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Primer removal during mammalian mitochondrial DNA replication

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ABSTRACT

The small circular mitochondrial genome in mammalian cells is replicated by a dedicated replisome, defects in which can cause mitochondrial disease in humans. A fundamental step in mitochondrial DNA (mtDNA) replication and maintenance is the removal of the RNA primers needed for replication initiation. The nucleases RNase H1, FEN1, DNA2, and MGME1 have been implicated in this process. Here we review the role of these nucleases in the light of primer removal pathways in mitochondria, highlight associations with disease, as well as consider the implications for mtDNA replication initiation.

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1. Introduction

Mitochondria are vital in providing cellular energy in the form of ATP through the oxidative phosphorylation pathway (OXPHOS). The OXPHOS system depends on expression of the mitochondrial genome that encodes key subunits of the respiratory chain. As a consequence, defects in mtDNA maintenance or sequence can lead to mitochondrial disorders comprising a heterogeneous range of clinical symptoms and syndromes [1–3]. Some patients carry mutations in components of the mitochondrial replication/transcription system, which can lead to mtDNA deletions and depletions [4,5]. MtDNA mutations are also implicated in normal ageing, neurodegenerative diseases and cancer [1,6].

Initiation and elongation of mammalian mtDNA replication is relatively well understood (recent reviews include [4,7,8]). Replication is initiated at two strand-specific origins and requires the priming activity of the mitochondrial RNA polymerase (POLRMT). Each mtDNA strand is then replicated continuously by POLγ, the mitochondrial DNA polymerase.

Termination of mtDNA replication is comparably less well studied. A key step in genome maintenance that is often overlooked is the removal of RNA replication primers and ligation of the nascent DNA ends. Without this, gaps and nicks will form in the DNA resulting in genome instability. While primer removal in nuclear DNA has been thoroughly studied for decades, few studies have addressed this in the mitochondrial genome. Here we review what nucleases and pathways are implicated in the processing of primers during mammalian mtDNA replication. We relate this to disease, draw comparisons with Okazaki fragment maturation, and discuss the implications for mtDNA replication initiation.

2. The mitochondrial genome

Mitochondrial DNA in humans is an approximately 16.6 kb long double-stranded circular molecule (Fig. 1). The two strands can be separated on denaturing cesium chloride gradients and are therefore referred to as the heavy strand (H-strand) and the light strand (L-strand). The mitochondrial genome is highly compact, containing no introns and little non-coding DNA. It codes for 13 subunits of the OXPHOS complexes, as well as 2 ribosomal RNAs and 22 transfer RNAs that are required for translation of the OXPHOS subunits. All other proteins (~99%), including those required for mtDNA replication and transcription, are encoded by nuclear genes and are imported from the cytoplasm.

Somatic cells contain between 1000–10,000 copies of mtDNA, with 2–10 copies per mitochondrion. MtDNA is packaged into DNA–protein complexes called nucleoids of which the major protein component is mitochondrial transcription factor A (TFAM) [9–12]. In addition to replication and transcription proteins, nucleoids also contain known components of the inner membrane, suggesting that mtDNA may be membrane associated [13].

MtDNA contains two regions of non-coding DNA: a ~1 kb sequence known as the non-coding region (NCR; Fig. 1 top panel), and a distant ~30 nt sequence containing the origin of replication for the L-strand (OriL). The NCR contains one transcription promoter for each strand (light strand promoter—LSP; heavy strand promoter—HSP). It also contains the origin of replication for the H-strand (OriH), classically annotated at position 191 nt, though less dominant origins have been reported nearby (reviewed in [14]).

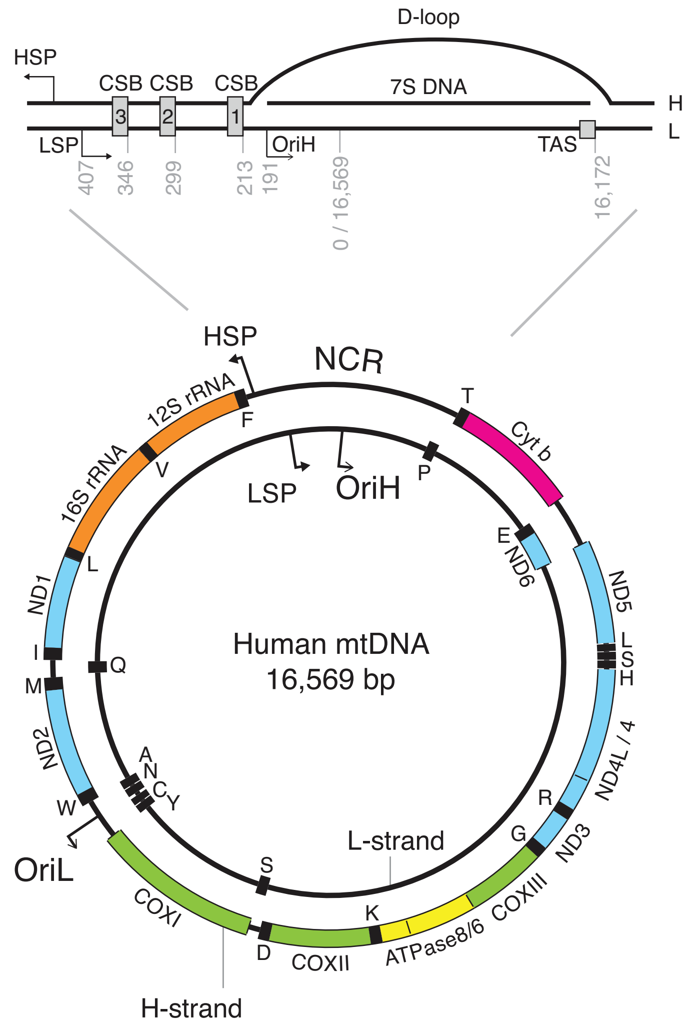

Fig. 1. Map of the human mitochondrial genome.
The major non-coding regions are the NCR (non-coding region), and OriL (origin of replication for the light strand). The outer and inner circles are the heavy (H) and light (L) strands, respectively. The NCR (enlargement shown above genome; nucleotide positions indicated in grey) contains the H- and L-strand promoters (HSP, LSP), three conserved sequence boxes (CSB1-3), the H-strand origin of replication (OriH) and the termination-associated sequence (TAS). Replication initiated at OriH often preterminates and the nascent strand (7S DNA) remains bound to the template creating a displacement loop (D-loop). Gene color coding: complex III cytochrome b (Cyt b)—pink; complex I NADH dehydrogenase (ND) genes—blue; complex IV cytochrome c oxidase (COX) genes—green; complex V ATP synthase (ATPase) genes—yellow; ribosomal RNA (rRNA)—orange; transfer RNA genes—black boxes.

