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Mitochondrial transcription: Lessons from mouse models

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Abstract

Mammalian mitochondrial DNA (mtDNA) is a circular double-stranded DNA genome of ~ 16.5 kilobase pairs (kb) that encodes 13 catalytic proteins of the ATP-producing oxidative phosphorylation system (OXPHOS), and the rRNAs and tRNAs required for the translation of the mtDNA transcripts. All the components needed for transcription and replication of the mtDNA are, therefore, encoded in the nuclear genome, as are the remaining components of the OXPHOS system and the mitochondrial translation machinery. Regulation of mtDNA gene expression is very important for modulating the OXPHOS capacity in response to metabolic requirements and in pathological processes. The combination of *in vitro* and *in vivo* studies has allowed the identification of the core machinery required for basal mtDNA transcription in mammals and a few proteins that regulate mtDNA transcription. Specifically, the generation of knockout mouse strains in the last several years, has been key to understanding the basis of mtDNA transcription *in vivo*. However, it is well accepted that many components of the transcription machinery are still unknown and little is known about mtDNA gene expression regulation under different metabolic requirements or disease processes. In this review we will focus on how the creation of knockout mouse models and the study of their phenotypes have contributed to the understanding of mitochondrial transcription in mammals.

2. Introduction

Mitochondria are the main sites of aerobic oxidation where important cellular functions including pyruvate and fatty acid oxidation, nitrogen metabolism and heme biosynthesis take place [1]. Electron transport chain (ETC) and the oxidative phosphorylation (OXPHOS) system, which are embedded in the inner mitochondrial membrane, provide most of cellular energy in the form of ATP [2, 3]. NADH and FADH₂ are generated in the mitochondrial matrix as reducing power for ATP biosynthesis or thermogenesis [1]. The mitochondria genome in mammalian cells codes for a small number of important factors. Both strands have their own promoter and are transcribed as polygenic transcription units, specifying multiple RNAs (mRNA, rRNA, or tRNA). Because of its limited coding capacity, the mitochondrial DNA (mtDNA) relies on nuclear genes for structural components and biological functions. Besides, nuclear-encoded genes also regulate mitochondrial transcription, translation, and mtDNA replication, thus the precise cooperation of nuclear

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and mtDNA expression is important to regulate OXPHOS capacity in response to different physiological demands and disease states [1]. Dysregulated mtDNA expression has been associated with human mitochondrial diseases [4-6] and is also observed in normal aging process [7, 8]. The machinery that regulates expression of mammalian mtDNA is only partially understood. In this review we focus on mouse models that have shed light on the mtDNA transcriptional machinery in mammals. These mouse models were built by knocking out nuclear-encoded genes in a whole-body or tissue-specific manner. Currently, knockout mice for the following genes have been described: *Tfam*, *Tfb1m*, *Lrrpprc*, *Mterf2*, *Mterf3*, and *Mterf4* (Table 1).

3. Mammalian mtDNA structure and mitochondrial transcripts

MtDNA in mammals consists of ~16.5 kb circular double-stranded DNA that encodes for only 37 genes: 13 subunits of the OXPHOS complexes, 2 rRNAs (12S rRNA and 16S rRNA), and 22 tRNAs [9]. The mitochondrial genome encodes for essential proteins for the complex I, III, IV and V of the ETC, but curiously complex II subunits are all encoded by the nuclear genome. The mtDNA genes are arranged very asymmetrically between the Heavy (H) and Light (L) strands, designated as such because of their different densities in a cesium chloride gradient. The H strand harbors most of the genes, 12 of the 13 mRNAs, the 2 rRNAs and 14 of the 22 tRNAs, whereas the L strand codes only for the subunit ND6 of complex I and 8 tRNAs. There are two noncoding regions (NCR) in the mtDNA that regulate mtDNA transcription and replication. The major NCR of about 900 bp of length, commonly known as D-loop, contains the transcription promoter of both strands and the origin of replication of the heavy strand (depicted OH, Figure 1). In addition, several studies have shown association of multiple proteins with the D-loop region, indicating that the D-loop is the major site of transcriptional regulation [1]. The minor NCR, located between the tRNA^{Cys} and tRNA^{Asn} coding site is relatively small (30 bp) and contains the origin of replication of the L strand. mtDNA is transcribed as polycistronic transcription units, that are subsequently processed to excise tRNAs, rRNAs and mRNAs [10]. The mitochondrial RNA processing, though still poorly characterized, has been reviewed recently [11].

Two promoter regions for mitochondrial transcription, designated HSP1 and HSP2 have been identified in the H strand [12]. There has been a great deal of controversy regarding the existence of HSP2, as it is not easily observed in *in vitro* assays. However, the *in vivo* evidence strongly suggests the existence of a second initiation site [13, 14]. The HSP1 and HSP2 promoters, determined by S1 nuclease protection assay, are located very close, spaced by about 100 bp in the D-loop region and transcribed in the same direction [15](Figure 1). Transcription initiated from the HSP2 promoter produces a full-length transcript covering the 2rRNAs (12S rRNA and 16S rRNA), 12mRNAs and 13 tRNAs. However, transcription from the HSP1 promoter has been shown to be prematurely terminated downstream of the 16S rRNA, transcribing only for the tRNA^{Val}, tRNA^{Phe} and the 2 rRNAs [16]. Transcription initiated from HSP1 promoter appears to terminate as a result of a site-specific binding of the mitochondrial termination factor MTERF1 (Figure 1)[17]. Termination of the transcripts initiated at the HSP2 promoter has been less studied, but a region rich in A/T content, called H2, was identified upstream of the tRNA^{Phe} gene and has been suggested as the termination region [18]. Recently several proteins have been identified to bind mtDNA in the mouse H2 termination region, including ATAD3 and the Leucine-rich pentatricopeptide-repeat containing protein (LRPPRC) [19].

