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Hitting the Brakes: Termination of Mitochondrial Transcription

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Abstract

Deficiencies in mitochondrial protein production are associated with human disease and aging. Given the central role of transcription in gene expression, recent years have seen a renewed interest in understanding the molecular mechanisms controlling this process. In this review, we have focused on the mostly uncharacterized process of transcriptional termination. We review how several recent breakthroughs have provided insight into our understanding of the termination mechanism, the protein factors that mediate termination, and the functional relevance of different termination events. Furthermore, the identification of termination defects resulting from a number of mtDNA mutations has led to the suggestion that this could be a common mechanism influencing pathogenesis in a number of mitochondrial diseases, highlighting the importance of understanding the processes that regulate transcription in human mitochondria. We discuss how these recent findings set the stage for future studies on this important regulatory mechanism.

Keywords

mitochondrial transcription termination; MTERF; mitochondria; gene expression

1. Introduction

Mitochondria are involved in a vast array of cellular functions [1] but are most widely known for their role in generating ATP through oxidative phosphorylation [2]. This process is strictly dependent on expression of the mitochondrial genome, which encodes a small set of proteins that are essential for functionality of the electron-transport chain, as revealed by a number of human diseases linked to deficiencies in their synthesis or assembly (reviewed in [3–5]). The mitochondrial genome is encoded in a super-coiled double stranded circular DNA molecule of approximately 16.5 kilobases [6, 7]. Mammalian and other eukaryotic cells typically contain hundreds to thousands of mitochondrial DNA (mtDNA) genomes assembled into supramolecular structures called nucleoids [8–11]. In humans, each mtDNA genome contains 37 genes encoding the RNA components of the mitochondrial translation machinery: two ribosomal RNAs and 22 transfer RNAs, as well as mRNAs for 13 subunits

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of the respiratory chain [12] (see Figure 1). The two mtDNA strands have different buoyant densities in a cesium chloride gradient, which led to them being denoted heavy (H) and light (L) [13]. The 37 genes encoded in mtDNA are asymmetrically distributed, with the L-strand encoding only 8 tRNAs and one mRNA while the H-strand encodes the remaining two rRNAs, 14 tRNAs and 12 mRNAs [2]. In most, but not all cases, the mRNA and rRNA genes are flanked by at least one tRNA gene. Hence, excision of tRNA molecules is thought to be required for production of mature mRNA and rRNA molecules. This mode of RNA processing is known as the “tRNA punctuation model” [14]. In metazoa, the mitochondrial genome is extremely compact – there are no introns, and encoded polypeptide, tRNA and rRNA genes are smaller than their counterparts in the nucleus or in prokaryotes [15]. Furthermore, all of the genes are very closely spaced and little or no 5′ or 3′ flanking sequences exist on the mature mRNAs. The only sizeable non-coding region is the control region, or D-loop regulatory region, named after the triple-stranded structure or displacement loop that is formed by association of the nascent heavy strand in this region [2, 13, 16]. The roughly 1 kb long D-loop region contains two of the three promoters involved in transcription initiation (the L-strand promoter, LSP, and one of the two H-strand promoters, HSP1), as well as evolutionarily conserved regulatory sequences involved in DNA replication, D-loop formation, and presumably termination of transcription (see Figure 1) [17].

2. Origins and initiation of mitochondrial transcription

The initial studies on mtDNA transcription were carried out about two decades ago, both in vitro and in vivo using cultured human and mouse cells. These studies characterized the mitochondrial RNAs in terms of their identity, structure and metabolic properties [18–22], and identified the cis-acting elements required for mitochondrial transcription [23–25]. Mapping studies of nascent transcripts and in vivo analysis of the kinetics of transcript synthesis suggested that transcription in human mitochondria starts at three different initiation points: one for the L-strand (LSP) and two for the H-strand (HSP1 and HSP2) [18, 19, 26].

The current model for transcription of the H-strand in humans involves two HSP promoters and two partially overlapping units. The first of these starts at the initiation site HSP1, which is located 19 bp upstream of the tRNA^{Phe} gene (within the D-loop) and ends at the 3′ end of the 16S rRNA gene. The HSP1 transcription unit is responsible for the synthesis of the two ribosomal RNAs, tRNA^{Phe} and tRNA^{Val} (see Figure 1). The second transcription unit, operating with a frequency about 20 times lower [19], starts at the initiation site HSP2 which is located 2 bp upstream of the 5′ end of the 12S rRNA gene. Transcription from HSP2 produces a polycistronic RNA molecule covering almost the whole H-strand, encoding the mRNAs for the 12 H-strand encoded polypeptides and 12 tRNAs. Transcription of the L-strand begins at the LSP promoter, also located in the D-loop about 150 bp away from HSP, and produces only 8 tRNAs, one polypeptide mRNA and the H-strand replication primer. These primary transcripts are processed to produce the individual mRNA, rRNA, and tRNA molecules [14, 27, 28]. This transcription model explains how differential regulation of rRNA versus mRNA transcription could be accomplished through initiation of H-strand transcription at the two alternative sites [19]. Analysis of the effects of different ATP concentrations and intercalating drugs such as ethidium bromide on in organello transcription and the results of footprinting experiments have also provided support for this model of two H-strand transcription units [29–31]. However, it is important to note that this model has not yet been verified in vivo.

