MTERFD3 is a Mitochondrial Protein that Modulates Oxidative Phosphorylation

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MTERFD3 IS A MITOCHONDRIAL PROTEIN THAT MODULATES OXIDATIVE PHOSPHORYLATION

By

Corneliu Luca

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MTERFD3 IS A MITOCHONDRIAL PROTEIN THAT MODULATES
OXIDATIVE PHOSPHORYLATION

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Mitochondrial function is critical for the survival of eukaryotes. Hence, mitochondrial dysfunctions are involved in numerous human diseases. An essential process for a normal mitochondrial function is mitochondrial gene expression which is tightly regulated in response to various physiological changes. The accurate control of mitochondrial gene expression is essential in order to provide the appropriate oxidative phosphorylation capacity for diverse metabolic demands. Recent findings in the basic mitochondrial replication and transcription regulation helped advance our understanding of organelle function and basic pathogenetic mechanisms of mitochondrial DNA mutations associated with oxidative phosphorylation defects.

Mitochondrial transcription is regulated by the mitochondrial transcription termination factor (mTERF) both at the initiation and termination levels. A protein family containing highly conserved mTERF motifs has been identified recently and its members named generically as “terfins.” In this work, one of these factors, mTERFD3, has been characterized in vitro and in vivo. The mTERFD3 protein is highly conserved throughout evolution. It is a mitochondrial protein localized to the matrix and is abundantly expressed in high energy demand tissues. We found that it contains 4 putative leucine
zippers and is able to form dimers \textit{in vitro}. We showed that mTERFD3 binds mtDNA at the transcription initiation site in the mtDNA regulatory region. These findings suggest that mTERFD3 may be involved in regulating mitochondrial gene expression at the transcriptional initiation level.

In order to study the functional significance of mTERFD3 \textit{in vivo} we developed a mouse deficient in mTERFD3 using a gene trapping strategy. The KO mice had a normal lifespan but showed decreased weight gain and decreased fat content in females. Fibroblasts isolated from KO mice displayed decreased growth rate when compared with WT in respiratory media, and had decreased complex IV activity. Consistent with the above findings, we found that muscle, one of the tissues with high energy demands, showed abnormal mitochondrial function, displaying features characteristic of mitochondrial myopathy such as decreased muscle strength and endurance. Muscle mitochondria of the KO mice showed a significant decrease in the complex II +III and complex IV activity. The decrease in OXPHOS complexes activity was associated with increased citrate synthase activity, suggesting mitochondrial proliferation, a feature typical for mitochondrial disorders. Another important finding was a decrease in the muscle mitochondrial transcripts in the KO animals associated with decreased steady state levels of OXPHOS subunits.

Together these data suggest that mTERFD3 is a mitochondrial protein involved in the regulation of mtDNA transcription. mTERFD3 KO is not embryonic lethal suggesting that it is involved in the fine tuning of mitochondrial transcription. We conclude that mTERFD3 is a mitochondrial protein that modulates oxidative phosphorylation function, probably by directed interactions with the mtDNA regulatory region. This work shows
the importance of mTERFD3, an mTERF family member, in the mitochondrial gene expression regulation.
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ABBREVIATIONS

ANT1  adenosine nucleotide translocator
COX   cytochrome oxidase
CSB   consensus sequence block
ETC   electron transport chain
HSP   heavy strand promoter
KSS   Kearns Sayre syndrome
LHON  Leber’s hereditary optic neuropathy
LSP   light strand promoter
MELAS Mitochondrial myopathy, encephalopathy, lactic acidosis with stroke like episodes
MERRF Myoclonus epilepsy with red-ragged fibers
MILON Mitochondrial Late-Onset Neurodegeneration Mice
MILS  maternally inherited Leigh syndrome
MNGIE Mitochondrial neurogastrointestinal encephalomyopathy
mTERF mitochondrial transcription termination factor
mTERFD3(mTERF2) mitochondrial transcription termination factor domain containing 3
mTERFD1(mTERF3) mitochondrial transcription termination factor domain containing 1
mtDNA mitochondrial DNA
MTSSB mitochondrial single stranded binding protein
NARP Neurogenic weakness, ataxia and retinitis pigmentosa
NRF nuclear respiratory factor
OXPHOS  oxidative phosphorylation

POLG mitochondrial DNA polymerase gamma

PEO Progressive external ophthalmoplegia

PGC1α (PPARγ coactivator) Peroxisome Proliferator Activating Receptor gamma coactivator 1 α

POLRMT mitochondrial RNA polymerase

SOD superoxide dismutase

ROS reactive oxidative species

TAS termination associated sequences

TFAM mitochondrial transcription factor A

TFB1M, TFB2M mitochondrial transcription factor B1 and B2
The mitochondrial transcription termination factor mTERF is an evolutionary conserved protein whose main function is believed to be associated with termination of transcription from the heavy strand promoter of mtDNA. Recently, mTERF was also shown to be involved in regulation of transcriptional initiation and replication suggesting that the mTERF function is much more complex than previously thought. mTERF is part of a family of well conserved proteins whose functions are not characterized yet. Bioinformatic tools identified 3 new genes in vertebrates coding for proteins homologues of mTERF. Aiming at shedding light on the function of mTERF protein family members, we have characterized in vitro and in vivo the function of one of the mTERF homologues namely mTERFD3.

In the first part of this dissertation we show that mTERFD3 is well conserved throughout evolution indicating that it has an important biological function. Using immunohistochemical methods we confirmed that mTERFD3 is indeed localized in the mitochondria. Mitochondrial fractionation experiments suggest that mTERFD3 is localized in the matrix compartment. The pattern of expression on northern blot is typical for a mitochondrial protein with high expression in heart, brain, liver and kidney. Next we have pursued extensive biochemical characterization and showed that mTERFD3 is able to form dimers in vitro. We also show that mTERFD3 binds specifically the heavy strand promoter of mtDNA. All these data make mTERFD3 a strong candidate protein for mitochondrial transcriptional regulation.
In the second part of this work we characterized the function of mTERFD3 in vivo. We have produced a mouse deficient in mTERFD3 using insertional mutagenesis. The KO mice had a normal life span but had a significantly reduced weight, and decreased fat content. Fibroblasts deficient in mTERFD3 grew slower in media requiring a functional OXPHOS suggesting a respiration defect. In accordance, the activity of complex IV was significantly reduced in KO fibroblasts. In vivo mitochondrial protein labeling showed that fibroblast deficient in mTERFD3 had decreased mitochondrial protein synthesis. This was likely due to decreased transcription from the heavy strand promoter since mitochondrial DNA transcripts were significantly decreased as shown by northern blot experiments.

In the muscle of mTERFD3 KO mice we have identified features suggestive of a mitochondrial myopathy. Mice deficient in mTERFD3 displayed decreased grip strength and decreased endurance when exercised on treadmill. Biochemical characterization showed decreased complex II+III and complex IV activity in muscle. The OXPHOS defect is associated with increased citrate synthase activity and increased succinate dehydrogenase staining indicating mitochondrial proliferation. Indeed, electron micrograph imaging of deficient muscle showed an increased number and size of mitochondria. This extensive proliferation is likely the result of a compensatory mechanism activated as a response to OXPHOS defect. This mechanism involves upregulation of PGC-1α, a well known transcriptional coactivator that regulates mitochondrial biogenesis, which was found to be upregulated in mTERFD3 KO mice. Analysis of mitochondrial transcripts in the mTERFD3 KO muscle revealed a decrease in
several mRNAs. The most significant decrease was found in ATPase 6 and ND6 subunits, suggesting the importance of mTERFD3 in mitochondrial transcription.

