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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 lose 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

13 subunits of oxidative phosphorylation (OXPHOS) complexes as well as tRNAs and rRNAs required for their synthesis. In mitochondria, mtDNA is packaged in nucleoprotein complexes called nucleoids. Due to its central role in encoding subunits of the OXPHOS complexes, mtDNA is essential for mitochondrial function and any perturbations involving its replication or repair can be detrimental for the host cell. Consequently, disorders involving disturbances of mtDNA replication or gene expression have often dramatic pathological phenotypes, in the worst case failure to thrive [1]. Devastating diseases caused by defects in mtDNA replication include progressive external ophthalmoplegia, ataxia-neuropathy, mitochondrial neurogastrointestinal encephalomyopathy and Alpers-Huttenlocher syndrome [2]. Impaired mitochondrial DNA integrity has been also linked to a range of common diseases and ageing [3], the latter likely connected to stem cell maintenance that affects tissue regeneration [4].

Despite its small size and apparent simplicity, studying mammalian mitochondrial DNA maintenance mechanisms has proven to be challenging. Not only there is disagreement regarding the replication mechanisms of mtDNA, but also our knowledge of the scale and scope of mitochondrial genome repair mechanisms is limited. While mechanisms such as base excision repair of mtDNA are relatively well characterized, other reported mtDNA maintenance mechanisms rely often on rather anecdotal observations [5]. Most of the suspected repair proteins mainly localize to the nucleus, and it is often difficult to unequivocally prove that the protein localizes into mitochondria under normal conditions, instead of being detected there only because of contamination from sample preparation procedures. These issues have been recently exemplified by a critical review regarding the proposed localization of various DNA polymerases in mitochondria [6]. Conversely, the difficulty of finding a rare repair protein in mitochondria does not exclude the protein from having a function in mtDNA maintenance. It might be that some of the mtDNA repair mechanisms operate only under specific circumstances or in a limited number of cell types. Again, demonstrating this can be technically challenging, especially if the protein is essential for nuclear genome maintenance, ruling out knockout studies. However, systematic analysis of the dual localized genes can reveal alternative AUGs [7] or non-conventional mitochondrial targeting signals [8,9], which can be targeted by gene knock-in to interrogate only the mitochondrial function. More focus should be put on the search for enzymes involved in DNA maintenance mechanisms that are necessitated by the nature of nucleic acid metabolism reactions, such as recombination or the repair of protein-DNA crosslinks [10].

The recent discovery of a mitochondrial isoform of tyrosyl-DNA phosphodiesterase 2 [11], capable of removing tyrosine-DNA adducts, could represent the first example of the existence of these DNA repair mechanisms in mitochondria.

Our motivation to write this review was to raise the awareness of still outstanding issues connected to the basic mtDNA maintenance mechanisms and of interesting observations that have not been properly pursued. Despite the apparent simplicity of the mitochondrial genome, its multiple unexplained features demonstrate that our knowledge of mtDNA maintenance is limited at best.

Recursive progress in elucidating mtDNA replication mechanisms

Mitochondria are constantly replicating their genome and this replication is thought to be independent of the cell cycle [12,13]. Cells need to replace the mtDNA lost due to mitochondrial turnover or produce additional mtDNA when mitochondrial biogenesis is upregulated. Additionally, mitotic cells have to replicate their mtDNA to maintain a constant copy number when the cytoplasmic compartment is partitioned between the daughter cells.

Due to the evolutionary history of mitochondria, the mtDNA replication machinery in eukaryotes is essentially prokaryotic in its constituents. For example, the mitochondrial single-strand binding protein mtSSB is a homologue of bacterial SSB [14], whereas the replicative helicase TWNK and the catalytic subunit of the mitochondria DNA polymerase Pol γ have resemblance to bacteriophage T7 gp4 primase-helicase and gp5 DNA polymerase [15-17]. The minimal mammalian mtDNA replisome can be reconstituted *in vitro* using these three proteins, with Pol γ being present as a heterotrimeric holoenzyme (one catalytic subunit Pol γA plus two accessory subunits, Pol γB) [18]. Intriguingly the mtDNA replication mechanisms seem to have evolved differently in different eukaryotic lineages, representing almost all possible replication modes including recombination-dependent replication [19], rolling-circle replication [20], strand-coupled replication [21] and strand-displacement mechanisms [22]. Even more complicated, more than one mechanism might co-exist in the same group of organisms. For instance at least three different mtDNA replication mechanisms have been proposed to exists in mammals [23]. Despite the ongoing controversies [24,25], there is a general agreement that the mammalian mtDNA replicates mostly in a highly asymmetric manner, with one strand (light, or the L-strand) replicated with