Mitochondria utilize unique enzyme systems responsible for mtDNA transcription (reviewed in [17, 32, 33]), which are nuclear-encoded, but distinct from those used in the nucleus. It is

now more or less generally accepted that the core machinery involved in initiation of transcription consists of the mitochondrial polymerase (POLRMT) and two transcription factors, mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M) [34, 35]. POLRMT is a single-subunit RNA polymerase of the T-odd bacteriophage RNA polymerase family [36, 37]. However, unlike these other phage polymerases, which do not require interactions with transcription factors to initiate transcription, mammalian POLRMT requires the methyltransferase-related transcription factor, TFB2M, to initiate promoter-specific transcription from LSP and HSP1 *in vitro* [38, 39]. Initially, the TFB2M orthologue, TFB1M, was also thought to be essential for initiation, but recent studies indicate that only TFB2M is absolutely required for efficient, promoter-specific transcription *in vitro* [39]. Initiation also involves the high-mobility group (HMG) box DNA-binding protein, TFAM, which was the first mitochondrial transcription factor identified [40]. Human TFAM binds upstream of the LSP and HSP1 promoters [41, 42], and facilitates initiation *in vitro* by binding to TFB2M in a manner that requires its C-terminal tail [43] and possibly interactions with one of its two HMG box domains [42].

3. Termination of transcription by human MTERF1

Transcription initiated at the HSP1 promoter is preferentially terminated within the tRNA^{Leu} gene immediately downstream of the 16S rRNA gene [19, 44]. This site-specific termination event is mediated by a DNA-binding protein, mitochondrial transcription termination factor 1 (MTERF1), first identified by Attardi and colleagues [45], and later shown to be sufficient to mediate transcriptional termination *in vitro* [46]. Subsequent *in vitro* work has demonstrated that termination by MTERF1 is bidirectional [46, 47], and shows even higher efficiency when POLRMT proceeds in the direction of L-strand transcription [46].

In addition to binding the termination site in tRNA^{Leu}, MTERF1 may also bind an additional site in the HSP1 promoter region and stimulate transcription [48]. In a manner that bears some similarities with prokaryotic transcription, it has been proposed that simultaneous binding of MTERF1 to the HSP1 site and the canonical tRNA^{Leu} termination site causes a looping-out of the rDNA. This loop would allow recycling of POLRMT and other transcription components from the tRNA^{Leu} site to the HSP1 initiation site for efficiently starting another transcription cycle after termination occurs. This “ribomotor” model [49] is one way that the greater abundance of rRNAs compared to the downstream H-strand mRNAs can be explained [50], although differential stability of rRNA and mRNA species is thought to be a significant factor [50]. Moreover, this model would also explain the selective termination of transcription originating at HSP1 and suggests a mechanism for regulating the balance between termination and read-through. In addition, some evidence also suggests that MTERF1 may be involved in transcription initiation from the HSP2 [48]. The mechanistic basis for this remains unknown as does the precise protein components required for regulation at HSP2, which is the least studied of the three known human mtDNA promoters. Recently, Jacobs and colleagues showed that MTERF1 binds several sites in mtDNA *in vivo* and that altered MTERF1 protein levels affect mtDNA replication pausing at these sites [51], leading to a model in which MTERF1 may mediate transcription and replication passage on the same mtDNA molecule. It is important to note that the existence of such alternative MTERF1 binding sites is controversial, for example, as subsequent studies have not been able to reproduce binding of MTERF1 to the HSP [52]. Furthermore, *in vitro* experiments indicate that the specificity of MTERF1 seems to be exquisite for the tRNA^{Leu} site [53], implying that the affinity for other sites (if they exist) would be lower. This hypothesis is consistent with the striking degree of protection of the MTERF1 tRNA^{Leu} site observed in *in vivo* methylation [54] and DNaseI footprinting assays [55], as well as with ChIP studies that have predominately found MTERF1 bound to the tRNA^{Leu} site.