We can conclude from our work that mTERFD3 is a mitochondrial protein that plays an important role in modulation of OXPHOS by regulating transcription at the initiation level. mTERFD3 is not embryonic lethal and no other defects in other tissues besides muscle have been identified. Its function may be related to fine tuning of OXPHOS in muscle. Further work is necessary to fully understand the involvement of mTERFD3 in transcriptional regulation. The KO mice will be an important tool in future studies to evaluate the mitochondrial transcription regulation in response to stress challenges and to understand the compensatory mechanisms activated as a response to OXPHOS defect.
CHAPTER 1

MITOCHONDRIAL FUNCTION AND OXPHOS DEFECTS

1.1 Mitochondria

Mitochondria are eukaryotic organelles that generate most of the energy required by cells through a process known as oxidative phosphorylation (OXPHOS). Besides ATP production mitochondria are also involved in other important functions such as fatty acid metabolism, programmed cell death, reactive oxygen species production, citric acid cycle and calcium buffering. They are descendants of α protobacteria which during evolution gained eukaryotic functions (Lang et al., 1999). In an endosymbiotic process, aerobic bacteria have been engulfed by larger anaerobic cells followed by gene transfer to the nucleus of host cell (Raven, 1970). The present mitochondria retain many prokaryotic characteristics such as chloramphenicol sensitive protein synthesis. These organelles have a unique structure; they consist of two membranes, an outer and inner membrane. The inner membrane invaginates and forms special structures called cristae in which most of the OXPHOS proteins are located (Griparic and van der Bliek, 2001). The mitochondrial matrix contains the mitochondrial DNA (mtDNA), bacterial like ribosomes and more than 600 different proteins (Taylor et al., 2003). Although mitochondria contain DNA, most of the mitochondrial proteins are encoded by nuclear DNA and later imported using specialized machinery. Mammalians cells contain a variable number of mitochondria ranging from 100 to 10000 per cell. Mitochondria form a dynamic network in which fusion and fission play an important role in redistribution of mitochondrial content. It is thought that the balance between mitochondrial fusion and fission is essential in mitochondrial function and maintenance (Chen et al., 2003). By definition
mitochondria are able to express an integral genome and generate ATP coupled with electron transport.

1.2 The structure and function of OXPHOS system

The OXPHOS is a fundamental biochemical process in which the proton gradient produced during the electron transport chain is used by the ATP synthase to produce ATP. The OXPHOS system consists of 5 multiprotein complexes (complex I-V) depicted schematically in figure 1.1. Most of the respiratory complexes subunits (approximately 90) are encoded by nuclear DNA and only 13 by mtDNA. During oxidation of carbohydrates in the citric acid cycle and lipids via beta oxidation, electrons carriers (NADH and FADH$_2$) are produced. These electrons are transported by electron transport chain (complexes I to IV). Based on the chemiosmotic theory proposed by Mitchell the redox reactions in the respiratory chain produce an electrochemical proton gradient that provides the thermodynamic link between respiration and ATP synthesis (Mitchell and Moyle, 1967).

Complex I, or NADH: ubiquinone oxidoreductase is the largest complex of the respiratory chain. It has an L shape and consists of 44 subunits, 7 of which are mitochondrial encoded. It contains 6 iron-sulfur clusters and one flavin mononucleotide cofactor. NADH is oxidized and two electrons are transferred to FMN center. These electrons are then transferred via iron sulfur clusters to the matrix side to reduce ubiquinone to ubiquinol and 4 protons are subsequently transferred to the intermembrane space (Saraste, 1999).
Figure 1.1 Schematic representation of respiratory chain. The oxidative phosphorylation system consists of a series of 4 complexes that transport electrons from one complex to another producing at the same time a proton gradient across the inner membrane. As a result ATP is produced by complex V. Most of the subunits are encoded by the nuclear genome, only 13 being encoded by the mitochondrial DNA (from Kegg pathways).
Complex II, or succinate dehydrogenase, is exclusively nuclear encoded. FADH$_2$ is oxidized and two electrons are transported from the iron/sulfur cluster to ubiquinone producing ubiquinol. During this step no protons are transferred.

Complex III is a ubiquinol-cytochrome $c$ reductase. It catalyses the transfer of electrons from ubiquinol to cytochrome $c$. It contains a membrane embedded cytochrome b, the Rieske Fe/S protein and cytochrome $c_1$. During electron transfer one electron is transferred via the Rieske iron sulfur protein while the second one is transferred using cytochrome b. Cyt b accepts 2 electrons that are transferred to ubiquinone generating ubiquinol and completing a process known as Q cycle. During this process ubiquinone is recycled and protons transferred from the matrix to the inner membrane side (Berry et al., 2000).

Complex IV, or cytochrome $c$ oxidase has 13 subunits. It transfers electrons from the reduced cytochrome $c$ to a pool of oxygen producing water. This complex uses heme groups $a$, $a_3$, and copper atoms to store electrons. For each electron transferred, four protons are released in the inner membrane space (Babcock, 1999).

Coupled to the electron transfer, protons are pumped from the matrix and transferred to the intermembrane space. This gradient is used by ATP synthase (complex V) to produce ATP.

The overall rate of OXPHOS depends on the availability of oxidizable substrates like NADH, the rate of electron transport and ADP concentration. OXPHOS rate also varies significantly in different tissues. It has been suggested that OXPHOS complexes exist in supercomplexes embedded in the inner mitochondrial membrane, thereby
facilitating the electron transfer between complexes and decreasing the chance for electron leaks that would produce excess of reactive oxidative species. The functional roles of respiratory chain supercomplexes are believed to be catalytic enhancement, substrate channeling, and stabilization of complexes (Schagger, 2001).

Under normal conditions 1-2% of electrons escape electron transport chain (ETC) and reduce oxygen to superoxide radical (O$_2^-$). Most of ROS is produced by complex I and complex III. The superoxide, highly toxic to cells, is detoxified using specialized mechanisms such as superoxide dismutase (SOD$_2$), catalase and glutathione peroxidase. When the ROS production exceeds the antioxidant capacity of cells, oxidative stress results. ROS are responsible for oxidative damage to lipids, proteins and DNA. Excess production of ROS has been implicated in carcinogenesis, aging and apoptosis (Halliwell and Cross, 1994).

1.3 Mitochondrial genetics

Mitochondria derive from prokaryotic organisms and their function depends on the coordination of two separate genomes. Most of the mitochondrial proteins are encoded by the nuclear genome and later imported using specialized machinery (Neupert and Brunner, 2002). However, mitochondria have their own DNA that encodes for only 13 polypeptides. During evolution a massive gene transfer from the endosymbiotic prokaryote to the nucleus took place, and mitochondria retained genetic information for only few essential components of the respiratory chain. The mitochondrial genetics has unique properties. First, the genome is maternally inherited (Giles et al., 1980), with all the mtDNA copies inherited from the oocyte. Second, the genome is polyploid. Each mitochondrion contains 2-10 copies of mtDNA molecules, and during cell division the
genome is redistributed randomly. Another important characteristic is a much faster evolution of mitochondrial genome compared to the nuclear genome due to an increased rate of mtDNA mutation (10 fold higher than nuclear DNA) (Cann et al., 1987). This is explained by less efficient repair mechanisms, increased exposure to ROS and lack of histones. Occasionally, wild type and mutant mtDNA can coexist in the same cell, a condition named heteroplasmy. During early mitosis the two mitochondrial genomes can segregate in two distinct cell lineages (Wallace, 1986). The rapid segregation is explained by the “bottleneck phenomenon”, that occurs during oogenesis when the amount of mtDNA is drastically reduced. The primordial germ cell that contains about 50 mtDNA copies will divide and segregate a reduced number of mtDNA so that, in the mature oocyte that contains 100,000 mtDNA copies, the mutation load is drastically reduced (Thorburn and Dahl, 2001). Another important characteristic of mitochondrial genetics is the “threshold effect”. For a given heteroplasmic mutation, a certain load of mutation is necessary to be accumulated in the cell in order to produce a deleterious effect.