considerable delay compared to the other strand (heavy, or the H-strand) (**Figure 1**). In this mechanism, the earlier initiated replication of the H-strand results in the displacement of the L-strand template as single-stranded DNA (ssDNA), hence initially dubbed as the strand-displacement model (SDM) [12].

Unbraiding the displaced strand identity

Mitochondrial DNA replication was initially studied using transmission electron microscopy (TEM) [26,27] and in fact, fully double-stranded replication intermediates were observed in the first TEM analyses [26,28]. Later studies revealed partially single-stranded replication intermediates [27,29], resulting in the establishment of the strand-displacement model (SDM) of mtDNA replication [12]. In this model, replication is unidirectional and asymmetric, with leading-strand synthesis displacing one strand, which is replicated with delay and resulting in persisting single-stranded intermediates. Decades later the mtDNA strand-displacement model was revisited by a series of studies from Ian Holt's group [30-33] using two-dimensional Brewer-Fangman agarose electrophoresis (2D-AGE) that separate nucleic acids according to their size and shape [34]. Although initially confusing the mitochondrial DNA replication field by introducing the concept of strand-coupled [30] and bi-directional [32,33] mtDNA replication, being opposite to the original model, the studies also provided evidence that the SDM intermediates can be generated from double-stranded replication intermediates by RNase digestion, indicating that the L-strand template is covered by RNA prior its replication [31,35,36]. Random hybridization of RNA on ssDNA during isolation procedures was excluded by experiments showing that RNA could be cross-linked on the displaced L-strand *in vivo* using psoralen [36]. On the other hand, chromatin immune precipitation experiments showed that the occupancy profile of mtSSB displays a distinct pattern that can be expected from replication intermediates that are replicated by the SDM, mtSSB fully covering the exposed single-stranded lagging-strand [24]. However, in the heat of the debate it is often missed that there is in fact much consensus on the mode of mtDNA replication, apart from the identity of L-strand replication intermediates. H-strand replication initiates first at the origin of H-strand replication (O_H) and proceeds unidirectional through the genome. The L-strand replication initiates at one major origin, the O_L, only when H-strand replication has reached it (**Figure 1B**) and the replication of both strands terminates when it they have reached the non-coding region (**Figure 1C**) [25,35,37,38]. The only practical

difference is that in the so-called bootlace model the displaced H-strand is covered by preformed RNA (**Figure 1D**) [25] instead of mtSSB (**Figure 1E**), as in the classical model [12]. Due to the similarity of DNA synthesis in the SDM and bootlace models, methods visualizing newly replicated DNA cannot differentiate between the two [39]. As far as known, the bootlace mechanism of replication would be unique to mitochondrial DNA. It has been proposed that covering the displaced strand with RNA could have a number of advantages: protection of the replication fork against breakage, elimination of mutations and prevention of secondary structure formation [40], but currently there is no experimental support or precedence for these ideas.

The main weakness of the bootlace model is that the recruitment and annealing of preformed RNA at the replication fork should be an active mechanism with a dedicated enzymatic machinery. Until its components are discovered, the issue can be considered unsettled. However, an interesting candidate for the RNA annealing could be the mitochondrial replicative helicase TWNK, which has been shown to have DNA annealing activity *in vitro* [41]. If the annealing activity is not limited to DNA, TWNK's central location at the replication fork would offer a simple mechanistic explanation for the rapid covering of the displaced strand with RNA. Furthermore, TWNK has 5′ to 3′ unwinding activity [42] and thus moves on the L-strand during DNA replication, making the annealing of RNA mechanistically plausible. The mechanism would probably have to exclude the usage of modified or structurally constrained RNAs, such as ribosomal RNA and tRNAs.

