Maintenance and Expression of Mammalian Mitochondrial DNA

Claes M. Gustafsson,[1] Maria Falkenberg,[1] and Nils-Göran Larsson[2,3]

[1]Department of Medical Biochemistry and Cell Biology, University of Gothenburg, 405 30 Gothenburg, Sweden; email: claes.gustafsson@medkem.gu.se, maria.falkenberg@medkem.gu.se

[2]Department of Mitochondrial Biology, Max Planck Institute for Biology of Ageing, 50931 Cologne, Germany; email: larsson@age.mpg.de

[3]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm, Sweden

Annu. Rev. Biochem. 2016. 85:133–60

First published online as a Review in Advance on March 24, 2016

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-060815-014402

Keywords

mitochondria, mtDNA, transcription, replication, respiratory chain, polymerase

Abstract

Mammalian mitochondrial DNA (mtDNA) encodes 13 proteins that are essential for the function of the oxidative phosphorylation system, which is composed of four respiratory-chain complexes and adenosine triphosphate (ATP) synthase. Remarkably, the maintenance and expression of mtDNA depend on the mitochondrial import of hundreds of nuclear-encoded proteins that control genome maintenance, replication, transcription, RNA maturation, and mitochondrial translation. The importance of this complex regulatory system is underscored by the identification of numerous mutations of nuclear genes that impair mtDNA maintenance and expression at different levels, causing human mitochondrial diseases with pleiotropic clinical manifestations. The basic scientific understanding of the mechanisms controlling mtDNA function has progressed considerably during the past few years, thanks to advances in biochemistry, genetics, and structural biology. The challenges for the future will be to understand how mtDNA maintenance and expression are regulated and to what extent direct intramitochondrial cross talk between different processes, such as transcription and translation, is important.

Contents

INTRODUCTION

The Origin of mtDNA

According to current theories, mitochondria originated from α-proteobacteria, and the eukaryotic cell was created when an α-proteobacterium entered an archaebacterium (1). This endosymbiosis provided the bioenergetic means for large variations in the shape, size, and function of eukaryotic cells to evolve, leading to the creation of multicellular organisms with specialized cell types (1). During evolution, most of the genes in the ancestral bacterial genome were lost or transferred to the nucleus, leaving only the compact mitochondrial DNA (mtDNA) molecule. Remarkably, in all organisms studied there is a perfect correlation between the presence of mtDNA and the existence of mitochondria with a functional respiratory chain (2). The reason for this interdependency between oxidative phosphorylation (OXPHOS) and mtDNA is unclear and could be explained by the evolutionary history of mitochondria or by strong selection for some regulatory features that necessitates the presence of mtDNA close to the respiratory chain (2). It is possible that the local regulation of gene expression is important for metabolic control; for example, the molecular machineries governing mtDNA expression could be directly influenced by the mitochondrial membrane potential (Ψ_m) or the reduction–oxidation (redox) status of the organelle, or both.

Inheritance and Segregation of mtDNA in Mammals

There are many copies of mtDNA per cell in both somatic tissues and germ cells, and a mutation may be present in only a fraction of all copies. In the homoplasmic state, a cell or a tissue has

mtDNA of only one genotype, whereas in a heteroplasmic state, there is a mixture of two or more genotypes. A heteroplasmic, pathogenic mtDNA mutation has to be present above a certain minimal threshold to cause respiratory-chain deficiency (3), and mutation levels typically vary vastly among different tissues and even among cells in a given tissue because of somatic segregation (4). Mitochondrial DNA is exclusively maternally inherited and two main processes may explain why the mtDNA present in sperm is not transmitted to the next generation. First, there is a significant downregulation of the mtDNA copy number during spermatogenesis (5, 6). Second, there is a mechanism that actively degrades sperm mitochondria after fertilization (7, 8). It has been estimated that a mammalian oocyte contains approximately 10^5 copies of mtDNA, which segregate into daughter cells after fertilization as the embryo starts to divide, and replication of mtDNA does not ensue until after implantation (9). During development, a small number of mtDNA molecules will be set aside for the germ line and will populate primordial germ cells to give rise to the future oocytes. It is important to recognize that although somatic tissues contain abundant mtDNA mutations (10), the germ line has several mechanisms to counteract maternal transmission of mtDNA mutations (2): (*a*) Only a subset of the pool of mtDNAs in the mother is transmitted to the next generation because of a bottleneck effect during development (11). (*b*) There is a purifying selection mechanism in the maternal germ line that decreases the transmission of mtDNA mutations causing amino acid changes in the encoded proteins (12). (*c*) High levels of mtDNA mutations that affect transfer RNA (tRNA) genes are strongly selected against in the developing embryo (12). (*d*) Mothers with high levels of mtDNA mutations in the germ line have decreased fertility (13).

Expression of Mammalian mtDNA

Mammalian mtDNA is a gene-dense, double-stranded DNA (dsDNA) molecule of 16.6 kb, which encodes 11 messenger RNAs (mRNAs) (translated to 13 proteins), 2 ribosomal RNAs (rRNAs) (12S and 16S rRNA), and 22 tRNAs (**Figure 1**). The OXPHOS system consists of approximately 90 proteins that have a dual genetic origin, that is, the subunits are either encoded by nuclear genes, translated on cytosolic ribosomes and imported into mitochondria, or encoded by mtDNA and translated on mitochondrial ribosomes (**Figure 2**). The 13 mtDNA-encoded subunits constitute only a minority of the OXPHOS subunits, but they are nevertheless essential because OXPHOS collapses in the absence of mtDNA expression (14). It has been estimated that mitochondria contain approximately 1,200 different proteins and, remarkably, several hundred of these are needed for mtDNA expression (15, 16). The regulation of mtDNA expression is quite complex and involves many different levels of control, such as mtDNA maintenance, mtDNA replication, mtDNA transcription, the processing of primary transcripts, RNA modification, RNA stability, the coordination of translation, translation by mitochondrial ribosomes, and the regulated insertion of translated proteins into the mitochondrial inner membrane (17). In this review, we focus on the molecular machineries involved in the maintenance, replication, and transcription of mtDNA. An extensive review of the mechanisms involved in RNA maturation and translation can be found in Reference 17.

MITOCHONDRIAL TRANSCRIPTION

Transcription Patterns

The two strands of mtDNA differ in their base composition: Because one strand is rich in guanines, the strands can be separated into a heavy (H) and a light (L) strand using density centrifugation in alkaline $CsCl_2$ gradients (18). The mammalian mtDNA genome is densely packed with coding

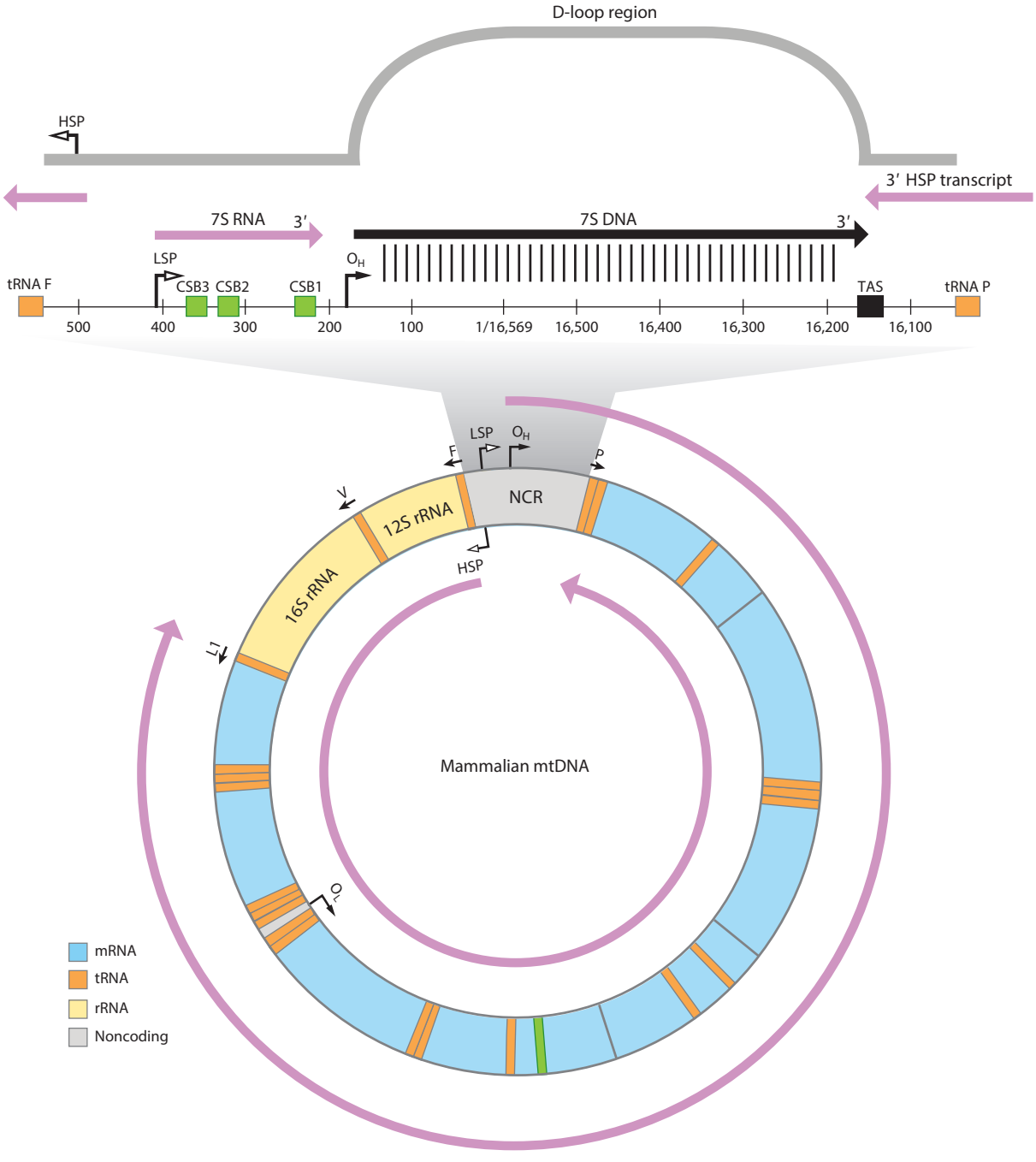
D-loop region
HSP
3′ HSP transcript
7S RNA
3′
7S DNA
3′
LSP
O_H
tRNA F
CSB3
CSB2
CSB1
TAS
tRNA P
500
400
300
200
100
1/16,569
16,500
16,400
16,300
16,200
16,100
F
LSP
O_H
P
NCR
V
12S rRNA
16S rRNA
HSP
L1
Mammalian mtDNA
mRNA
tRNA
rRNA
Noncoding

Figure 1

Organization of the human mitochondrial genome. An enlarged version of the NCR is shown at the top. Characteristic features include the 7S DNA, which associates with mtDNA to create a displacement loop (D-loop) in the NCR. Transcripts initiated from the LSP are frequently terminated at CSB1, creating the 7S RNA. These transcripts should not be confused with the primers formed by transcription termination at CSB2 (not shown in this figure). Full-length LSP transcripts are terminated just downstream of the 16S rRNA, whereas full-length HSP transcripts are terminated at the 3′ end of the D-loop region. Abbreviations: CSB, conserved sequence block; HSP, heavy-strand promoter; LSP, light-strand promoter; mRNA, messenger RNA; mtDNA, mitochondrial DNA; NCR, noncoding control region; O_H, heavy-strand origin; rRNA, ribosomal RNA; tRNA, transfer RNA; TAS, termination-associated sequence.

information and contains 37 genes in just 16.6 kb of sequence. All genes in mammalian mtDNA lack introns, and there is only one longer noncoding region, which is referred to as the control region. The control region contains a dedicated promoter for the transcription of each strand of mtDNA, that is, the light-strand promoter (LSP) and the heavy-strand promoter (HSP), as well as regulatory sequences controlling mtDNA replication (**Figure 1**). Transcription initiation at LSP or HSP produces near-genome-length polycistronic transcripts that encompass all of the coding information on each strand. There are transcription termination events that prevent transcription from proceeding into the control region, where transcription was initiated (**Figure 1**). The primary transcripts are processed to release the individual RNA molecules. The steady-state levels of the longer unprocessed transcripts are low and, therefore, processing is likely to occur cotranscriptionally. In 1981, mouse and human mtDNA were sequenced and it was noted that tRNA