Mammalian mitochondrial DNA is a circular double stranded DNA of 16.5 kb in human and 16.3 kb in mouse (Anderson et al., 1981). mtDNA are associated with nucleoids and anchored to the inner membrane (Satoh and Kuroiwa, 1991). The mtDNA organization is conserved throughout evolution. mtDNA is very compact, with virtually no introns.

As previously mentioned, mammalian mtDNA encodes for 13 proteins, 22 tRNA and 2 rRNA (see figure 1.2). All 13 proteins encoded by mtDNA are part of OXPHOS complexes, seven are subunits of complex I (ND1, ND2, ND3, ND4, ND4L, ND5, ND6),
Figure 1.2 Mitochondrial DNA is a 16.5 kb circular double-stranded DNA. It contains a heavy and a light strand that encode for 13 proteins, 22 tRNAs and 2 rRNAs. Heavy-strand transcription is initiated from two sites, HSP1 and HSP2. The HSP1 transcript (in red) terminates at the 3’ end of the 16S rRNA, in a region bound by the mTERF protein. The HSP2 transcript produces a polycistronic molecule that corresponds to almost the entire heavy strand (blue). Transcription from the light-strand promoter (LSP) produces the ND6 mRNA molecule (yellow), some tRNAs and primers for initiation of DNA synthesis at the heavy-strand origin of DNA replication (OH). Non-coding regions are indicated in green (from Asin-Cayuela et al, 2007)
one part of complex III (cyt b), three part of complex IV (COXI, COXII, COXIII), and two part of complex V (ATPase 6 and ATPase 8). The rest of the proteins that compose the OXPHOS complexes are encoded by nuclear DNA and imported in the mitochondria.

Cesium chloride gradient centrifugation experiments demonstrated that mtDNA strands have different buoyant densities due to different GC content. Due to this characteristics mtDNA strands are named heavy or H-strand and light or L-strand. The H-strand is responsible for coding most of the genes whereas the L-strand codes for only one protein and 8 tRNAs. There are only few noncoding regions, the most important being the D-loop, a 1kb region that contains elements important in regulation of transcription and replication. This region, situated between tRNA_{Phe} and tRNA_{Pro}, has a triple stranded structure (Walberg and Clayton, 1981). It contains the origin of replication for the heavy strand O_H, the promoters for transcription of H-strand and L-strand (HSP and LSP respectively), conserved sequence blocks (CSB) and termination associated sequences (TAS). CSB are sites where RNaseMRP cleaves the primer used for initiation of replication. (Chang et al., 1985). Termination associated sequences (TAS) are sites where nascent H strands terminate frequently and form the D-loop structure (Madsen et al., 1993). The small nucleic acid strand that displaces heavy strand is named 7S DNA. The other important noncoding region is a 30 nucleotide region that is thought to be the origin of replication for L strand (O_L).

The mtDNA genetic code is different from the nuclear code: the UGA codon which is a termination signal in universal genetic code, encodes tryptophene in mitochondria, AUA codes for methionine instead of isoleucine and AGG and AGA are termination codons (Bonitz et al., 1980).
1.3.1 Mitochondrial replication. Replication of mitochondria is independent of the cell cycle. The half life of mtDNA varies from 7 days in heart to 30 days in brain (Gross et al., 1969). Each mtDNA molecule replicates independently of others in the nucleoid, a DNA-protein complex associated with the inner mitochondrial membrane (Kaufman et al., 2007). Mitochondria has its unique set of proteins involved in replication. The regulation of mtDNA copy number is tightly controlled as suggested by rapid restoration of mtDNA levels after depletion in culture (Moraes et al., 1999). Many of the components of the replication machinery have been identified but there is still controversy on how exactly the replication occurs. Replication is a slow process and it takes about 2 hours to complete (Clayton, 1982).

Mitochondrial replication requires a mitochondrial DNA polymerase $\gamma$ (POLG). POLG is a 140 kDa heterotrimer formed from one catalytic $\alpha$ subunit and 2 accessory $\beta$ subunits (Gray and Wong, 1992). The $\alpha$ subunit has 5’-3’ DNA polymerase activity and 3’-5’ exonuclease activity responsible for high fidelity of transcription, whereas the $\beta$ subunit is responsible for processivity. Mutation in the POLG gene have been associated with progressive external ophthalmoplegia, a disease associated with multiple, large mtDNA deletions (Van Goethem et al., 2001).

Mitochondrial single strand binding protein MTSSB is a 15kDa protein that is present as a tetramer and is essential for maintenance of mtDNA. It stabilizes ssDNA in the D-loop and other replicative intermediates (Takamatsu et al., 2002).

Mitochondrial DNA helicase, Twinkle, is a hexamer that unwinds dsDNA and provides ssDNA template for POLG. Mutations in Twinkle are associated with
progressive external ophthalmoplegia. POLG, Twinkle and MTSSB act together at the replication fork to form the replisome (Korhonen et al., 2004).

There are 2 replication initiation sites, one for the heavy strand $O_H$ situated in the D-loop, and one for the light strand $O_L$. In order to initiate the replication the formation of an RNA primer is necessary. The RNA primer is thought to be processed by RNAse MRP. The conserved sequence blocks I, II, and III situated in the D-loop downstream of LSP, are associated with RNA-DNA transition. Recently, CSB II was identified as a strong transcription termination element important in the RNA primer formation (Pham et al., 2006).

The nascent H strand is frequently terminated, with the resulting formation of 7S DNA responsible for the triple stranded structure called D-loop. Termination associated sequences (TAS) have been identified at the 3’ end of D-loop and have been proposed to regulate mtDNA replication (Madsen et al., 1993). The proteins involved in this event are not known but there is some speculation that mTERF proteins may be involved in this process. This idea is plausible since mTERF homologue in sea urchin is able to stop replication and has contrahelicase activity (Loguercio Polosa et al., 1999).

There are two models proposed for the replication of mtDNA in mammals. In the strand asynchronous model the replication occurs unidirectionaly with the synthesis of the daughter H strand starting at $O_H$. A transcription event from LSP promoter forms the primer necessary for initiation at $O_H$. When replication of heavy strand reaches 2/3 of the genome, $O_L$ is activated and the replication of the light chain starts in the opposite direction (Clayton, 1982). Both strands are replicated continuously, starting from different points, without forming lagging strands. Most replication events initiated from
O₁₁ are aborted soon after initiation producing a triple strand or displacement loop named D-loop.

The strand synchronous model proposes the classic leading and lagging strand replication forks as detected in two dimensional gel electrophoresis by Holt and Jacobson in 2000. This model suggests that replication occurs symmetrically in the leading-lagging strand and starting from different points (Holt et al., 2000). If the replication occurs in this mode, a primase is also necessary to remove the primer used for the lagging strand, and this remains unidentified so far. It is thought that both mechanism are present in mammalian cells. The asynchronous model is used in normal condition, whereas when mtDNA copy number is altered the cells are using the synchronous model.

Mitochondrial DNA replication usually takes about 2 hours and is cell cycle independent. After the synthesis is complete, RNA primers are removed, DNA ligated and negative supercoils formed.

1.3.2 Mitochondrial transcription is regulated both at the initiation and termination through cis and trans acting elements.

a) Transcriptional initiation. The basic mtDNA transcription machinery was identified in the 1980s. Transcription from mitochondrial promoters produces polycistronic transcripts that encode for 2 ribosomal RNA, 13 messenger RNA and 22 transfer RNA. Mitochondrial transcription takes place from two heavy strand promoters (HSP) and a light strand promoter (LSP) located within 150 bp of one another in the D-loop (see figure 1.3), and in opposite orientation (Chang and Clayton, 1984). Enhancer elements located upstream of these promoters are required for optimal transcription and are regulated by trans acting elements.
The heavy strand of mouse mtDNA contains two initiation sites, HSP1 and HSP2. HSP1 is situated at the 5’ of tRNA\textsuperscript{Phe} (position 16282) and is responsible for the transcription of a short transcript that terminates at the 3’ end of 16S RNA (Sbisa et al., 1990). This transcript is responsible for the production of the majority of rRNA and tRNA\textsuperscript{Leu}. The HSP2 initiation site located downstream (position16290) is used less frequently and is responsible for the production of a long polycistronic molecule. This long transcript is subsequently processed by RNAse P and other unknown enzymes to excise the tRNAs and produce mRNAs in a model known as the tRNA punctuation model. Maturation of the excised tRNAs is completed by addition of the sequence CCA to their 3’-end. Also, mitochondrial mRNAs are polyadenylated by a mitochondrial poly(A) polymerase.