Transcripts originated from the LSP promoter are nearly full-genome length, coding for one protein (ND6) and a subset of tRNAs. It is widely accepted that light strand transcription termination takes place at the D-loop regulatory region [1]. Besides that, transcripts emanating from LSP have been shown to prime mtDNA replication [20]. Recently,

polyadenylation events have been detected at positions 160-185 nt, downstream of the LSP promoter [20][1]. This region coincides with the abortion of transcription, as observed by deep sequencing [21]. Moreover this region showed high protection against DNaseI digestion, suggesting that protein binding to this region could induce transcription termination [21]. This premature termination could be important to prime mitochondrial DNA replication, therefore the identification of the proteins that can bind this D-loop region became an important goal.

4. Mitochondrial DNA transcription: what we have learned from mouse gene knockouts

4.1. Basic concepts of mtDNA transcription in mammals

The central mammalian mtDNA transcription machinery includes three proteins expressed in all cell types: mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM) [22] and mitochondrial transcription factor B2 (TFB2M) (Figure 1)[23, 24]. However, significant mitochondrial transcription initiated from HSP1 and LSP promoters has been reported in the absence of the human-TFAM (h-TFAM) *in vitro*, suggesting that TFAM may be dispensable for mitochondrial transcription basal levels, at least *in vitro* [25]. Moreover, in the presence of TFAM, transcription from both promoters were enhanced *in vitro*, and interestingly, transcription started from the LSP promoter was activated at a lower levels of TFAM when compared to the HSP1 promoter [25]. Because the LSP promoter primes mtDNA replication [20], it has been postulated that TFAM differentially regulates mitochondrial replication versus transcription, depending on the ratio of TFAM: mtDNA [26]. According to this model, low TFAM:mtDNA ratios would favor mtDNA replication because of the preferential activation of LSP promoter versus mtDNA transcription.[27][28][26].

The mitochondrial transcription factor A or TFAM belongs to the high-mobility-group (HMG) box domain protein family [22], which has the ability to bind, unwind and bend DNA without sequence specificity [29]. TFAM protein in mammals plays important dual functions in mtDNA transcription [30] and mtDNA maintenance [31]. mtDNA is organized in nucleoprotein particles called nucleoids [32]. Moreover, TFAM is the main protein component of the nucleoids, playing a key role in mtDNA packaging [20, 33]. Several studies showed that mammalian TFAM regulates mtDNA transcription *in vitro* [23, 25, 34] and *ex vivo* [28]. Mutational analysis has revealed that the essential domain for TFAM dependent transcriptional activation *in vitro* is the carboxyl-terminal (C-term) tail [35]. A C-term truncated version of TFAM can bind DNA *in vitro*, but has little transcriptional activity [35]. The yeast TFAM homologue, ABF2, lacks the C-term transcriptional activation domain and accordingly, is not required for transcription of yeast mtDNA but is essential for mtDNA maintenance [36]. TFAM binds specific sequence upstream of the HSP and LSP promoter, mostly as a dimer and the exact distance to the promoter seems to be essential (Figure 1) [35, 37]. It has been postulated that TFAM could unwind the promoter region of the mtDNA, increasing the accessibility of the others components. In agreement, direct protein-protein interaction has been observed between both TFB1M and TFB2M and TFAM C-terminal tail [38]. Recent findings, obtained by X-ray crystallography show that human TFAM induces promoter DNA bending, reversing the direction of the DNA helix [39]. Moreover, fluorescence resonance energy transfer assays showed that TFAM bends promoter DNA to a greater degree than non-specific DNA [40]. TFAM lacking the C-terminal distorted both promoter and generic DNA to a significantly reduced degree, in agreement with markedly decreased transcriptional activation capacity at LSP and HSP1 promoters *in vitro* [40]. Thus the enhanced bending of promoter DNA induced by the C-

terminal domains seems to be a critical component of the ability of TFAM to activate promoter-specific initiation by the basal mitochondrial transcription machinery[40].

Mammalian mitochondria appears to have a unique RNA polymerase (POLRMT) that efficiently transcribes both mtDNA strands *in vitro* [23]. POLRMT is a single subunit polymerase and belongs to the T7 bacteriophage RNA polymerase family [41]. The mitochondrial transcription factor B2 and its homolog B1 (TFB2M and TFB1M) are dual-function proteins that can activate mtDNA transcription *in vitro* [23] and also act as rRNA methyltransferases *in vivo* [42-44]. In *in vitro* assays, TFB2M was reported to stimulate transcription initiation 10 fold higher than TFB1M [23]. On the other hand, TFB1M showed more rRNA methyltransferase activity than TFB2M in an *in vivo* assay performed in *E. coli* [43]. The study of the heart-specific *Tfb1m* knockout mice indicated a major role of TFB1M as rRNA dimethyltransferase *in vivo* [44]. This same study also showed that methylation of the 12S rRNA mediated by TFB1M is required for assembly of the mitochondrial ribosome and is essential for viability in mice [44]. Phylogenetic analysis indicates that the existence of the two mitochondrial transcription factor B in metazoans is the result of a gene duplication event that occurred early in the evolution, prior to the divergence of fungi and metazoans [43]. Thus, it has been proposed that although both transcription factors conserve the transcription activation and rRNA methyltransferase activities, they have evolved to specialize in specific functions. Moreover, mutations in the rRNA methyltransferase domain do not affect TFB1M transcription activation ability, indicating that the two functions are independent [38].