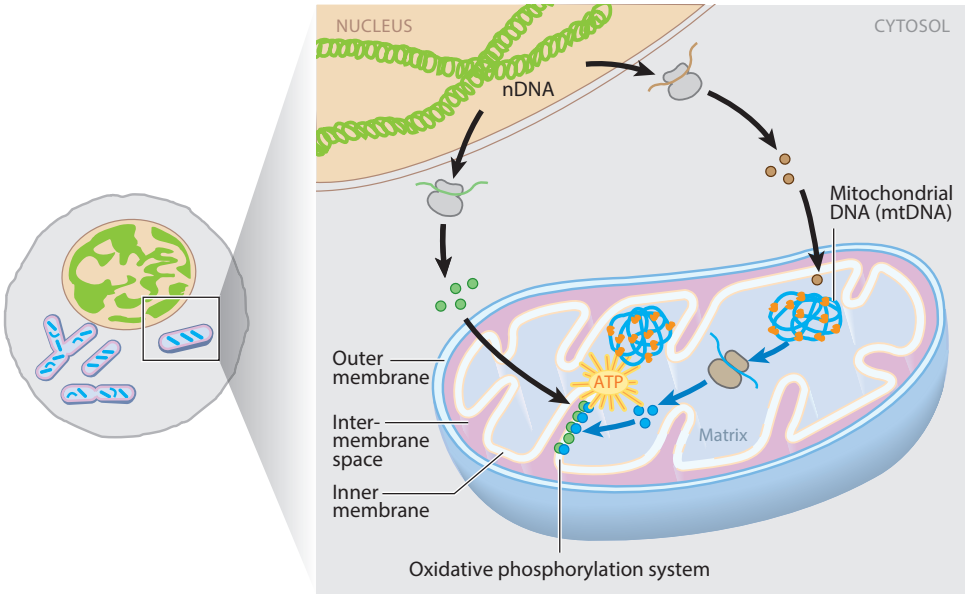

Figure 2

Respiratory-chain subunits and ATP synthase are the products of two different genomes. The mitochondrial genome encodes for 13 subunits of the oxidative phosphorylation system, as well as for transfer RNAs and ribosomal RNAs required for their synthesis. All other proteins, including those required for the maintenance and expression of mitochondrial DNA, are encoded in the nucleus, synthesized in the cytosol, and transported into mitochondria. Abbreviations: ATP, adenosine triphosphate; nDNA, nuclear DNA.

genes often flank rRNA and protein-coding genes (19, 20), which led to the postulation of the "tRNA punctuation model" (21). According to this model, tRNAs are specifically recognized and cleaved in the polycistronic transcripts, thus leading to the release of tRNAs, mRNAs, and rRNAs (21), which subsequently undergo further maturation by, for example, base modifications, CCA addition, and polyadenylation (17). In mammalian mitochondria, the 5′ ends of tRNAs are cleaved in the polycistronic transcript by an all-protein version of ribonuclease (RNase) P, consisting of three subunits (MRPP1–3), whereas the 3′ ends of tRNAs are processed by RNase Z (ELAC2) (17).

The enzymatic machineries for the maintenance and expression of mammalian mtDNA are completely distinct from those found in the nucleus, which is consistent with the α-proteobacterial origin of mitochondria (22). However, many of the involved proteins are dissimilar to the corresponding α-proteobacterial components, and instead are similar to proteins present in the T-odd lineage of bacteriophages, including factors such as the mitochondrial DNA-directed RNA polymerase (POLRMT), the catalytic subunit of mtDNA polymerase (POLγA), and the replicative mitochondrial helicase (TWINKLE) (23, 24). Thus, there are several examples of bacteriophage-derived replication and transcription factors that have replaced the original α-proteobacterial enzymes during the endosymbiosis process (25).

Mitochondrial RNA Polymerase

The single subunit mitochondrial RNA polymerase was first identified in yeast (26), and later, based on sequence similarity, in human cells (27). Mammalian POLRMT is related to the RNA polymerase (RNAP) encoded by bacteriophage T7 (T7 RNAP). A mitochondrial-targeting signal with a length of 41 amino acids is cleaved off from human POLRMT after mitochondrial import, and the mature protein corresponds to 1,189 amino acids. X-ray structure analysis has demonstrated that human POLRMT contains a catalytic domain in the C terminus of the protein (amino acids 647–1,230) and an N-terminal domain (amino acids 368–647) with similarity to the promoter-binding, AT-rich recognition loop of T7 RNAP (28).

Although structurally similar and with a common evolutionary origin, T7 RNAP and POLRMT differ in their mechanisms of transcription elongation. In fact, the structure of the elongating form of POLRMT is strikingly different from that reported for T7 RNAP (29). Whereas the phage polymerase refolds extensively during transition to elongation, the elongating form of POLRMT does not. Furthermore, the N-terminal domain contains a conserved, intercalating, hairpin-like structure, which melts promoter DNA during the initiation of transcription by T7 RNAP; the corresponding region in POLRMT has a different function, as it has a role in separating RNA from DNA during transcription elongation (29).

The N-terminal extension (NTE; amino acids 42–368) is a unique feature of POLRMT that is not found in phage polymerases and for which there are only limited high-resolution structural data. Sequence analysis has identified a pentatricopeptide repeat (PPR) domain known to bind RNA, but its function remains unclear. The PPR domain is located next to the point where the newly synthesized RNA exits the catalytic domain of POLRMT (29). Hypothetically, the PPR domain may prevent reannealing of newly synthesized RNA to the template DNA in order to avoid blocking subsequent rounds of transcription initiation. POLRMT binds sequence specifically to promoter elements, but it is unable to initiate transcription on its own and requires the help of mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M).

Mitochondrial Transcription Factor B2

TFB2M was originally discovered based on its primary sequence similarities to the yeast transcription factor mtTFB (30). TFB2M can support the initiation of transcription from HSP and LSP in a purely recombinant, in vitro system containing POLRMT and TFAM (30). TFB2M interacts transiently with POLRMT and forms part of the catalytic site during the initiation of transcription (31). In the initiation complex, TFB2M interacts directly with the priming substrate but, as has been demonstrated with yeast mtTFB, the protein is probably lost from the complex once POLRMT leaves the promoter and enters elongation (32). Mammalian cells also contain a second mtTFB homolog, denoted TFB1M. Both TFB1M and TFB2M are similar in sequence to a large family of rRNA methyltransferases present in bacteria, archaea, and eukaryotes (30). TFB1M likely represents the ancestral methyltransferase, whereas TFB2M is the result of a gene duplication that has allowed it to evolve into a mitochondrial transcription factor (33–35). Interestingly, a similar gene-duplication event may have given rise to the accessory subunit of mtDNA polymerase, which is related to a family of tRNA synthetases (36). Results from biochemical experiments and the characterization of conditional knockout mice have shown that TFB1M dimethylates two highly conserved adenines at the 3′ end of the mitochondrial 12S rRNA (37), and the loss of TFB1M in mice impairs biogenesis of the small ribosomal subunit but transcription is unaffected (37).

Mitochondrial Transcription Factor A

TFAM is the third essential component of the core mitochondrial transcription machinery (38). TFAM binds sequence-specifically to mitochondrial promoters and creates a stable U-turn in DNA (39, 40). Similar to other members of the high mobility group (denoted HMG)-box domain family, TFAM can bind, unwind, and bend DNA without sequence specificity (22). In fact, TFAM has been shown to coat the entire mtDNA molecule, forming a compact nucleoid structure (41–44). Biophysical studies have also generated important new insights into the ways in which the protein changes the structure of mtDNA. These studies have shown that TFAM may slide over DNA and form longer patches upon collision with other TFAM monomers (45). When TFAM binds to DNA, the protein induces DNA unwinding of the short surrounding region (45). Coalescence of the bubbles formed by neighboring TFAM monomers may drive protein aggregation into long patches (46). Such DNA-mediated allosteric interactions may also be important for activating transcription. TFAM-induced melting bubbles in the promoter region may coalesce with a bubble created by POLRMT and TFB2M at the transcription start site (46).

A Model for the Initiation of Mitochondrial Transcription

A combination of studies using in vitro biochemistry and structural biology, as well as biophysical studies, has provided us with a model for how transcription is initiated in mitochondria (**Figure 3**). The reaction is initiated by TFAM binding to a high-affinity site situated 10–15 base pairs (bp) upstream of the start site for transcription. Next, TFAM interacts with POLRMT and recruits the protein to the promoter. POLRMT recruitment depends on direct interactions with TFAM, but it also involves structural changes in the promoter region, which facilitate POLRMT–DNA interactions (47–49). In agreement with this notion, conditions that induce promoter breathing can circumvent the strict TFAM requirement for transcription initiation (38). In the absence of TFB2M, POLRMT–promoter interactions are mainly restricted to sequences around position

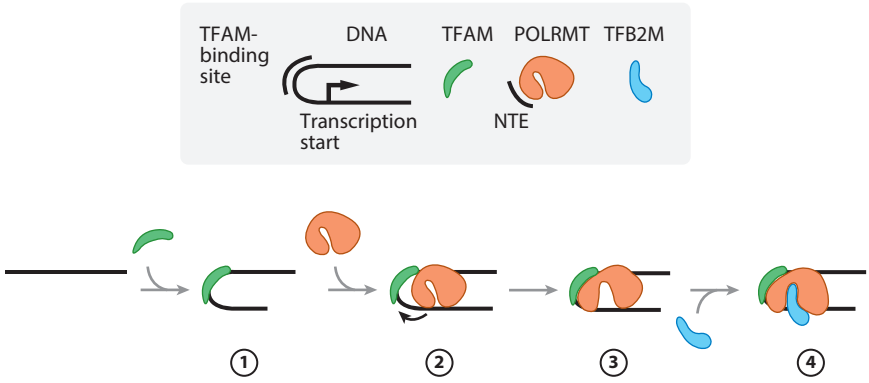

Figure 3

A sequential model of the initiation of mammalian mitochondrial DNA transcription. ① TFAM interacts with a high-affinity binding site just upstream of the transcription start site and introduces a 180° bend in the DNA. ② POLRMT is recruited by both TFAM and sequence-specific interactions with DNA. POLRMT interacts with DNA around the transcription start site and with DNA upstream of the TFAM-binding site. ③ In complex with DNA and TFAM, POLRMT undergoes a conformational change, which enables ④ the binding of TFB2M and the formation of a fully assembled initiation complex. Abbreviations: NTE, N-terminal extension; POLRMT, mitochondrial DNA-directed RNA polymerase; TFAM, mitochondrial transcription factor A; TFB2M, mitochondrial transcription factor B2.

−50 to −60 (48). This interaction is also evident from cross-linking experiments that have demonstrated direct contacts between POLRMT and the upstream promoter region (50). The contacts formed are explained from structural data demonstrating that TFAM induces a sharp bend in the promoter DNA, which helps to juxtapose POLRMT and the −50 to −60 region (39, 40). In the next step, TFB2M enters the transcription complex and the fully assembled initiation complex encircles the promoter (47, 48). This observation is in agreement with data revealing that TFB2M is required for the structural changes that occur around the transcription start site (31, 48) and for the formation of the first phosphodiester bond (51). How the NTE of POLRMT affects transcription initiation is poorly understood, but the domain appears to have a repressive effect on transcription. The loss of the NTE generates a hyperactive polymerase, which can also initiate transcription in the absence of TFAM (48).

Mitochondrial Transcription Elongation Factor

The mitochondrial transcription elongation factor (TEFM) was identified based on its sequence similarity to previously characterized transcription elongation factors from other systems (52). TEFM interacts with the catalytic, C-terminal part of POLRMT, and depletion of TEFM impairs transcription elongation both in vitro (53, 54) and in knockdown cell lines (52). TEFM stimulates POLRMT interactions with an elongation-like DNA:RNA template, and the protein is present at the promoter before the initiation of transcription (53, 54). Thus, it could be debated whether TEFM should be regarded as a second subunit of mitochondrial RNAP or as an accessory elongation factor. TEFM helps the polymerase to transcribe longer stretches of RNA and to bypass regions generating highly structured RNA (e.g., tRNA clusters and the strong G-quadruplex-forming region of conserved sequence block-2 (CSB2) (53, 54). TEFM also stimulates transcription past oxidative lesions, such an 8-Oxo-2′-deoxyguanosine, which otherwise may cause premature transcription termination (53, 54).

