
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40 **RNA incorporation vs. incorporation of ribonucleotides on** 41 **mitochondrial DNA** 42

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44 The presence of ribonucleotides in mtDNA was documented as early as the 1970s [43,44].
45 These single ribonucleotides (rNMPs) embedded in mature mtDNA are not related to
46 incorporation of longer RNA species on the displaced strand. While the proposed bootlace
47 intermediates, which consist of longer fragments of RNA, are sensitive to cleavage by RNase
48 H1 [45], mature mtDNA containing single, or at most two consecutive rNMPs, is not.
49 Instead, these molecules are readily degraded using alkali treatment or RNase H2, cleaving at
50 single rNMPs [46].
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3 rNMPs are frequently incorporated in both the nuclear and mitochondrial DNA during
4 normal replication processes due to their abundance and chemical similarity to dNMPs [47-
5 49]. The ribonucleotides inserted into DNA change the chemical and structural properties of
6 the DNA due to the additional hydroxyl group on the 2' carbon of the sugar moiety.
7 Consequently, rNMPs that persist in in nuclear DNA can lead to severe replication stress
8 [47,49,50]. Besides blocking replication, the presence of the extra OH group on the 2' carbon
9 of ribonucleotides makes rNMPs more prone to hydrolysis, especially at alkaline pH. This is
10 potentially relevant for mitochondria, as their pH is thought to be more basic (pH 8.0)
11 compared to the nucleus (pH 7.2)[51]. Consequently, strand breakage resulting from alkaline
12 hydrolysis at rNMPs should be relatively common for mtDNA. In contrast to this idea,
13 mitochondrial gene expression seems tolerant to the accumulation of rNMPs in the mtDNA
14 template.
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24 In the nucleus, the ribonucleotide excision repair (RER) pathway is responsible for the
25 complete removal of ribonucleotides and essential to avoid genome instability and disease
26 [50,52]. The RER pathway is initiated by the RNase H2, a highly efficient enzyme removing
27 virtually all rNMPs from nuclear DNA under normal cellular conditions [48,50]. In contrast,
28 mitochondria have relatively stable levels of incorporated rNMPs all along the genome
29 [31,43,46,53]. Based on *in vitro* biochemical studies, these ribonucleotides are not attributed
30 to higher rNMP incorporation during replication than in the nucleus. For example, yeast Pol γ
31 (Mip1) has an rNMP incorporation rate comparable to its nuclear counterparts (1 rNTP per
32 640 dNTPs) [52]. The human Pol γ has an even higher discrimination ability against rNTPs
33 with a 1 100- to 77 000-fold preference for dNTPs, depending on the base [54], but still
34 sufficient to incorporate approximately 1 rNTP per 2300 dNTPs [46]. The proofreading (3'-
35 exonuclease) activity of Pol γ does not contribute to ribonucleotide removal, as shown also
36 for the nuclear DNA polymerases ϵ and δ [55]. Thus, the most likely explanation for the
37 persistence of rNMPs on mtDNA is the lack of RER in mitochondria. Evidence for this
38 hypothesis comes from genome-wide ribonucleotide mapping approaches, which showed that
39 deletion of RNase H2 does not increase rNMPs in mtDNA [56]. Furthermore, the rNMP
40 frequency on mtDNA correlates with the expected incorporation rate during replication, when
41 the ratio between free rNTPs and the dNTPs available for DNA synthesis are taken into
42 account [57]. mtDNA is especially vulnerable to high rNTP levels, since in contrast to
43 nuclear DNA replication mtDNA replication is not restricted to S phase of the cell cycle [13],
44 when dNTP pools are high [58]. Consistent with this idea, mtDNA from post-mitotic tissues,
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3 expected to have high rNMP/dNMP ratios, contains more frequent ribonucleotides than
4 mtDNA from rapidly dividing cells [59].
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8 **Making the ends meet at mitochondrial replication initiation**