Other factors with a role in the control of transcription of mtDNA have been identified. MTERF1 is believed to promote transcription termination downstream of the rRNA genes [17]. A putative mitochondrial transcription elongation factor (TEFM) has been recently identified [45]. Human cells depleted of TEFM showed decreased mitochondrial transcript levels, suggesting a role in mitochondrial transcription elongation. Moreover TEFM has been shown to interact with the POLRMT [45]. The mitochondrial ribosomal protein L12 (MRPL12) was identified as a binding partner of the POLRMT in human cells, therefore suggesting that MRPL12 plays a part in mitochondrial transcription [46]. To clarify the roles of these proteins, more detailed studies *in vivo* will be needed. In addition, several groups have reported a mitochondrial localization of nuclear receptors and nuclear transcription factors, suggesting they might directly influence mitochondrial transcription in mammals. Data including estrogen hormone receptor, the tumor suppressor p53 and the previous documented thyroid hormone receptor among others, have been recently reviewed in [11, 47].

The transcription factor STAT3 has also been reported in mitochondria and there is evidence suggesting that it controls the activity of the respiratory chain [48] and supports Ras-dependent oncogenic transformation [49]. Supporting the effect on OXPHOS, a transgenic mouse expressing a mitochondria-targeted STAT3 showed protection against ischemia and ROS damage [50]. The potential physiological role of STAT3 directly on mtDNA is still poorly understood and will not be discussed in this review.

4.2. TFAM: coupling mtDNA maintenance and transcription

TFAM function *in vivo* has been extensively studied in full body and in several tissue specific *Tfam* knockout mouse models (Table 1). Germ line disruption of the mouse *Tfam* (*Tfam*^{-/-}) gene leads to severe mtDNA depletion, which causes a profound respiratory chain deficiency and embryonic lethality between E8.5 and E10.5 [51]. This result indicates that TFAM is an essential protein for mtDNA maintenance *in vivo* and essential for embryonic viability in mice. Interestingly the heterozygous *Tfam*^{+/-} animals, which have 50% TFAM protein levels of the controls, present a similar reduction in the mtDNA levels, suggesting a

linear correlation between TFAM and mtDNA levels [51]. Despite the general mtDNA depletion exhibited in the heterozygous *Tfam*^{+/-} mice, only heart and kidney tissues showed a decrease in the mitochondrial transcripts levels while in other tissues, such as liver and muscle, mitochondrial transcripts remain unchanged [51]. Besides, only heart tissue showed an OXPHOS deficiency. These results indicate the existence of compensatory transcriptional and/or translational mechanisms that can counteract the mitochondrial depletion at certain time points in some tissues but not in the heart [51]. Moreover, tissue-specific *Tfam* knockout mice have been shown to retain mitochondrial transcripts levels for weeks after the lack of TFAM, and the consequent mtDNA depletion, supporting the idea of a transcriptional compensatory mechanism [52].

TFAM has been selectively disrupted in a tissue specific manner to study its function in mice, by using the cre-loxP recombination system (listed in Table 1). Conditional knockout mouse models were generated lacking TFAM in heart and skeletal muscle [52-55], in pancreatic-β cells [56] and in the central nervous system [57-59]. All the tissues where TFAM was ablated showed a clear mtDNA depletion and thus respiratory enzyme deficiency, strongly indicating that TFAM protein is essential to maintain mtDNA copy number *in vivo* in mammals.

Interestingly a transgenic mouse model overexpressing the human TFAM protein (cloned in an artificial Chromosome, P1) was generated [31], based on a previous study showing that the human TFAM protein is a poor activator of mouse mtDNA transcription *in vitro*, despite its ability to bind unspecifically the mouse mtDNA [28, 60]. Thus, it has been possible to study TFAM chaperone function *in vivo*, independently of its role in mitochondrial transcription [31]. Accordingly with the data obtained *in vitro*, the overexpression of human TFAM in a control mouse caused an increase in the mtDNA content in all tissues analyzed, but without a relative increase in the mitochondrial transcripts levels and respiratory enzyme levels [31]. This finding indicates that TFAM protein levels directly regulate mtDNA copy number *in vivo* without altering mitochondrial gene expression [31]. The general mechanism proposed is that TFAM regulates mtDNA copy number by stabilization of the mtDNA molecules, supported by the main presence of TFAM protein in the mitochondrial nucleoids [20]. However, the exact mechanisms where mtDNA levels can be maintained in the absence of mtDNA transcription remain unknown.

In parallel, human TFAM transgene expression was induced in a mouse strain carrying germ line *Tfam* ablation to study its effect in absence of endogenous TFAM protein [31]. Expression of the human TFAM protein was not able to rescue the embryonic lethality of *Tfam*^{-/-} mice. However, the *Tfam*^{-/-} knockout E8.5-E9.5 embryos, expressing human TFAM presented an increase in the mtDNA content, corroborating the observation that human TFAM induces an increase in mtDNA levels *in vivo* also in a *Tfam* knockout background [31]. Moreover the results showed a clear correlation between mtDNA levels and human *TFAM*/mouse *Tfam* gene dosage [31]. The *Tfam*^{-/-} knockout embryos expressing the human TFAM presented low levels of mitochondrial transcripts suggesting that although mtDNA content was increased, it could not be transcribed efficiently due to the lack of transcriptional function of the human TFAM protein and consequently the embryo lethality was not rescued [31]. This suggests that the lethality observed in the homozygous *Tfam*^{-/-} knockout embryos is originally caused by the lack of mitochondrial transcription, which is required to prime mtDNA replication [20].