3.1 Structure of MTERF1

The x-ray crystallographic structure of human MTERF1 reveals that the 342-residue protein (mature form) has an all- α -helical structure that binds as a monomer to a 22-nucleotide termination sequence in the tRNA^{Leu} gene [53, 56]. The MTERF1 protein structure is modular, being configured around a motif of two α -helices and a 3_{10} helix repeat known as the MTERF repeat. This MTERF repeat configuration was previously predicted by Roberti et al [57] and is similar in structure to other all- α -helical domains such as the HEAT domain [58], suggested to play a role in duplex DNA binding in DNA PK-cs [59], and the PUM/PUF domain, which is involved in RNA binding by PUMILIO proteins [60]. The 8 MTERF repeats found in MTERF1 constitute a helical fold that allows the protein to bind the major groove of duplex DNA containing the termination sequence. The extensive surface interactions observed between MTERF1 and the substrate DNA in the crystal structure indicate that the protein fold is likely dedicated to duplex DNA binding.

The MTERF1 crystal structure demonstrates that upon binding its target sequence, the protein alleviates the DNA duplex twist and promotes duplex melting and eversion of three nucleotide bases, leading to a novel and unique DNA binding mode (Figure 2). Although the mechanism by which it promotes this eversion, or base-flipping is not yet clear, MTERF1 stabilizes these three nucleotides in an extra-helical conformation through stacking interactions with three amino acid residues (Phe243, Tyr288 and Arg162) (see Figure 2 inset). We have demonstrated, using an MTERF1 triple mutant, that these three side chains are essential to maintaining the conformation observed in the crystal structure and although the MTERF1 triple mutant can still bind to the termination sequence, affinity for this termination site is significantly reduced. Moreover, the ability of the triple mutant MTERF1 to promote transcriptional termination in vitro is dramatically reduced.

With the exception of these stacking interactions and a few other important contacts (unpublished data), the majority of the interactions observed between MTERF1 and DNA involve the phosphate backbone of the substrate DNA sequence, and are therefore mostly electrostatic and non-specific. Sequence specificity appears to be determined in large part by a small number of key interactions between six arginine residues and guanine bases in the termination sequence. This type of major groove interaction is frequently seen in sequence-specific DNA binding proteins and is thought to be critical for MTERF function – we have shown that eliminating even a single one of these interactions can drastically affect both DNA binding and transcription termination [53]. Interestingly, this mechanism of sequence recognition implies that while the interaction between MTERF1 and its binding sequence involves contacts with 20 base pairs, only six of the 40 bases appear to be initially actively recognized by the protein. The extensive total number of protein-DNA interactions suggest that the entirety of the MTERF1 fold is involved in binding the termination sequence, but it is not immediately apparent how a single MTERF1 molecule could simultaneously bind both the HSP initiation and termination sites in the transcriptional loop model [48]. One possibility is that an additional molecule of MTERF1 or other factor(s) may mediate the association of these two sites and facilitate loop formation.

3.2 Model for termination by MTERF1

The MTERF1 crystal structure suggests a binding mechanism that involves establishment of site-specific interactions for sequence recognition followed by melting and unwinding of the DNA duplex. This unwinding would presumably destabilize base-pairing of the central nucleotides in the recognition sequence and facilitate subsequent base flipping, thereby stabilizing MTERF1 on the substrate DNA. We have demonstrated that stable MTERF1 binding and termination in vitro is dependent on base flipping, and the ability of MTERF1 to promote termination appears to be at least partially dependent on the strength of the

cumulative interactions between MTERF1 and the termination sequence. This would suggest a model of transcription in which MTERF1 acts as a “roadblock,” preventing or interfering with transcriptional elongation. This model is consistent with the observation that MTERF1 terminates transcription bi-directionally and can arrest elongation by heterologous polymerases ([47] and our own unpublished results). However, in vitro termination by MTERF1 displays a distinct polarity, with MTERF1 being more efficient when terminating transcription originating from the light strand promoter than the heavy strand promoter [46, 53]. This polarity may be a result of the majority of protein-DNA interactions being established with the light strand [53] (ie, the strand transcribed from the LSP promoter) as well as the higher affinity of MTERF1 for this strand, as observed by Nam et al [61]. This suggests a mechanism in which MTERF1 might transiently bind to single-stranded DNA, although the measured affinity of MTERF1 for single-stranded DNA is extremely low ([61] and our own unpublished observations). Nevertheless, except for the asymmetrical distribution of interactions, no obvious structural feature provides an explanation for the observed polarity of termination. One possibility is that the observed orientation dependence of termination activity is due to the unique conformation of MTERF1 on DNA, although interactions between MTERF1 and mitochondrial POLRMT (or additional elongation factors) may also influence the polarity of termination events. Furthermore, it is not yet known whether other proteins can modulate termination polarity in vivo.