RNA incorporation vs. incorporation of ribonucleotides on mitochondrial DNA

The presence of ribonucleotides in mtDNA was documented as early as the 1970s [43,44]. These single ribonucleotides (rNMPs) embedded in mature mtDNA are not related to incorporation of longer RNA species on the displaced strand. While the proposed bootlace intermediates, which consist of longer fragments of RNA, are sensitive to cleavage by RNase H1 [45], mature mtDNA containing single, or at most two consecutive rNMPs, is not. Instead, these molecules are readily degraded using alkali treatment or RNase H2, cleaving at single rNMPs [46].

rNMPs are frequently incorporated in both the nuclear and mitochondrial DNA during normal replication processes due to their abundance and chemical similarity to dNMPs [47-49]. The ribonucleotides inserted into DNA change the chemical and structural properties of the DNA due to the additional hydroxyl group on the 2´ carbon of the sugar moiety. Consequently, rNMPs that persist in in nuclear DNA can lead to severe replication stress [47,49,50]. Besides blocking replication, the presence of the extra OH group on the 2´ carbon of ribonucleotides makes rNMPs more prone to hydrolysis, especially at alkaline pH. This is potentially relevant for mitochondria, as their pH is thought to be more basic (pH 8.0) compared to the nucleus (pH 7.2)[51]. Consequently, strand breakage resulting from alkaline hydrolysis at rNMPs should be relatively common for mtDNA. In contrast to this idea, mitochondrial gene expression seems tolerant to the accumulation of rNMPs in the mtDNA template.

In the nucleus, the ribonucleotide excision repair (RER) pathway is responsible for the complete removal of ribonucleotides and essential to avoid genome instability and disease [50,52]. The RER pathway is initiated by the RNase H2, a highly efficient enzyme removing virtually all rNMPs from nuclear DNA under normal cellular conditions [48,50]. In contrast, mitochondria have relatively stable levels of incorporated rNMPs all along the genome [31,43,46,53]. Based on *in vitro* biochemical studies, these ribonucleotides are not attributed to higher rNMP incorporation during replication than in the nucleus. For example, yeast Pol γ (Mip1) has an rNMP incorporation rate comparable to its nuclear counterparts (1 rNTP per 640 dNTPs) [52]. The human Pol γ has an even higher discrimination ability against rNTPs with a 1 100- to 77 000-fold preference for dNTPs, depending on the base [54], but sill sufficient to incorporate approximately 1 rNTP per 2300 dNTPs [46]. The proofreading (3´-exonuclease) activity of Pol γ does not contribute to ribonucleotide removal, as shown also for the nuclear DNA polymerases ε and δ [55]. Thus, the most likely explanation for the persistence of rNMPs on mtDNA is the lack of RER in mitochondria. Evidence for this hypothesis comes from genome-wide ribonucleotide mapping approaches, which showed that deletion of RNAse H2 does not increase rNMPs in mtDNA [56]. Furthermore, the rNMP frequency on mtDNA correlates with the expected incorporation rate during replication, when the ratio between free rNTPs and the dNTPs available for DNA synthesis are taken into account [57]. mtDNA is especially vulnerable to high rNTP levels, since in contrast to nuclear DNA replication mtDNA replication is not restricted to S phase of the cell cycle [13], when dNTP pools are high [58]. Consistent with this idea, mtDNA from post-mitotic tissues,

expected to have high rNMP/dNMP ratios, contains more frequent ribonucleotides than mtDNA from rapidly dividing cells [59].

Making the ends meet at mitochondrial replication initiation

O_H was assigned as the leading strand origin based on the mapping of free 5′-ends in the mitochondrial non-coding region (NCR) [35,60,61], while POLRMT has been proposed to prime H-strand replication by initiating transcription from the L-strand promoter [61], which terminates specifically at CSBII [62-64]. It should be noted that CSBII is located up to 100 bp upstream from the most prominent 5′-ends of DNA at O_H [35,60,65] (**Figure 2**). As an *ad hoc* explanation for this gap, it has been proposed that efficient primer-removing activity in mitochondria could also degrade the nascent DNA molecules [63]. Some support for this explanation comes from the observation that Pol γ can also displace a few (<10) nucleotides from the 5′-end of the preceding strand, generating 5′-flaps, which are subsequently removed by 5′-exonuclease MGME1 [66]. Although these overhangs *in vitro* are not long enough to explain the discrepancy between the alleged priming site and the main 5′-end of the H-strand DNA, the loss of MGME1 *in vivo* shifts some of these ends close to CSBII [67]. The NCR has also additional H- and L-strand 5′-ends downstream of O_H, [35,68], whose priming mechanisms relation to mtDNA replication have not been addressed. Contrary to the earlier model of SDM [12], the four-way junctional replication intermediates at the non-coding region show that replication of both strands terminates around O_H [37,68] (**Figure 1B**). In order to obtain these termination intermediates, additional, hitherto unknown L-strand origins upstream of O_H are required.