Mitochondrial Transcription Termination Factor 1

Early studies led to the idea that H-strand transcription involves two overlapping transcription units and that it is initiated from two separate transcription start sites, denoted HSP1 and HSP2 (55). HSP1 is situated just upstream of the $tRNA^{Phe}$ gene, and transcription from this promoter produces transcripts that span the $tRNA^{Phe}$, 12S rRNA, 16S rRNA, and $tRNA^{Val}$ genes. In contrast, the HSP2 transcription initiation site is located approximately 100 bp further downstream, at the boundary between the $tRNA^{Phe}$ and 12S rRNA genes, and transcription from this second promoter supposedly proceeds along almost the entire length of the H-strand (55). There is no consensus on whether the HSP2 promoter exists. Some reports have failed to observe initiation from HSP2 in reconstituted transcription systems, whereas others have suggested that transcription initiation at HSP2 can be observed in vitro (30, 56–59). However, the data are not congruent because the precise HSP2 transcription start site mapped in vitro differs from the one that has been mapped in vivo (55, 59).

One key feature of the H-strand two-transcription-unit model is the regulation of transcription termination. Transcription initiated from HSP1 is supposedly terminated within the $tRNA^{Leu(UUR)}$ gene at a position immediately downstream of the 16S rRNA gene, whereas transcription initiation from HSP2 continues past the termination site (60, 61). This model has been suggested to explain why the steady-state levels of the rRNAs are about 50-fold higher than those of the mRNAs produced downstream of the termination site. The responsible termination factor was identified based on protein purification and is called mitochondrial transcription termination factor 1 (MTERF1) (62, 63). Recently, the two-transcription-unit hypothesis was challenged by a knockout of *Mterf1* in mice (64). The loss of the MTERF1 protein did not create any noticeable phenotype, and the relative levels of rRNA and mRNA remained unaffected. Instead, MTERF1 helped to reduce antisense transcription of the rRNA genes. The in vivo observations and conclusions are in excellent agreement with in vitro biochemical analyses of the action of the MTERF1 protein in transcription assays. In such assays, MTERF1 only partially terminates H-strand transcription, whereas transcription in the opposite direction (L-strand transcription) is almost completely blocked (64, 65).

Other Members of the MTERF Family

Three additional proteins have been identified based on their sequence similarities to MTERF1, namely MTERF2, MTERF3, and MTERF4 (66). X-ray structure determinations have demonstrated that these MTERF proteins are shaped like half-doughnuts, with a positively charged path on the convex side (40, 67–70). In MTERF1, the charged path helps to unwind the DNA helix, and three nucleotides are everted at the MTERF1 recognition site. Base flipping is necessary for a stable interaction between MTERF1 and DNA and for promoting transcriptional termination (67). The MTERF2, MTERF3, and MTERF4 proteins were initially investigated for their ability to bind sequence-specifically to mtDNA and to promote transcription or termination of mtDNA replication, or both. However, none of these proteins has been shown to regulate transcription termination. Instead, they seem to have adopted other roles in regulating transcription initiation and in ribosomal assembly (71–73).

MTERF2 is the least well characterized of the MTERF-family proteins. This protein is associated with the mitochondrial nucleoid (72), and deletion of the *Mterf2* gene in mice causes no obvious phenotype (N-G Larsson, unpublished observation). The function of MTERF2 remains to be determined. MTERF3 was originally identified as a negative regulator of mitochondrial transcription (71). The loss of *Mterf3* causes embryonic death in mid-gestation, whereas tissue-specific depletion of the MTERF3 protein in the heart causes massive upregulation of mtDNA transcription initiation and impaired respiratory-chain function. Chromatin immunoprecipitation

experiments have shown that MTERF3 can interact with the mitochondrial promoter region, but an exact binding site has never been defined (71). Interestingly, MTERF3 has also been shown to interact with 16S rRNA to facilitate the assembly of the large mitochondrial ribosomal subunit necessary for translation (74). The exact mechanisms of MTERF3 function have not been determined. The effect on translation is intriguing, but the exact binding site and the functional effect of MTERF3 on ribosome assembly remain unclear. The dramatic increase of de novo transcription in the absence of MTERF3 could potentially be occurring secondary to a general induction of mitochondrial biogenesis in response to decreased translation. Alternatively, MTERF3 could directly act to coordinate transcription and translation, and thus simultaneously affect both of these levels of gene transcription (71, 74). Clearly, more work is needed to define how MTERF3 may interact with mtDNA and the 16S rRNA sequences.

The last member of the mammalian MTERF family of proteins is MTERF4. Similar to MTERF3, the loss of MTERF4 causes a phenotype that is lethal to mouse embryos (73). The protein forms a stable, heterodimeric complex with NSUN4, a methyltransferase that methylates cytosine 911 in the mouse 12S rRNA (68, 75). Initially, it was believed that MTERF4 functions by targeting NSUN4 to the mitochondrial ribosome (73), but later experiments demonstrated that NSUN4 also methylates the 12S rRNA in the absence of the MTERF4 protein (75). Instead, the MTERF4–NSUN4 heterodimer seems to help to assemble the small and large ribosomal subunits into a functional monosome (75). The loss of MTERF4 not only impairs translation but also causes a massive increase in mitochondrial transcription, which is linked to a decrease in MTERF3 (73). It remains to be determined how MTERF3 and MTERF4 may cooperate to affect mitochondrial transcription and translation.

mtDNA REPLICATION

Mammalian mtDNA is replicated by a set of proteins that is distinct from the nuclear replication machinery. Mutations of mtDNA accumulate in somatic tissues with age (10), and many mutations seem to be formed by replication errors during embryogenesis, rather than by unrepaired damage (76, 77). Also, the patterns of polymorphisms in mice and humans are consistent with the idea that replication errors are the main source of mtDNA variation (12). Thus, the mtDNA replication process is of great importance for understanding sequence variation in mtDNA in mammalian evolution, as well as in disease and aging. We summarize briefly what is known about the factors involved.

The mtDNA Replisome

Similar to mitochondrial transcription machinery, many mtDNA replication factors are related to the simple replication machineries found in bacteriophages (25). At the core of mtDNA replication is DNA polymerase-γ (POLγ), the only replicative polymerase in mitochondria. Human POLγ is a heterotrimer, with one catalytic subunit (POLγA) and two accessory subunits (POLγB) (78–80); mouse knockouts lacking either POLγA or POLγB are lethal to the embryo (81, 82). A second DNA polymerase, referred to as PrimPol (primase polymerase), has been reported to act in both the nucleus and mitochondria. PrimPol is not essential for mtDNA maintenance and may serve a more specialized role, for example, by facilitating mtDNA replication fork progression at lesions or replication blocks, or both (83, 84).

POLγA has a molecular mass of 140 kDa (78) and belongs to the family-A DNA polymerases. Other members of this family are bacteriophage T7 DNA polymerase and bacterial DNA polymerase I (85–87). POLγA contains a 3′ to 5′ exonuclease domain that allows efficient proofreading

of a newly synthesized DNA strand, making POLγ one of the most accurate DNA polymerases known, with an error frequency of less than 1×10^{-6} per nucleotide (88). The enzyme also harbors a 5′-deoxyribose phosphate lyase activity, which may contribute to base excision repair, further lowering the mtDNA mutational load (89, 90).

The accessory POLγB subunit has a molecular mass of 55 kDa and shares structural similarity with class IIa aminoacyl-tRNA synthetases (36, 80). The protein has a dsDNA-binding activity (91) that is dispensable for DNA synthesis on a single-stranded DNA (ssDNA) template, but is required for replication on dsDNA (92). POLγB serves to increase both the catalytic activity and the processivity of POLγA by enhancing interactions with DNA substrates. POLγ is conserved in eukaryotes (79, 80), but the structural composition of the enzyme complex varies in metazoans. In contrast to that in mammals, *Drosophila melanogaster* POLγ is a heterodimer consisting of one POLγA and one POLγB subunit (80). In *Saccharomyces cerevisiae*, the accessory POLγB subunit is missing and POLγA is fully active as a monomer (93).

POLγ is, on its own, unable to use dsDNA as a template (92, 94, 95) and requires the DNA helicase TWINKLE for DNA synthesis to occur (94). The TWINKLE protein is essential because the mouse knockout is lethal to embryos (96). During mtDNA replication, TWINKLE travels together with POLγ at the replication fork and catalyzes nucleotide triphosphate-dependent unwinding of the mtDNA duplex in the 5′ to 3′ direction (94). TWINKLE is homologous to the T7 phage gene 4 protein (97). The phage protein also has a primase activity in its amino-terminal region, but this activity has been lost in the metazoan TWINKLE protein (98). Instead, the primers required for initiating mtDNA synthesis are likely produced by the mitochondrial RNA polymerase POLRMT (99, 100). TWINKLE requires a fork structure (a single-stranded 5′-DNA loading site and a short 3′-tail) to initiate unwinding, and just as in the gp4 protein, TWINKLE forms a hexamer in solution (97, 101, 102). The mitochondrial ssDNA-binding protein (mtSSB) stimulates TWINKLE's helicase activity and stabilizes the long stretches of single-stranded DNA formed at the replication fork (94, 101). The mtSSB protein has a molecular mass of 16 kDa and binds to ssDNA as a tetramer. In addition to coating ssDNA, mtSSB also stimulates mtDNA synthesis by facilitating POLγ primer recognition and enhancing POLγ processivity (103).

Topoisomerases are essential for replication, as well as transcription, because they allow changes in DNA topology (104). Mitochondrial topoisomerase I (TOP1mt) belongs to the type 1 class of topoisomerases, which catalyzes transient single-stranded DNA breaks (105). The protein has a molecular mass of about 72 kDa and can relax supercoiled DNA generated by replication or transcription (106, 107). Recently, two nuclear type IIA topoisomerases, TOP2α and TOP2β, which catalyze transient double-stranded DNA breaks, were found to be present and active in mammalian mitochondria (108). Interestingly, TOP2α forms a complex with mtDNA at both ends of 7S DNA and may thus be involved in regulating the stability of the D-loop structure.

Mutations affecting the function of the basic mtDNA replication machinery are important causes of human mitochondrial disease. A large number of pathogenic mutations have been reported in the genes encoding POLγA and TWINKLE, and some mutations have also been found in the gene encoding POLγB (109). Affected patients display deletions or depletion of mtDNA that leads to respiratory-chain deficiency and neuromuscular symptoms: Progressive external ophthalmoplegia, exercise intolerance, and muscle weakness are common symptoms (109).