3.3 Significance of termination at tRNA^{Leu}

The strong polarity observed in termination assays in vitro combined with the lack of in vivo evidence supporting a role in HSP termination suggest that MTERF1-mediated HSP termination might only be a secondary role and that the main function of MTERF1 is termination of LSP transcription at the tRNA^{Leu} site. This hypothesis is supported by the fact that the light-strand does not encode any additional genes beyond tRNA^{Leu}. Recent observations in vivo have shown that manipulation of MTERF1 expression levels is strongly correlated with alterations in the relative amounts of antisense transcripts on both sides of the tRNA^{Leu} termination site [62]. These findings are consistent with the notion that termination at this site may be important for preventing the accumulation of antisense transcripts that would otherwise interfere with the assembly of the rRNAs into ribosomes. This idea would imply that MTERF1 function is, as originally thought, important for ribosome biogenesis, albeit by a different mechanism than first proposed.

3.4 Termination of HSP transcription at the distal site

Any discussion of mitochondrial termination is incomplete when only considering termination at the tRNA^{Leu} site. Evidently some degree of read-through must occur at the tRNA^{Leu} site so that HSP transcription can progress beyond this site and generate most of the mitochondrial mRNAs. How such read-through is regulated, and whether it is specific to transcription originating from HSP2 is in itself an interesting question for which no answer yet exists. In any case, read-through HSP transcription is thought to ultimately terminate at a distal site within the D-loop. The mouse HSP distal termination site was originally identified as a 22 bp region within the D-loop containing a conserved A/T rich sequence motif. Initial in vitro studies suggested that termination at this site is unidirectional and requires sequence-specific DNA binding proteins [63], but the mechanisms controlling distal termination in human mitochondria are far from being well understood. Since MTERF1 predominantly binds to the tRNA^{Leu} site, it is possible that distal termination may not depend on MTERF1, therefore raising the question of how termination is achieved at that site. Recent work by Wanrooij et al suggests that the mitochondrial polymerase, POLRMT, utilizes a mechanism similar to bacterial rho-independent termination to generate the RNA primers necessary for mitochondrial replication [64]. In T7 bacteriophages transcription termination occurs as a consequence of the polymerase interacting with spontaneously generated double-stranded

RNA hairpins and falling off DNA due to the weak affinity of T7 RNA polymerase for double stranded structures [65]. In the case of mitochondrial POLRMT, Wanrooij et al have shown that stable G-quadruplex structures can form in the nascent RNA, suggesting that these structures then mediate transcription termination in a way reminiscent of RNA hairpins in rho-independent termination [64]. It is therefore possible that a similar mechanism is responsible for termination of transcription at the HSP distal site. Nevertheless, it cannot be excluded that MTERF1 might influence in termination at that site. As mentioned earlier, alternative MTERF1 binding sites have been described in the proximity of the distal site [51], and these sites will need to be further characterized in order to elucidate how termination is achieved and what role, if any, MTERF1 plays in the process. Finally, it is also possible that proteins other than MTERF1 might contribute to distal HSP termination. Preliminary experiments have identified several proteins that appear to bind at the mouse HSP distal termination region, including the Leucine-rich pentatricopeptide-repeat containing protein (LRPPRC) [66]. This protein is essential for the expression of mtDNA encoded respiratory chain subunits, and LRPPRC deficient cells were reported to have a reduction in both oxygen consumption and expression of mRNA and tRNA [66, 67]. Recent *in vivo* work in an RNAi system further supports the concept that LRPPRC is involved in regulating expression of mitochondrial mRNAs [68]. Moreover, several homologues of MTERF1 have been identified in recent years, and like LRPPRC, they too have been implicated in the regulation of mitochondrial transcription and gene expression.

4. The MTERF family of proteins

MTERF1 is the founding member of a family of related proteins that are widely conserved throughout evolution (although notoriously absent from yeast) and that are defined by the presence of several copies of the MTERF motif. Three human MTERF1 paralogues, MTERF2-4, were identified in 2005 by Linder et al. and were all predicted to localize to mitochondria [69]. Three of these proteins, MTERF1-3, have since been demonstrated to influence the transcription process [52, 53, 70], while recent work suggests that MTERF4 may play a role in mitochondrial translation [71]. Interestingly, the structural similarity that MTERF1 shares with MTERF3 (rmsd of 2.7 Å over 218 C-α atoms), despite the latter being crystallized in the absence of substrate [72, 73], implies that MTERF proteins share a common fold and supports the idea that they have evolved to bind nucleic acids.