The best characterized L-strand replication origin is O_L, which is also primed by POLRMT, recognizing the stem-loop structure formed by the tRNA cluster in this region of the genome [38,39,69]. Evidence of additional L-strand origins between O_H and O_L have been obtained both by atomic force microscopy [22] as well as mapping of free 5′-ends [35,68]. Apart from these putative origins, L-strand initiation seems highly flexible, since ssDNA intermediates are rapidly converted to dsDNA throughout the mitochondrial genome when the H-strand procession is halted [70-72]. This promiscuous L-strand priming is carried out by an archaic primase-polymerase, PrimPol [73], making it a key enzyme for replication restart in mitochondria. It is noteworthy that PrimPol preferentially inserts DNA primers and that the

priming occurs preferentially on specific sequence, 3′-GTCC-5′, present every 100–1000 nt on both strands of the mammalian mtDNA genome [73]. Interestingly however, PrimPol is expendable for mtDNA maintenance [74] and therefore unlikely to play a major role in mtDNA replication initiation under normal physiological conditions.

Promiscuous L-strand priming vs. strand-coupled replication

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

Replication mechanisms and the origin of pathological mtDNA rearrangements

Nearly all of the mechanistic studies have focused on the SDM mechanism (eg. [18,38,62,69]) and apart for a single transmission electron microscope observation [72], the evidence for bidirectional replication initiating outside of O_H is based almost entirely on 2D-AGE data. The focus on SDM could generate an unintentional bias in the interpretation of

mtDNA maintenance disorders. For example, pathological mtDNA deletions caused by mutations in Pol γ and TWNK are exclusively seen in tissues such as skeletal muscle and brain [1], having mainly dsDNA mtDNA replication intermediates [75,77]. The rearrangements seen in these patients are associated with replication stalling due to the defective replisome [70,78] or imbalanced nucleotide pools [79]. Interestingly, imbalance in mitochondrial dNTPs leads to a 3- to 4-fold increase in rNMPs incorporated in the mtDNA [57]. The increased rNMP content has been suggested to block the mtDNA replication machinery and contribute to genomic instability (Berglund et al currently ref 57). However, later it was shown that processivity or fidelity of Pol γ is virtually unaffected by single ribonucleotides in the DNA template [46,54], by contrast, its DNA synthesis is strongly inhibited by the presence of free rNTPs during DNA replication at physiological levels of both rNTPs and dNTPs [46]. Although a similar inhibition has been reported for other polymerases [52], Pol γ seems to be exceptionally sensitive to high rNTP/dNTP ratios. The inhibition of Pol γ by rNTPs, resulting in low processivity and frequent replication stalling, may therefore at least partly explain the mtDNA depletion and deletions in patients suffering from defects in the mitochondrial dNTP supply [80].

Stalled replication forks are prone to collapse and cause double-strand breaks [81], whose insufficient repair could explain the deletion formation. This mechanism of deletion formation is also supported by the fact that mice expressing a mitochondrially targeted restriction endonuclease acquire similar deletions as seen in the disorders involving mtDNA maintenance genes [82]. Interestingly, broken mtDNA is rapidly lost in mitotic cells, providing a putative explanation why some tissues do not acquire deletions. Some insight into the actual mechanism has been obtained from recent studies involving MGME1 [83]. MGME1 is a mitochondrial exonuclease, degrading most efficiently free 5´- ends of DNA [84]. Although MGME1 is not essential for the upkeep of mtDNA, its impairment results in a typical mtDNA maintenance disorder in humans [84] and mice [83]. Apart for its function in the processing of DNA flaps [66], MGME1 works together with Pol γ and TWNK to rapidly degrade linear mtDNA fragments, including broken, partially replicated molecules [85]. In *Mgme1* knockout mice, the broken mtDNA is maintained linear in tissues with mainly SDM-type replication, such as liver, while being converted into circular deletions in heart [83]. Intriguingly, the fate of the linear molecules correlates with the preferred replication mechanism as liver mtDNA replicates by SDM, while heart has mainly dsDNA replication intermediates [75]. This correlation does not need to be mechanistically related, but further

indicates that the mtDNA maintenance mechanisms are adapted to their cellular environment. Rapid turnover of linear mtDNA could be a suitable strategy for tissues with low damage rates, whereas tissues exposed to higher levels of stress would risk depleting their mtDNA copy number without additional repair mechanisms.