The Mode of mtDNA Replication

According to the strand-displacement model (SDM) of mtDNA replication, DNA synthesis occurs continuously on both strands and no Okazaki fragments are formed (**Figure 4**) (110). To ensure proper coordination of DNA synthesis for the two strands, mtDNA contains a dedicated origin

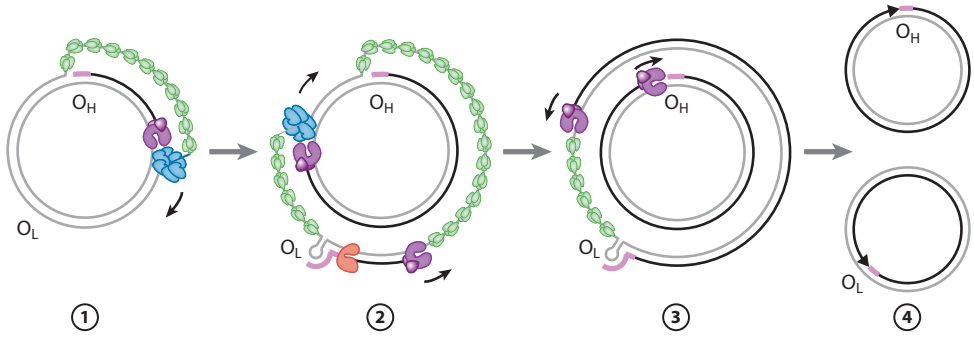

Figure 4

The strand-displacement model of mitochondrial DNA (mtDNA) replication. ① After initiation at the heavy-strand origin (O_H), the replisome proceeds unidirectionally to produce the nascent H-strand. The displaced, parental H-strand is bound and stabilized by the mitochondrial single-stranded DNA-binding protein (mtSSB) (*green*). ② When the H-strand replication machinery passes the light-strand origin (O_L), a stem-loop structure is formed. The mitochondrial DNA-directed RNA polymerase POLRMT (*orange*) synthesizes short primers at the stem loop, which are used to initiate L-strand DNA synthesis. Please note that TWINKLE (*blue*) is required only for the H-strand because the template for L-strand synthesis is single-stranded DNA covered with mtSSB. ③, ④ After completion of mtDNA strand synthesis, replication is terminated at either O_H or O_L, depending on where DNA synthesis was initiated.

of DNA replication on each strand, the heavy-strand origin (O_H) and the light-strand origin (O_L) (**Figures 4** and **5**). Replication is initiated at O_H, and DNA synthesis proceeds in one direction to produce a new H-strand. During the first phase of replication, there is no simultaneous synthesis of the complementary L-strand. During the synthesis of the new H-strand, mtSSB covers the displaced, parental H-strand and blocks POLRMT transcription (111). Thus, mtSSB prevents the random initiation of RNA primer synthesis on the displaced strand. When the replication machinery has synthesized about two-thirds of the mtDNA molecule, it passes O_L, which becomes single-stranded and folds into a stem-loop structure (**Figures 4** and **5**). The stem hinders mtSSB binding and leaves the single-stranded loop region accessible for POLRMT, which initiates primer synthesis from a poly-T stretch (100, 111). After about 25 nucleotides (nt) of primer synthesis, POLRMT is replaced by POLγ, and L-strand DNA synthesis begins. The replication of the two strands is interconnected because H-strand synthesis is required for the initiation of L-strand synthesis. Once initiated, H- and L-strand synthesis proceed continuously in opposite directions until the two events reach a full circle. Support for the SDM has been provided by using a number of different techniques, including atomic force microscopy, mapping of free 5′ ends to O_H and O_L, as well as in vitro reconstitution of replication-dependent initiation of mtDNA synthesis at O_L (18, 100, 112–114). The O_L sequence is strongly conserved in all vertebrates, and in vivo saturation mutagenesis of mouse mtDNA has demonstrated that O_L is essential for mtDNA maintenance. The structure and the sequence requirement for human O_L have also been elucidated in detail in vitro. A functional human O_L must include a stable double-stranded stem region with a pyrimidine-rich template strand and a single-stranded loop of at least 10 nt (114).

An interesting consequence of the SDM is that the sites for the initiation and termination of mtDNA synthesis differ between the two daughter molecules. On one, initiation and termination will take place at O_H, and on the other it will be at O_L (**Figure 4**). In addition, the factors required for producing the two daughter molecules will also be distinct. Replication initiating at O_H uses a dsDNA template and, therefore, depends on TWINKLE for DNA unwinding. In contrast, DNA synthesis initiated at O_L uses ssDNA as a template and, therefore, TWINKLE is not required.

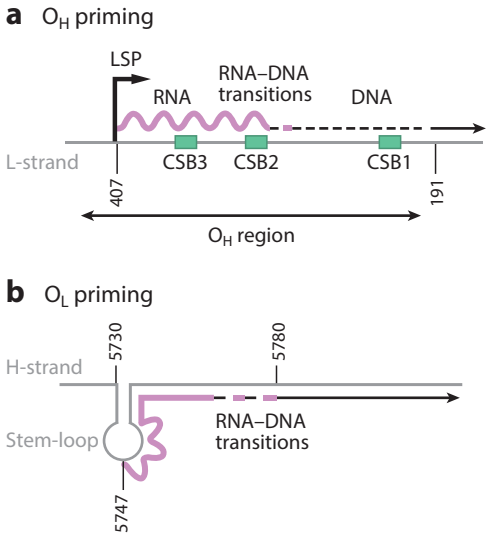

Figure 5

Models for initiation of mitochondrial DNA synthesis at the heavy-strand origin (O_H) and the light-strand origin (O_L). (*a*) Transcription initiated at the light-strand promoter (LSP) is prematurely terminated at conserved sequence block-2 (CSB2). The termination is directed by G-quadruplex structures formed in the nascent RNA. The primer remains stably associated with template DNA, forming an R-loop structure. Once DNA synthesis has been initiated, primer processing removes the RNA and about 100 nucleotides (nt) of downstream DNA. As a consequence, the free 5′ end of nascent H-strand DNA is placed at position 191. (*b*) When exposed in its single-stranded conformation, the O_L adopts a stem-loop structure, and the mitochondrial DNA-directed RNA polymerase POLRMT initiates primer synthesis from a poly-dT stretch in the single-stranded loop region. After about 25 nt, POLRMT is replaced by POLγ, and L-strand DNA synthesis commences.

These strand-specific differences may influence the relative rate of H- and L-strand synthesis and could explain why there are strand-specific mtDNA mutation patterns, for example, as observed in cancer cells (115, 116).

In addition to the SDM, two alternative models for mtDNA replication have been suggested, that is, the model of ribonucleotide incorporation throughout the lagging strand (RITOLS) and the model of strand-coupled mtDNA replication (117–121). Both of these models are primarily based on mtDNA replication intermediates observed using neutral two-dimensional agarose gel electrophoresis (known as 2D-AGE). The generality of the strand-coupled DNA replication mechanism has been questioned because products interpreted as intermediates from this type of replication mechanism do not become rapidly labeled *in organello* (121, 122). The RITOLS replication model and the SDM are similar, with the exception of the requirement for mtSSB. According to the SDM, the displaced H-strand is coated with mtSSB, whereas the RITOLS model argues that the parental H-strand is coated with RNA. The RNA intermediates in the RITOLS model are processed transcripts (including tRNAs and rRNAs), which are successively threaded onto the lagging-strand template as the replication fork advances, and they remain hybridized until displaced, degraded, or processed further during lagging-strand DNA synthesis (121). A weakness of this so-called bootlace model is that the enzymes required for the process—that is, the hybridization of RNA intermediates to DNA and the subsequent RNA displacement—have not been defined (122). Furthermore, it is unclear how well highly structured and modified tRNAs and rRNAs can be melted to anneal to ssDNA (122). Another

strong argument against the RITOLS model is that the in vivo occupancy profile of mtSSB is in good agreement with the SDM (111). According to this later model, the parental H-strand close to the control region will remain single-stranded for a much longer time than it will in regions closer to O_L. In agreement with this prediction, the highest levels of mtSSB are found close to the mitochondrial control region and they gradually decline towards O_L (111).

Initiation of mtDNA Replication at O_H

The initiation of mtDNA replication can be regarded as a committed step and should, therefore, be under strict control. François Jacob, Sidney Brenner, and François Cuzin (123) formulated a general model of the initiation of DNA replication in 1963. In their model, replication is regulated by specific DNA sequences called replicators. The binding of an initiator to the replicator triggers the initiation of DNA replication. The replicator model has turned out to be valid for many types of genomes, but it fails to explain how the initiation of mtDNA replication is regulated in mitochondria. The structure of the origins of replication varies among organisms. In *Escherichia coli*, oriC corresponds to 3 A–T rich 13-mer repeats and 4 9-mer repeats, whereas a typical budding yeast origin is about 100 bp and contains a common 11-bp consensus sequence that is essential for origin activity (85). In human mitochondria, O_H has traditionally been defined as a single position and has classically been annotated at nucleotide position 191 because of the existence of a prominent free 5′ DNA end at this site (22). We believe that this nomenclature has confounded our thinking about how replication is initiated in human mitochondria and that the mitochondrial O_H needs to be redefined (**Figure 5**). First, primer formation is initiated at LSP, which is located approximately 200 nt upstream of O_H. Transcription initiated from LSP not only generates polycistronic near-genome-length transcripts but also supposedly generates the primers required to initiate H-strand DNA replication (22). Therefore, the promoter region is an integral part of O_H function. Second, one of the classical hallmarks of an origin is the transition from RNA to DNA, that is, the point at which the DNA polymerase initiates DNA synthesis from the 3′ end of an RNA primer. In human mitochondria, this point of transition has been mapped to the CSB2 region, which is located approximately 100 nt downstream of LSP (**Figure 5**). CSB2 is one of three conserved sequence blocks (CSB1–3) located downstream of LSP (124–126). The CSB2 region is G-rich and during its transcription, a G-quadruplex structure is formed in the nascent RNA, which stimulates transcription termination and the formation of primers that may be used to initiate H-strand DNA synthesis (127, 128). The CSB2 region is, therefore, an essential element of O_H function.

Even if RNA–DNA transitions map to CSB2, the 5′ ends of nascent DNA map to about 100 bp further downstream, at the classical O_H position. This discrepancy implies that the nascent H-strand undergoes considerable 5′ end processing to remove the RNA primer and approximately 100 nt of DNA (110). Based on the considerations we mention here, we suggest that O_H should be redefined as the region containing LSP, the CSBs, and the classical O_H position. We suggest that this area of mtDNA should be referred to as the O_H region (**Figure 5**). Such a definition would be more in line with the classical replicator model and in line with how origins are defined in other systems.

Termination of mtDNA Replication

Once POLγ has completed the replication of both strands of the mtDNA circle, it needs to produce juxtaposed 5′ and 3′ DNA ends at, respectively, O_H and O_L to allow efficient ligation (95). Ligation is performed by DNA ligase III, and the loss of this enzyme causes mtDNA depletion

and embryonic lethality in mice (129, 130). When POLγ encounters a 5′ end at the termination of DNA replication, the polymerase starts to idle, that is, POLγ initiates successive cycles of polymerization and 3′ to 5′ exonuclease degradation at the nick (95, 131). The idling activity is required for proper ligation because POLγ lacking exonuclease activity will continue DNA synthesis into dsDNA, thereby creating a 5′ flap that cannot be used as a substrate for DNA ligase III (95). The failure to create DNA ends that can be ligated explains why the exonuclease-deficient POLγ mouse model (the mtDNA mutator mouse) has strand-specific nicks at O_H (95). The replication of such nicked mtDNA templates causes the formation of linear, deleted mtDNA fragments, spanning O_H and O_L, in mice expressing exonuclease-deficient POLγ (132).

Primer Processing in mtDNA Replication

The RNA primers used to initiate mtDNA synthesis must be removed, likely by degradation by ribonuclease H1 (RNASEH1). In vitro studies have demonstrated that RNASEH1 cuts the RNA part of a chimeric DNA:RNA strand annealed to DNA, but it is unable to cut the DNA part. *RnaseH1* knockout mice show mtDNA depletion and embryonic lethality. Furthermore, RNA primers are retained in the O_H and O_L regions in embryonic fibroblasts lacking RNASEH1 (133, 134).

Even if RNASEH1 removes the RNA part of the primer in the O_H region (from LSP to CSB2), there must also be a second factor processing the DNA part of the primer (from CSB2 to O_H). A candidate for this task has recently been identified in patients with impaired mtDNA replication and multisystemic mitochondrial disease caused by mutations in the gene encoding the mitochondrial genome maintenance exonuclease-1 (MGME1). MGME1 is a mitochondrial RecB-type exonuclease belonging to the PD-(D/E)XK nuclease superfamily (135, 136). The nuclease activity of MGME1 has been confirmed in vitro and the enzyme can cut both ssDNA and DNA-flap substrates. Interestingly, affected patients have increased ratios of an abortive H-strand replication product spanning the control region (7S DNA) in comparison with full-length mtDNA. In addition, the 5′ ends of the 7S DNA are extended and map near CSB2 instead of at O_H. These findings are consistent with incomplete processing of the DNA part of the RNA–DNA primer at O_H in the absence of MGME1, which suggests that MGME1 is the deoxyribonuclease required for primer processing at O_H. As a consequence, MGME1 deficiency impairs ligation at O_H, and cells from MGME1 patients contain the same linear, deleted mtDNA fragment as the one observed in the exonuclease-deficient POLγ mouse (132).