The light chain of mouse mtDNA has one promoter located on the D-loop region with the initiation site at 16184 (LSP). From this promoter several tRNA and one mRNA (ND6) are produced. It is important to mention that this promoter is used for synthesis of a primer essential for initiation of replication.

The basic transcriptional machinery requires a mitochondrial RNA polymerase (POLRMT), transcription factors TFAM and TFB1M or TFB2M. The human mitochondrial RNA polymerase was identified by homology with the yeast polymerase (Tiranti et al., 1997). Interestingly, the mitochondrial RNA polymerase (POLRMT) in eukaryotes is not a multisubunit enzyme but rather a single protein similar with T7 bacteriophage. Also, POLRMT is not able to initiate transcription alone. It requires additional transcription factors such as the mitochondrial transcription factor A and either the mitochondrial transcription factor B1 or B2.
Figure 1.3 Schematic representation of the D-loop regulatory region. The three conserved sequence blocks (CSB I, CSB II, and CSB III) are located just downstream of light-strand promoter (LSP). The conserved termination-associated sequence (TAS) elements are located at the 3’ of the nascent D-loop strands and are proposed as a major regulation point of mtDNA replication. Abbreviations: HSP, heavy-strand promoter; LSP, light-strand promoter; $O_H$, origin of H-strand DNA replication (from Larsson et al, 2007)
TFAM is a high mobility group protein able to bend and wrap the mtDNA. In vitro DNase protection assays indicated that TFAM binds enhancer elements located upstream of the initiation sites for heavy and light chain promoters (Topper and Clayton, 1989). TFAM induces conformational changes in the HSP and LSP and favors the access of POLRMT to the template for transcription (Fisher and Clayton, 1985). TFAM is not able to initiate transcription alone with POLRMT. TFB1M and TFB2M are transcription factors identified by homology with yeast transcription factors. Either one of this transcription factors can initiate transcription in vitro together with POLRMT and TFAM (Falkenberg et al., 2002). Interestingly, these factors display homology to RNA methyltransferases and are able to donate S-adenosyl methionine. Thus these proteins seem to have dual function acting as both transcription factors and an rRNA modification enzyme (McCulloch and Shadel, 2003).

**b) Transcriptional termination.** Transcriptional termination for HSP₁ transcript has been identified at the 3’ end of 16S RNA as suggested by single stranded nuclease protection assays (Dubin et al., 1982). Later, a protein fraction was isolated from HeLa cell lysates based on affinity to a 28bp region situated at the end of 16S RNA molecule (Kruse et al., 1989). Mutagenesis of this region showed that a tridecamer sequence is essential for transcriptional termination (Christianson and Clayton, 1988). The mitochondrial transcription termination factor (mTERF) was cloned and showed to be a highly conserved protein that can form trimers, although only the monomer is able to bind DNA (Asin-Cayuela et al., 2004). It contains three leucine zipper domains necessary for intramolecular folding and two basic domains necessary for DNA binding.
(Fernandez-Silva et al., 1997). Recently phosphorylation of mTERF was shown to be necessary for transcriptional termination (Prieto-Martin et al., 2004). Also mTERF is able to form trimers, however only the monomer is able to bind DNA (Asin-Cayuela et al., 2004).

A pathogenic mutation in the tRNA\textsuperscript{Leu(UUR)} in the 3243 position, present in the MELAS syndrome, was associated with decreased in vitro binding of mTERF to the termination site (Chomyn et al., 1992). However the mutation did not affect the mRNA to rRNA ratio.

mTERF simultaneously binds initiation and transcription sites forming a loop. The loop has been visualized by electron microscopy and may help reinitiation of transcription (Martin et al., 2005). In this model the RNA polymerase and the other factors involved in transcription are shuttled between the initiation and transcription sites and reused for a rapid and efficient transcription of ribosomal RNA. The data suggest that this process involves formation of a DNA loop in which RNA polymerase complexes are recycled around the rDNA segment of the genome (see figure 1.4).

The natural mTERF monomer, extracted from HeLa lysate was shown to bind simultaneously the initiation and termination of transcription sites. However, it is important to notice that recombinant mTERF is not able to bind the initiation site suggesting the need for additional factors or posttranslational modifications. It is possible that the mTERF molecule binds to the termination site directly and to the H\textsubscript{i} site with the participation of auxiliary proteins.
Figure 1.4 Proposed model for mTERF mediated ribosomal DNA looping: mTERF simultaneously binds initiation (H₁), and termination (mTERM) site in mtDNA, forming a loop. H₁ promoter is used for rRNA synthesis whereas H₂ promoter is used for synthesis of a long polycistronic transcript. Mitochondrial transcription requires a RNA polymerase (orange), transcription factor A (TFAM), depicted in green and either one of TFB1M or TFB2M. mTERF is mediating the formation of a rDNA loop that promotes recycling of transcription factors (from Martin et al., 2005).
Recent data support the idea that mTERF is not only involved in transcriptional regulation but also in replication modulation. mTERF binding at its canonical binding site in the tRNA$^{\text{Leu(UUR)}}$ gene influences replication pauses near this site (Hyvarinen et al., 2007). New binding sites for mTERF have been identified elsewhere in the genome, in the noncoding region and ND1 gene. Binding at these sites was weaker than in the tRNA$^{\text{Leu(UUR)}}$ gene, but replication pausing in these regions was nevertheless influenced by mTERF overexpression. The authors concluded that mTERF acts as a replication pausing system that evolved to limit genomic instability by preventing collisions of the replication and transcription machineries, and that mTERF is a possible candidate gene for involvement in genetic disorders mediated by mtDNA rearrangements such as progressive external ophthalmoplegia (PEO).

A termination site for the HSP$_2$ transcript has been described on the D-loop region upstream of tRNA$^{\text{Phe}}$ but the proteins involved are not known (Camasmudram et al., 2003). The termination of the light strand was not studied so far, but the fact that mTERF can terminate transcription bidirectionally suggests that is a possible candidate for transcriptional termination in the light strand.

mTERF protein family was recently identified as a large family of proteins containing 4 subgroups (Linder et al., 2005). MTERF1 and 2 are conserved in vertebrates whereas 3 and 4 are present in lower species. Interestingly there are not yeast homologues of mTERF proteins. The plant homologue termed MOC1, is targeted to the mitochondrion, and its expression is up-regulated in response to light (Schonfeld et al., 2004). Loss of MOC1 causes a high light-sensitive phenotype and disrupts the
transcription and expression profiles of the mitochondrial respiratory complexes causing, as compared with wild type, light-mediated changes in the expression levels of nuclear and mitochondrial encoded cytochrome c oxidase subunits and ubiquinone-NAD subunits. An mTERF orthologue in Paracentrotus lividus (mtDBP) stops transcription bidirectionally in the D-loop region and has contrahelicase activity (Loguercio Polosa et al., 1999). It has been speculated that a similar mechanism may function in mammals and mTERF homologues would be involved in transcription termination in the TAS regions.