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11 O_H was assigned as the leading strand origin based on the mapping of free 5'-ends in the
12 mitochondrial non-coding region (NCR) [35,60,61], while POLRMT has been proposed to
13 prime H-strand replication by initiating transcription from the L-strand promoter [61], which
14 terminates specifically at CSBII [62-64]. It should be noted that CSBII is located up to 100
15 bp upstream from the most prominent 5'-ends of DNA at O_H [35,60,65] (**Figure 2**). As an *ad*
16 *hoc* explanation for this gap, it has been proposed that efficient primer-removing activity in
17 mitochondria could also degrade the nascent DNA molecules [63]. Some support for this
18 explanation comes from the observation that Pol γ can also displace a few (<10) nucleotides
19 from the 5'-end of the preceding strand, generating 5'-flaps, which are subsequently removed
20 by 5'-exonuclease MGME1 [66]. Although these overhangs *in vitro* are not long enough to
21 explain the discrepancy between the alleged priming site and the main 5'-end of the H-strand
22 DNA, the loss of MGME1 *in vivo* shifts some of these ends close to CSBII [67]. The NCR
23 has also additional H- and L-strand 5'-ends downstream of O_H , [35,68], whose priming
24 mechanisms relation to mtDNA replication have not been addressed. Contrary to the earlier
25 model of SDM [12], the four-way junctional replication intermediates at the non-coding
26 region show that replication of both strands terminates around O_H [37,68] (**Figure 1B**). In
27 order to obtain these termination intermediates, additional, hitherto unknown L-strand origins
28 upstream of O_H are required.
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42 The best characterized L-strand replication origin is O_L , which is also primed by POLRMT,
43 recognizing the stem-loop structure formed by the tRNA cluster in this region of the genome
44 [38,39,69]. Evidence of additional L-strand origins between O_H and O_L have been obtained
45 both by atomic force microscopy [22] as well as mapping of free 5'-ends [35,68]. Apart from
46 these putative origins, L-strand initiation seems highly flexible, since ssDNA intermediates
47 are rapidly converted to dsDNA throughout the mitochondrial genome when the H-strand
48 procession is halted [70-72]. This promiscuous L-strand priming is carried out by an archaic
49 primase-polymerase, PrimPol [73], making it a key enzyme for replication restart in
50 mitochondria. It is noteworthy that PrimPol preferentially inserts DNA primers and that the
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3 priming occurs preferentially on specific sequence, 3'-GTCC-5', present every 100–1000 nt
4 on both strands of the mammalian mtDNA genome [73]. Interestingly however, PrimPol is
5 expendable for mtDNA maintenance [74] and therefore unlikely to play a major role in
6 mtDNA replication initiation under normal physiological conditions.
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10 11 **Promiscuous L-strand priming vs. strand-coupled replication**

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15 The number of L-strand replication origins will have an impact on the SDM, as frequent
16 priming of the L-strand replication would eventually give rise to dsDNA replication
17 intermediates, resembling strand-coupled replication (**Figure 1F**). There is, however, one
18 additional difference that concerns the directionality of the replication, as true strand-coupled
19 replication is essentially bi-directional. Fittingly, bi-directional replication intermediates have
20 been observed using 2D-AGE [32,33], although they typically represent the minority of all
21 replication intermediates in the most commonly studied tissues and cultured cells. These
22 intermediates are best recognized by the occurrence of replication bubbles as a sign of
23 initiation in regions outside of O_H . However, these alternative initiation sites are located
24 maximally several thousand base pairs downstream of O_H , and once the one end of the
25 replication reaches O_H , the replication will be effectively unidirectional [32,33,68]. In
26 contrast to mitotic cells, mtDNA from most post-mitotic tissues contain mainly dsDNA
27 replication intermediates [75]. Notably, the type of replication intermediates correlates also
28 with the levels of mtSSB, which is highly abundant only in cells or tissues having SDM
29 intermediates [75]. Interestingly, only SDM intermediates incorporate radiolabeled
30 nucleotides during *in organello* replication [25], raising doubts if the fully dsDNA
31 intermediates are replicative DNA at all. Alternatively, fork-like DNA structures might
32 represent strand-invasion events as in yeast [76] and therefore be related to repair processes
33 rather than replication.
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47 **Replication mechanisms and the origin of pathological mtDNA** 48 **rearrangements**