Also present in the NCR are three conserved sequence blocks (CSB1-3) between LSP and OriH.

A region between OriH and the termination-associated sequence (TAS) at the end of the NCR can form a displacement-loop (D-loop), called such because it forms a triple-stranded structure created by prematurely terminated replication from OriH. The preterminated nascent H-strand, called 7S DNA (~650 nt), remains annealed to the template strand, though its function remains largely unknown [15].

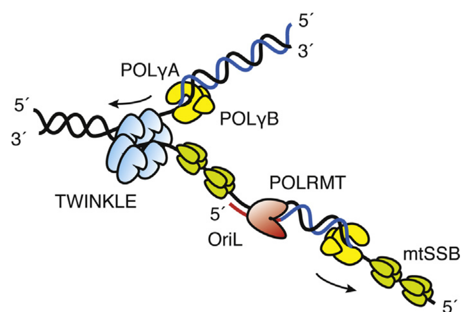

Fig. 2. The mtDNA replication fork.
TWINKLE helicase (blue) unwinds the dsDNA in a 5′–3′ direction. POLRMT (orange) is shown synthesizing the RNA primer (red) at OriL. The DNA polymerase POLγ (yellow) is made of one A subunit and two B subunits. Tetrameric mtSSB (green) stabilizes single stranded DNA. Template DNA—black; nascent DNA—blue.

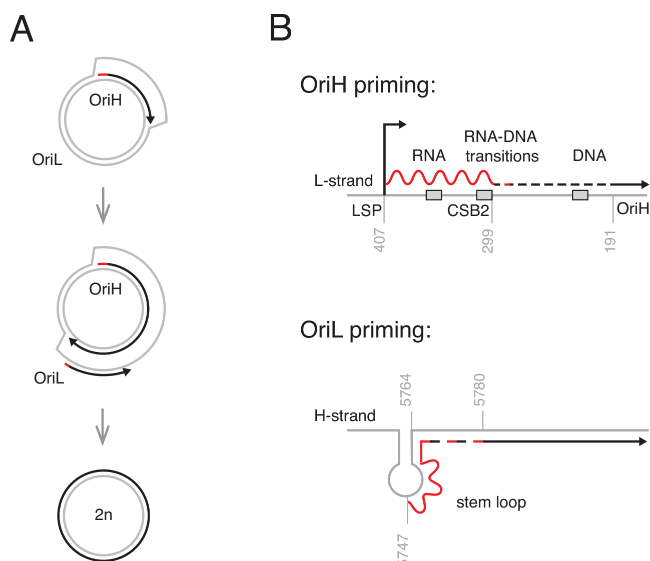

Fig. 3. Initiation and replication of mtDNA.
(A) In the strand displacement replication mode, replication initiates from an RNA primer (red) at OriH. DNA synthesis (black arrows) of the H-strand proceeds unidirectionally, displacing the parental H-strand. When OriL is exposed, L-strand synthesis is initiated and proceeds in the opposite direction. (B) Schematic of OriH and OriL priming. Upper panel: an RNA primer (red wavy line) is transcribed from LSP. RNA–DNA transitions (dashed red/black line) occur near the 3′-end of CSB2. 5′-end processing of the DNA (black dashed line) generates a mature 5′-end of the nascent DNA (solid black) at OriH. Nucleotide positions are indicated in grey. Lower panel: an RNA primer (red wavy line) is transcribed from a stem-loop formed in the single stranded H-strand template. RNA–DNA transitions (dashed red/black line); nascent L-strand (solid black). Nucleotide positions are indicated in grey.

3. Overview of mitochondrial primer formation and DNA replication

The core factors required for mitochondrial primer formation and DNA replication are distinct from those in the nucleus (Fig. 2). POLRMT, a single subunit RNA polymerase, is responsible for all mitochondrial transcription including primer formation. POLγ, the only known replicative DNA polymerase in mitochondria, comprises the catalytic A subunit and two accessory B subunits. In addition to its polymerase activity, POLγA also harbors 3′–5′ exonuclease activity and lyase activity [16,17]. The duplex DNA at the replication fork is unwound by the hexameric 5′–3′ TWINKLE helicase, while mitochondrial single stranded DNA binding protein (mtSSB) protects the single stranded DNA created in its wake. Most probably because mitochondria originated from an alphaproteobacterium that invaded a primordial eukaryotic cell, several of the mtDNA replication/transcription factors bear similarity to bacteriophage or bacterial proteins, including POLγA, POLRMT, TWINKLE, and mtSSB [14].

Three models have been proposed to explain how mtDNA is primed and replicated (reviewed in [7,14]). According to the strand displacement model proposed over 30 years ago, replication is initiated at OriH (Fig. 3A). DNA synthesis is primed by transcription from LSP located ∼200 nt upstream of OriH (Fig. 3B). LSP transcription can either produce a near full-length polycistronic transcript for gene expression, or it can be pre-terminated near CSB2 and serve as the RNA primer for replication initiation at OriH [18–20]. The mechanism regulating the switch between genome transcription and primer formation involves a G-quadruplex forming sequence at CSB2, located ∼100 nt downstream of LSP [21,22].

Once initiated, H-strand synthesis proceeds unidirectionally and displaces the parental H-strand (Fig. 3A). When the replication machinery passes the second origin, OriL, it becomes single-stranded and forms a stem-loop structure from which POLRMT initiates primer synthesis (∼20–30 nt; Fig. 3B). L-strand DNA synthesis initiates from OriL and proceeds in a continuous mode in the opposite direction to H-strand synthesis. This means that both H-strand and L-strand DNA synthesis only require a single priming event each, after which the strands are continuously synthesized until coming full circle. L-strand replication may in some cells/tissues also be primed from cryptic L-strand sites [23]. Support for the OriH and OriL origins includes the mapping of free 5′-ends to these sites [24], reconstitution of replication initiation at OriL *in vitro* [25,26], and the abnormal occurrence of an 11 kb linear replication product, the ends of which map to these origins [27,28].

4. Overview of primer processing

Due to the circular nature of mtDNA, replication termination most likely occurs at or very close to the origins OriH and OriL. What happens when POLγ completes DNA synthesis and reaches the RNA primers is not clear, but in principle, the primers must at some point be replaced with DNA. The factors and pathways required for primer processing are beginning to emerge.

4.1. Lessons from primer processing in the nucleus

Much insight can be gained from studies on primer removal during Okazaki fragment maturation of lagging-strand replication in the nucleus (recent reviews include [29,30]). Notably, primer removal pathways here all involve displacement of the primer by the replicating DNA polymerase (Polδ) when it reaches the 5′-end of the RNA primer of the previously formed Okazaki fragment (Fig. 4A). In the absence of a helicase, Polδ can synthesize a few nucleotides into the duplex region by strand displacement synthesis, creating a short 5′-primer flap. This flap can then be cleaved away by nucleases, creating a ligatable 5′-DNA end through which the Okazaki fragments can be ligated.