4.1 Mitochondrial transcription termination factor 2 (MTERF2)

MTERF2 (also known as mTERFL or mTERF.D3), was first characterized several years ago [74, 75]. Initial *in vivo* studies demonstrated that MTERF2 overexpression inhibits cell growth and that its expression is regulated in a reciprocal manner with that of MTERF1 [74]. Subsequent work has shown that MTERF2 is able to bind nonspecifically to DNA and is present in nucleoids [76]. Knocking out MTERF2 in mice is not lethal, but the mice exhibit respiratory defects when metabolically challenged with a high fat/low carbohydrate diet [77]. Under these conditions, the mice exhibit decreased steady-state levels of most mRNA and tRNA transcripts and reduced translation of several proteins. When fed a standard diet, however, the mice have normal levels of most mitochondrial RNA transcripts, with the exception of promoter-proximal tRNA species, which are increased, and promoter-distal tRNAs, which are decreased. Interestingly, it was observed that MTERF2 exhibited a ~10-fold preference for binding in the HSP promoter region that was confirmed *in vivo* by ChIP analysis [77]. These results may suggest a role for MTERF2 as a positive modulator of mitochondrial transcription, but the current evidence is unclear and the situation may be more complex. For instance, sequence-specific mtDNA binding by MTERF2 was not observed in cell culture experiments [78]. In addition, overexpression of MTERF2 in cells resulted in modest mtDNA copy number depletion (as well as an accumulation of specific

replication intermediates), while MTERF2 knockout appears to cause an increase in mtDNA copy number. These findings may suggest an alternative or additional role for MTERF2 in regulating replication fork progression. While the effect of overexpressing MTERF2 on mtDNA copy number has since been confirmed by an independent study [79], further studies are needed to investigate the functions of MTERF2 in replication and transcription.

4.2 Mitochondrial termination factor 3 (MTERF3)

The x-ray crystallographic structure of MTERF3 (also known as mTERF.D1 or CGI-12) in the absence of DNA has been solved [72, 73] and shows striking similarity to the MTERF1 structure. Both proteins have nearly identical half-doughnut shapes consisting of MTERF motif repeats, suggesting that MTERF family members have similar folds and bind nucleic acids. However, there are also interesting differences, as only one of the five arginines necessary for sequence-specific DNA binding and only one of the three amino acids that stabilize base flipping are present in MTERF3 [73].

The *in vivo* function of this protein has recently been addressed through gene knockout studies in mice [52, 80]. Mammalian MTERF3 has been shown to localize to mitochondria, and while a global knockout of MTERF3 in mice is embryonic lethal, Park et al have analyzed a heart-specific knockout in detail [52]. Heart tissue from these mice exhibits an aberrant mitochondrial transcript profile leading to impaired respiration and subsequent demise. Specifically, both mRNA and tRNA transcripts proximal to the promoters are increased, whereas those more distal to the promoters are decreased. This imbalance in the steady-state level of promoter-proximal and promoter-distal tRNA species is reminiscent of the situation in the MTERF2 knockout mice. In addition, the authors observed that MTERF3 is able to bind mtDNA in the promoter region and that its immunodepletion from mitochondrial extracts leads to increased transcription. These findings led the authors to propose that increased transcription initiation in the absence of MTERF3 leads to collision of transcription complexes on opposite mtDNA strands and, hence, incomplete transcription of each strand. The *Drosophila* homolog of MTERF3, known as D-MTERF3, has been identified and it was recently reported that overexpression of this protein results in a modest decrease in mRNA levels [70]. Overall, the current evidence seems to point to a role for MTERF3 as a negative regulator of mitochondrial transcription. Interestingly, Jacobs and colleagues have also recently observed that MTERF3 overexpression has an inhibitory effect on mitochondrial replication, similar to that of MTERF2 [78].

4.3 Mitochondrial transcription termination factor 4 (MTERF4)

To date, MTERF4 (also known as mTERF.D2) has been the least studied MTERF family member but recent work has provided the first insight into the function of this protein in mitochondria. *In vivo* and *in vitro* studies have confirmed that MTERF4 localizes to mitochondria and demonstrated that this protein, like MTERF2 and -3, is essential to embryonic development in the mouse [71]. Furthermore, like MTERF3, a heart-specific conditional knockout of MTERF4 results in mitochondrial cardiomyopathy and respiratory chain deficiency. A substantial increase in the steady state levels of all mRNAs and rRNAs was observed (in some cases ~300% of normal levels). LSP transcripts encoding tRNAs were also increased for promoter-proximal genes, while those for promoter-distal genes were either unchanged or decreased. Despite the increased levels of transcripts, the MTERF4 knockout cells showed impaired translation and defective ribosome assembly, suggesting that the increase in *de novo* transcription is likely a response to the severe impairment in translation. MTERF4 is predicted to have a fold similar to MTERF1 and MTERF3, with positively charged surface areas for binding of nucleic acids [72]. Consistently, it was recently shown that MTERF4 binds the 16S rRNA and forms a stable stoichiometric complex with NSUN4, a mitochondrial rRNA methyltransferase, therefore

targeting this protein to the large ribosomal subunit [71]. While this evidence points to a primary role in regulating translation, it is tempting to speculate that MTERF4 may also modulate mtDNA transcription, particularly in the context of the prokaryote-like organization of mitochondria, with no compartmentalization between transcription and translation, which may enable direct crosstalk between these two processes [81].