It appears that both RNASEH1- and MGME1-dependent processing in the O_H region take place prior to the completion of H-strand DNA synthesis (137). In support of this notion, it should be noted that the 5′ end of the 7S DNA is also located at O_H, which implies that prematurely terminated replication products are processed in the same manner as full-length products. Why is a nearly 100-nt-long fragment removed from the 5′ end of the nascent H-strand DNA? We favor a model in which the shift of the free 5′ end from CSB2 to O_H helps secure proper ligation of the H-strand after replication. The region between LSP and the start of the displacement loop (D-loop) is actively transcribed, producing primers for the initiation of DNA synthesis, 7S RNA transcripts, and full-length polycistronic transcripts. The function of the 7S RNA is not known, but it is mostly discussed in terms of being a primer for the initiation of DNA replication at O_H (22, 138, 139). It appears unlikely that the 7S RNA could actually prime DNA synthesis because the transcript is polyadenylated at its 3′ end and not associated with mtDNA. Instead, we propose that the 7S RNA is the product of active termination at CSB1 (139). Ligation is a delicate process and DNA ligase III function may be disturbed by ongoing transcription. By moving the ligation away from CSB2 into a less-transcribed D-loop region, interference between transcription and ligation

at O_H may be minimized. Transcription termination at CSB1 may also secure the triple-stranded DNA structure of the D-loop region. In fact, the levels of transcription over the D-loop region may directly regulate the turnover rate of the D-loop because POLRMT will probably displace the annealed 7S DNA strand when initiating transcription at LSP and transcribing beyond the CSB2 region.

Other Factors That May Have a Role in Processing Mitochondrial Primers

In the nucleus, primer removal pathways typically involve displacement of the primer by the replicating DNA polymerase as it reaches the 5′ end of the RNA primer of a downstream Okazaki fragment (140). This could also be true for the processing of the primer at O_L, but not at O_H, because the processing seems to takes place prior to the completion of H-strand DNA synthesis, as discussed above. In the absence of a helicase, DNA polymerases have limited strand-displacement activity, and DNA synthesis can typically proceed for only a few nucleotides into the downstream dsDNA region, which leads to the creation of a short 5′ flap. This flap is cleaved by nucleases, such as FEN1 or DNA2, and these proteins have been localized to both mitochondria and the nucleus (141, 142). It is unclear whether these nucleases affect primer processing in mitochondria and, if so, how this occurs (137).

Regulation of mtDNA Replication and Copy Number Control

Replication of mtDNA may be regulated at four different levels, at least: (*a*) Regulation may occur through the initiation of transcription at LSP because transcription is a prerequisite for primer formation (30); (*b*) regulation may occur through termination or processing of the LSP transcript to generate a 3′ end in the CSB2 region to initiate DNA replication (53, 54); (*c*) regulation may occur by terminating DNA synthesis after approximately 650 nt to form the 7S DNA (139, 143); and (*d*) the regulation of the fraction of mtDNA molecules that are available for the initiation of DNA replication may occur by controlling the degree of mtDNA compaction (41, 144).

About two-thirds of all transcription events initiated at LSP in vitro are prematurely terminated at CSB2 by the formation of G-quadruplex structures in the nascent RNA (126, 127). The termination at CSB2 is also stimulated by a poly-dT stretch found just downstream of CSB2. The transcripts remain stably associated with DNA, forming an R-loop structure, and the stability of this structure depends on the formation of a G-quadruplex structure between the nascent DNA and the nontemplate strand (127, 128). It appears likely that the prematurely terminated transcripts are used as primers for initiating H-strand mtDNA synthesis because in this region their 3′ ends overlap with the transition points from RNA to DNA (126). However, we would emphasize that replication initiation at CSB2 has not yet been reconstituted in vitro, despite substantial efforts. Therefore, there may be missing factors that are essential for primer maturation.

Primer formation may be regulated by TEFM because this elongation factor strongly reduces transcription termination at CSB2 in vitro (53, 54). Potentially, the levels of active TEFM could govern the ratio between primer formation and full-length, productive transcription. Additional in vivo evidence is needed to substantiate this idea because experiments in cell lines have demonstrated that knockdown of TEFM has only very limited effects on the mtDNA copy number and mitochondrial replication intermediates (52).

As noted above, most replication events (95%) are terminated after about 650 nt; this occurs in a region termed the termination-associated sequence (TAS) (145, 146). The balance between abortive and genome-length mtDNA replication is, therefore, likely to be regulated at the end of the D-loop region rather than at O_H (139, 143). The 7S DNA resulting from abortive replication remains bound to its parental L-strand, whereas the nontemplate H-strand is displaced. As a

result, a triple-stranded D-loop structure is formed (110). Comparative genomics has identified two closely related 15-nt palindromic sequence motifs ($ATGN_9CAT$) on each side of the D-loop. The motifs are strongly conserved among vertebrates. One copy is located just upstream of the 5′ end of the 7S DNA, thus forming part of CSB1, whereas the other motif, denoted core-TAS, is located just downstream of the 3′ end of the 7S DNA (139). The exact function of these sequences is still not known, but palindromic sequences are often binding sites for sequence-specific DNA-binding proteins. Protein binding to TAS sequences has been identified by *in organello* footprinting analyses in both mouse and human mitochondria, and a yet-to-be-identified 48-kDa protein has been reported to bind the D-loop region in bovine mitochondria (147). The regulatory potential of the TAS region has also been underscored by the use of mitochondrial chromatin immunoprecipitation analysis to determine POLγ and TWINKLE occupancy (139). The two proteins are enriched in the D-loop region, which is consistent with this region being replicated much more frequently than the rest of the genome. Interestingly, TWINKLE levels are low at the 3′ end of the D-loop, but upon mild depletion of mtDNA, increased TWINKLE occupancy at this site correlates with decreased levels of the 7S DNA. This indicates that TWINKLE can be reloaded at the 3′ end of the D-loop, allowing the mitochondrial replication machinery to reinitiate DNA replication at the 3′ end of the 7S DNA, if required (139). Genetic experiments in the mouse also support the idea that TWINKLE is important for control of the mtDNA copy number. Increased expression of TWINKLE in the muscle and heart of mice increases mtDNA copy numbers up to threefold, whereas reduced expression of TWINKLE by RNA interference in human cells, or by conditional knockout of *Twinkle* in mice, leads to a profound drop in mtDNA copy numbers (96, 148–150).

PACKAGING mtDNA INTO MITOCHONDRIAL NUCLEOIDS

Mammalian mtDNA has a contour length of approximately 5 μm, whereas mitochondria typically have a width of approximately 0.5 μm (151). Thus, it is evident from space constraints alone that mtDNA needs to be compacted to fit inside mitochondria. In bacteria, genomes are packed into nucleoid structures by abundant small basic proteins, such as the DNA-bending integration host factor (152). Nucleoids in mammalian mitochondria have been visualized with many different microscopic approaches involving fluorescent dyes that bind DNA and fluorescently tagged nucleoid proteins (153). Initially, conventional light microscopy suggested that nucleoids contained several copies of mtDNA and had a diameter of approximately 200–300 nm (153), but the development of superresolution microscopy led to a radically changed view (43, 154). It was recognized that in a variety of mammalian cell types the nucleoid had a diameter of only approximately 100 nm (43, 154). Because nucleoids frequently cluster together, previous light microscopy studies had failed to properly resolve them individually (43). In fact, when the clustering was taken into account, approximately 60% more nucleoids were identified per mammalian cell in comparison with previous estimates (43). The number of mtDNA molecules per nucleoid observed has now been estimated to be approximately 1.4 in human cells and approximately 1.1–1.5 in mouse cells (43). Measuring mouse cells using superresolution microscopy has shown that they have a slightly elongated nucleoid shape, with a ratio between the lengths of the long and short axes of approximately 1.0–1.5 (43). Importantly, an increase in mtDNA copy numbers in mouse cells resulted in more nucleoids per cell, but the nucleoid size and shape, as assessed by superresolution microscopy, were unaltered (43).

The abundant TFAM protein is the major structural protein of the mammalian nucleoid. Other proteins also associate with the nucleoid, such as transcription and replication factors, but TFAM is present at a ratio of 1 subunit per 16–17 bp of mtDNA (155). The in vitro reconstitution of nucleoids—obtained by mixing TFAM and mtDNA, and followed by the use of rotary shadowing electron microscopy—has shown that TFAM alone can fully compact mtDNA into nucleoids

(42). Interestingly, single TFAM molecules bind mtDNA in patches (42), consistent with a cooperative binding mode (41, 45). TFAM seems to compact mtDNA by cross-strand binding and loop formation (**Figure 6**). Cryo–electron tomographic imaging of nucleoids in situ in bovine mitochondria has shown that they have an irregular shape, with dimensions of approximately 115 nm × 80 nm × 80 nm. Thus, the data predict that a single copy of mtDNA is fully coated and

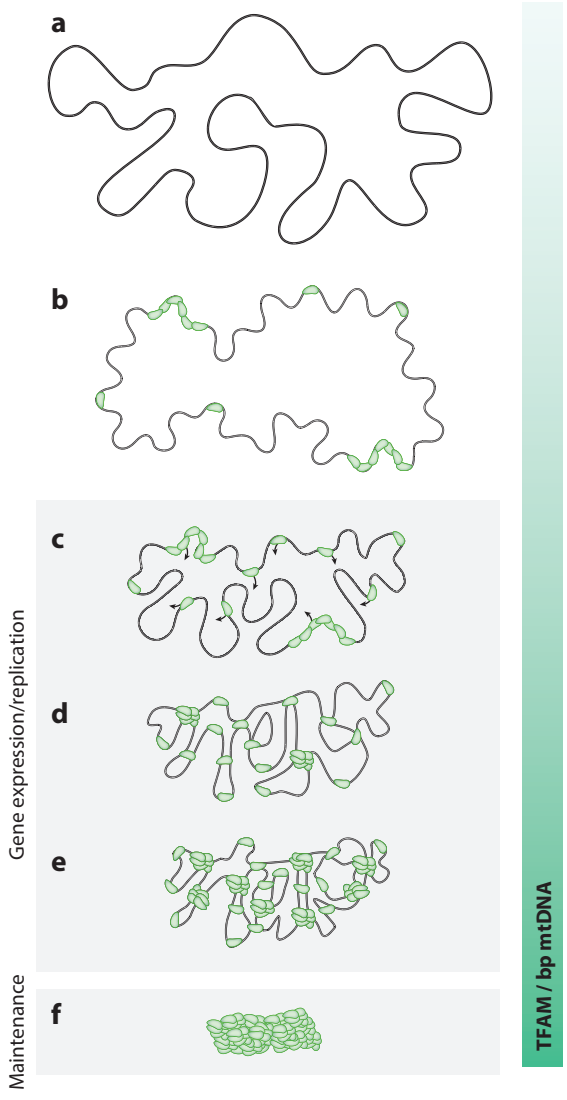

Figure 6

Model for packaging mtDNA into the mitochondrial nucleoid. (*a*) Naked mtDNA. (*b*) TFAM molecules (*green*) bind to mtDNA in short patches. (*c*) TFAM bridges neighboring mtDNA duplexes (*arrows*) by cross-strand binding. (*d*,*e*) In combination, mtDNA duplex bending and cross-strand binding by TFAM compact mtDNA. At this stage, mtDNA can still be used for replication and gene expression. (*f*) The final, tightly packaged mtDNA in the mitochondrial nucleoid. TFAM blocks DNA unwinding and makes mtDNA inaccessible to the replication and transcription machineries. Abbreviations: bp, base pairs; mtDNA, mitochondrial DNA; TFAM, mitochondrial transcription factor A.