This mechanism of primer flap formation and cleavage only favors ligation if Polδ strand displacement is limited to a few nucleotides. Otherwise, flaps can regenerate after cleavage, or uncleavable secondary structures may form in long flaps. Strand displacement is kept low by the 3′–5′ exonuclease activity of Polδ. Here, the nucleotides added during strand displacement can be subsequently 3′–5′ degraded until Polδ returns to the nick position. This cycle is repeated in a process called idling and is essential for maintaining a ligatable nick [31]. Nucleases therefore have repeated opportunities to cleave the transiently formed flaps. Once

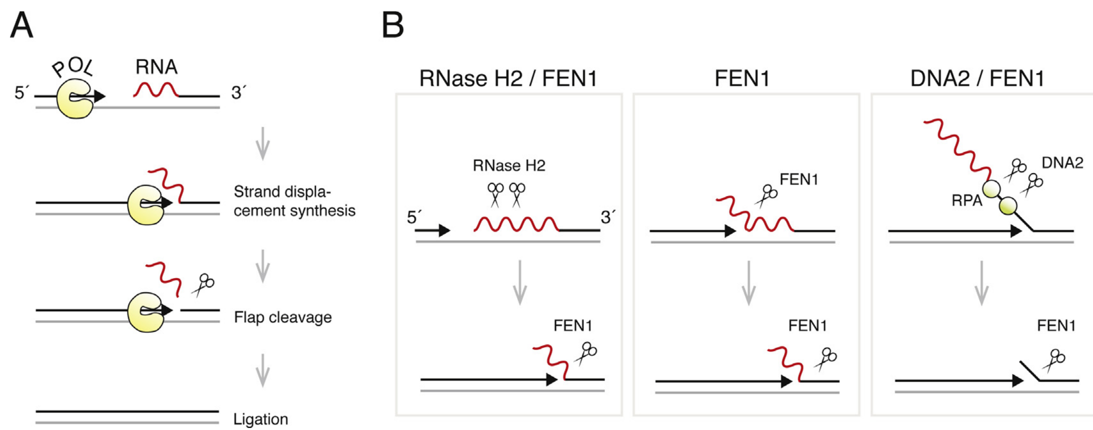

Fig. 4. Primer removal pathways in the nucleus.
(A) Primer removal involves strand displacement synthesis and cleavage of primer flap intermediates. Replicating DNA polymerase (yellow) reaches the 5′-end of a downstream RNA primer (red wavy line). The polymerase synthesizes a few nucleotides beyond the 5′-end, creating a single stranded flap that is cleaved away by nucleases (scissors). (B) Models of nuclease pathways during Okazaki fragment maturation. RNase H2/FEN1pathway: RNase H2 degrades most of the annealed RNA before strand displacement synthesis (black arrow) creates a flap that is cut by FEN1. FEN1 pathway: strand displacement synthesis creates reiterative short flaps that are cleaved by FEN1. DNA2/FEN1 pathway: a long flap is formed and is covered by RPA (circles). DNA2 cleaves the flap, leaving a short RPA-free flap that is cut by FEN1.

the RNA primer is completely removed and replaced with DNA, ligase seals the nick.

What are the nucleases? Three nucleases—ribonuclease H2 (RNase H2), flap structure-specific endonuclease 1 (FEN1), and DNA replication helicase/nuclease 2 (DNA2)—are proposed to operate through three main pathways (Fig. 4B). In the RNase H2/FEN1 pathway, RNase H2 first degrades most of the annealed RNA primer. The final RNase H2 resistant ribonucleotides are then displaced into a flap by Polδ and cleaved away by the flap endonuclease FEN1. In the FEN1 pathway (also called short flap pathway), Polδ strand displacement synthesis generates a 1–2 nt primer flap which is then cleaved by FEN1 [31]. This process is reiterated through nick translation until the RNA is completely removed. In the DNA2/FEN1 (or long flap) pathway, single stranded DNA binding protein RPA (replication protein A) binds to a long flap that has escaped FEN1 cleavage due to helicase activity for example. RPA-bound DNA is inhibitory to FEN1, necessitating the activity of a second nuclease, DNA2. Cleavage by DNA2 leaves behind a short flap that FEN1 can cleave away.

4.2. *Mitochondrial primer processing*

Not all DNA polymerases possess strand displacement activity. Interestingly, the mitochondrial DNA polymerase POLγ, like Polδ, possesses limited strand displacement activity [32,33], suggesting that primer flap intermediates are likely to be involved in primer removal in mitochondria too. POLγ has also been shown to idle at the 5′-end of a primer [33] and as for Polδ, the intrinsic 3′–5′ exonuclease activity of POLγ is essential for limiting its strand displacement activity and promoting ligation [34]. Thus, upon reaching the 5′-end of a primer, the polymerase and 3′–5′ exonuclease activities of POLγ are delicately balanced to favor RNA primer removal and DNA ligation. This balance can become disrupted by mutations in the exonuclease domain, some of which are associated with human mitochondrial disease [34].

The discovery of nucleases within mitochondria, deficiencies in which are associated with mtDNA defects, further implies that primer flap pathways operate here. In the past ten years in fact, all of the primary factors implicated in Okazaki fragment maturation, FEN1, DNA2, and RNase H2, have been suggested to have mitochondrial counterparts. We will discuss the possible roles of these, as well as the recently identified mitochondrial genome maintenance exonuclease 1 (MGME1) protein, in mtDNA maintenance.

4.3. *Identifying mitochondrial factors*

To qualify as a credible primer processing factor, several lines of evidence should be considered. It must firstly be shown that the candidate protein has mitochondrial localization. Well-established cytological and biochemical assays are routinely used for this purpose. In addition, the purified factor must possess enzymatic activity that can process primer substrates *in vitro*, ideally with the mtDNA replication system. Finally, loss of function of a primer processing factor should cause mtDNA defects *in vivo*. How to predict, detect and interpret defects that reflect primer removal deficiency is challenging though.

One can expect that incomplete primer removal will lead to ligation defects, since the mitochondrial DNA ligase (Lig3) discriminates against RNA [35]. Cellular depletion of Lig3 causes increased mtDNA nicks and single stranded gaps, as well as reduced mtDNA levels [36,37]. Lig3 deletion in mice causes embryonic lethality with development arresting at embryonic day E8.5 [38], while deletion in the nervous system leads to loss of mtDNA and premature death [39]. Furthermore, recent data suggest that the linear 11 kb mtDNA fragment that is found in the exonuclease-deficient POLγ mutator mouse, is caused by ligation problems of the nascent DNA strand [34]. Upon completion of H-strand replication, exonuclease-deficient POLγ displaces the downstream 5′-end of the nascent strand excessively and creates a persistent unligatable flap at OriH (Fig. 5, left panels). The resulting nick leads to a double stranded break during the next round of L-strand replication initiated from OriL (Fig. 5, right panels). Based on these findings, improper primer removal may cause similar defects in mtDNA.