More recently, human TFAM protein has been overexpressed in a heart specific *Tfam* knockout mouse [27]. Because of the disruption of *Tfam* in mouse cardiomyocytes, these animals showed dilated cardiomyopathy with heart conduction block, dying by 10-14 weeks of age [53]. Mutant animals showed severe mtDNA depletion in heart and consequently

reduced mitochondrial transcripts levels and respiratory chain proteins deficiency [53]. However the expression of hTFAM protein in the heart of mutant mice restored mtDNA content near to control levels at the age of 12 weeks and animals lived until 52 weeks, indicating a rescue of the cardiomyopathy phenotype [27]. The rescued mice have severely decreased *de novo* transcription of mtDNA in the heart, in agreement with the previous results showing that hTFAM does not activate mitochondrial transcription in mouse [31]. Interestingly, despite the decrease in *de novo* transcription they contained normal steady-state levels of mitochondrial transcripts at the age of 12 weeks, suggesting the existence of compensatory mechanism that could increase transcripts stability or reduce transcription termination [27].

In summary: studies in mice clearly showed that mammalian TFAM is vital to maintain mtDNA content and to maintain mtDNA transcript levels [31]. Moreover, these studies revealed the importance of mitochondrial transcription modulation as a compensatory mechanism during mitochondrial dysfunction.

4.3. TFB1M: coupling mtDNA transcription and translation?

TFB1M was initially reported as a mitochondrial transcription factor *in vitro* [23, 38]. However, the study of a conditional knockout mouse model revealed that TFB1M is an rRNA methyltransferase necessary for mitochondrial translation *in vivo* [44]. Germ line disruption of the *Tfb1m* in mice induced lethality at the embryonic stage E8.5 [44]. To study TFB1M function *in vivo*, the authors disrupted *Tfb1m* specifically in the heart and skeletal muscle. Those animals had a cardiomyopathy and shorted life span caused by a respiratory chain defect in the heart [44]. The heart of the *Tfb1m* knockout mice showed a dramatic decline of 12S rRNA levels, while the rest of mitochondrial transcripts remained similar or increased compared to control mice [44]. Lack of MTFB1 resulted in loss of dimethylation of 12S rRNA in the heart of the mice, impaired mitochondrial ribosomal assembly and caused a decrease in the levels of the respiratory complexes [44]. Accordingly, mitochondria isolated from *Tfb1m* knockout hearts showed a clear decrease in the rate of mitochondrial translation [44]. Increased steady-state mtDNA and TFAM levels were detected in the heart of the *Tfb1m* knockout mice, suggesting compensatory mechanisms. In addition, *Tfb1m* knockout mice showed reduction of MTERF3 protein levels (a mtDNA transcription repressor) and increased TFB2M protein levels, which could counteract the mitochondrial translation defect. Finally, although data obtained in mice indicates that the main function of TFB1M is essential for ribosomal biogenesis and therefore mitochondrial translation *in vivo* [44], it cannot be ruled out the possibility that TFB1M could regulate mtDNA transcription *in vivo*, for example under disease/stress condition.

4.4. LRPPRC: regulation the stability of the mitochondrial transcripts

The LRPPRC, leucine rich pentatricopeptide repeat containing protein, also known as LRP130, belongs to the pentatricopeptide repeat (PPR) family [19]. This motif is usually found in proteins involved in the metabolism of the RNA [61]. Mutations in *Lrpprc* have been linked to Leigh Syndrome French-Canadian (LSFC) characterized by cytochrome c oxidase deficiency [62]. Several studies suggest that LRPPRC protein is involved in the regulation of the mitochondrial gene expression. First, LRPPRC was found to interact in the nucleus with PGC1 α [63] and recent studies suggest that LRPPRC affects the stability of mitochondrial mRNA [64, 65].

The function of LRPPRC *in vivo*, was studied in a mouse “genetrap” model, where the C-terminus (120 amino acids) of LRPPRC protein was replaced by a selectable marker [66]. The homozygous mutant mice, expressing only the fusion protein (LRPPRC-marker) die before E 12.5 stage. Thus, to study its function, the authors used mouse embryonic

fibroblasts (MEFs) isolated from homozygous mutants. MEFs isolated from 8 day mutant embryos showed a marked decrease in cytochrome c oxidase activity (more than 60%) and in the levels of the COX-I and COX-III mitochondrial transcripts [66]. However the levels of other mitochondrial transcripts remained unchanged [66]. In addition, recombinant LRPPRC protein bound to a specific segment of COX1 mRNA, encoded by mouse mtDNA nucleotides 5961-6020. The available data suggest that LRPPRC is involved in the stabilization of mitochondrial mRNAs encoding COX transcripts.