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51 Nearly all of the mechanistic studies have focused on the SDM mechanism (eg.
52 [18,38,62,69]) and apart for a single transmission electron microscope observation [72], the
53 evidence for bidirectional replication initiating outside of O_H is based almost entirely on 2D-
54 AGE data. The focus on SDM could generate an unintentional bias in the interpretation of
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3 mtDNA maintenance disorders. For example, pathological mtDNA deletions caused by
4 mutations in Pol γ and TWNK are exclusively seen in tissues such as skeletal muscle and
5 brain [1], having mainly dsDNA mtDNA replication intermediates [75,77]. The
6 rearrangements seen in these patients are associated with replication stalling due to the
7 defective replisome [70,78] or imbalanced nucleotide pools [79]. Interestingly, imbalance in
8 mitochondrial dNTPs leads to a 3- to 4-fold increase in rNMPs incorporated in the mtDNA
9 [57]. The increased rNMP content has been suggested to block the mtDNA replication
10 machinery and contribute to genomic instability (Berglund et al currently ref 57). However,
11 later it was shown that processivity or fidelity of Pol γ is virtually unaffected by single
12 ribonucleotides in the DNA template [46,54], by contrast, its DNA synthesis is strongly
13 inhibited by the presence of free rNTPs during DNA replication at physiological levels of
14 both rNTPs and dNTPs [46]. Although a similar inhibition has been reported for other
15 polymerases [52], Pol γ seems to be exceptionally sensitive to high rNTP/dNTP ratios. The
16 inhibition of Pol γ by rNTPs, resulting in low processivity and frequent replication stalling,
17 may therefore at least partly explain the mtDNA depletion and deletions in patients suffering
18 from defects in the mitochondrial dNTP supply [80].
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30 Stalled replication forks are prone to collapse and cause double-strand breaks [81], whose
31 insufficient repair could explain the deletion formation. This mechanism of deletion
32 formation is also supported by the fact that mice expressing a mitochondrially targeted
33 restriction endonuclease acquire similar deletions as seen in the disorders involving mtDNA
34 maintenance genes [82]. Interestingly, broken mtDNA is rapidly lost in mitotic cells,
35 providing a putative explanation why some tissues do not acquire deletions. Some insight into
36 the actual mechanism has been obtained from recent studies involving MGME1 [83].
37 MGME1 is a mitochondrial exonuclease, degrading most efficiently free 5'- ends of DNA
38 [84]. Although MGME1 is not essential for the upkeep of mtDNA, its impairment results in a
39 typical mtDNA maintenance disorder in humans [84] and mice [83]. Apart for its function in
40 the processing of DNA flaps [66], MGME1 works together with Pol γ and TWNK to rapidly
41 degrade linear mtDNA fragments, including broken, partially replicated molecules [85]. In
42 *Mgme1* knockout mice, the broken mtDNA is maintained linear in tissues with mainly SDM-
43 type replication, such as liver, while being converted into circular deletions in heart [83].
44 Intriguingly, the fate of the linear molecules correlates with the preferred replication
45 mechanism as liver mtDNA replicates by SDM, while heart has mainly dsDNA replication
46 intermediates [75]. This correlation does not need to be mechanistically related, but further
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3 indicates that the mtDNA maintenance mechanisms are adapted to their cellular environment.
4 Rapid turnover of linear mtDNA could be a suitable strategy for tissues with low damage
5 rates, whereas tissues exposed to higher levels of stress would risk depleting their mtDNA
6 copy number without additional repair mechanisms.
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11 Because of its central role in the turnover of linear mtDNA, the knockout of *Mgme1* provides
12 a perfect tool to study mitochondrial double-strand repair. The inability to degrade broken
13 mtDNA could force the cells to employ other DSB repair mechanisms. However, MGME1
14 clearly has also other roles in mtDNA maintenance, complicating the picture. As mentioned
15 before, one of the hallmarks of MGME1 insufficiency are specific linear mtDNA deletions
16 spanning the region between O_H and O_L , corresponding to broken partially replicated
17 molecules [67,85] and are identical to the ones seen in mice expressing exonuclease deficient
18 (exo^-) Pol γ [86,87]. Interestingly, O_H is also prominent replication pause site on the
19 mitochondrial genome [88] and this pausing is strongly enhanced in both exo^- Pol γ [87] and
20 *Mgme1* knockout mice [83]. This sequence specific pausing or stalling is likely to be related
21 to double-strand break formation at the next replication origin due to incomplete processing
22 of replication termination intermediates. After 3'-end of the replicating strand has met the
23 preceding 5'-end at the terminus, the concerted action of MGME1 and Pol γ 3'-exonuclease
24 activity is required to remove short flap structures to create ends suitable for DNA ligase
25 [66]. Defects in the ligation step would leave a nick in the origin region just upstream of the
26 priming site, resulting in a double-strand break during a second round of replication (**Figure**
27 **3A**). Enhancement of the pause sites would in this case represent a replication intermediate
28 stalled at one fork due double-strand break at the other end of the molecule. Due to the
29 unidirectional nature of mtDNA replication, double-strand break at O_L will automatically
30 result in molecules with the opposite end fixed at O_H (**Figure 3B**) and resulting in apparent
31 site-specific stalling, reported in patients and *Mgme1* knockout mice [84,85]. This partially
32 replicated molecule would persist until repaired or broken also at the other end to generate
33 the linear deletion. Similar breakpoint is expected to occur also at O_H , as previously
34 suggested to be behind the generation of the linear deletion [89]. However, as the deletion
35 breakpoint is in the vicinity of O_H [67], it is difficult to imagine how priming over the nicked
36 H-strand template would work. Also unless replication stalls at O_L (**Figure 3C**), additional L-
37 strand origins or PrimPol dependent priming upstream of it would allow the replication of the
38 whole genome, resulting in fully replicated linear molecules (**Figure 3D**).
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3 Surprisingly little is known about the repair of mtDNA double-strand breaks. Although
4 recombination is an important DSB repair mechanism for almost all known genetic entities,
5 its existence in mitochondria remains controversial [90]. Mitochondrial DNA rearrangement
6 breakpoints form typically between microhomologies, indicating intramolecular
7 recombination or microhomology-mediated end joining [2,67,82,85,91-94]. In addition,
8 replication slippage has also been suggested as a mechanism [95], but this fails to explain
9 how rearrangements are obtained from restriction endonuclease cleavage of mtDNA [82,92].
10 Replication coupled repair of double-strand breaks, similar to the one occurring T7 phage,
11 would neatly combine the two models [39]. In this model, mitochondrial replisome switches
12 templates by taking advantage of short regions of homology at the broken molecule. In T7
13 phage the strand exchange is dependent on the ssDNA annealing activity associated with
14 gp2.5 single-strand binding protein [96]. Intriguingly, TWNK has the same strand annealing
15 activity [97] and is capable in improving mtDNA integrity under stress conditions, while
16 generating circular rearrangements [91]. Notably, the existence of replication-coupled DSB
17 repair does not exclude recombination as a genome maintenance mechanism as both are
18 present in the T7 phage [98]. Mechanistically the two are operate similarly and it is only a
19 matter of definition when strand-exchange is considered as molecular recombination. T7
20 phage is possibly a suitable model for mtDNA recombination as it operates independently of
21 RecA or similar dedicated recombinase.
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34 **True love can wait – revisiting the faithful nucleoid hypothesis**