5. Nucleases implicated in mitochondrial primer processing

5.1. *RNase H1*

RNase H endonucleases have long been implicated in primer removal during nuclear lagging-strand DNA replication because they hydrolyze the RNA strand of RNA/DNA heteroduplexes and their expression correlates with DNA synthesis [40]. In mammals there are two classes of RNase H protein, RNase H1 and H2, (called RNase HII and HI respectively until the early 00s), which are ubiquitously expressed in cells and tissues. Both are present in the nucleus, but only RNase H1 localizes to mitochondria [41–46]. The

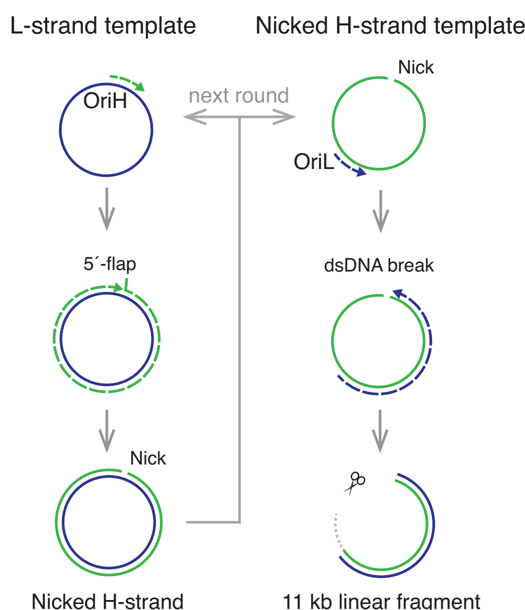

Fig. 5. Model on how ligation failure at OriH generates an 11 kb linear fragment. Replication of the nascent H-strand (green dashed arrow) initiates from OriH (top left panel). When POLγ reaches the 5′-end of the nascent strand, a transient 5′-flap is formed. A persistent flap (due to exonuclease-deficient POLγ strand displacement or nuclease deficiency) will lead to ligation failure, resulting in a daughter molecule with a nick on the H-strand near OriH (bottom left panel). In the next replication round, replication initiated from the intact L-strand template will lead to regeneration of the nicked molecule (left panels), while replication initiated from OriL on the nicked H-strand template will generate an 11 kb linear fragment (right panels). Here, L-strand replication will terminate when the nick on the H-strand is reached, leading to a double stranded DNA (dsDNA) break near OriH (right middle panel). The unstable single stranded region of the H-strand template is degraded (dotted grey line and scissors, lower right panel), leaving a double stranded 11 kb linear fragment.

human RNase H1 open reading frame encodes a 286 residue long protein that contains a mitochondrial targeting sequence (MTS). RNase H1 was first cloned and purified in 1998 and shown to be homologous to RNase HI from *Escherichia coli* [42,47,48]. Eukaryotic RNases H1 have a conserved N-terminus for enhanced RNA/DNA hybrid binding, a spacer region, and a conserved C-terminal catalytic domain that is also essential for substrate binding.

5.1.1. *In vitro* biochemical activity of RNase H1

Human RNase H1 was first isolated from placenta and shown to specifically cleave RNA hybridized to DNA [44]. Here we review features of its activity that are relevant for primer removal (for a more detailed review, see [43]). RNase H1 cleaves endonucleolytically to generate 3′-OH and 5′-phosphate ends and requires at least 3–4 ribonucleotides to be active [44,49].

The cleavage pattern of purified recombinant human RNase H1 has been studied using model RNA primer substrates comprised of an RNA:DNA chimeric strand annealed to a DNA strand. Here, RNase H1 cleaves at numerous sites only between the ribonucleotides, leaving behind 2 ribonucleotides attached to the 5′-end of the DNA [50]. These residual ribonucleotides would be incompatible with ligation [35]. An RNase H1 pathway in mitochondrial primer processing would therefore necessitate a second nuclease, analogous to the nuclear RNase H2/FEN1 pathway.

Using long hybrids it has also been found that mammalian RNases H1, unlike prokaryotic RNase HI, is a processive enzyme, conferred by protein dimerization after substrate binding [51]. RNase H1 may therefore have evolved to process long RNA/DNA hybrids in higher organisms. This is significant given that the RNA primer at OriH is ~100 nt in length (Fig. 3B, upper panel).

5.1.2. *Role of RNase H1 in mtDNA primer removal*

An RNase H1 mouse knockout made over 10 years ago revealed that RNase H1 has an essential, non-redundant function in mtDNA replication [52]. Loss of RNase H1 in mice is embryonic lethal, with embryos arresting development at E8.5 due to mtDNA depletion. The timing corresponds to when the maternal mtDNA contribution becomes diluted and new mtDNA synthesis must begin as cells start relying on the OXPHOS system for energy. While there was a dramatic lack of mtDNA replication, it was not possible to pinpoint at what step in replication the defect arises. Rather paradoxically, RNases have also been hypothesized to cleave the RNA transcript initiated by the transcription machinery at LSP to generate the mature 3′-primer end for replication initiation. RNase H has been shown to be involved in primer formation in the bacterial ColE1 plasmid [53], but if impaired primer maturation can explain the lack of mtDNA replication in mammalian cells lacking RNase H1 still needs clarifying.

To help resolve this issue, a study on cultured cells suggests that RNase H1 is involved in primer removal rather than primer formation [37]. MtDNA replication was studied in cells after mtDNA was transiently depleted with the use of the cytidine analogue dideoxycytidine (ddC), an inhibitor of POLγ. When the drug is removed, cells undergo intense mtDNA replication to repopulate the mtDNA pool, providing an attractive system to study mtDNA replication. While simple knockdown of core replication factors causes mtDNA depletion, knockdown of accessory factors often requires the ddC approach to elicit effects (for example [54]). Using this system combined with siRNA mediated RNase H1 knockdown, it was found that normal recovery of mtDNA levels required RNase H1 activity [37].

Furthermore, using 2D gel analysis to study replication intermediates during the recovery phase, an increased number of intermediates in the RNase H1-knockdown cells was observed. These were more sensitive to S1 nuclease, indicating increased mtDNA nicks and gaps. This phenotype is more consistent with a defect in RNA primer removal after DNA replication, rather than failure to initiate replication due to any lack of primer maturation. It would be interesting to examine, using specific probes, whether the nicks/gaps are enriched at the origins where RNA primers would be expected to be located. For an overview of mtDNA defects associated with depletion of RNase H1, see Table 1.