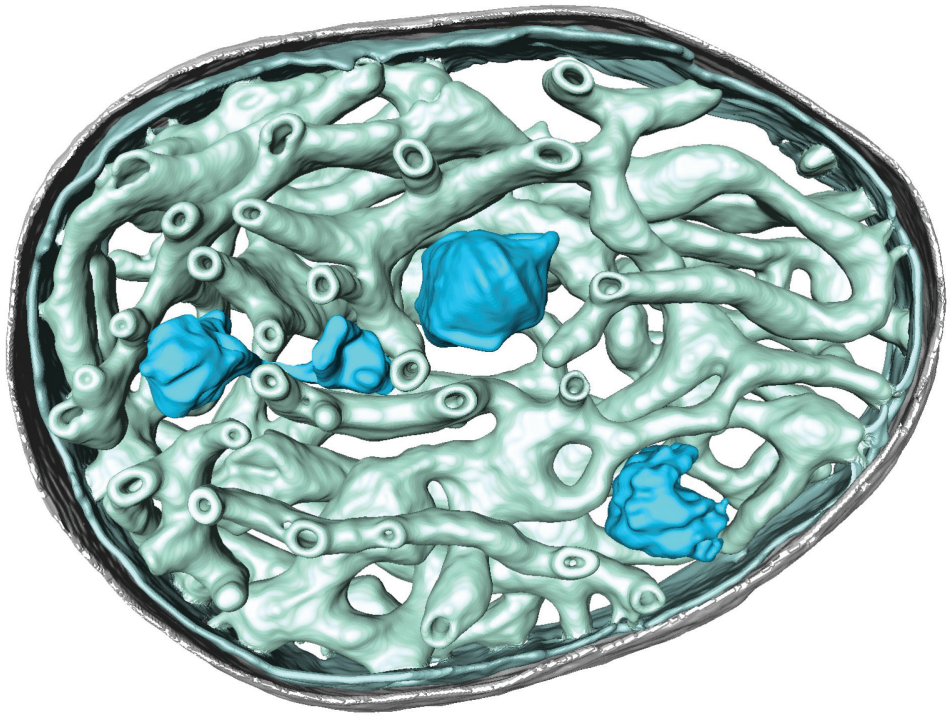

Figure 7

Mitochondrial nucleoids observed by cryo–electron tomography. The figure is a segmented, surface representation of a tomographic slice through the mitochondrion of a bovine heart. The mitochondrial nucleoids are shown in blue, and the green structures represent cristae. Figure kindly provided by Dr. Karen M. Davies and Dr. Werner Kühlbrandt.

compacted by TFAM to form an irregularly shaped, slightly elongated nucleoid (**Figure 7**). It will be important for future studies to determine whether mitochondrial nucleoids have specialized functions. It could be, perhaps, that the more compacted, slightly elongated nucleoids observed by superresolution microscopy represent a DNA storage form, whereas more elongated nucleoids harbor ongoing transcription or replication of mtDNA, or both. In support of this notion, at physiological ratios of TFAM to mtDNA, there are large variations in mtDNA compaction, from fully compacted nucleoids to naked DNA (41, 44). In compacted nucleoids, TFAM forms stable protein filaments on DNA that block DNA melting by POLRMT and TWINKLE. As a consequence, TFAM compaction can prevent the progression of the replication and transcription machineries (41). Thus, mtDNA compaction could be a way to regulate the number of mtDNA molecules involved in active transcription or mtDNA replication, or both. Small changes in the levels of TFAM protein may strongly affect the ratio between compacted nucleoids and open mtDNA molecules.

CONCLUSIONS AND FUTURE PROSPECTS

Great progress has been made during the past decade in defining the key components of the transcription and replication machineries in mammalian mitochondria. Not only have fundamental processes—such as transcription initiation, transcription elongation, transcription termination,

replication of the leading and lagging strands of mtDNA, and compaction of mtDNA into nucleoids—been reconstituted in vitro, but the importance of many of the key components has been validated in conditional mouse knockouts. Furthermore, an increasing number of atomic structures of components of the mtDNA transcription and replication machineries are available, and these have yielded an increased mechanistic understanding of these processes. The challenges for the future will be to understand how the activity of the transcription and replication machineries is regulated in response to physiological demand and disease. Does regulation simply depend on the regulated mitochondrial import of key components or is there direct, intramitochondrial cross talk that coordinates the different levels of control of mtDNA expression? How are the maintenance and expression of mtDNA regulated in response to the activity of the OXPHOS system and bioenergetic demands? There is a perfect correlation between the existence of a mitochondrial respiratory chain and the existence of mtDNA in all known eukaryotes, which implies that the control of the expression of key components of the OXPHOS system must occur in proximity to the respiratory chain.

SUMMARY POINTS

1. The mammalian mtDNA transcription-initiation machinery is a three-component system, consisting of POLRMT, TFB2M, and TFAM, which interacts with TEFM to promote transcription elongation.
2. Transcription is initiated from a dedicated promoter on each strand of mtDNA, that is, LSP and HSP.
3. Experimental data have called into question the existence of a second H-strand promoter.
4. MTERF1 promotes the termination of LSP transcription to prevent elongating transcription from generating antisense transcripts to the rRNA genes and to control regional interference.
5. The minimal mammalian mtDNA replisome consists of POLγA, POLγB, TWINKLE, and mtSSB, and it can replicate both strands of mtDNA.
6. POLRMT is likely the primase needed for the initiation of mtDNA replication at O_H and O_L.
7. There is strong experimental support for the hypothesis that mtDNA replication occurs by an asymmetric, strand-displacement mode.
8. Single molecules of mtDNA are packaged by TFAM into a slightly elongated, irregularly shaped nucleoid structure of approximately 80–100 nm.

FUTURE ISSUES

1. Additional experiments are needed to determine whether increased overall mtDNA transcription involves transcriptional activators, which stimulate the basal mtDNA transcription machinery or the recruitment of the transcription machinery to additional mtDNA templates, or both.
2. Molecular definition is needed of the transcription termination event that prevents transcription initiated at HSP from reaching the control region.

3. The role of the puzzling N-terminal domain region of POLRMT should be clarified using combined biochemical and genetic approaches.
4. It should be elucidated if intramitochondrial molecular cross talk exists to coordinate processes such as replication, transcription, and translation.
5. The mechanism regulating the degree of compaction of the mitochondrial nucleoid needs to be defined to gain an understanding of how a subset of nucleoids is selected for transcription or replication in mtDNA.
6. Additional experiments must determine whether a specific molecular machinery is needed for the distribution of mitochondrial nucleoids.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by the Swedish Research Council (grants to M.F., N.-G.L., and C.M.G.), the Swedish Cancer Society (grants to C.M.G. and M.F.), Deutsche Forschungsgemeinschaft, SFB 829 (grant to N.-G.L.), a European Research Council Advanced Investigator Grant (to N.-G.L. and C.M.G.), a European Research Council Starting Investigator Grant (to M.F.), and the Knut and Alice Wallenberg foundation (grants to M.F., N.-G.L., and C.M.G.). We thank Jennifer Uhler and Annika Röhl for the illustrations.

LITERATURE CITED

1. Martin WF, Garg S, Zimorski V. 2015. Endosymbiotic theories for eukaryote origin. *Philos. Trans. R. Soc. Lond. Ser. B* 370:20140330
2. Stewart JB, Larsson NG. 2014. Keeping mtDNA in shape between generations. *PLOS Genet.* 10:e1004670
3. Hayashi J, Ohta S, Kikuchi A, Takemitsu M, Goto Y, Nonaka I. 1991. Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *PNAS* 88:10614–18
4. Larsson NG, Clayton DA. 1995. Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet.* 29:151–78
5. Larsson NG, Garman JD, Oldfors A, Barsh GS, Clayton DA. 1996. A single mouse gene encodes the mitochondrial transcription factor A and a testis-specific nuclear HMG-box protein. *Nat. Genet.* 13:296–302
6. Larsson NG, Oldfors A, Garman JD, Barsh GS, Clayton DA. 1997. Down-regulation of mitochondrial transcription factor A during spermatogenesis in humans. *Hum. Mol. Genet.* 6:185–91
7. Kaneda H, Hayashi J, Takahama S, Taya C, Lindahl KF, Yonekawa H. 1995. Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *PNAS* 92:4542–46
8. Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. 1999. Ubiquitin tag for sperm mitochondria. *Nature* 402:371–72
9. Ebert KM, Liem H, Hecht NB. 1988. Mitochondrial DNA in the mouse preimplantation embryo. *J. Reprod. Fertil.* 82:145–49
10. Larsson NG. 2010. Somatic mitochondrial DNA mutations in mammalian aging. *Annu. Rev. Biochem.* 79:683–706

11. Hauswirth WW, Laipis PJ. 1982. Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. *PNAS* 79:4686–90
12. Stewart JB, Freyer C, Elson JL, Wredenberg A, Cansu Z, et al. 2008. Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLOS Biol.* 6:e10
13. Ross JM, Stewart JB, Hagstrom E, Brene S, Mourier A, et al. 2013. Germline mitochondrial DNA mutations aggravate ageing and can impair brain development. *Nature* 501:412–15
14. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, et al. 1998. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* 18:231–36
15. Sickmann A, Reinders J, Wagner Y, Joppich C, Zahedi R, et al. 2003. The proteome of *Saccharomyces cerevisiae* mitochondria. *PNAS* 100:13207–12
16. Foster LJ, de Hoog CL, Zhang Y, Zhang Y, Xie X, et al. 2006. A mammalian organelle map by protein correlation profiling. *Cell* 125:187–99
17. Hallberg BM, Larsson NG. 2014. Making proteins in the powerhouse. *Cell Metab.* 20:226–40
18. Berk AJ, Clayton DA. 1974. Mechanism of mitochondrial DNA replication in mouse L-cells: asynchronous replication of strands, segregation of circular daughter molecules, aspects of topology and turnover of an initiation sequence. *J. Mol. Biol.* 86:801–24
19. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, et al. 1981. Sequence and organization of the human mitochondrial genome. *Nature* 290:457–65
20. Bibb MJ, Van Etten RA, Wright CT, Walberg MW, Clayton DA. 1981. Sequence and gene organization of mouse mitochondrial DNA. *Cell* 26:167–80
21. Ojala D, Montoya J, Attardi G. 1981. tRNA punctuation model of RNA processing in human mitochondria. *Nature* 290:470–74
22. Falkenberg M, Larsson NG, Gustafsson CM. 2007. DNA replication and transcription in mammalian mitochondria. *Annu. Rev. Biochem.* 76:679–99
23. Cermakian N, Ikeda TM, Cedergren R, Gray MW. 1996. Sequences homologous to yeast mitochondrial and bacteriophage T3 and T7 RNA polymerases are widespread throughout the eukaryotic lineage. *Nucleic Acids Res.* 24:648–54
24. Gray MW, Burger G, Lang BF. 1999. Mitochondrial evolution. *Science* 283:1476–81
25. Shutt TE, Gray MW. 2006. Bacteriophage origins of mitochondrial replication and transcription proteins. *Trends Genet.* 22:90–95
26. Kelly JL, Lehman IR. 1986. Yeast mitochondrial RNA polymerase: purification and properties of the catalytic subunit. *J. Biol. Chem.* 261:10340–47
27. Masters BS, Stohl LL, Clayton DA. 1987. Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* 51:89–99
28. Ringel R, Sologub M, Morozov YI, Litonin D, Cramer P, Temiakov D. 2011. Structure of human mitochondrial RNA polymerase. *Nature* 478:269–73
29. Schwinghammer K, Cheung AC, Morozov YI, Agaronyan K, Temiakov D, Cramer P. 2013. Structure of human mitochondrial RNA polymerase elongation complex. *Nat. Struct. Mol. Biol.* 20:1298–303
30. Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM. 2002. Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat. Genet.* 31:289–94
31. Sologub M, Litonin D, Anikin M, Mustaev A, Temiakov D. 2009. TFB2 is a transient component of the catalytic site of the human mitochondrial RNA polymerase. *Cell* 139:934–44
32. Mangus DA, Jang SH, Jaehning JA. 1994. Release of the yeast mitochondrial RNA polymerase specificity factor from transcription complexes. *J. Biol. Chem.* 269:26568–74
33. Shutt TE, Gray MW. 2006. Homologs of mitochondrial transcription factor B, sparsely distributed within the eukaryotic radiation, are likely derived from the dimethyladenosine methyltransferase of the mitochondrial endosymbiont. *Mol. Biol. Evol.* 23:1169–79
34. Moustafa IM, Uchida A, Wang Y, Yennawar N, Cameron CE. 2015. Structural models of mammalian mitochondrial transcription factor B2. *Biochim. Biophys. Acta* 1849:987–1002
35. Guja KE, Venkataraman K, Yakubovskaya E, Shi H, Mejia E, et al. 2013. Structural basis for S-adenosylmethionine binding and methyltransferase activity by mitochondrial transcription factor B1. *Nucleic Acids Res.* 41:7947–59