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37 Double-strand break repair by recombination, including the replication-coupled strand-
38 exchange is usually highly efficient and unless gene conversion or illicit recombination
39 between non-homologous DNA regions has taken place, the repaired molecules are identical
40 to the original copy. As mitochondria typically have only one mtDNA genotype,
41 recombination-dependent repair is impossible to detect. Main evidence against biologically
42 significant mtDNA recombination comes from the failure to detect recombinant
43 mitochondrial genomes from mice with two different mtDNA haplotypes (BALB and NZB)
44 [90]. However, this study analyzed only liver and kidney which have no four-way junctional
45 mtDNA [75]. In contrast the junctional molecules present in brain, muscle and brown fat
46 have all the characteristics of recombination junctions [77] and their levels correlate with
47 mtDNA rearrangements and dimers [91]. The induction of double-strand breaks in
48 BALB/NZB mouse tissues resulted mainly in typical rearrangements between intramolecular
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3 sequence homologies, but generated also recombinant BALB/NZB sequences from
4 intermolecular repair events [92]. The outcome of any recombination event will be dependent
5 on the availability of intact molecules for homology-dependent repair. As only one [99] or
6 few [77] copies of mtDNA exist per nucleoid, the composition of these nucleoids will dictate
7 whether intermolecular recombination is possible at all. If a single nucleoid contains only
8 progeny of one ancestral nucleoid, all genotypically identical as proposed by the so-called
9 “faithful nucleoid” model [100], successful intermolecular recombination between different
10 haplotypes will be impossible to detect. The ability of nucleoids to exchange genetic material
11 is an open question waiting to be addressed. Promisingly, some data suggests that nucleoids
12 aggregate upon mtDNA damage [101]. It remains to be seen whether this reflects a general
13 problem in mtDNA replication and segregation or an active repair process.
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23 **Conclusions**