5.2. FEN1

FEN1 is a structure specific 5′–3′ nuclease that cleaves single stranded 5′-flaps and also has weak exonuclease activity. Human FEN1 is 380 amino acids in length and belongs to the XPG/RAD2 family of endonucleases, the activity of which is conserved from bacteria to mammals. FEN1 is a key factor in nuclear DNA metabolism, including replication and repair, and has been extensively studied and reviewed (for recent reviews, see [55,56]). Biochemical and genetic studies, particularly in yeast, have unraveled the role of FEN1 in Okazaki fragment maturation where it operates in all three proposed pathways either alone, or in combination with RNase H2 or DNA2 (Fig. 4B). Since the initial isolation of mammalian FEN1 homologs from nuclei in the late 1980s, it was not until 2008 that a possible role in mtDNA metabolism first emerged. FEN1 lacks a classical MTS and although its mitochondrial localization is contentious [57–60], FEN1 is implicated in mitochondrial base excision repair and primer processing [57,59]. In addition to full length FEN1, there have been reports of a shorter, mitochondrial specific isoform [57,58]. Termed FENMIT, it is generated from a downstream alternative translation initiation site [58]. FENMIT lacks nuclease activity but binds preferentially to RNA flaps, though its physiological role in mitochondria remains unclear [58].

Table 1
Reported mtDNA defects and diseases associated with deficiency in RNase H1, FEN1, DNA2 and MGME1.

Sample source	RNase H1	FEN1	DNA2	MGME1
Human patients	No data	No data	Deletions [79] (Southern blot, long PCR) Mitochondrial myopathy	Depletion, deletions- incl. 11 kb linear fragments, duplications, increased 7S DNA [17,80] (Southern blot, qPCR, long PCR) Multisystemic mt disease
Whole body mouse KO	MtDNA depletion [46] (qPCR) Embryonic lethal (arrest E8.5)	No data [66] Embryonic lethal (arrest E4)	No data [78] Embryonic lethal (arrest before E7.5)	No data
SiRNA in cultured cells	Normal levels [35] (qPCR)	Normal levels [35,54] (qPCR, Southern blot) Normal number of lesions [66] (long PCR)	Normal levels [35,54] (qPCR, Southern blot, 2D-AGE) Reduced replication intermediates [73] (2D-AGE) Transient increase in lesions [67] (long PCR) Reduced repair of oxidative damage [73] (long PCR)	Normal levels [54,80] (Southern blot) Increased replication intermediates [80] (2D-AGE) Increased 7S DNA [54,80] (Southern blot)
SiRNA in transiently mtDNA-depleted cells	No mtDNA recovery [35] (PCR) Increased nicks/gaps [35] (2D-AGE)	Normal replication intermediates [35] (2D-AGE)	Normal replication intermediates [35] (2D-AGE)	No data

KO—knockout; qPCR quantitative PCR; 2D-AGE—two dimensional agarose gel electrophoresis.

5.2.1. In vitro *biochemical activity of FEN1*

Though its identity was not known at the time, FEN1 was originally purified in the 1980s and 90s from a variety of cells and tissues for its ability to cleave flap substrates (for details see [61]). Since then, its biochemical activity and crystal structure on templates have been intricately studied [55,56]. FEN1 is a structure specific nuclease that preferentially recognizes and cleaves single stranded 5′-flaps (DNA or RNA) that are flanked by dsDNA, substrates that mimic primer flap (or repair) intermediates.

FEN1 cleaves at the flap base, releasing the intact flap [55,56,62]. However, longer flaps that can be covered by the nuclear single stranded DNA binding protein RPA are not ideal substrates for FEN1. Efficient cleavage can be elicited by increased FEN1 concentrations, or by the addition of DNA2 (discussed in Section 5.3). It has not however been tested whether the mitochondrial counterpart of RPA, mtSSB, has a similar inhibitory effect on FEN1 cleavage. Interestingly, in a yeast reconstituted Polδ DNA replication system, primer flaps coated with *E. coli* SSB, which is homologous to mtSSB, are cleaved as inefficiently as those covered with RPA [63].

5.2.2. *Role of FEN1 in mitochondrial primer removal*

Loss of FEN1 in mice is embryonic lethal [64,65] causing defects in nuclear DNA replication and cell proliferation [65]. Since these embryos die at a stage (∼E4) before they initiate mtDNA replication from the maternal pool, it was not possible to examine FEN1's role in mtDNA replication. Alternative approaches using siRNA mediated FEN1 knockdown in cultured cells have also been used, with none of these studies uncovering any effect on mtDNA (see Table 1). Cells with FEN1 knockdown showed no increases in single stranded breaks [66], or abnormal mtDNA copy number [54]. Moreover, knockdown of FEN1 (even in combination with DNA2) after transient mtDNA depletion did not result in slower repopulation, nor increases in replication stalling or single stranded breaks/gaps [37].

In vitro approaches have been used to isolate flap removal activity from mitochondrial extracts, with conflicting results regarding FEN1. While mitochondria do contain 5′-flap removal activity, two studies excluded FEN1 [60,67] and two did not [57,59]. In the latter studies, mitochondrial 5′-flap endonuclease activity could be abolished by FEN1 immunodepletion and restored by adding back purified recombinant FEN1.

Another biochemical approach used an *in vitro* mtDNA replication system with mitochondrial extracts and a gapped substrate containing a downstream 5′-flap [59]. In such assays, the gap in the substrate is filled by DNA synthesis, creating a ligatable nick only if the 5′-flap is removed. DNA ligation was reduced or abolished if FEN1 was immunodepleted from the mitochondrial extracts, but could be restored by adding back purified recombinant FEN1 [59]. Similar results were also obtained using only purified proteins (FEN1, POLγ, and Lig3) instead of extract. These assays relied on preformed flaps, so whether FEN1 together with POLγ strand displacement synthesis is sufficient to remove a fully annealed RNA primer, as shown in a reconstituted nuclear system with Polδ, remains to be shown.

5.3. *DNA2*

DNA2 is a multifaceted DNA processing enzyme conserved in eukaryotes that contains both nuclease and ATPase/helicase domains. Human DNA2, a 1060 residue long protein, is a member of the PD-(D/E)XK superfamily with homology to bacterial RecB nuclease. Studies largely in yeast have shown its involvement in nuclear DNA replication, repair and telomere replication. Human DNA2 was not successfully purified until 2006, when two groups showed it had similar activity to the yeast protein, possessing 5′–3′ and 3′–5′ ssDNA endonuclease activity, ATPase activity, but weak 5′–3′ helicase activity [68,69]. It also possesses single strand annealing, strand exchange, and strand resection activities [70,71]. DNA2, together with FEN1, is implicated in the long flap pathway of Okazaki maturation (Fig. 4B), and in mitochondrial primer flap removal and base excision repair [59]. DNA2 contains a non-canonical internal MTS and several approaches have shown it localizes to mitochondria [59,72].