Because of its central role in the turnover of linear mtDNA, the knockout of *Mgme1* provides a perfect tool to study mitochondrial double-strand repair. The inability to degrade broken mtDNA could force the cells to employ other DSB repair mechanisms. However, MGME1 clearly has also other roles in mtDNA maintenance, complicating the picture. As mentioned before, one of the hallmarks of MGME1 insufficiency are specific linear mtDNA deletions spanning the region between O_H and O_L, corresponding to broken partially replicated molecules [67,85] and are identical to the ones seen in mice expressing exonuclease deficient (exo$^-$) Pol γ [86,87]. Interestingly, O_H is also prominent replication pause site on the mitochondrial genome [88] and this pausing is strongly enhanced in both exo$^-$ Pol γ [87] and *Mgme1* knockout mice [83]. This sequence specific pausing or stalling is likely to be related to double-strand break formation at the next replication origin due to incomplete processing of replication termination intermediates. After 3′-end of the replicating strand has met the preceding 5′-end at the terminus, the concerted action of MGME1 and Pol γ 3′-exonuclease activity is required to remove short flap structures to create ends suitable for DNA ligase [66]. Defects in the ligation step would leave a nick in the origin region just upstream of the priming site, resulting in a double-strand break during a second round of replication (**Figure 3A**). Enhancement of the pause sites would in this case represent a replication intermediate stalled at one fork due double-strand break at the other end of the molecule. Due to the unidirectional nature of mtDNA replication, double-strand break at O_L will automatically result in molecules with the opposite end fixed at O_H (**Figure 3B**) and resulting in apparent site-specific stalling, reported in patients and *Mgme1* knockout mice [84,85]. This partially replicated molecule would persist until repaired or broken also at the other end to generate the linear deletion. Similar breakpoint is expected to occur also at O_H, as previously suggested to be behind the generation of the linear deletion [89]. However, as the deletion break point is in the vicinity of O_H [67], it is difficult to imagine how priming over the nicked H-strand template would work. Also unless replication stalls at O_L (**Figure 3C**), additional L-strand origins or PrimPol dependent priming upstream of it would allow the replication of the whole genome, resulting in fully replicated linear molecules (**Figure 3D**).

Surprisingly little is known about the repair of mtDNA double-strand breaks. Although recombination is an important DSB repair mechanism for almost all known genetic entities, its existence in mitochondria remains controversial [90]. Mitochondrial DNA rearrangement breakpoints form typically between microhomologies, indicating intramolecular recombination or microhomology-mediated end joining [2,67,82,85,91-94]. In addition, replication slippage has also been suggested as a mechanism [95], but this fails to explain how rearrangements are obtained from restriction endonuclease cleavage of mtDNA [82,92]. Replication coupled repair of double-strand breaks, similar to the one occurring T7 phage, would neatly combine the two models [39]. In this model, mitochondrial replisome switches templates by taking advantage of short regions of homology at the broken molecule. In T7 phage the strand exchange is dependent on the ssDNA annealing activity associated with gp2.5 single-strand binding protein [96]. Intriguingly, TWNK has the same strand annealing activity [97] and is capable in improving mtDNA integrity under stress conditions, while generating circular rearrangments [91]. Notably, the existence of replication-coupled DSB repair does not exclude recombination as a genome maintenance mechanism as both are present in the T7 phage [98]. Mechanistically the two are operate similarly and it is only a matter of definition when strand-exchange is considered as molecular recombination. T7 phage is possibly a suitable model for mtDNA recombination as it operates independently of RecA or similar dedicated recombinase.