4.5 The mitochondrial transcription termination factor, MTERF, family

MTERF proteins have been implicated in regulation of mitochondrial transcription [17, 67, 68], replication [69] and translation [70, 71]. Like the rest of the proteins implicated in mtDNA metabolism, MTERF proteins are encoded in the nuclear genome and therefore must be imported into the mitochondria [16]. Four members of the MTERF family have been identified in vertebrates, designated as MTERF1-4 [72]. Of the four, MTERF1 and MTERF2 are exclusively found in vertebrates, whereas MTERF3 and MTERF4 proteins are found also in worms and insects, thus appearing to be the ancestral genes in metazoans [72]. All family members display the conserved MTERF motif [16]. A detail structure-function analysis of the *in vitro* synthesized human MTERF1 protein support MTERF motif characterized by leucine zipper-like domains, likely to confer DNA binding capacity [73]. [16, 73] Recently, the crystal structure of MTERF1 and MTERF3 protein have been resolved and the MTERF motif was suggested to comprise three α -helices domains, separated by loops, in clear contradiction with the earlier leucine zipper model [74, 75]. The several MTERF motifs, that are conserved in this family, form a half-doughnut-shaped right-handed superhelix that is thought to mediate the DNA interaction [74].

MTERF1, traditionally referred as MTERF, was the first family member identified as a mitochondrial transcription terminator factor in human mitochondria [17]. Transcription starting from the HSP1 promoter encodes for the 2 rRNA, tRNA^{Val} and tRNA^{Phe}, and is usually terminated downstream of the 16S rRNA, possibly by the action of MTERF1 protein. It has been shown that MTERF1 binds to a 28 bp region at the 3' end of the tRNA^{Leu(UUR)} gene, downstream of the 16S rRNA, commonly known as transcription termination region (TERM) region [17, 73]. The binding of MTERF1 to TERM region has been corroborated using an *in vivo* footprinting approach in human cells [76]. Recently, the specific sequence recognition of the TERM region has been attributed to five arginine residues that are conserved on MTERF1 proteins between species, but not on others MTERFs [75]. The A3243G mutation in the MTERF1 binding site at the tRNA^{Leu(UUR)} gene causes the mitochondrial disease MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes). The presence of the mutation has been shown to reduce MTERF1 binding capacity *in vitro* [77] but not *in vivo* [78]. Even though the affinity of MTERF1 to the A3243G containing site is lower, no differences were found in the rRNA/mRNA transcripts ratio *in vitro* [77]. More studies will be needed to determine if there is any role for MTERF1 in MELAS.

In addition to its role in transcription termination, MTERF1 has been shown to promote transcription initiation by binding in transcription promoter region, near to the HSP1 promoter [15]. The authors proposed a model where MTERF1 binds simultaneously to the HSP1 and TERM regions, generating a mtDNA loop that would promote the recycling of transcription [15]. This model would explain for the higher ratios of rRNA:mRNA found in cells. MTERF1 mediated transcription activation has not been reproduced *in vitro* [79], indicating the presence of other factors. Recently, MTERF2 and MTERF3 have been shown to bind the HSP region, suggesting a possible role in the regulation of transcription initiation (Figure 1)[68].

Besides its role in transcription initiation and termination, MTERF1 appears to be able to modulate mtDNA replication by modulating replication pausing [69]. Electrophoretic mobility shift assays (EMSA) in human protein extracts overexpressing MTERF1 showed additional binding sites, in the D-loop region, OL, ND1 and the adjacent IQM tRNA gene cluster, suggesting that MTERF1 affects mtDNA replication pausing at these sites [69]. Furthermore, overexpression of recombinant MTERF2 and MTERF3 resulted in accumulation of specific mtDNA replication intermediates suggesting a defect in the terminal steps of the replication [80].

MTERF1 is the most studied of the MTERF family members, but little is known about its regulatory function *in vivo*. Studies with *Mterf2-3* and *4* knockout mice have been described, but at the moment no *Mterf1* knockout mouse model has been published. In agreement with phylogenetic studies, knockout of the ancestral *Mterf* family member genes, *Mterf3* and *Mterf4* in mice are embryonic lethal, indicating that these are essential genes for viability in mammals [67, 71]. In contrast, mice lacking of *Mterf2* gene are viable and display very mild phenotypes [68].

4.5.1 Mterf2 knockout mouse—MTERF2 can be found in the literature referred also as MTERF.D3, MTERF domain containing protein number 3 and MTERFL, MTERF like protein. MTERF2 protein was first identified as a mitochondrial localized polypeptide in human cells [81]. MTERF2 is exclusively found in vertebrate species as is its homolog MTERF1 [72]. The expression of MTERF2 was repressed after serum addition to starved cells and overexpression of MTERF2 suppressed cell growth, suggesting a role in the regulation of cell cycle [81]. In contrast, MTERF1 expression increased after serum addition to starved cells indicating that MTERF1 and 2 levels are regulated in opposite directions [81]. The physiological relevance of these data remains to be elucidated.

Knockout of the *Mterf2* gene in mice was not lethal, but the mice gained less weight than wild-type controls, developed a mild myopathy and showed memory deficits, suggesting an important role for MTERF2 protein in muscle and brain, two tissue that are highly dependent on the mitochondrial energy production [68]. Surprisingly, OXPHOS levels in brain remained unaffected and *Mterf2* knockout animals only showed a mild OXPHOS deficit (indicated by lower COX and CI+III activities) in muscle and liver tissues [68]. However, when the animals were fed with a high fat–low carbohydrates diet, the *Mterf2* knockout mice showed defects in OXPHOS complexes activities in multiple tissues, with the only exception of the heart [68]. These defects were associated with decreased steady-state levels of mitochondrial proteins, which in turn were caused by decreased mitochondrial mRNA levels, suggesting that MTERF2 positively modulates mitochondrial transcription [68]. The *Mterf2* knockout animals showed increased mitochondrial mass as indicated by increased citrate synthase activity and mtDNA levels in all tissues but the heart, probably as a compensatory mechanism to counteract the OXPHOS deficiency. In addition, a general increase in the levels of the mRNAs involved in mtDNA transcription, including MTERF1, 3 and 4 family members was detected in muscle of the *Mterf2* knockout mice [68].