5.3.1. In vitro *biochemical activity of DNA2*

Until recently, due to difficulties in purifying human DNA2, the vast majority of biochemical characterizations have been carried out on yeast DNA2. Nonetheless, their activities appear to be quite similar. When presented with model primer flap substrates, human DNA2 has been found to preferentially bind to and cleave longer flaps [74]. It cuts the flaps at numerous positions, leaving behind a short flap that is resistant to DNA2 cleavage.

DNA2 cleaves substrates with 5′- or 3′-flaps with equal efficiency [68,69], though it cleaves closer to the flap base when presented with a 5′-flap [68]. Addition of RPA stimulates its 5′–3′ nuclease activity [68], thereby switching DNA2 into a 5′–3′ nuclease optimized for primer removal [75]. No studies have addressed the

impact of mtSSB, the mitochondrial counterpart of RPA, on DNA2 activity. The fact that yeast DNA2 can cleave yeast RPA coated flaps, but not *E. coli* SSB coated flaps [63], suggests that flaps covered with mtSSB (homologous to *E. coli* SSB) might be DNA2-resistant. This would be consistent with the specific binding between DNA2 and RPA [63,76]. In terms of mitochondrial primer removal it will be important to establish what effect mtSSB has on its activity.

5.3.2. *Role of DNA2 in mitochondrial primer removal*

Whether DNA2 contributes to the cleavage of a gapped substrate with a downstream 5′-flap has been studied in mitochondrial extracts. Immunodepletion of DNA2 from these extracts reduced, but did not abolish, the production of ligatable ends [59]. Using only purified proteins (POLγ, Lig3 and DNA2) no ligated products were formed, however, DNA2 did stimulate DNA ligation in the presence of FEN1 [59]. These findings are consistent with previous studies showing that DNA2 is unable to cleave short flaps.

It has also been shown by co-immunoprecipitation and pull-down assays that DNA2 interacts with POLγ [59]. This interaction stimulated POLγ DNA synthesis on double stranded circular DNA, presumably *via* the DNA2 helicase domain [59]. In the presence of DNA2, POLγ synthesized DNA up to 400 nt in length [59], well above the length of the primers at OriL or OriH. Thus, DNA2 may help to remove RNA primers either by cleaving pre-formed flaps, and/or by facilitating RNA primer displacement and flap formation as POLγ completes DNA synthesis.

Knockout of DNA2 in mice is embryonic lethal, with development arresting before E7.5 and the initiation of mtDNA replication, precluding meaningful mtDNA analysis [77]. Recently, different DNA2 mutations in patients with muscle mitochondrial dysfunction were uncovered [78]. Southern blot and long PCR on mtDNA from patient muscle biopsies revealed multiple mtDNA deletions [78]. *In vitro* assays on the purified recombinant proteins showed that the mutant DNA2 proteins had reduced nuclease, ATPase, and/or abnormal helicase activity. However, whether DNA2 is involved in primer removal at the origins, or has another function in mitochondria such as in DNA repair, still needs to be determined.

There is little agreement concerning the effects on mtDNA after siRNA mediated DNA2 knockdown in cultured cells (see Table 1 for an overview). One study observed no effect on mtDNA levels measured by Southern blot [54], while another reported a mild reduction based on replication intermediates in 2D gel analysis [72]. Using ddC induced transient mtDNA depletion, DNA2 knockdown had no effect on mtDNA recovery or the abundance of single stranded gaps and nicks, not even in combination with FEN1 depletion [37]. On the other hand, DNA2 knockdown has been reported to elicit increased mtDNA lesions and hamper DNA repair [66,72].

5.4. *MGME1*

The identification of MGME1, also known as Ddk1, was first reported in 2013 by two different groups and was shown to be an exclusively mitochondrial protein [54,79]. It belongs to the RecB subfamily of PD-(D/E)XK nucleases and contains a classical MTS. Mutations in the protein cause mitochondrial disease with patients exhibiting a range of abnormal mtDNA phenotypes [79]. The human MGME1 protein has been characterized *in vitro* and shown to be a DNA endo-/exonuclease with strong preference for ssDNA [54,79]. MGME1 is the least evolutionarily conserved of the nuclease candidates—orthologs are found only in Metazoa, Capsaspora and Monosiga, but not in some insects such as *Drosophila* [54].

5.4.1. In vitro *biochemical activity of MGME1*

Recombinant MGME1 has been expressed and purified from *E. coli* and cultured human cells, and shown to have a strong preference for cleaving single stranded over double stranded DNA [54,79]. It has no activity on RNA, nor when hybridized to DNA. Using substrates that resemble primer flap intermediates, it was shown that MGME1 efficiently cleaves 5′-DNA flaps [79]. Final cleavages center at or near the flap base [79]. Though MGME1 cannot cut RNA, it can cut within the DNA of an RNA–DNA chimera, a few nucleotides downstream of the RNA–DNA junction [54,79]. MGME1 flap cleavage has only been tested on long flaps (30 nt), and it would be worth examining its activity on short flaps.

Like FEN1 and DNA2, MGME1 absolutely requires a free end for cleavage [79]. A preference for 5′-flap versus 3′-flap cleavage is significant for primer removal. Whether MGME1 has a preference is not entirely clear. By comparing the cleavage pattern of 5′ and 3′-flap substrates, or using biotin-streptavidin blockage of either the 5′ or 3′-end of ssDNA, it was concluded that MGME1 cleaves 5′-ends more efficiently [79]. However, another study found that MGME1 had a 3′-end or no end preference, depending on the substrate used [54]. It would be interesting to test whether a factor such as mtSSB would shift MGME1's polarity in the same way as RPA does for DNA2.

5.4.2. *Role of MGME1 in mitochondrial primer removal*

Loss-of-function mutations in MGME1 can cause mitochondrial disease in humans [20,79]. Analysis of mtDNA from patient muscle biopsies or fibroblast cells showed a spectrum of mtDNA defects, including deletions, depletions, rearrangements, and 7S DNA accumulation (Table 1). Patient fibroblasts transiently exposed to ddC in order to artificially deplete mtDNA showed greatly impaired mtDNA repopulation. Knockdown of MGME1 using siRNA in normally growing HeLa cells did not yield any mtDNA loss [54,79], not even in combination with FEN1 or DNA2 depletion [54]. 2D-AGE analysis did however reveal increased levels of replication intermediates, consistent with similar findings in patient fibroblasts [79].