True love can wait – revisiting the faithful nucleoid hypothesis

Double-strand break repair by recombination, including the replication-coupled strand-exchange is usually highly efficient and unless gene conversion or illicit recombination between non-homologous DNA regions has taken place, the repaired molecules are identical to the original copy. As mitochondria typically have only one mtDNA genotype, recombination-dependent repair is impossible to detect. Main evidence against biologically significant mtDNA recombination comes from the failure to detect recombinant mitochondrial genomes from mice with two different mtDNA haplotypes (BALB and NZB) [90]. However, this study analyzed only liver and kidney which have no four-way junctional mtDNA [75]. In contrast the junctional molecules present in brain, muscle and brown fat have all the characteristics of recombination junctions [77] and their levels correlate with mtDNA rearrangements and dimers [91]. The induction of double-strand breaks in BALB/NZB mouse tissues resulted mainly in typical rearrangements between intramolecular

sequence homologies, but generated also recombinant BALB/NZB sequences from intermolecular repair events [92]. The outcome of any recombination event will be dependent on the availability of intact molecules for homology-dependent repair. As only one [99] or few [77] copies of mtDNA exist per nucleoid, the composition of these nucleoids will dictate whether intermolecular recombination is possible at all. If a single nucleoid contains only progeny of one ancestral nucleoid, all genotypically identical as proposed by the so-called "faithful nucleoid" model [100], successful intermolecular recombination between different haplotypes will be impossible to detect. The ability of nucleoids to exchange genetic material is an open question waiting to be addressed. Promisingly, some data suggests that nucleoids aggregate upon mtDNA damage [101]. It remains to be seen whether this reflects a general problem in mtDNA replication and segregation or an active repair process.

Conclusions

The last five decades of research on mtDNA maintenance mechanisms have provided a collection of puzzle pieces, some more compatible with other bits than with others. Regarding the mechanisms of mtDNA replication, a waiting challenge is to mechanistically consolidate the existing findings regarding the role of RNA or mtSSB on the displaced strand. Additional L-strand origins as well as their relation to the more promiscuous priming seen in replicative stress should be elucidated in detail by old-fashioned 5′-end mapping or perhaps by applying Next Generation sequencing-based methods [102]. Further studies of the fully dsDNA replication intermediates should be aimed to reveal whether there is more than one replication mechanism operating in mammalian mitochondria, or whether they represent only variations of SDM with more frequent L-strand priming. The bi-directional replication origins detected by 2D-AGE remain intriguing and suggest that mammalian mtDNA maintenance is not as simple as thought. Future research should study and confirm these replication intermediates with different experimental techniques. Furthermore, the correlation between the type of replication intermediates and tissue specific occurrence of mtDNA rearrangements suggests that the two are linked. Understanding the mtDNA maintenance mechanisms will be the key to understand also the pathological mechanisms of various mitochondrial disorders. The biological significance of rNMPs in mtDNA and their role in mitochondrial genome maintenance is intriguing. Accumulation of rNMPs in the nuclear DNA is detrimental for cell survival, but for a yet unknown reason, the mitochondrial

replication and transcription machineries seem to be adapted to the RNA-rich nature of their template. Manipulation of Pol γ's fidelity to incorporate rNMPs could provide experimental tools to address a potential role of ribonucleotides in mtDNA stability.