Overall, these new findings on MTERF family members underscore the importance of these proteins in regulating mitochondrial gene expression. The results strongly suggest that these proteins have evolved to bind nucleic acids, although with remarkable differences in their substrate specificity, being capable of specific and/or unspecific binding to ssDNA, dsDNA and RNA in the case of MTERF4. Furthermore, the different genetic experiments highlight their potential to serve multiple functional roles.

5. Termination of mitochondrial transcription in invertebrates

The MTERF protein family includes two invertebrate mitochondrial transcription termination factors, sea urchin mtDBP and *Drosophila* DmTTF. Both are involved in terminating transcription, and have also been implicated in transcription initiation and regulation of mtDNA replication. This multiplicity of functions appears to be a theme in the MTERF protein family, and our current understanding of these two invertebrate termination factors can provide insight into the roles of MTERF protein family members as well as the termination process.

5.1 Sea urchin mitochondrial transcription termination factor (mtDBP)

The mtDNA of the sea urchin *Paracentrotus lividus* encodes the same genes found in mammalian mtDNA, although the genetic organization is slightly different – the ribosomal RNA genes are separated by a 3.3 kb region that contains a cluster of 15 tRNA genes as well as genes for two polypeptides [82]. Like mammalian mtDNA, the sea urchin mtDNA also contains a non-coding D-loop region, although it is very short (~130 bp) and is located in the tRNA gene cluster downstream of the 12S rRNA gene. Mitochondrial transcription is thought to occur via multiple transcription units [83], but the precise initiation sites have not yet been defined. The sea urchin mitochondrial transcription termination factor, mtDBP, has been studied in detail by Cantatore and colleagues, and is known to bind with high affinity to two sites in the sea urchin mtDNA, one of which is located in the D-loop region, near the origin of replication [84]. The observation that mtDBP displays a significant homology with human MTERF1 led to the idea that it could possess a transcription termination activity [85], and initial in vitro experiments showed that indeed, mtDBP exhibits bidirectional transcription termination activity in the presence of human mitochondrial RNA polymerase, and unidirectional termination activity with bacteriophage polymerases [86]. More recently, an in vitro transcription assay using mtDBP and the endogenous sea urchin mitochondrial polymerase (mtRNAP) showed that termination of transcription occurred in a mtDBP-dependent manner when the enzyme approached the D-loop region binding site in the direction of L-strand transcription [87]. However, when the enzyme encountered the binding site in the opposite direction, termination occurred independently of mtDBP. This has led to the suggestion that, like human MTERF1, mtDBP acts as a polar termination factor, and that mitochondrial transcription termination in sea urchin may take place by two alternative modes [70]. In vitro experiments have also shown that mtDBP exhibits bidirectional contrahelicase activity [88], suggesting a role as a negative regulator of replication. Furthermore, Roberti et al have observed that H-strand read-through transcription and simultaneous sequence-dependent termination at the mtDBP binding site causes mtDBP to fall off the mtDNA, an event which may relieve helicase impairment and therefore allow mtDNA replication to resume [70]. Thus, it is likely that mtDBP may help regulate the balance between mtDNA transcription and replication in sea urchin mitochondria. Similarly,

a role in mammalian mtDNA replication has also been suggested for MTERF1, based in part on the observation that the level of its expression in cultured human cells influences replication pausing in the vicinity of MTERF1 binding sites [89]. In a manner similar to that of mtDBP, it has been proposed that MTERF1 may facilitate the orderly passage of oppositely oriented transcription and replication complexes, helping to prevent collapse of the replication fork and subsequent generation of recombinogenic ends that could facilitate detrimental rearrangements in the mtDNA [89].

5.2 *Drosophila* transcription termination factor (DmTTF)