MGME1 patient cells also contain an 11 kb truncated linear mtDNA fragment, the ends of which map near OriH and OriL [20]. The OriL end of the linear deletion maps precisely within the RNA primer-DNA transition zone, while the more heterogeneous OriH ends span largely within a 100 nt range between OriH and CSB2. The fact that both MGME1-deficiency and POLγ exonuclease deficiency (mutator mice) give rise to a very similar subgenomic linear fragment has valuable implications for how mtDNA is replicated and primers are removed. Given the biochemical properties of MGME1, it is conceivable that the fragment is generated due to incomplete primer flap processing at OriH (discussed in Section 7). Furthermore, 7S DNA contains 5′ extended ends in MGME1 deficient patient cells. This suggests that 7S DNA is normally processed to the mature form through single stranded flap intermediates and MGME1 cleavage [20].

Finally, reciprocal co-immunoprecipitation studies revealed that MGME1 and POLγ interact [20]. Conceivably, a primer flap processing factor would physically interact with the DNA polymerase in order to coordinate flap cleavage and strand displacement synthesis.

6. Models of mitochondrial primer removal pathways

In mitochondria, the primers at OriH and OriL differ substantially both in length and genomic context (Fig. 3B), and we suggest that they are possibly removed by different mechanisms. Sufficient data in the disparate literature allows us to piece together three speculative models on how primer processing might occur, using Okazaki fragment maturation models as a framework (Fig. 6).

6.1. RNase H1 primer removal model

RNase H1 is an essential mtDNA replication protein that causes mtDNA defects that are consistent with a role in primer removal. RNase H1 can efficiently process RNA primers, but it cannot remove the last two ribonucleotides of an RNA–DNA chimera. Based on the limited strand displacement activity of POLγ we propose that once POLγ reaches the 5′-end of the remaining di-ribonucleotide primer, the polymerase creates short flaps. These short 5′-flaps can then be cleaved away by a single stranded nuclease, such as FEN1. MGME1 could potentially also cleave away the remaining di-ribonucleotide primer flap, but MGME1 short flap cleavage remains to be tested and would require the flap to include downstream DNA since MGME1 is unable to cut RNA.

6.2. Short flap model

In the absence of RNase H1 activity, RNA primers would need to be displaced into flaps for nucleolytic cleavage. A short flap model could operate at OriL, where the primer is only ~25 nt long, but would seem inefficient at OriH where the primer is much longer. Of all the candidate nucleases, FEN1 is the only one that can cleave RNA flaps. POLγ together with FEN1 could conceivably engage in coordinated nick translation to remove the annealed RNA primer, as shown for Polδ and FEN1, but this possibility needs to be experimentally addressed *in vitro*.

6.3. Long flap model

DNA2 in combination with FEN1 has been shown to be able to process longer flaps (20 nt) in an *in vitro* mtDNA replication system [59]. Also, MGME1 can cleave long flaps (30 nt) in nuclease assays [79]. Some questions remain however, for example, what effect does mtSSB binding to very long flaps have on DNA2, FEN1 and MGME1, and what creates the long flaps in the first place? What is clear from *in vitro* data is that the strand displacement activity of POLγ is insufficient. However, DNA2 can stimulate POLγ DNA synthesis on duplex DNA [59] and the yeast protein has been shown to unwind DNA/RNA hybrids [76]. Other helicases, the formation of secondary DNA structures, or pre-terminated transcription, could conceivably also facilitate long flap formation (see Section 7).

7. OriH primer processing and implications for replication initiation

Processing of the unusually long OriH primer in the complex genomic context of the NCR presents interesting problems as well as informative implications. Due to its considerable length of 100 nt, we favor RNA primer removal *via* an effective pathway such as RNase H1 degradation over a short flap pathway that could entail up to ~100 cycles of POLγ-dependent flap formation and nucleolytic cleavage.

An unresolved peculiarity in mtDNA initiation is that the RNA–DNA transitions map just after CSB2, yet the 5′-ends of nascent DNA map ~100 nt downstream of CSB2, at OriH (Fig. 3B). It is not typically possible to distinguish between the 5′-ends of various nascent H-strands (pre-terminated 7S DNA or full-length products), but it is generally accepted that the positions of the 5′-ends are shared [14,15]. Regardless, the discrepancy between DNA–RNA transitions and OriH implies that the nascent H-strand undergoes considerable 5′-end processing which remains an unresolved enigma.

We propose that the RNA primer between LSP and CSB2 is firstly removed by RNase H1 shortly after replication initiation (Fig. 7A). The remaining ~100 nt of DNA between CSB2 and OriH are then removed by a flap pathway. Evidence for a flap pathway comes from patients who are deficient in MGME1 where the 5′-ends of the nascent strands are extended towards the CSB regions [20].

What creates the flaps between CSB2 and OriH is unclear, but a long-flap pathway might involve a helicase, POLRMT, or secondary structures (Fig. 7A). One of the several reported mitochondrial helicases may fulfill this role, however, the replicative helicase TWINKLE is unlikely to be involved since TWINKLE itself requires a flap to be active [80,81]. In support of POLRMT, a highly abundant polyadenylated transcript known as 7S RNA is initiated from LSP and its 3′-end maps at CSB1, just upstream of OriH (see Fig. 1 for positions) [82,83]. Finally, several predicted secondary DNA structures in the L-strand of the D-Loop have been reported, which could cause displacement of the nascent strand into a flap [84,85].

Meticulous mapping of the 11 kb linear fragment that is found both in mutator mice and MGME1 patients is very revealing [20,28]. In a model proposed by us, the linear fragment is an abnormal replication product that arises due to failed ligation of the nascent H-strand at OriH (see Fig. 5) [34]. Undesirable creation of flaps by exonuclease-deficient POLγ in the mutator mice or failure to remove flaps by MGME1 in the patients, will lead to ligation failure and a nick around OriH. When this nicked mtDNA molecule is replicated, initiation of H-strand synthesis is not disturbed because the L-strand is intact (Fig. 5, left panels). However, when replication from OriL initiates on the nicked H-strand, the nascent L-strand will terminate at the nick, generating a double stranded break near OriH (Fig. 5, right panels).

In mutator mice, one end of the linear fragment maps downstream of OriH (in a ~600 nt range between OriH and TAS) [28], but in MGME1 patients, the equivalent end maps mainly upstream of OriH (in a ~100 nt region between CSB2 and OriH) [20] (Fig. 7B). This difference can be explained by the above model: MGME1 deficiency leads to failed 5′-end processing of the nascent H-strand upstream of OriH, while in mutator mice, excessive strand displacement synthesis by exonuclease-deficient POLγ shifts the 5′-end downstream of OriH.

Nucleases other than MGME1, such as FEN1, also play a likely role in 5′-end processing since MGME1 is not essential and the 11 kb fragment is much less abundant in MGME1 patients than in mutator mice, suggesting that the ligation failure is not complete.