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Conflict of interest

The authors declare no conflict of interest

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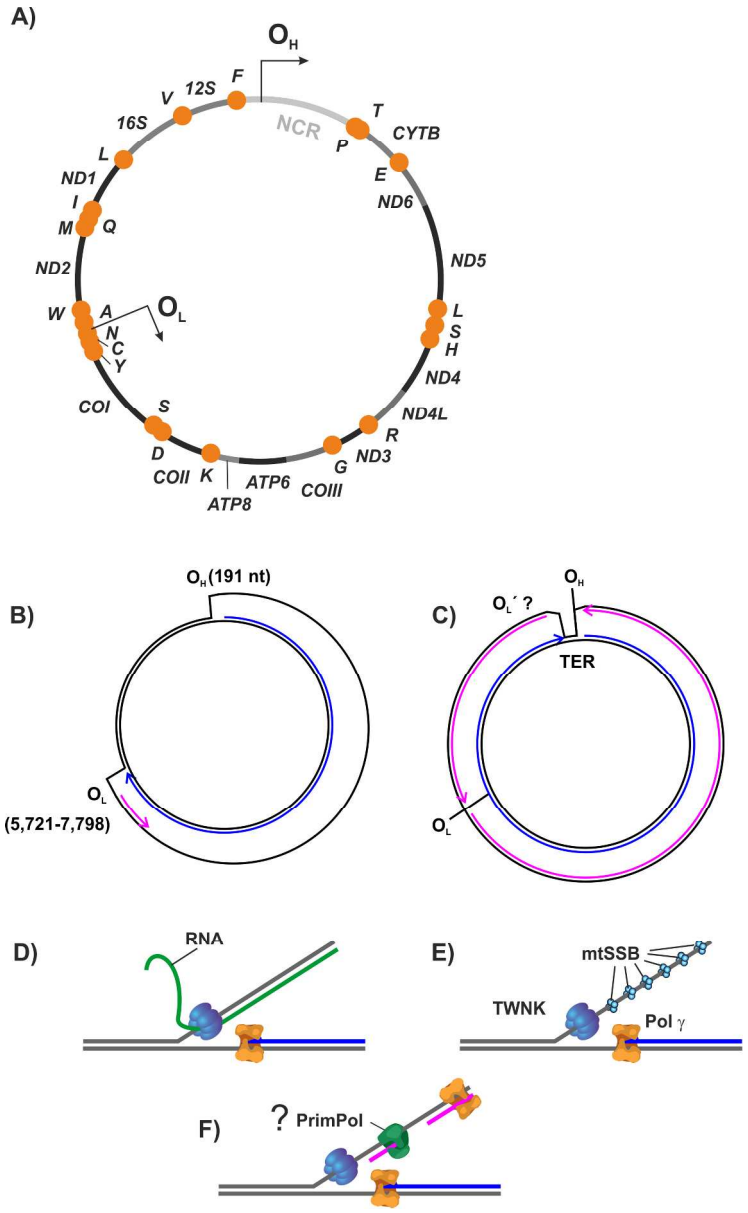
Figure legends

Figure 1. Mammalian mitochondrial DNA replication. (A) A schematic map of mammalian mitochondrial DNA. Most genes are located on the H-strand (outer side of the circle) and apart for ND6, the L-strand (inner side) has only tRNA genes (orange circles). Non-coding region (NCR) has transcriptional control regions and the origin(s) of H-strand replication (O_H). The main origin for L-strand replication (O_L) is located in the WANCY tRNA cluster. (B) The best characterized mechanism of mtDNA replication is highly asymmetric, with the L-strand replication initiating at O_L with considerable delay compared to the H-strand replication. Only DNA intermediates shown. (C) Replication termination (TER) of both strands has been suggest to occur at the NCR, close to the O_H [37], necessitating additional L-strand origins upstream of O_L. (D) According to the classical mtDNA replication model, the displaced strand is excessively coated by mitochondrial single-strand binding protein (mtSSB). (E) According to the so-called bootlace model, preformed RNA is incorporated at the replication fork on the displaced strand, explaining the observed RNA:DNA hybrids on mtDNA replication intermediates. (F) Fully double-stranded DNA replication intermediates do not need to represent strand-coupled replication but could be also generated by frequent priming of the L-strand replication.

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.

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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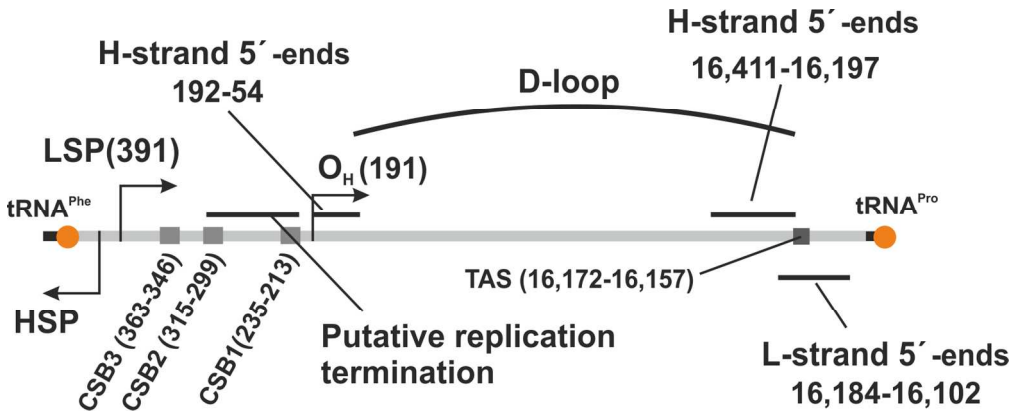