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26 The last five decades of research on mtDNA maintenance mechanisms have provided a
27 collection of puzzle pieces, some more compatible with other bits than with others.
28 Regarding the mechanisms of mtDNA replication, a waiting challenge is to mechanistically
29 consolidate the existing findings regarding the role of RNA or mtSSB on the displaced
30 strand. Additional L-strand origins as well as their relation to the more promiscuous priming
31 seen in replicative stress should be elucidated in detail by old-fashioned 5'-end mapping or
32 perhaps by applying Next Generation sequencing-based methods [102]. Further studies of the
33 fully dsDNA replication intermediates should be aimed to reveal whether there is more than
34 one replication mechanism operating in mammalian mitochondria, or whether they represent
35 only variations of SDM with more frequent L-strand priming. The bi-directional replication
36 origins detected by 2D-AGE remain intriguing and suggest that mammalian mtDNA
37 maintenance is not as simple as thought. Future research should study and confirm these
38 replication intermediates with different experimental techniques. Furthermore, the correlation
39 between the type of replication intermediates and tissue specific occurrence of mtDNA
40 rearrangements suggests that the two are linked. Understanding the mtDNA maintenance
41 mechanisms will be the key to understand also the pathological mechanisms of various
42 mitochondrial disorders. The biological significance of rNMPs in mtDNA and their role in
43 mitochondrial genome maintenance is intriguing. Accumulation of rNMPs in the nuclear
44 DNA is detrimental for cell survival, but for a yet unknown reason, the mitochondrial
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3 replication and transcription machineries seem to be adapted to the RNA-rich nature of their
4 template. Manipulation of Pol γ 's fidelity to incorporate rNMPs could provide experimental
5 tools to address a potential role of ribonucleotides in mtDNA stability.
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8 9 **Acknowledgements**

10
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18 19 **Conflict of interest**

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22 The authors declare no conflict of interest
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16 Figure legends