Surprisingly, no OXPHOS-related phenotype was found in the heart of the *Mterf2* knockout animals. Further experiments will be needed to address the exact mechanism and the possible interactions with the other MTERF proteins. These results highlighted that mammalian mitochondrial transcription is a very complex process, highly regulated, probably in a tissue specific manner.

MTERF2 was also found to bind nonspecifically the entire mtDNA [82], which contrasts the finding that MTERF2 has a preferential affinity to the HSP promoter region [68]. *In vivo* co-immunoprecipitation assays showed that MTERF2, MTERF1 and MTERF3 proteins may

interact but probably not directly, as the interaction was observed only in the presence of the mtDNA [68].

In conclusion data obtained with the MTERF2 mouse model indicate that although MTERF2 function is not essential for mtDNA transcription in mammals, it functions in modulating mitochondrial transcription under stress conditions.

4.5.2 Mterf3 knock-out mouse—MTERF3 member, also referred in the literature as MTERF.D1, was identified as a MTERF family member using comparative genetic analysis [72]. The function of MTERF3 protein *in vivo* has been addressed by knockout studies in mice [67]. Homozygous *Mterf3* knockout mouse embryos exhibit growth retardation and die around embryonic day 8.5, indicating that it is essential in mammals [67]. Mouse model with *Mterf3* gene ablated specifically in cardiac and skeletal muscle showed a shorter lifespan and a cardiomyopathy phenotype with several features consistent with marked mitochondrial respiratory chain dysfunction [67]. OXPHOS activities and respiratory chain protein levels were decreased in the heart of the *Mterf3* specific knockout mice [67]. In addition, *Mterf3* knockout hearts presented alteration in the steady state levels of the mtDNA transcripts [67]. In the *Mterf3* knockout hearts there was an accumulation of transcripts containing the genes more proximal to the promoter regions, suggesting that MTERF3 protein is important for RNA processing [67]. It has been shown using chromatin immunoprecipitation assay, that MTERF3 binds to the HSP promoter in the mtDNA, thus suggesting that MTERF3 might regulate transcription initiation [67]. Accordingly, *de novo* transcription of isolated mitochondria from *Mterf3* knockout heart was increased from both LSP and HSP promoters compared to controls, suggesting that MTERF3 is a negative regulator of the mitochondrial transcription *in vivo* [67]. These data were corroborated in an *in vitro* transcription assay, showing a 50% increase in mitochondrial transcript levels in MTERF3 depleted cells [67]. However addition of recombinant MTERF3 did not restore transcription to control levels, indicating that depletion of MTERF3 also leads to loss of additional factor required for MTERF3 function. Curiously, MTERF3 protein in flies has been implicated in mitochondrial translation [70].

4.5.3 Mterf4 knock-out mouse—*Mterf4*, (also designated as MTERF.D2) was identified by phylogenetic analysis as a member of the MTERF family encoding a protein with predicted mitochondrial localization [72]. According to sequence alignment, *Mterf4* is found not only in vertebrates, but also in worms and insects, and thus is an ancestral *Mterf* gene, similar to *Mterf3*. MTERF4 is a nuclear-encoded mitochondrial protein [71]. So far, very little data have been published regarding MTERF4 function *in vitro* [71].

A systemic *Mterf4* knockout mouse was reported by Camara and colleagues as embryonic lethal. Embryos at E8.5 showed significant mutant phenotypes as observed in knockouts of other genes that are essential for mtDNA expression, e.g., *Tfam*, *Tfb1m*, or *Mterf3* [71]. Tissue-specific knockout of *Mterf4* in the heart led to a progressive and lethal mitochondrial cardiomyopathy phenotype with maximal life span shortened to 21 weeks. A significant increase in mtDNA and dramatic increases in mitochondrial transcripts were found in the end-stage *Mterf4* knockout hearts. However, complexes assembly and all of the mtDNA-encoded respiratory chain subunits were decreased. *In vitro* RNA immunoprecipitation assay identified the preferential crosslinking of MTERF4 to 16S rRNA, 12S rRNA, and 7S rRNA. Further mass spectrometry and size-exclusion chromatography analysis revealed that MTERF4 also interacts with the mitochondrial RNA methyltransferase NSUN4 to form a heterodimer. The MTERF4-NSUN4 complex was found to co-migrate with the large ribosomal subunit in a sedimentation gradient assay, while targeting of NSUN4 to the large ribosomal subunit was strongly decreased or abolished in the absence of MTERF4, both *in*

vitro and *in vivo*. This data suggest that MTERF4 regulates mitochondrial gene expression by binding to and targeting NSUN4 to the large ribosomal subunit [71].

The systemic and tissue-specific knockouts of *Mterf4* mouse models indicate that it has an important role in regulating mitochondrial translation. In these models, increased levels of TFAM and TFB2M, thus mitochondrial biogenesis, are suggested as compensatory mechanisms to rescue the severe respiratory chain deficiency. It is not clear whether MTERF4 directly signal to repress these processes. Conditional knockout of *Mterf4*, also in a tissue-specific manner, could help us understand its role beyond development. On the other hand, MTERF4 does contain DNA binding domains and one cannot rule out the possibility that MTERF4 could bind mtDNA and regulate gene transcription.