The *Drosophila melanogaster* mtDNA genome is larger than that of humans or sea urchin, mostly due to a large non-coding region of about 4.6 kbp that is A–T rich [90]. The genomic architecture is also markedly different from that of human and sea urchin genomes, with four gene clusters being equally distributed between the light and heavy strands. Initial mapping studies suggested that *Drosophila* mtDNA could be transcribed by multiple transcription units and several transcription termination sites were hypothesized [91]. In 2003, a *Drosophila* homologue of human MTERF1 and sea urchin mtDBP, known as DmTTF (for *Drosophila* mitochondrial transcription termination factor) was identified and characterized [92]. DmTTF binds two non-coding sequences located at the end gene blocks that are transcribed in opposite directions – one sequence between the tRNA^{Glu} and tRNA^{Phe} genes and another between tRNA^{Ser} and a polypeptide gene. The locations of these DmTTF binding sequences coincide with two previously predicted transcription termination sites, which led to the suggestion that DmTTF may function as a transcription termination factor [91]. Subsequently, this function was confirmed *in vitro* by measuring the ability of DmTTF to arrest mitochondrial transcription in the presence of human POLRMT and a chimeric DNA template containing both the human HSP promoter and DmTTF binding site [93]. Like mtDBP, DmTTF acts *in vitro* to terminate transcription with directional properties depending on the polymerase used [92]. Although the mechanism of sequence recognition by DmTTF is not yet known, it is interesting to note that DmTTF binds to a sequence containing only A–T base pairs, indicating that sequence recognition is unlikely to involve arginine-guanine interactions as seen for MTERF1, further highlighting the flexibility of the protein-DNA interaction in different MTERF family members. Analyses of DmTTF function in cultured cells have shown that manipulation of its expression leads to complex effects on transcript levels [94], and whether or not DmTTF plays a role in replication (like its homologues MTERF1 and mtDBP) has not yet been determined.

6. Termination defects and implications for disease

Pathogenic mtDNA mutations were first reported in human patients more than two decades ago [95–97], and these important discoveries provided a genetic basis for the classification of mitochondrial disease. To date, a significant number of pathogenic mtDNA mutations have been identified and many of them have associated clinical phenotypes that have been characterized (reviewed in refs [98–100]). These mutations in the mtDNA often result in altered gene expression and therefore contribute to respiratory defects [98, 99, 101] and other mitochondrial pathologies that have been implicated in aging [102–104] as well as human disease. Most mtDNA mutations are thought to result in either defects in mitochondrial DNA maintenance or defects in mitochondrial translation. Such pathogenic defects in mitochondrial translation are frequently associated with mutations in tRNA genes, and interestingly, the tRNA^{Leu} gene contains the highest number of identified mutations of any mitochondrial tRNA gene. Not surprisingly, several of these mutations occur in the MTERF1 binding sequence. One such mutation, A3243G, is associated with mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), a syndrome which is characterized by lactic acidosis, episodic vomiting, seizures and recurrent cerebral insults resembling strokes that lead to hemiparesis, hemianopsia or

cortical blindness [105]. The A3243G mutation, which has been shown to affect transcription in vitro [106], is the most common mtDNA mutation with a prevalence of more than 200 per 100,000 in a recent Australian study [107], and accounts for the majority (80%) of MELAS cases [108, 109]. Despite the observed in vitro termination defect, in vivo studies have shown that the A3243G mutation does not affect the balance between mitochondrial HSP transcripts upstream and downstream of the termination sequence [110]. Moreover, it has been shown that A3243G leads to defects in tRNA function, suggesting that these are the main cause of MELAS pathology rather than transcriptional alterations [111]. However, it is important to note that the transcript ratios measured in these in vivo studies only provide a measure of HSP termination, and therefore it is possible that defects in LSP termination might exist in A3243G carriers and contribute to MELAS pathogenesis. Furthermore, it has also been suggested that termination may be regulated in a tissue-specific manner in vivo [112], such that termination defects occur predominately in particular cell types that have not yet been studied in vivo.

After the MTERF1 structure was solved, revealing the basis for MTERF1 sequence specificity, transcription termination assays performed on seven other mutations within the termination sequence also revealed effects on transcriptional termination (see Table 1). Of these, two showed an effect that was significantly stronger than that observed with the A3243G mutation [53]. These mutations include the G3249A mutation that causes a variant of Kearns-Sayre syndrome [113] as well as the G3242A mutation, associated with an uncharacterized mitochondrial disorder [114]. Although tRNA mutations are generally thought to predominately affect the function of the mature tRNAs [115], these in vitro observations raise the possibility that the pathogenic effects of these mutations are also related to their effect on termination by MTERF1. Furthermore, given that MTERF1 appears to be needed for initiation at HSP2 and recycling of transcription at HSP1, it is possible that MTERF1 might play a role in promoter regulation, which could also be impacted by A3243G and other mtDNA mutations.