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19 **Figure 1.** Mammalian mitochondrial DNA replication. (A) A schematic map of mammalian
20 mitochondrial DNA. Most genes are located on the H-strand (outer side of the circle) and
21 apart for ND6, the L-strand (inner side) has only tRNA genes (orange circles). Non-coding
22 region (NCR) has transcriptional control regions and the origin(s) of H-strand replication
23 (O_H). The main origin for L-strand replication (O_L) is located in the WANCY tRNA cluster.
24 (B) The best characterized mechanism of mtDNA replication is highly asymmetric, with the
25 L-strand replication initiating at O_L with considerable delay compared to the H-strand
26 replication. Only DNA intermediates shown. (C) Replication termination (TER) of both
27 strands has been suggest to occur at the NCR, close to the O_H [37], necessitating additional L-
28 strand origins upstream of O_L . (D) According to the classical mtDNA replication model, the
29 displaced strand is excessively coated by mitochondrial single-strand binding protein
30 (mtSSB). (E) According to the so-called bootlace model, preformed RNA is incorporated at
31 the replication fork on the displaced strand, explaining the observed RNA:DNA hybrids on
32 mtDNA replication intermediates. (F) Fully double-stranded DNA replication intermediates
33 do not need to represent strand-coupled replication but could be also generated by frequent
34 priming of the L-strand replication.
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47 **Figure 2.** The non-coding region of human mtDNA. Primers for H-strand replication are
48 suggested to be formed by POLRMT initiation transcription from light-strand promoter
49 (LSP) that prematurely terminates at conserved sequence block 2 (CSB). The closest 5'-ends
50 of DNA are located at O_H , but there is an additional cluster of 5'-ends further downstream
51 close to the termination associated sequence (TAS). Replication terminating at TAS is
52 suggested to generate the so-called 7S DNA, which remains hybridized to with the template
53 DNA forming a triple-stranded D-loop structure. There are reported L-strand 5'-ends
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3 downstream of O_H , but these are likely not related to the replication terminus for both strand,
4 mapped further upstream of O_H .
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8 **Figure 3.** Linear deletion could involve strand break and replication stalling. (A) Inability to
9 ligate the newly replicated strand results in a nick close to the replication origin, explaining
10 the linear deletions observed in *Mgme1* knockout or Pol γ mutator mice span between O_H and
11 O_L , which arise from the next round of replication. (B) Nick close to the O_L on L-strand will
12 result in double-strand break when H-strand is replicated. Because O_H is unidirectional
13 origin, these broken molecules will remain stalled after the strand break (schematic
14 illustration on the right). (C) If the strand break occurs at O_H , the replication fork seems to
15 stall at O_L [83,84], although the mechanism is unclear. (D) Stalling at O_L is necessary for
16 linear deletions, as additional L-strand origins or PrimPol priming activity upstream of O_L ,
17 also suggested by the termination dsDNA intermediates [37], strand-break at O_H would
18 generate full-length linear replication intermediates.
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For Peer Review

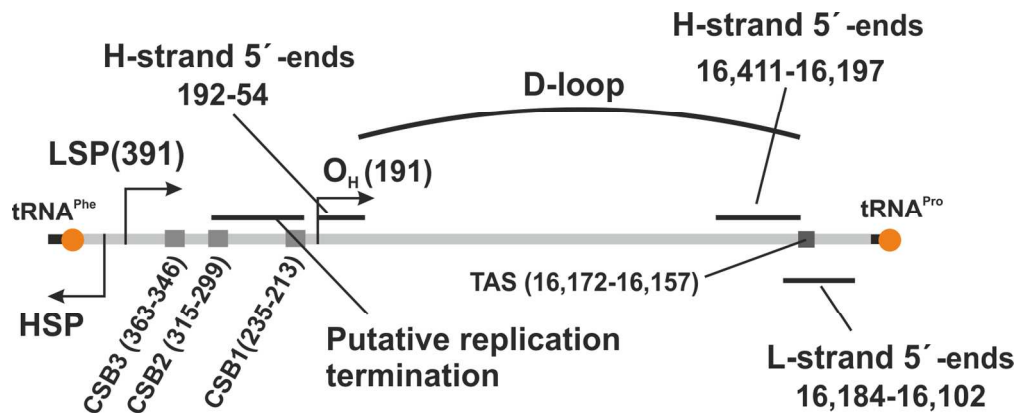


Figure 2. The non-coding region of human mtDNA. Primers for H-strand replication are suggested to be formed by POLRMT initiation transcription from light-strand promoter (LSP) that prematurely terminates at conserved sequence block 2 (CSB). The closest 5'-ends of DNA are located at O_H, but there is an additional cluster of 5'-ends further downstream close to the termination associated sequence (TAS). Replication terminating at TAS is suggested to generate the so-called 7S DNA, which remains hybridized to with the template DNA forming a triple-stranded D-loop structure. There are reported L-strand 5'-ends downstream of O_H, but these are likely not related to the replication terminus for both strand, mapped further upstream of O_H.