So far few reports about mTERF homologues are available. MTERFL (MTERF2) is a human homologue of mTERF that is localized to mitochondria and is highly expressed in heart, muscle and liver (Li et al., 2005). mTERF3 homologue in Drosophila has been implicated in regulation of mitochondrial protein synthesis (Roberti et al., 2006). Recently, the mTERF3 (mTERFD1) has been characterized in vivo. mTERFD1 KO is embryonic lethal. Tissue specific inactivation of mTERFD1 in heart was associated with cardiomyopathy, decreased OXPHOS activity and mitochondrial proliferation. The authors showed that the mitochondrial transcripts from the heavy strand were upregulated in the KO animals and concluded that mTERFD1 acts as a repressor of mitochondrial gene expression (Park et al., 2007).

1.3.3 Mitochondrial DNA maintenance. Maintenance of the mitochondrial genome is essential for aerobic metabolism. The number of mtDNA molecules vary within different tissues of mammals and is dependent on the OXPHOS capacity of a given tissue. It is thought that the mass of mtDNA rather than copy number is maintained constant (Tang et al., 2000). While one the limiting factor for mtDNA mass is the mitochondrial nucleotide
pool size, the fine tuning of mtDNA copy number is mediated by DNA binding transcription factors such as nuclear respiratory factor NRF1 and NRF2 and nuclear coactivators like peroxisome proliferator activated receptor gamma coactivator PGC-1. NRFs are transcription factors that increase expression of genes like TFAM, TFB1M and TFB2M that are directly involved in mtDNA transcription and maintenance (Scarpulla, 2002). PGC-1α is a transcriptional coactivator that is upregulated in response to physiological stimuli like cold, fasting and exercise (Puigserver et al., 1998).

Mitochondrial transcription factor A, TFAM, was originally described as essential for transcriptional initiation. Later it was clear that it is also essential for the regulation of mtDNA copy number. TFAM is an abundant protein that is closely associated with mtDNA. Heterozygous TFAM KO mice have decreased mtDNA copy number (Larsson et al., 1998) and overexpression of TFAM is associated with increased mtDNA number (Ekstrand et al., 2004). TFAM promoter is under tight control of NRF1, suggesting that NRF1 controls mtDNA copy number through TFAM.

1.4 OXPHOS defects in human diseases

Oxidative phosphorylation capacity is highly variable and diverse, as determined by different combinations of the mitochondrial content, the amount of respiratory chain complexes, and their intrinsic activity (Rossignol et al., 2000). Skeletal muscle and heart present with the highest OXPHOS capacity and a low resistance against the occurrence of respiratory chain perturbations. Conversely, liver and kidney are characterized by a lower OXPHOS capacity and a lower sensitivity to OXPHOS defects. The brain is between the first and second group regarding the OXPHOS capacity and flux response.
Mitochondrial diseases present with a wide spectrum of clinical manifestations. The prevalence of mitochondrial diseases is 12 in 100,000 individuals (Chinnery et al., 2000). They are caused by either mtDNA mutations or nuclear DNA mutations of genes involved in respiratory chain. The complexity of the respiratory chain accounts for a wide range of clinical manifestations of mitochondrial disorders. Because muscle and central nervous system are often affected, mitochondrial disorders affecting these tissues are called encephalomyopathies. They are characterized by a variable combination of muscle weakness, myalgia, ophthalmoplegia and ptosis, pigmentary retinopathy, optic atrophy, sensorineural hearing loss, seizures, ataxia, myoclonus and stroke like episodes. Histological features include mitochondrial proliferation seen as red-ragged fibers with the gomori-trichrome staining.

To be pathogenic a mtDNA mutation has to reach a certain level as the WT can compensate for the mutated DNA in heteroplasmic conditions. This threshold effect can vary from 60% to 95% mutant. To date there are more than 200 different mtDNA mutations broadly classified as point mutations or deletions. The mtDNA point mutations are usually maternally inherited and can affect protein, tRNA, and rRNA encoding genes. The mtDNA deletions and duplications are sporadic whereas defects in nuclear genes associated with mtDNA replication and maintenance show mendelian inheritance pattern. All these different diseases cause OXPHOS defects that manifests at mitochondrial and cellular level. At the mitochondrial level the ATP production, calcium homeostasis and ROS production are affected.

In addition to well defined mutations in mtDNA there is also evidence that mitochondrial dysfunction and possibly age-related mtDNA mutations play a role in
neurodegeneration, diabetes and heart failure. Also the accumulation of somatic mtDNA deletions has been hypothesized to play a role in normal aging.

1.4.1 mtDNA point mutations are maternally inherited. The majority of the pathogenic mtDNA point mutations are heteroplasmic and the mutant DNA is inherited in a variable proportion from the maternal germline.

Leber’s hereditary optic neuropathy (LHON) is a disease characterized by adult onset blindness due to retinal ganglia cell death followed by optic nerve atrophy (Wallace et al., 1988). Mutations in subunits of complex I are associated with LHON, and these mutations are usually homoplasmic. The most common mutations are in ND1 subunit (3460), ND4 subunit (11778) and ND6 (14484). These mutations affect highly conserved aminoacids, producing complex I deficiency and hence increased ROS production. Besides mtDNA mutations, other factors are necessary to produce disease. As a consequence, the penetrance is incomplete and controlled by environmental factors, other mitochondrial factors or nuclear genes. Optic nerves of patient with LHON show degenerative features in axons and myelin sheet. However it is not known what makes retinal ganglion cells selectively vulnerable to this defect. The disease affects predominantly young adults, often males. Visual acuity deteriorates rapidly over a period of days-weeks usually followed by the other eye (Nikoskelainen et al., 1983). We have found that high doses of macrolides antibiotics, which affect mitochondrial protein synthesis, can also precipitate the onset of LHON (Luca et al., 2004).

Sensorineural hearing loss can be associated with A1555G transition in the 12S rRNA gene. Like LHON this disease is homoplasmic and with incomplete penetrance. This mutation affects a highly conserved region in 12S RNA, homologous to the bacterial
domain of aminoglycoside binding and makes it more susceptible to antibiotic binding (Prezant et al., 1993). However, deafness is also encountered in subjects with 1555 mutation who were never exposed to aminoglycosides. The hair cell and cochlea like optic nerve are tissues that require a lot of ATP, making them susceptible to damage when mitochondrial dysfunction is present.

Neurogenic weakness, ataxia and retinitis pigmentosa (NARP) is a disease caused by T8993G mutation in ATPase 6 gene (Holt et al., 1990). Clinical syndrome is evident when the level of heteroplasmy is above 60%. In these patients there is a significant defect in the rate of ATP synthesis. When the level of heteroplasmy is above 90% the disease is more severe and called maternally inherited Leigh syndrome (MILS).

Mitochondrial myopathy, encephalopathy, lactic acidosis with stroke like episodes (MELAS) is a disease associated with a mutation in tRNA_{Leu(UUR)} gene. It is characterized by stroke like episodes due to focal brain lesions, lactic acidosis and red ragged fibers. Other associated manifestations are headache, vomiting, ataxia, diabetes or cardiomyopathy. The most frequent mutation is the A3243G mutation (Goto et al., 1990). When the mutation reaches a certain threshold (usually more than 80%) it produces a severe defect in mitochondrial protein synthesis associated with defect in complex I and IV.

Myoclonus epilepsy with red-ragged fibers (MERRF) is caused by a mutation in tRNA_{Lys} in the position 8344 (Shoffner et al., 1990). Patients display a severe defect in mitochondrial protein synthesis similar to MELAS pathology. The clinical presentation is epilepsy, ataxia and myopathy, deafness and dementia.
1.4.2 Mitochondrial DNA deletions. Deletions and duplications of mtDNA are associated with severe mitochondrial diseases that are progressive and usually cause death. These mutations are sporadic and thought to arise de novo early in embryogenesis. Random mtDNA segregation events during embryogenesis are responsible for different heteroplasmic levels of deletions in different tissues. Single mtDNA deletions have been associated with three major clinical groups: Progressive external opthalmoplegia, Kearns-Sayre syndrome and Pearson’s syndrome.