5. Mitochondrial transcription regulation in disease

Transcriptional compensation for partial mtDNA depletion was first observed in the heterozygous *Tfam* knockout mice (*Tfam*^{+/-}) by Larsson and coworkers [51]. In *Tfam*^{+/-} knockout mice, all tissues analyzed presented similar levels of mitochondrial DNA depletion (~ 40% reduction), however no significant decreases in the levels of mitochondrial transcripts and mitochondrial encoded proteins levels were found, with the only exception of the heart [51]. Moreover, the fact that only the heart of *Tfam*^{+/-} showed a mt-transcript/proteins deficiency, while the levels in other tissues remained unchanged, clearly indicated that some tissue are more sensitive than others to the same mitochondrial defect [51]. The molecular mechanisms that make some tissues more sensitive than others and the existence of tissue specific compensatory mechanism are clinically very relevant questions, but unfortunately unanswered at the moment.

Loss of mtDNA leads to mitochondrial depletion syndrome (MDS), a clinically heterogeneous diseases in humans [83]. Most MDS are caused by mutations in the nuclear-encoded proteins responsible for mtDNA maintenance and in genes coding for important proteins for the maintenance of the mitochondrial dNTP pool [84]. However, some MDS have been associated to nuclear genes, such as *Mpv17*, in which the function of the corresponding protein remains unknown [85].

Transcriptional compensation for partial mtDNA depletion has been shown in two different mouse models of human mitochondrial depletion syndrome (MDS): in *Mpv17* knockout mice [86] and in *Tk2*^{-/-} knockin mouse model [87, 88], as well as in skeletal muscle of patients with *Tk2* mutations [89, 90].

In humans, mutations in *MPV17* gene are responsible for a hepatocerebral form of MDS [85]. *Mpv17*^{-/-} mice showed profound reduction of mtDNA levels in liver and muscle, but interestingly a mild OXPHOS deficiency compared with the severe mtDNA depletion [86]. Pulse-chase *in organello* experiment showed that efficiency of transcription was much higher in isolated mitochondria of liver of *Mpv17*^{-/-} mice versus the *Mpv17*^{+/+} mice littermates [86], strongly indicating the existence of transcriptional compensatory mechanism in the liver. In addition, MTERF1 transcripts and protein levels were significantly decreased exclusively in tissues that showed profound mtDNA depletion (liver and muscle) of *MPV17*^{-/-} but not in the brain, suggesting that the compensatory mechanisms may be mediated by down regulation of MTERF1 [86]. MTERF1 is known to be regulated by the PGC1 α -NRF1/2 axis, however, no differences were found in the transcript levels of PGC1 α -NRF1/2 in the liver of *Mpv17*^{-/-} mice. Also no differences were detected in the transcript levels of the mtDNA transcription related factors, POLRMT, TFAM, TFB1M and TFB2M.

In clear agreement with this result, recent data has showed a transcriptional compensatory mechanism in the homozygous thymidine kinase 2 (H126N) knockin mouse (*Tk2*^{-/-}), a human MDS mouse model [87, 88]. TK2 is a ubiquitous enzyme, required for the salvage pathway of deoxypyrimidine nucleoside triphosphates synthesis, and for mtDNA replication in post mitotic cells [91]. In proliferating cells, TK2 activity has minor relevance because of the presence of the cell cycle regulated enzymes of the *de novo* synthesis pathway of dNTPs and of the cytosolic isoform thymidine kinase 1 (TK1). Recent data with the *Tk2*^{-/-} mouse related the onset of mtDNA depletion in each tissue with the levels of TK1 activity and with mtDNA transcriptional compensation mechanisms [88]. *Tk2*^{-/-} mice showed mtDNA depletion in multiple tissues with the brain and heart among the most affected. However, only the brain showed significant OXPHOS deficiency, while the heart was spared in this mouse model [87]. Mitochondrial transcript levels relative to the mtDNA content showed a marked increase in both brain and heart, indicating a compensatory mechanism to the mtDNA depletion. The transcriptional repressor MTERF3 transcript levels were significantly decreased in the brain and heart of the *Tk2*^{-/-} animals, suggesting that this transcriptional compensation was mediated by down-regulating the expression of the MTERF3 [88]. Thus it was suggested that down-regulation of MTERF3 could compensate for the mtDNA depletion in the heart but not in the brain in the *Tk2*^{-/-} animals, where mtDNA depletion was more severe. The up-regulation of mtDNA transcripts seems not to be due by activation of the PGC1 α -NRF1/2 axis because no differences in these transcripts were detected. Accordingly, previous data showed that MTERF3 is not regulated by the PGC1 α -NRF1/2 pathway [92]. No differences were detected in the transcript levels for TFAM, TFB1M and TFB2M, and neither in the MTERF1 and MTERF2 levels in the *Tk2*^{-/-} mice [88]. Unfortunately, the signals that trigger the mtDNA transcriptional compensatory mechanisms remain unknown.

All these results suggest that compensation at the transcriptional and probably at the translational level may be a general mechanism in response to mtDNA depletion and emphasize the importance of mtDNA transcription regulation in relation to the onset, tissue specificity and severity of mitochondrial diseases.