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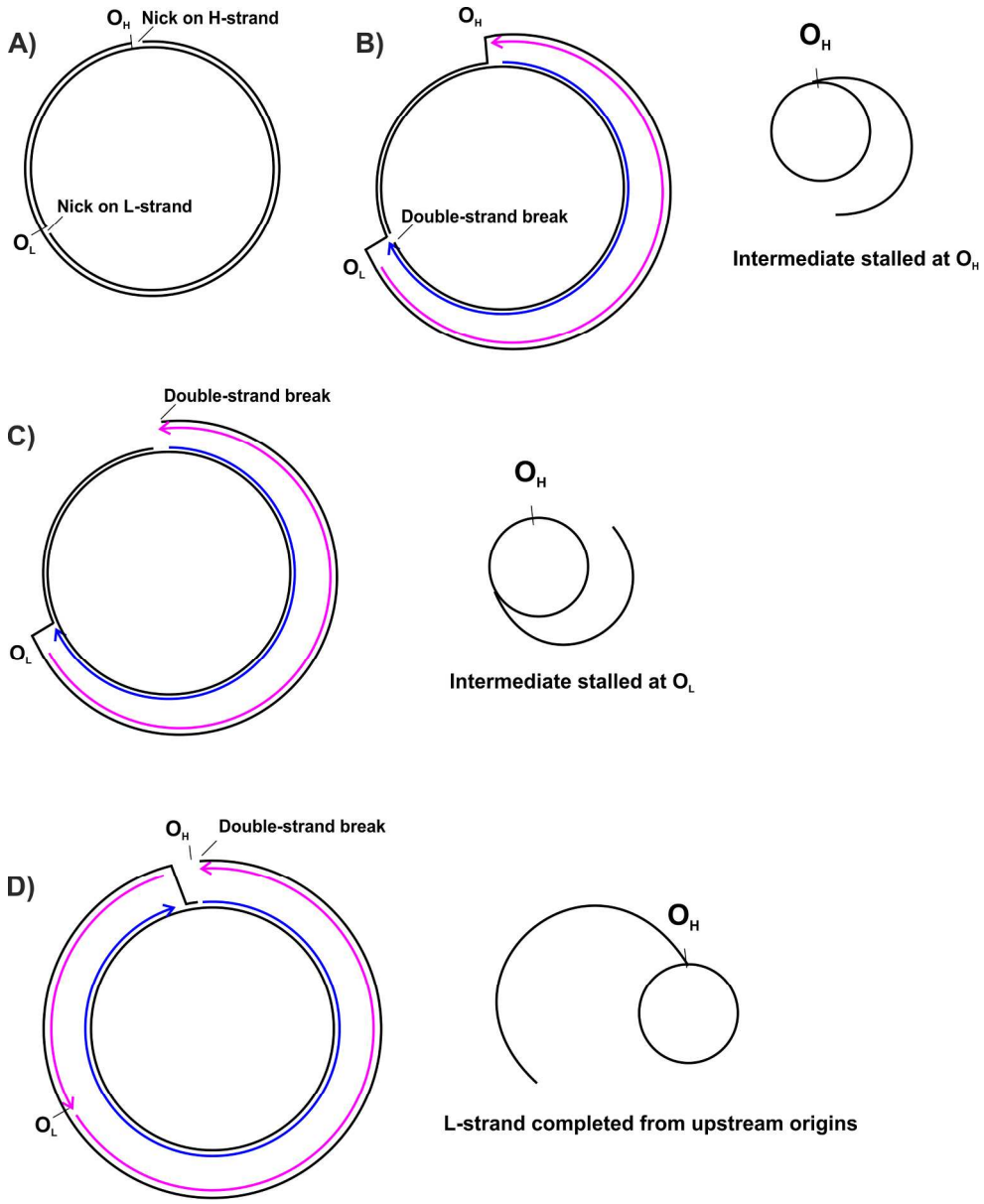

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