Regardless of the mechanism, what is the purpose of shifting the 5′-end of the nascent DNA from the RNA–DNA transition point at CSB2 to OriH? We speculate that it may be to facilitate ligation of the newly replicated DNA. The NCR is a complex region with many regulatory sequences and activities, particularly between LSP and CSB1. By moving the delicate and essential step of ligation away from this area, interference can be minimized. How 5′-end processing terminates specifically at OriH remains an unsolved question. Our understanding of primer processing is in its infancy, and more studies on how primers are processed will inevitably also shed more light on the initiation of mtDNA replication.

8. Concluding remarks

There has been a boom in the mtDNA replication field during recent decades, stimulated by the realization that mtDNA defects underlie numerous diseases and can be linked to biological ageing. However, with the focus on initiation and elongation, few studies have yet specifically addressed the issue of primer removal. Currently, there are virtually no studies that have reconstituted mtDNA replication, primer removal and ligation in the test tube. These types of assays have contributed immensely to the field of Okazaki fragment maturation. Moreover, mtDNA defects that are relevant to primer removal are typically overlooked and difficult to predict.

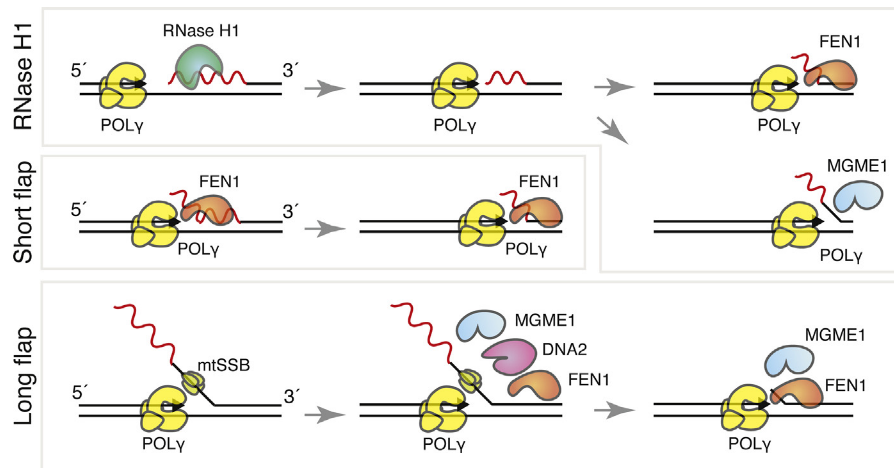

Fig. 6. Models of mitochondrial primer removal.
RNase H1 pathway—RNase H1 degrades most of the primer (red wavy line), the remaining ribonucleotides are displaced into a short flap by POLγ and then cleaved by FEN1, or possibly MGME1 in which case the flap must also contain DNA. Short flap pathway—short RNA flaps created by POLγ strand displacement synthesis are cleaved by FEN1 through reiterative cycles. Long flap pathway—a long RNA:DNA flap is formed and covered by mtSSB. DNA2, MGME1 or FEN1 are possible nuclease candidates. Any remaining short flap is cleaved by FEN1, or possibly MGME1. Black lines: DNA; red wavy lines: RNA primer. See text in Section 6 for further details.

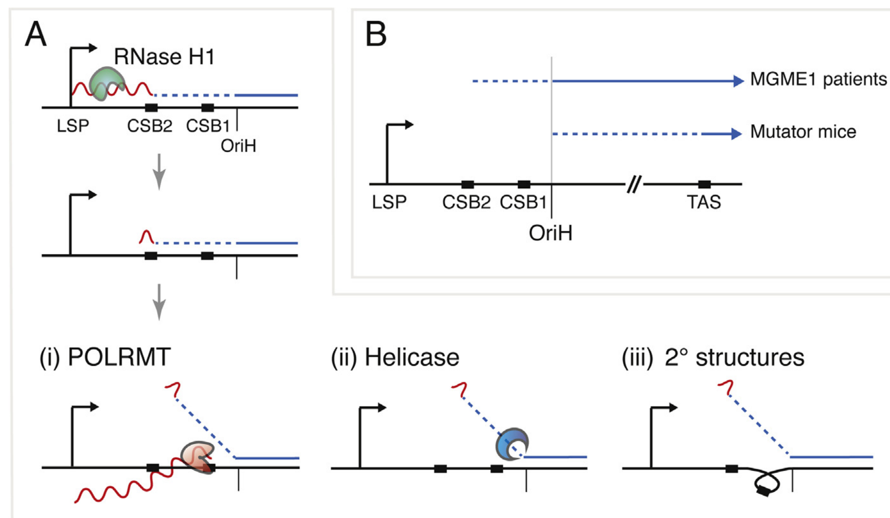

Fig. 7. Primer removal and 5′-end processing at OriH.
(A) Model on how the mature 5′-end of the nascent H-strand is shifted to OriH. RNase H1 removes the RNA primer (red wavy line), leaving behind 1–2 ribonucleotides attached to the 5′-end of the nascent H-strand (blue line). The dashed blue line represents the region of DNA that is then removed, possibly by three alternative long-flap pathways: (i) POLRMT transcription from LSP terminates near OriH; (ii) a 5′–3′ helicase unwinds the DNA; (iii) secondary structures form in the template strand. (B) Mapping of the OriH ends of the linear 11-kb mtDNA fragment found in MGME1 patient cells and mutator mice. Dashed lines represent the range in which ends were found: in MGME1 patients this is between CSB2 and OriH, in mutator mice the range is between OriH and TAS. The classical 5′-end (OriH) of the nascent H-strand is indicated with a grey vertical line.

What seems likely is that primer removal involves several factors and pathways. The *in vivo* evidence combined with their biochemical activity make RNase H1 and MGME1 two of the strongest candidates. Although there is good *in vitro* evidence for FEN1's role, there are no clear effects on mtDNA when it is depleted and there is disagreement about its mitochondrial localization. It may however be a question of time before more evidence emerges. It was not long ago for example, that DNA2 was accepted to be mitochondrial and mutations in patients with mitochondrial disease were mapped to DNA2. Due to the essential nuclear function of DNA2 and FEN1, it would be worth investigating the design of new knockout models to specifically address the mitochondrial function of these proteins. Also when analyzing mtDNA, more discerning tools would be valuable to better distinguish between replication and repair defects, as it is not clear to what extent DNA2, FEN1 and MGME1 are involved in either. Many other questions remain, for example, what effect does mtSSB have on the activity of these proteins? Moreover, in Okazaki fragment maturation the DNA clamp and processivity factor, PCNA (proliferating-cell nuclear antigen), serves as a platform to direct nuclease and ligase activities [86]. What, if any, factor coordinates these activities in mitochondria, which lack PCNA, remains to be established.

Conflict of interest

The authors declare that there are no conflicts of interest.

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