Progressive external opthalmoplegia (PEO) is caused by large deletion of mtDNA in muscle, characterized by bilateral ptosis and opthalmoplegia, muscle weakness and wasting. In response to severe COX deficiency mitochondria proliferate and form red ragged fibers.

Kearns Sayre syndrome (KSS) is a sporadic severe disorder characterized by progressive external opthalmoplegia, pigmentary retinopathy, and cardiac conduction defects with onset before age of 20. KSS patients have large deletions in muscle and severe OXPHOS defects. Neuroradiologically KSS patients present with distinctive abnormalities of the deep structures of the brain and subcortical white matter.

Pearson bone marrow-pancreas syndrome is a rare disorder of infancy characterized by pancytopenia and pancreatic insufficiency. Infants surviving infancy may present with KSS features later in life.

1.4.3 Mutations in the nuclear genome. Mutations in different complexes are associated with Leigh syndrome. Mutations in the SURF1 gene that participates in complex IV assembly is associated with cytochrome oxidase deficiency and Leigh syndrome (Tiranti et al., 1998).
Multiple mtDNA deletions are also associated with nuclear gene mutations. In these cases inheritance is mendelian. Autosomal dominant progressive ophtalmoplegia is characterized by progressive muscle weakness affecting external ocular muscles. Three genes have been associated with this disease: POLG, helicase Twinkle and nucleotide transporter ANT1 (Van Goethem et al., 2002).

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disease associated with both deletions and mtDNA depletion due to a mutation in thymidine phosphorylase causing an imbalance in mitochondrial nucleotide pool (Bardosi et al., 1987).

1.5 Animal models of OXPHOS defects

OXPHOS defects have been associated with a wide range of human diseases. In order to understand the respiratory chain dysfunction a number of animal models have been created. Tissue specific knock out approach has been used to produce various respiratory chain disorders that mimic human diseases. Another approach used is production of mice containing a heteroplasmic mtDNA population. Transmitochondrial mice are produced by injecting cytoplasts containing mutated mtDNA in a one cell embryo and subsequent implantation as a 2 cell embryo in pseudopregnant mother (Pinkert and Trounce, 2002).

ANT1, adenosine nucleotide translocator is responsible for nucleotide exchange along the inner mitochondrial membrane. Mice deficient in ANT1 showed cardiac hypertrophy, and mitochondrial myopathy with marked mitochondrial proliferation, decreased respiration and exercise tolerance (Graham et al., 1997). By knocking out ANT1, ADP transport in the cell was impaired, respiration severely reduced and ROS
Mitochondrial superoxide dismutase (SOD2) is an oxygen radical scavenger essential for survival. It converts superoxide radical to hydrogen peroxide which is further processed inside mitochondria by glutathione peroxidase. Mice lacking SOD2 die at day 4-10 postnatally. Accumulation of ROS as a result of a partial SOD2 deficiency results in loss of fatty acid oxidation, fatty liver and cardiac myopathy (Li et al., 1995). Due to circumstantial evidence that increased ROS production is associated with mitochondrial defects, antioxidant therapies were tested to see if in the SOD2 deficient mice the deleterious effects could be reversed. Indeed treatment with MnTBAP, an oxygen radical scavenger, the life span of KO SOD2 animals was increased to 3 weeks (Melov et al., 2001). The SOD2 heterozygous knockouts provided a model for chronic oxidative stress. The result of increased ROS production in heterozygous SOD2 is increased proton leakage across the membrane, oxidation of mitochondrial lipids, sensitization of mitochondrial permeability transition pore and apoptosis (Kokoszka et al., 2001). This study supports the hypothesis that increased ROS production is associated with mitochondrial dysfunction and results in apoptosis, a common feature of aging.

TFAM, an essential regulator of mtDNA gene expression is also important for mtDNA maintenance. The TFAM KO is embryonic lethal whereas heterozygous animals are viable and have reduced mtDNA copy number (Larsson et al., 1998). The heart specific TFAM KO displayed dilative cardiomyopathy and death around day 20,
decreased mtDNA levels and decreased mtDNA transcripts (Wang et al., 1999). This animal model reproduced many features of mitochondrial diseases: decreased mtDNA expression and decreased COX activity, abnormally enlarged mitochondria and severe cardiomyopathy.

Disruption of TFAM in pancreatic beta cells was associated with insulin deficiency and diabetes. Together with decreased mtDNA copy number, the pancreatic beta cells displayed decreased mtDNA expression, respiratory chain deficiency, abnormal mitochondria and decreased blood insulin concentration (Silva et al., 2000). Decreased ATP concentration in the beta cells is responsible for dysfunction of K+ channel and hence decreased insulin secretion.

The Mitochondrial Late-Onset Neurodegeneration Mice (MILON) is another mouse model for mitochondrial disease has been produced by ablation of TFAM in brain. At 6 months the mice deficient in TFAM showed behavioral abnormalities and increased sensitivity to neuronal stress (Sorensen et al., 2001).

Recently, mTERF and its homologues have been the focus of investigation. The recent identification of mTERF family of transcription factors opened the quest for functional characterizations of proteins that may be involved in the mitochondrial transcription regulation. mTERF3 (mTERFD1) is a member of mTERF protein family that is extremely well conserved throughout evolution. Inactivation of this gene is lethal in mice. Tissue specific inactivation in heart was associated with severe respiratory deficiency, cardiomyopathy and increased transcription, suggestion a possible role of mTERF3 as a transcriptional repressor (Park et al., 2007). The increased transcription
initiation was associated with decreased expression of critical promoter-distal tRNA genes.

mTERFD3 (mTERF2) is another member of this highly conserved family of proteins whose function was not investigated. Would be interesting to know if mTERFD3, like his homologues mTERF and mTERFD1, is involved in transcriptional regulation of mtDNA. To elucidate the possible involvement of mTERFD3 in mitochondrial gene expresion we studied the function of this mTERF family member in vitro and in vivo. In the first part of this work we showed that mTERFD3 is localized to mitochondria and that binds specifically the heavy strand promoter suggesting that is involved in regulation of mitochondrial transcription initiation. To determine the effects of mTERFD3 deficiency in vivo we have produced a mouse that lacks mTERFD3. Our results showed that mTERFD3 deficiency is responsible for decreased mitochondrial transcription resulting in decreased OXPHOS activity in muscle and mitochondrial myopathy.
CHAPTER 2

BIOCHEMICAL CHARACTERIZATION OF THE MOUSE mTERFD3

2.1 BACKGROUND

Oxidative phosphorylation (OXPHOS) is an essential process that occurs in mitochondria and is responsible for ATP production. A tight regulation of mitochondrial gene expression is required in order to adapt the energy production to the various requirements of the mammalian metabolism. Mitochondrial DNA transcription is regulated both at the initiation and termination level but these processes are not completely understood. The initiation of transcription requires a specific mitochondrial RNA polymerase (POLRMT), the transcription factor A (TFAM) and either TFB1M or TFB2M. Only one transcription terminator was identified so far. This protein, the mitochondrial transcription termination factor (mTERF) was isolated from HeLa lysate based on its affinity to mtDNA (Kruse et al., 1989). mTERF binds mtDNA at the boundary between 16S rRNA and tRNA^Leu(UUR) and is responsible for termination of transcription at this site (Daga et al., 1993).

Interest in studying mTERF increased after the report that A3243G point mutation present in MELAS syndrome was associated with decreased affinity binding of mTERF to the mtDNA. mTERF has 3 leucine zipper domains that are used for intramolecular folding (Fernandez-Silva et al., 1997) and help bring together the basic domains that interact with mitochondrial DNA (mtDNA). Although mTERF can form oligomers, it seems that the monomer only is able to bind to the termination site (Asin-Cayuela et al., 2004).
A recent report demonstrated that mTERF is able to bind mtDNA not only at the termination but also at the initiation site suggesting a more complex mechanism than previously envisioned. Binding at the initiation site was not possible with recombinant mTERF but only with extracted fraction from cells suggesting a requirement for additional factors (Martin et al., 2005).