In addition to compensate for a loss of mtDNA, a transcriptional stabilization mechanism has been proposed to compensate for a mitochondrial transcriptional deficiency, [27]. As mentioned above, specific loss of TFAM protein in the heart of the mice leads to a cardiomyopathy phenotype [27]. The expression of the human TFAM protein in the heart of TFAM knockout mice restored mtDNA levels, but did not recover the *de novo* transcription [31]. Interestingly the rescued hearts contained transcript of around 1 kb length, complementary to the heavy strand that encompassed the mtDNA control region (termed anti-control region, ACR), that were not detected in control mice neither in other tissues of rescued mice besides heart [27]. This result clearly indicates that mitochondrial transcription is altered under stress/pathological conditions *in vivo* [27].

Further studies are needed to address whether mitochondrial transcription compensatory mechanisms are triggered exclusively upon a loss of mtDNA or mtDNA-derived transcript levels or if it is a general mechanism, that could partially compensate for different types of damaged mtDNA, including large deletions, point mutations and other genetic defects associated with OXPHOS function. Besides, it will be important to identify retrograde signals that trigger transcriptional compensatory mechanisms.

6. Concluding remarks

The use of mouse models has provided unique insights into the control of mtDNA gene expression. Recently, Mercer and colleagues analyzed the mitochondrial transcriptome of

several cell lines and tissues by deep sequencing [21]. Their results revealed a complex pattern suggesting an equally complex regulation. Besides differences in levels of transcripts they found variable processing and the presence of several small RNAs. They also performed DNase I sensitivity assay through the genome revealing areas of potential protein binding. Clearly, the regulation of mtDNA gene expression still requires further investigation as it may have key roles in healthy and diseased conditions.

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Abbreviations used in this paper

HSP1 and HSP2	heavy strand promoter 1 and 2
LRPPRC	leucine rich pentatricopeptide repeat containing
LSP	light strand promoter
mtDNA	mitochondrial DNA
MTERF	mitochondrial transcription termination factor
POLRMT	mitochondrial RNA polymerase
rRNA	ribosomal RNA
tRNA	transfer RNA
TFAM	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
TFB2M	mitochondrial transcription factor B2
ETC	electron transport chain
OXPHOS	oxidative phosphorylation system

Highlights

- Genetically modified mice have helped us understand mechanisms of mitochondrial gene expression.
- We review the knowledge acquired in mtDNA gene expression using mouse models.
- Tfam, Tfb1m, and Mterf family members were studied in details using mouse models.
- These models will uncover other regulators of mtDNA transcription.

Figure 1. mtDNA transcription initiation machinery and promoters

Mitochondrial transcription is bidirectional and starts in the D-loop region where the promoters HSP1, HSP2 and LSP are located. Transcription initiation requires the cooperation of TFAM, TFB2M and the RNA polymerase POLRMT. TFAM protein preferentially binds the mtDNA upstream of the promoters. Transcription initiated in the HSP1 promoter is terminated at the tRNA^{Leu(UUR)}, transcribing only for the tRNA^{Val}, the tRNA^{Phe} and the 2 ribosomal RNA (12S and 16S). However transcription initiated from the HSP2 promoter transcribes the full-length mtDNA complementary to the heavy strain. MTERF family members, MTERF1, MTERF2 and MTERF3 bind to the promoter region and modulate mtDNA transcription. MTERF1 also binds to the tRNA^{Leu(UUR)} inducing transcription termination. Transcripts originated from LSP promoter proceed through the entire mtDNA molecule or could be terminated prematurely to prime mtDNA replication. OH indicates the origin of replication on the heavy strand.

Table 1

Mouse models of proteins involved in mitochondrial DNA expression.

Gene	Mitochondrial function	Genetic manipulation	Mitochondrial DNA expression phenotype	References
<i>Tfam</i>	Transcription initiation/regulation Replication mtDNA maintenance	knockout in: Whole body Heart/Skeletal muscle, Pancreatic β cells, CNS Transgenic expression of human TFAM in a control background Transgenic expression of human TFAM in <i>Tfam</i> knockout mice specific for the heart	Embryonic lethal/Heterozygous $\downarrow$ mtDNA $\downarrow$ mtDNA/ $\downarrow$ Transcripts/ $\downarrow$ Proteins Normal mtDNA levels $\uparrow$ L-Transcripts Normal levels H-Transcripts Normal mtDNA levels $\downarrow$ Transcripts	[50] [52-59] [31] [27]
<i>Tfb1m</i>	Translation regulator	knockout in: Whole body Heart/skeletal muscle	Embryonic lethal Impaired translation Normal levels most mRNA $\downarrow$ 12S rRNA $\uparrow$ 16S rRNA $\uparrow$ ND1 $\uparrow$ tRNA	[44]
<i>Mterf2</i>	Positive transcription regulator	knockout in: Whole body/viable	$\downarrow$ Most transcript and tRNAs imbalance $\downarrow$ Protein levels	[68]
<i>Mterf3</i>	Negative transcription regulator	knockout in: Whole body Heart/skeletal muscle	Embryonic lethal $\uparrow$ Most transcript and tRNAs imbalance $\downarrow$ Protein levels	[67]
<i>Mterf4</i>	Translation regulator	knockout in: Whole body Heart/skeletal muscle	Embryonic lethal Impaired translation $\uparrow$ Most transcript and tRNAs imbalance transcribed from LSP	[71]
<i>Lrpprc</i>	mRNA stabilization	knockout in: Whole body	Embryonic lethal	[66]