36. Carrodeguas JA, Theis K, Bogenhagen DF, Kisker C. 2001. Crystal structure and deletion analysis show that the accessory subunit of mammalian DNA polymerase γ, Polγ B, functions as a homodimer. *Mol. Cell* 7:43–54
37. Metodiev MD, Lesko N, Park CB, Camara Y, Shi Y, et al. 2009. Methylation of 12S rRNA is necessary for in vivo stability of the small subunit of the mammalian mitochondrial ribosome. *Cell Metab.* 9:386–97
38. Shi Y, Dierckx A, Wanrooij PH, Wanrooij S, Larsson NG, et al. 2012. Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. *PNAS* 109:16510–15
39. Ngo HB, Kaiser JT, Chan DC. 2011. The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA. *Nat. Struct. Mol. Biol.* 18:1290–96
40. Jimenez-Menendez N, Fernandez-Millan P, Rubio-Cosials A, Arnan C, Montoya J, et al. 2010. Human mitochondrial mTERF wraps around DNA through a left-handed superhelical tandem repeat. *Nat. Struct. Mol. Biol.* 17:891–93
41. Farge G, Mehmedovic M, Baclayon M, van den Wildenberg SM, Roos WH, et al. 2014. In vitro-reconstituted nucleoids can block mitochondrial DNA replication and transcription. *Cell Rep.* 8:66–74
42. Kukat C, Davies KM, Wurm CA, Spahr H, Bonekamp NA, et al. 2015. Cross-strand binding of TFAM to a single mtDNA molecule forms the mitochondrial nucleoid. *PNAS* 112:11288–93
43. Kukat C, Wurm CA, Spahr H, Falkenberg M, Larsson NG, Jakobs S. 2011. Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *PNAS* 108:13534–39
44. Kaufman BA, Durisic N, Mativetsky JM, Costantino S, Hancock MA, et al. 2007. The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures. *Mol. Biol. Cell* 18:3225–36
45. Farge G, Laurens N, Broekmans OD, van den Wildenberg SM, Dekker LC, et al. 2012. Protein sliding and DNA denaturation are essential for DNA organization by human mitochondrial transcription factor A. *Nat. Commun.* 3:1013
46. Traverso JJ, Manoranjan VS, Bishop AR, Rasmussen KO, Voulgarakis NK. 2015. Allostery through protein-induced DNA bubbles. *Sci. Rep.* 5:9037
47. Yakubovskaya E, Guja KE, Eng ET, Choi WS, Mejia E, et al. 2014. Organization of the human mitochondrial transcription initiation complex. *Nucleic Acids Res.* 42:4100–12
48. Posse V, Hoberg E, Dierckx A, Shahzad S, Koolmeister C, et al. 2014. The amino terminal extension of mammalian mitochondrial RNA polymerase ensures promoter specific transcription initiation. *Nucleic Acids Res.* 42:3638–47
49. Morozov YI, Agaronyan K, Cheung AC, Anikin M, Cramer P, Temiakov D. 2014. A novel intermediate in transcription initiation by human mitochondrial RNA polymerase. *Nucleic Acids Res.* 42:3884–93
50. Morozov YI, Parshin AV, Agaronyan K, Cheung AC, Anikin M, et al. 2015. A model for transcription initiation in human mitochondria. *Nucleic Acids Res.* 43:3726–35
51. Lodeiro MF, Uchida AU, Arnold JJ, Reynolds SL, Moustafa IM, Cameron CE. 2010. Identification of multiple rate-limiting steps during the human mitochondrial transcription cycle in vitro. *J. Biol. Chem.* 285:16387–402
52. Minczuk M, He J, Duch AM, Ettema TJ, Chlebowski A, et al. 2011. TEFM (c17orf42) is necessary for transcription of human mtDNA. *Nucleic Acids Res.* 39:4284–99
53. Posse V, Shahzad S, Falkenberg M, Hallberg BM, Gustafsson CM. 2015. TEFM is a potent stimulator of mitochondrial transcription elongation in vitro. *Nucleic Acids Res.* 43:2615–24
54. Agaronyan K, Morozov YI, Anikin M, Temiakov D. 2015. Mitochondrial biology: replication-transcription switch in human mitochondria. *Science* 347:548–51
55. Montoya J, Christianson T, Levens D, Rabinowitz M, Attardi G. 1982. Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. *PNAS* 79:7195–99
56. Litonin D, Sologub M, Shi Y, Savkina M, Anikin M, et al. 2010. Human mitochondrial transcription revisited: Only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro. *J. Biol. Chem.* 285:18129–33
57. Hixson JE, Clayton DA. 1985. Initiation of transcription from each of the two human mitochondrial promoters requires unique nucleotides at the transcriptional start sites. *PNAS* 82:2660–64

58. Christianson TW, Clayton DA. 1986. In vitro transcription of human mitochondrial DNA: Accurate termination requires a region of DNA sequence that can function bidirectionally. *PNAS* 83:6277–81
59. Lodeiro MF, Uchida A, Bestwick M, Moustafa IM, Arnold JJ, et al. 2012. Transcription from the second heavy-strand promoter of human mtDNA is repressed by transcription factor A in vitro. *PNAS* 109:6513–18
60. Christianson TW, Clayton DA. 1988. A tridecamer DNA sequence supports human mitochondrial RNA 3′-end formation in vitro. *Mol. Cell. Biol.* 8:4502–9
61. Camasamudram V, Fang JK, Avadhani NG. 2003. Transcription termination at the mouse mitochondrial H-strand promoter distal site requires an A/T rich sequence motif and sequence specific DNA binding proteins. *Eur. J. Biochem.* 270:1128–40
62. Kruse B, Narasimhan N, Attardi G. 1989. Termination of transcription in human mitochondria: identification and purification of a DNA binding protein factor that promotes termination. *Cell* 58:391–97
63. Fernandez-Silva P, Martinez-Azorin F, Micol V, Attardi G. 1997. The human mitochondrial transcription termination factor (mTERF) is a multizipper protein but binds to DNA as a monomer, with evidence pointing to intramolecular leucine zipper interactions. *EMBO J.* 16:1066–79
64. Terzioglu M, Ruzzenente B, Harmel J, Mourier A, Jemt E, et al. 2013. MTERF1 binds mtDNA to prevent transcriptional interference at the light-strand promoter but is dispensable for rRNA gene transcription regulation. *Cell Metab.* 17:618–26
65. Asin-Cayuela J, Schwend T, Farge G, Gustafsson CM. 2005. The human mitochondrial transcription termination factor (mTERF) is fully active in vitro in the non-phosphorylated form. *J. Biol. Chem.* 280:25499–505
66. Linder T, Park CB, Asin-Cayuela J, Pellegrini M, Larsson NG, et al. 2005. A family of putative transcription termination factors shared amongst metazoans and plants. *Curr. Genet.* 48:265–69
67. Yakubovskaya E, Mejia E, Byrnes J, Hambardjieva E, Garcia-Diaz M. 2010. Helix unwinding and base flipping enable human MTERF1 to terminate mitochondrial transcription. *Cell* 141:982–93
68. Spahr H, Habermann B, Gustafsson CM, Larsson NG, Hallberg BM. 2012. Structure of the human MTERF4–NSUN4 protein complex that regulates mitochondrial ribosome biogenesis. *PNAS* 109:15253–58
69. Yakubovskaya E, Guja KE, Mejia E, Castano S, Hambardjieva E, et al. 2012. Structure of the essential MTERF4:NSUN4 protein complex reveals how an MTERF protein collaborates to facilitate rRNA modification. *Structure* 20:1940–47
70. Spahr H, Samuelsson T, Hallberg BM, Gustafsson CM. 2010. Structure of mitochondrial transcription termination factor 3 reveals a novel nucleic acid-binding domain. *Biochem. Biophys. Res. Commun.* 397:386–90
71. Park CB, Asin-Cayuela J, Camara Y, Shi Y, Pellegrini M, et al. 2007. MTERF3 is a negative regulator of mammalian mtDNA transcription. *Cell* 130:273–85
72. Pellegrini M, Asin-Cayuela J, Erdjument-Bromage H, Tempst P, Larsson NG, Gustafsson CM. 2009. MTERF2 is a nucleoid component in mammalian mitochondria. *Biochim. Biophys. Acta* 1787:296–302
73. Camara Y, Asin-Cayuela J, Park CB, Metodiev MD, Shi Y, et al. 2011. MTERF4 regulates translation by targeting the methyltransferase NSUN4 to the mammalian mitochondrial ribosome. *Cell Metab.* 13:527–39
74. Wredenberg A, Lagouge M, Bratic A, Metodiev MD, Spahr H, et al. 2013. MTERF3 regulates mitochondrial ribosome biogenesis in invertebrates and mammals. *PLOS Genet.* 9:e1003178
75. Metodiev MD, Spahr H, Loguercio Polosa P, Meharg C, Becker C, et al. 2014. NSUN4 is a dual function mitochondrial protein required for both methylation of 12S rRNA and coordination of mitoribosomal assembly. *PLOS Genet.* 10:e1004110
76. Ameur A, Stewart JB, Freyer C, Hagstrom E, Ingman M, et al. 2011. Ultra-deep sequencing of mouse mitochondrial DNA: mutational patterns and their origins. *PLOS Genet.* 7:e1002028
77. Zheng W, Khrapko K, Coller HA, Thilly WG, Copeland WC. 2006. Origins of human mitochondrial point mutations as DNA polymerase γ-mediated errors. *Mutat. Res.* 599:11–20
78. Gray H, Wong TW. 1992. Purification and identification of subunit structure of the human mitochondrial DNA polymerase. *J. Biol. Chem.* 267:5835–41

79. Yakubovskaya E, Chen Z, Carrodeguas JA, Kisker C, Bogenhagen DF. 2006. Functional human mitochondrial DNA polymerase γ forms a heterotrimer. *J. Biol. Chem.* 281:374–82
80. Fan L, Kim S, Farr CL, Schaefer KT, Randolph KM, et al. 2006. A novel processive mechanism for DNA synthesis revealed by structure, modeling and mutagenesis of the accessory subunit of human mitochondrial DNA polymerase. *J. Mol. Biol.* 358:1229–43
81. Hance N, Ekstrand MI, Trifunovic A. 2005. Mitochondrial DNA polymerase γ is essential for mammalian embryogenesis. *Hum. Mol. Genet.* 14:1775–83
82. Humble MM, Young MJ, Foley JF, Pandiri AR, Travlos GS, Copeland WC. 2013. *Polg2* is essential for mammalian embryogenesis and is required for mtDNA maintenance. *Hum. Mol. Genet.* 22:1017–25
83. Garcia-Gomez S, Reyes A, Martinez-Jimenez MI, Chocron ES, Mouron S, et al. 2013. PrimPol, an archaic primase/polymerase operating in human cells. *Mol. Cell* 52:541–53
84. Martinez-Jimenez MI, Garcia-Gomez S, Bebenek K, Sastre-Moreno G, Calvo PA, et al. 2015. Alternative solutions and new scenarios for translesion DNA synthesis by human PrimPol. *DNA Repair* 29:127–38
85. Kornberg A, Baker TA. 1992. *DNA Replication*. New York: Freeman. 2nd ed.
86. Fridlender B, Weissbach A. 1971. DNA polymerases of tumor virus: specific effect of ethidium bromide on the use of different synthetic templates. *PNAS* 68:3116–19
87. Beese LS, Derbyshire V, Steitz TA. 1993. Structure of DNA polymerase I Klenow fragment bound to duplex DNA. *Science* 260:352–55
88. Longley MJ, Nguyen D, Kunkel TA, Copeland WC. 2001. The fidelity of human DNA polymerase γ with and without exonucleolytic proofreading and the p55 accessory subunit. *J. Biol. Chem.* 276:38555–62
89. Pinz KG, Bogenhagen DF. 2000. Characterization of a catalytically slow AP lyase activity in DNA polymerase γ and other family A DNA polymerases. *J. Biol. Chem.* 275:12509–14
90. Pinz KG, Bogenhagen DF. 2006. The influence of the DNA polymerase γ accessory subunit on base excision repair by the catalytic subunit. *DNA Repair* 5:121–28
91. Carrodeguas JA, Pinz KG, Bogenhagen DF. 2002. DNA binding properties of human pol γB. *J. Biol. Chem.* 277:50008–14
92. Farge G, Pham XH, Holmlund T, Khorostov I, Falkenberg M. 2007. The accessory subunit B of DNA polymerase γ is required for mitochondrial replisome function. *Nucleic Acids Res.* 35:902–11
93. Ravichandran V, Vasquez GB, Srivastava S, Verma M, Petricoin E, et al. 2004. Data standards for proteomics: mitochondrial two-dimensional polyacrylamide gel electrophoresis data as a model system. *Mitochondrion* 3:327–36
94. Korhonen JA, Pham XH, Pellegrini M, Falkenberg M. 2004. Reconstitution of a minimal mtDNA replisome in vitro. *EMBO J.* 23:2423–29
95. Macao B, Uhler JP, Siibak T, Zhu X, Shi Y, et al. 2015. The exonuclease activity of DNA polymerase γ is required for ligation during mitochondrial DNA replication. *Nat. Commun.* 6:7303
96. Milenkovic D, Matic S, Kuhl I, Ruzzenente B, Freyer C, et al. 2013. TWINKLE is an essential mitochondrial helicase required for synthesis of nascent D-loop strands and complete mtDNA replication. *Hum. Mol. Genet.* 22:1983–93
97. Spelbrink JN, Li FY, Tiranti V, Nikali K, Yuan QP, et al. 2001. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.* 28:223–31
98. Shutt TE, Gray MW. 2006. Twinkle, the mitochondrial replicative DNA helicase, is widespread in the eukaryotic radiation and may also be the mitochondrial DNA primase in most eukaryotes. *J. Mol. Evol.* 62:588–99
99. Wanrooij S, Fuste JM, Farge G, Shi Y, Gustafsson CM, Falkenberg M. 2008. Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. *PNAS* 105:11122–27
100. Fuste JM, Wanrooij S, Jemt E, Granycome CE, Cluett TJ, et al. 2010. Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication. *Mol. Cell* 37:67–78
101. Korhonen JA, Gaspari M, Falkenberg M. 2003. TWINKLE has 5′ → 3′ DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J. Biol. Chem.* 278:48627–32
102. Korhonen JA, Pande V, Holmlund T, Farge G, Pham XH, et al. 2008. Structure–function defects of the TWINKLE linker region in progressive external ophthalmoplegia. *J. Mol. Biol.* 377:691–705