Bioinformatic tools have helped identify a family of highly evolutionary conserved mTERF like proteins (Linder et al., 2005). mTERF1 (mTERF) and mTERF2 (mTERFD3) are 2 groups present only in vertebrates, whereas mTERF3 (mTERFD1) and mTERF4 (mTERFD2) genes are encountered not only in vertebrates but also in worms and insects. mTERF1 (mTERF) group contains the previously characterized mTERF protein, whereas mTERF2 contains the mouse mTERFD3 gene characterized in this work. The characterization of mTERF family members is just emerging so that few reports about the function of mTERF like proteins are available. In sea urchin, the mTERF homologue (mtDBP) was shown to stop bidirectionally the mitochondrial RNA elongation (Loguercio Polosa et al., 1999). mtDBP has also a negative regulatory role in mtDNA synthesis since it inhibits a replicative helicase in Paracentrotus lividus. mTERF homologue in Drosophila melanogaster (DmTTF) binds mtDNA in two noncoding regions and was proposed to stop transcription (Roberti et al., 2003). Another mTERF homologue, MOC1, was identified in the green alga Chlamydomonas reinhardtii (Schonfeld et al., 2004). The loss of the MOC1 gene causes a high light-sensitive phenotype and disrupts mitochondrial gene transcription.

Human mTERFL (part of mTERF2 group) was cloned recently and proposed to be a serum-inhibitory factor that participates in the regulation of cell growth through the
modulation of mitochondrial transcription (Chen et al., 2005). The Drosophila mTERF3 homologue was shown to be involved in mitochondrial protein synthesis (Roberti et al., 2006). Recently mouse mTERF3 (mTERFD1) was shown to be involved in transcriptional repression of mtDNA. Mice deficient in mTERFD1 showed a decreased OXPHOS activity associated with increased rate of mitochondrial transcripts (Park et al., 2007).

In the present work we have characterized one of the mouse mTERF homologue, mTERFD3, member of the mTERF2 group that is unique to vertebrates. We showed that mTERFD3 is a mitochondrial protein that binds mitochondrial DNA in the heavy strand promoter region and that is able to form dimers. It also binds mtDNA in the promoter region suggesting that participates in the control of mtDNA expression.

2.2 METHODS

Bioinformatics. Alignments of mTERF homologues were performed using ClustalW server. Coiled-coils were predicted using COILS program. mTERF motifs were predicted using SMART software. Mitochondrial localization of mTERFD3 was predicted using TargetP prediction program.

Antibody production. A partial peptide with high antigenicity index was produced in E. coli and purified using a GST column (Amersham). Two rabbits were immunized by injecting the purified peptide, followed by a boost 4 weeks later. After exanguination and complement inactivation the antibodies were affinity purified. Western blots were performed using 20-40 µg of proteins separated on 4-20% SDS Page. After transferring onto PVDF, the membranes were incubated in blocking buffer and later with primary
antibody (1:500) overnight. The secondary antibody IRDye 680 or 800 (1:3000) (Rockland, Gilbertsville, PA), were subsequently detected by the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). The quantification of protein signals was performed by using the Odyssey software provided by LI-COR.

**Mitochondria preparation and submitochondrial fractionation** Standard methods were used for the preparation of mitochondrial and postmitochondrial fractions in cultured cells and tissue homogenates. Mitochondria were obtained by homogenization and differential centrifugation of muscle tissue taken from the hind limbs of different aged animals. Muscle homogenates were prepared in 10 mM Hepes, 0.5 mM EDTA, 0.5 mM EGTA and 250 mM sucrose (pH 7.4) that contained a complete protease inhibitor cocktail (Roche Diagnostics), using a motor-driven teflon-pestle homogenizer. Samples were centrifuged at 2,000 g for 3 min. The supernatant was centrifuged at 12,000 g for 10 min. The mitochondrial fraction was frozen in liquid nitrogen and stored at –80°C until needed. For suborganellar localization, freshly isolated mitochondria from heart were treated with digitonin 0.1%. Mitoplasts were obtained and further treated with Lubrol for 15 min. After high speed centrifugation the pellet was washed twice and labeled IM (inner membrane), whereas the supernatant was considered matrix proteins mix.

**Immunohistochemistry.** COS cells were grown on coverslips and transfected using an expression plasmid containing the full length cDNA for mTERFD3 and an HA tag. After 48 hours mitochondria were labeled with 200 nM MitoTracker (CMXRos; Molecular Probes, Eugee, OR) for 30 min at 37°C, washed with PBS and fixed with 2% paraformaldehyde, permeabilized with cold methanol, and then incubated with anti-HA-
Alexa Fluor 488-conjugated monoclonal antibody (Molecular Probes) for 2h. Coverslips were mounted with an antifade aqueous mounting gel (Biomeda, Foster City, CA), and the fluorescence was analysed in a Carl Zeiss confocal microscope.

**Expression and purification of full length mTERFD3.** Expression of recombinant mTERFD3 with a 6x Histidine tag was done in *E. coli* BL21. Expression was induced at 37°C with 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG). Cells were lysed using a French press and the supernatant was loaded on a Talon metal affinity column (Clonetech, Palo-Alto USA). Recombinant proteins were eluted as indicated by the manufacturer and fractions were dialyzed in a storage buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 5% glycerol), and stored at −80°C.

**EMSA** Electrophoresis mobility shift assays were carried out using γP³² end labeled probes. For the mTERF termination site we use a 44 oligo duplex as previously described. For HSP we used a double stranded oligo of approximately 50bp (16230-16284) and for the LSP a duplex corresponding to the initiation of transcription on the light chain (16183-16230). The reaction was performed in 25 mM HEPES, pH 7.5, 50 mM KCl, 12.5 mM MgCl₂, 1 mM dithiothreitol, 20% glycerol, 0.1% Tween 20, 0.5 µg of poly(dI-dC)·(dI-dC), 20 fmol of 5'-end ³²P-labeled probe, 5 µg of bovine serum albumin and 100-500 ng of recombinant mTERFD3. The reaction mixture was incubated on ice for 30 min, and immediately loaded and run in a 10% polyacrylamide gel in the cold room as described. After the run, the gel was dried and analyzed by autoradiography.

**Gel filtration chromatography.** The S-100 fraction from mouse heart mitochondria was loaded on a FPLC system (Pharmacia) in the cold room using a Sephadex S-200 column (Pharmacia). Approximately 1.5-2 ml of S-100 were loaded into the column, and the
eluate was collected in 1-ml fractions, frozen in liquid nitrogen, and stored at -80 °C until further analysis. Analysis of the fractions by SDS-PAGE followed by immunoblotting, using anti-mTERFD3 antiserum as primary antibody, and antirabbit IDRdye 800 as secondary antibody, allowed the determination of the elution volume of mTERFD3. The logarithm of molecular weight was plotted against $K_{av}$ that was calculated by subtracting the void volume from elution volume. The molecular weight was finally estimated by interpolation of its $K_{av}$ in the calibration curve.

**Statistical analysis** Statistical significance was determined for differences between a control group and an experimental group with two-tailed, unpaired Student $t$-test. * indicates $P < 0.05$. The error bars in each figure represent standard deviations.

### 2.3 RESULTS

#### 2.3.1 mTERFD3 is a matrix mitochondrial protein

Recent studies suggested that additional factors are involved in mediating binding of mTERF to the heavy strand promoter. Possible partners for mTERF interaction are factors that share similar structure and facilitate protein-protein interactions. With the aim of identifying such factors we performed genome wide searches to assess the evolutionary conservation of mTERF and identify homologues. Using bioinformatic approaches we identified in mouse, two mTERF homologues mTERFD1 (mTERF3) and mTERFD3 (mTERF2). Both of these proteins are predicted to be mitochondrial and display leucine zippers domains similar to the ones present in mTERF. mTERFD1 (mTERF3) was recently characterized and showed to be involved in transcriptional
repression of mtDNA (Park et al., 2007). However the function of mTERFD3 was not elucidated so far, and this reason we focused our attention on this protein.