143x57mm (300 x 300 DPI)

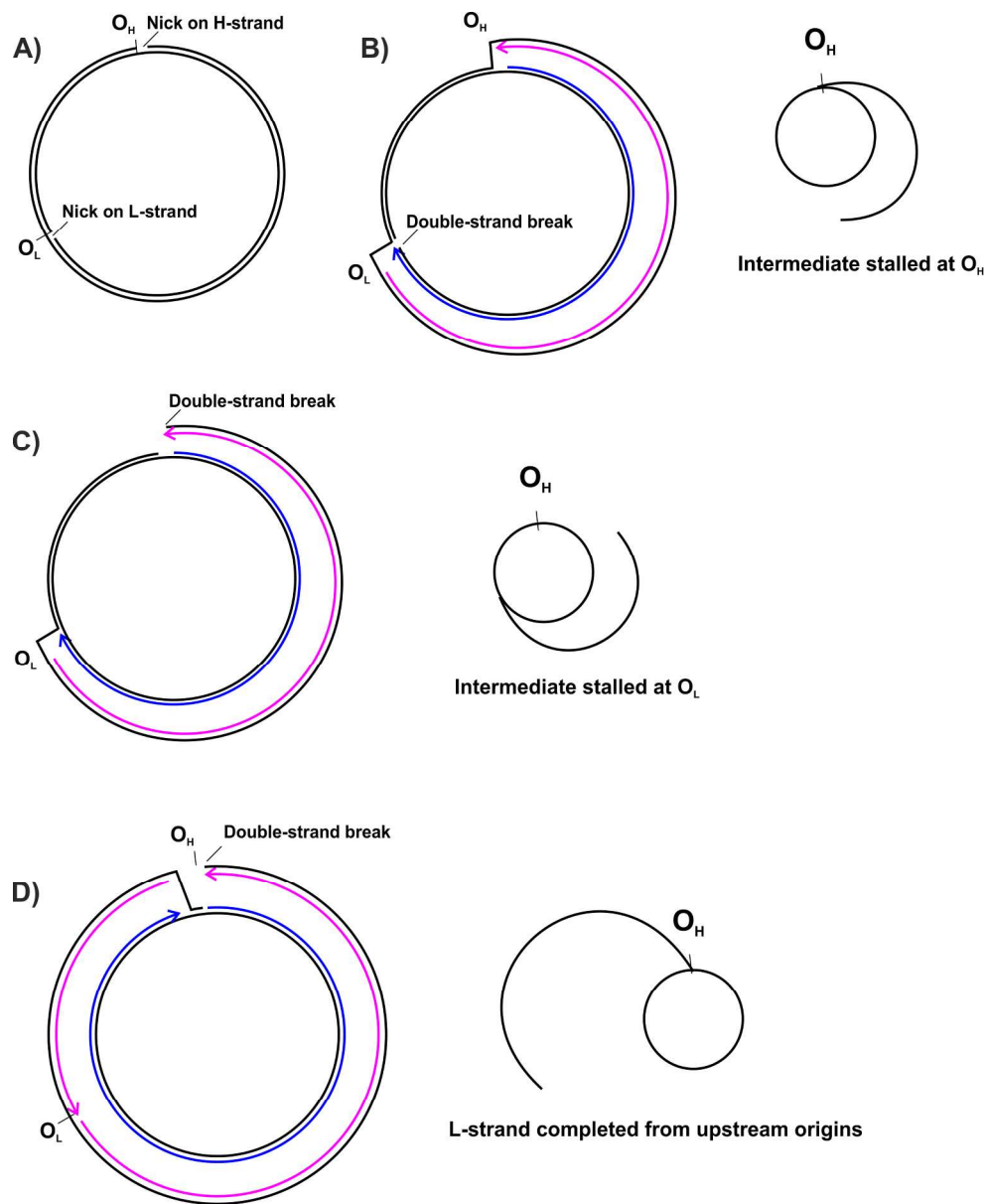


Figure 3. Linear deletion could involve strand break and replication stalling. (A) Inability to ligate the newly replicated strand results in a nick close to the replication origin, explaining the linear deletions observed in *Mgme1* knockout or Pol γ mutator mice span between O_H and O_L , which arise from the next round of replication. (B) Nick close to the O_L on L-strand will result in double-strand break when H-strand is replicated. Because O_H is unidirectional origin, these broken molecules will remain stalled after the strand break (schematic illustration on the right). (C) If the strand break occurs at O_H , the replication fork seems to stall at O_L [83,84], although the mechanism is unclear. (D) Stalling at O_L is necessary for linear deletions, as additional L-strand origins or PrimPol priming activity upstream of O_L , also suggested by the termination dsDNA intermediates [37], strand-break at O_H would generate full-length linear replication intermediates.

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