In considering the effects of these mutations it is important to keep in mind that, in addition to potential effects in transcription, MTERF1 appears to be able to modulate replication and therefore affect mitochondrial DNA maintenance [89]. Moreover, patients harboring pathogenic mtDNA mutations often are heteroplasmic, i.e. have a mixture of wild-type and mutated mtDNA molecules [116]. Furthermore, the appearance and severity of clinical symptoms seems to correlate with the mutation load [117]. Interestingly, it has been reported that long-term culture of human cells with the A3243G mtDNA mutation results in an increase in the relative levels of A3243G mutant mtDNA [118, 119], suggesting the mutation confers a replicative advantage. With this in mind, it is tempting to speculate that in some cases, the primary deficiency caused by mutations in the tRNA^{Leu} MTERF1 binding site may be a defect in tRNA function, but the additional effect of these mutations on MTERF1 binding and/or function results in more rapid replication of the mutated genomes, thus facilitating an increase in the mutation load. This, in turn, would contribute to the appearance of clinical symptoms and/or an increase in their severity.

Given the unusual and important roles of MTERF family members in transcription termination and initiation, as well as replication, more in-depth study of this interesting class of proteins is clearly warranted. A more detailed molecular understanding of how these proteins interact with the known transcription machinery, how they interact with each other, and how they are utilized differentially to control mtDNA expression in vivo will also provide insight into the pathogenesis of MELAS and other mitochondrial diseases.

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HIGHLIGHTS

- Termination of transcription in mitochondria is not well characterized.
- Human MTERF1 mediates termination at a major site in tRNA^{Leu}.
- MTERF1 utilizes a unique binding mechanism to promote termination.
- There are connections between transcription termination and mtDNA replication.
- Other MTERF family members have been implicated in termination and replication.

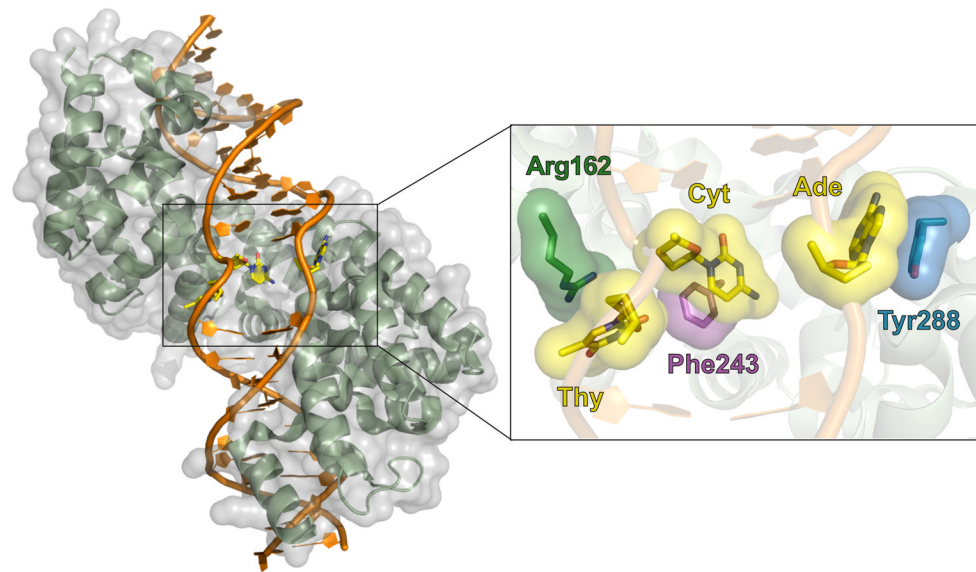


Figure 2. The X-ray Crystallographic Structure of the Mitochondrial Transcription Termination Factor MTERF1 Bound to the tRNA^{Leu} Mitochondrial DNA Termination Sequence MTERF1 utilizes a unique DNA binding mode that results in duplex unwinding and eversion of three nucleotide bases. MTERF1 is shown in green with its molecular surface shown in transparent grey; the DNA termination site is shown in orange; everted bases are shown as yellow sticks. *Inset:* π -stacking interactions with the MTERF1 residues (dark green, purple, and blue) stabilize the three corresponding DNA bases (yellow) in an extrahelical position. These interactions are essential for termination activity.

Table 1Pathogenic mtDNA mutations in the mitochondrial tRNA^{Leu} termination sequence

mtDNA Mutation	Termination defect	Functional tRNA defect	Clinical disease
A3236G	+ [53]	–	Sporadic bilateral optic neuropathy [119]
G3242A	++ [53]	–	Uncharacterized mitochondrial myopathy [113]
A3243G	+++ [53, 105]	+ [110, 120, 121]	MELAS (>80% of cases) [107, 108]
A3243T	++ [53]	+ [122]	Mitochondrial encephalopathy [123]
G3244A	++ [53]	+ [110]	Associated with MELAS, not fully characterized [113]
G3249A	+++ [53]	–	Variant of Kearns-Sayre syndrome [112]
T3250C	+ [53]	+ [124]	Mitochondrial myopathy [125]
A3251G	+ [53]	–	Mitochondrial myopathy [126]
A3252G	– [53]	–	MELAS [127]