103. Kaguni LS. 2004. DNA polymerase γ, the mitochondrial replicase. *Annu. Rev. Biochem.* 73:293–320
104. Champoux JJ. 2001. DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.* 70:369–413
105. Zhang H, Barcelo JM, Lee B, Kohlhagen G, Zimonjic DB, et al. 2001. Human mitochondrial topoisomerase I. *PNAS* 98:10608–13
106. Zhang H, Pommier Y. 2008. Mitochondrial topoisomerase I sites in the regulatory D-loop region of mitochondrial DNA. *Biochemistry* 47:11196–203
107. Sobek S, Dalla Rosa I, Pommier Y, Bornholz B, Kalfalah F, et al. 2013. Negative regulation of mitochondrial transcription by mitochondrial topoisomerase I. *Nucleic Acids Res.* 41:9848–57
108. Zhang H, Zhang YW, Yasukawa T, Dalla Rosa I, Khiati S, Pommier Y. 2014. Increased negative supercoiling of mtDNA in TOP1mt knockout mice and presence of topoisomerases IIα and IIβ in vertebrate mitochondria. *Nucleic Acids Res.* 42:7259–67
109. Copeland WC. 2014. Defects of mitochondrial DNA replication. *J. Child Neurol.* 29:1216–24
110. Clayton DA. 1991. Replication and transcription of vertebrate mitochondrial DNA. *Annu. Rev. Cell Biol.* 7:453–78
111. Miralles Fuste J, Shi Y, Wanrooij S, Zhu X, Jemt E, et al. 2014. In vivo occupancy of mitochondrial single-stranded DNA binding protein supports the strand displacement mode of DNA replication. *PLOS Genet.* 10:e1004832
112. Berk AJ, Clayton DA. 1976. Mechanism of mitochondrial DNA replication in mouse L-cells: topology of circular daughter molecules and dynamics of catenated oligomer formation. *J. Mol. Biol.* 100:85–92
113. Bogenhagen DF, Clayton DA. 2003. The mitochondrial DNA replication bubble has not burst. *Trends Biochem. Sci.* 28:357–60
114. Wanrooij S, Miralles Fuste J, Stewart JB, Wanrooij PH, Samuelsson T, et al. 2012. In vivo mutagenesis reveals that OriL is essential for mitochondrial DNA replication. *EMBO Rep.* 13:1130–37
115. Stewart JB, Alaei-Mahabadi B, Sabarinathan R, Samuelsson T, Gorodkin J, et al. 2015. Simultaneous DNA and RNA mapping of somatic mitochondrial mutations across diverse human cancers. *PLOS Genet.* 11:e1005333
116. Ju YS, Alexandrov LB, Gerstung M, Martincorena I, Nik-Zainal S, et al. 2014. Origins and functional consequences of somatic mitochondrial DNA mutations in human cancer. *eLife* 3:e02935
117. Holt IJ, Lorimer HE, Jacobs HT. 2000. Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell* 100:515–24
118. Yasukawa T, Yang MY, Jacobs HT, Holt IJ. 2005. A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. *Mol. Cell* 18:651–62
119. Yasukawa T, Reyes A, Cluett TJ, Yang MY, Bowmaker M, et al. 2006. Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *EMBO J.* 25:5358–71
120. Yang MY, Bowmaker M, Reyes A, Vergani L, Angeli P, et al. 2002. Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. *Cell* 111:495–505
121. Reyes A, Kazak L, Wood SR, Yasukawa T, Jacobs HT, Holt IJ. 2013. Mitochondrial DNA replication proceeds via a 'bootlace' mechanism involving the incorporation of processed transcripts. *Nucleic Acids Res.* 41:5837–50
122. Holt IJ, Jacobs HT. 2014. Unique features of DNA replication in mitochondria: a functional and evolutionary perspective. *BioEssays* 36:1024–31
123. Jacob F, Brenner S, Cuzin F. 1963. On the regulation of DNA replication in bacteria. *Cold Spring Harb. Symp. Quant. Biol.* 28:329–48
124. Kang D, Miyako K, Kai Y, Irie T, Takeshige K. 1997. In vivo determination of replication origins of human mitochondrial DNA by ligation-mediated polymerase chain reaction. *J. Biol. Chem.* 272:15275–79
125. Xu B, Clayton DA. 1995. A persistent RNA–DNA hybrid is formed during transcription at a phylogenetically conserved mitochondrial DNA sequence. *Mol. Cell. Biol.* 15:580–89
126. Pham XH, Farge G, Shi Y, Gaspari M, Gustafsson CM, Falkenberg M. 2006. Conserved sequence box II directs transcription termination and primer formation in mitochondria. *J. Biol. Chem.* 281:24647–52

127. Wanrooij PH, Uhler JP, Simonsson T, Falkenberg M, Gustafsson CM. 2010. G-quadruplex structures in RNA stimulate mitochondrial transcription termination and primer formation. *PNAS* 107:16072–77
128. Wanrooij PH, Uhler JP, Shi Y, Westerlund F, Falkenberg M, Gustafsson CM. 2012. A hybrid G-quadruplex structure formed between RNA and DNA explains the extraordinary stability of the mitochondrial R-loop. *Nucleic Acids Res.* 40:10334–44
129. Lakshmipathy U, Campbell C. 1999. The human DNA ligase III gene encodes nuclear and mitochondrial proteins. *Mol. Cell. Biol.* 19:3869–76
130. Puebla-Osorio N, Lacey DB, Alt FW, Zhu C. 2006. Early embryonic lethality due to targeted inactivation of DNA ligase III. *Mol. Cell. Biol.* 26:3935–41
131. He Q, Shumate CK, White MA, Molineux IJ, Yin YW. 2013. Exonuclease of human DNA polymerase γ disengages its strand displacement function. *Mitochondrion* 13:592–601
132. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, et al. 2004. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 429:417–23
133. Cerritelli SM, Frolova EG, Feng C, Grinberg A, Love PE, Crouch RJ. 2003. Failure to produce mitochondrial DNA results in embryonic lethality in *Rnaseh1* null mice. *Mol. Cell* 11:807–15
134. Holmes JB, Akman G, Wood SR, Sakhuja K, Cerritelli SM, et al. 2015. Primer retention owing to the absence of RNase H1 is catastrophic for mitochondrial DNA replication. *PNAS* 112:9334–39
135. Kornblum C, Nicholls TJ, Haack TB, Scholer S, Peeva V, et al. 2013. Loss-of-function mutations in *MGME1* impair mtDNA replication and cause multisystemic mitochondrial disease. *Nat. Genet.* 45:214–19
136. Nicholls TJ, Zsurka G, Peeva V, Scholer S, Szczesny RJ, et al. 2014. Linear mtDNA fragments and unusual mtDNA rearrangements associated with pathological deficiency of MGME1 exonuclease. *Hum. Mol. Genet.* 23:6147–62
137. Uhler JP, Falkenberg M. 2015. Primer removal during mammalian mitochondrial DNA replication. *DNA Repair* 34:28–38
138. Nicholls TJ, Minczuk M. 2014. In D-loop: 40 years of mitochondrial 7S DNA. *Exp. Gerontol.* 56:175–81
139. Jemt E, Persson O, Shi Y, Mehmedovic M, Uhler JP, et al. 2015. Regulation of DNA replication at the end of the mitochondrial D-loop involves the helicase TWINKLE and a conserved sequence element. *Nucleic Acids Res.* 43:9262–75
140. Garg P, Stith CM, Sabouri N, Johansson E, Burgers PM. 2004. Idling by DNA polymerase δ maintains a ligatable nick during lagging-strand DNA replication. *Genes Dev.* 18:2764–73
141. Liu P, Qian L, Sung JS, de Souza-Pinto NC, Zheng L, et al. 2008. Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria. *Mol. Cell. Biol.* 28:4975–87
142. Zheng L, Zhou M, Guo Z, Lu H, Qian L, et al. 2008. Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. *Mol. Cell* 32:325–36
143. Brown TA, Clayton DA. 2002. Release of replication termination controls mitochondrial DNA copy number after depletion with 2′,3′-dideoxycytidine. *Nucleic Acids Res.* 30:2004–10
144. Pohjoismaki JL, Wanrooij S, Hyvarinen AK, Goffart S, Holt IJ, et al. 2006. Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells. *Nucleic Acids Res.* 34:5815–28
145. Doda JN, Wright CT, Clayton DA. 1981. Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. *PNAS* 78:6116–20
146. Bogenhagen D, Clayton DA. 1978. Mechanism of mitochondrial DNA replication in mouse L-cells: kinetics of synthesis and turnover of the initiation sequence. *J. Mol. Biol.* 119:49–68
147. Roberti M, Musicco C, Polosa PL, Milella F, Gadaleta MN, Cantatore P. 1998. Multiple protein-binding sites in the TAS-region of human and rat mitochondrial DNA. *Biochem. Biophys. Res. Commun.* 243:36–40
148. Tyynismaa H, Sembongi H, Bokori-Brown M, Granycome C, Ashley N, et al. 2004. Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number. *Hum. Mol. Genet.* 13:3219–27
149. Ylikallio E, Tyynismaa H, Tsutsui H, Ide T, Suomalainen A. 2010. High mitochondrial DNA copy number has detrimental effects in mice. *Hum. Mol. Genet.* 19:2695–705
150. Ikeda M, Ide T, Fujino T, Arai S, Saku K, et al. 2015. Overexpression of TFAM or Twinkle increases mtDNA copy number and facilitates cardioprotection associated with limited mitochondrial oxidative stress. *PLOS ONE* 10:e0119687

151. Nass MM. 1966. The circularity of mitochondrial DNA. *PNAS* 56:1215–22
152. Dame RT, Espeli O, Grainger DC, Wiggins PA. 2012. Multidisciplinary perspectives on bacterial genome organization and dynamics. *Mol. Microbiol.* 86:1023–30
153. Kukat C, Larsson NG. 2013. mtDNA makes a U-turn for the mitochondrial nucleoid. *Trends Cell Biol.* 23:457–63
154. Brown TA, Tkachuk AN, Shtengel G, Kopek BG, Bogenhagen DF, et al. 2011. Superresolution fluorescence imaging of mitochondrial nucleoids reveals their spatial range, limits, and membrane interaction. *Mol. Cell. Biol.* 31:4994–5010
155. Bogenhagen DF. 2012. Mitochondrial DNA nucleoid structure. *Biochim. Biophys. Acta* 1819:914–20