ClustalW alignment showed high conservation of mTERFD3 (accession number NM_028832) in mammals, birds and fish (figure 2.1, panel A). The mouse mTERFD3 homology varies from 90.9% identity with rat mTERFD3 to 44.7% with the zebra fish homologue. It is interesting to note that mTERFD3 and mTERF are present only in vertebrates whereas mTERFD1 is also present in worms and insects, suggesting that mTERFD3 was retained during evolution for a specialized function characteristic to higher eukaryotes.

In the next step we have analyzed the structure of the protein using the SMART web tool and found that mTERFD3 contains mTERF motifs. These motifs consist of leucine repeats every other 7 aminoacid (XXXLXXX) and are present in all members of mTERF protein family. Figure 2.1, panel B, shows a graphic representation of these leucine zippers. We determined that mTERF and mTERFD1 contain 6 putative mTERF motifs whereas mTERFD3 and mTERFD2 have 4 motifs. We have also used COIL program and identified in mTERFD3 2 coil coils domains (not shown). All these data suggests that mTERFD3 is bona fide mTERF like protein and it may be involved in transcriptional process.

The mouse mTERFD3 is a 43 KDa protein encoded by a 3 exon gene in the chromosome 10. TargetP program showed a high probability of mitochondrial localization for mouse mTERFD3 (score 0.82). In order to confirm the subcellular
Figure 2.1 Evolutionary conservation of mTERFD3. A. ClustalW alignment of mTERFD3 homologues in *Homo sapiens, Mus musculus, Rattus norvegicus, Canis familiaris* and *Danio rerio*. The red bars show leucine zipper domains as predicted by SMART web tool. B. mouse mTERF homologues have conserved leucine zipper domains. mTERF and mTERFD1 contain 6 putative mTERF motifs whereas mTERFD3 and mTERFD2 have 4 motifs.
localization of mTERFD3 the full length protein was expressed in COS cells with an HA tag. Using immunohistochemistry, colocalization of mTERFD3 signal with mitotraker (a specific mitochondrial marker) was observed (figure 2.2, panel A). To confirm these results we produced a polyclonal antibody against mouse mTERFD3 and performed immunostaining on a mouse cell line (LMTK). We observed clear colocalization of the mTERFD3 signal with mitochondrial staining suggesting that mTERFD3 is indeed localized in the mitochondria.

In order to identify the suborganellar localization of mTERFD3 we have analyzed heart mitochondria using a polyclonal antibody against mTERFD3 (described in methods section). First we have prepared mitoplasts from heart mitochondria using mild digitonin treatment. Under these conditions the outer membrane was disrupted leaving the inner membrane intact. When mitoplasts were treated with proteinase K, mTERFD3 was protected from degradation, indicating a matrix or inner membrane localization (figure 2.2, panel B). Proteinase K degraded all mitoplast content when the inner membrane was disrupted by addition of Triton X100. Further submitochondrial fractionation was performed to separate the mitochondrial inner membrane from matrix compartment. mTERFD3 was found mostly in the matrix fraction, similar with HSP60, a known mitochondrial matrix protein. COX IV, a subunit of cytochrome oxidase and VDAC were found predominantly in the inner membrane fraction. We conclude from this data that mTERFD3 is a mitochondrial protein localized in the matrix compartment.
Figure 2.2. mTERFD3 is a matrix mitochondrial protein. A. mTERFD3 expressed in COS cells and colocalizes with MitoTracker signal. B. Mitoplasts prepared from heart mitochondria are resistant to proteinase K, whereas Triton X100 makes all proteins accessible to proteinase digestion. In the last two lanes matrix (MX) and inner membrane (IM) were separated. mTERFD3 is predominantly in matrix fraction as HSP60 whereas COXIV and VDAC are membrane associated. C. Northern Blot of mouse mTERFD3 shows the expression profile in mouse.
2.3.2 mTERFD3 is expressed abundantly in heart, brain, liver and testis.

To determine the expression profile of mTERFD3 in mouse we performed northern blot analysis and we found a pattern typical for mitochondrial proteins. mTERFD3 was expressed highly in tissues with increased metabolic demand such as heart, brain, and testis (figure 2.2, panel C). Surprisingly, the amount of RNA in the muscle is rather low.

2.3.3 mTERFD3 is able to form dimers in vitro.

It was previously shown that mTERF is able to form trimers. However the functional significance of trimer formation is not known. Since mTERFD3 contains conserved leucine zipper domains, we asked if mTERFD3 was also able to form oligomers. In a first set of experiments we used differential centrifugation in sucrose gradients. Proteins were extracted from heart mitochondria using 0.5% digitonin and 0.2M KCl and then subjected to differential centrifugation in sucrose gradients. mTERFD3 was identified by immunoblotting in the 80 kDa fraction (figure 2.3, panel A). Since mTERFD3 has a predicted molecular weight of 39 kDa, the presence in a higher molecular weight complex suggested that mTERFD3 is able to form oligomers. When the mitochondrial proteins were extracted with high salt (0.7M KCl) the mTERFD3 shifted to the fraction corresponding to 40 kDa (figure 2.3, panel B).

To confirm these findings we performed gel filtration chromatography using heart mitochondrial protein extracted with 0.2M KCl and 0.5% digitonin. Each fraction obtained was immunoblotted using an anti mTERFD3 polyclonal antibody. Using appropriate molecular weight markers we have identified mTERFD3 in two peaks,
**Figure 2.3.** mTERFD3 is able to form dimers *in vitro*. A. Differential centrifugation of mTERFD3 extracted with mild salt condition (0.2M KCl) from heart mitochondria followed by immunoblotting with anti mTERFD3 antibody. B. High salt (0.7M KCl) was used to extract mTERFD3. As MW control lactate dehydrogenase activity and Hemoglobin absorption were determined. C. Separation of heart mitochondria by size exclusion chromatography with Superdex 200 column shows mTERFD3 present in 2 peaks, one corresponding to 80 kDa (fraction 21) and one corresponding to 43 kDa (fractions 27-31). As molecular weight control markers we used aldolase (158 kDa), hemoglobin (67 kDa) and ovalbumin (43 kDa). D. Gel filtration chromatography showed elution of recombinant mTERFD3 in a peak corresponding to 90 kDa, double the mTERFD3 molecular weight.
corresponding to approximately 40 and 90 KDa (figure 2.3, panel C) suggesting that mTERFD3 is indeed forming protein complexes under this extraction conditions.

To identify if mTERFD3 is able to form heterodimers we repeated gel filtration chromatography experiments using recombinant mTERFD3. mTERFD3 was eluted in a peak that corresponds to 90kDa suggesting that mTERFD3 was able to form dimers in vitro (figure 2.3, panel D). Our findings are in concordance with the presence of coil-coiled domains in mTERFD3 structure that are likely involved in protein-protein interactions. However, the functional significance of mTERFD3 dimers remains to be determined.

2.3.3 mTERFD3 binds the mitochondrial DNA promoter region.

Since mTERFD3 is part of mTERF family of transcription factors it is plausible to hypothesize that it binds mtDNA. The possibility of a DNA-mTERFD3 interaction was explored initially using heparin binding chromatography. mTERFD3 from mouse heart mitochondrial extract was eluted from a heparin binding column between 0.3 and 0.7 M KCl suggesting the ability of this proteins to bind polyanions (figure 2.4, panel A). To determine if mTERFD3 is able to bind mtDNA, mTERFD3 with a 6x Histidine tag was expressed in E. coli and purified using a metal affinity chromatography. The recombinant protein was used in electromobility shift assays in which different mtDNA fragments were assessed for possible interactions. First we have focused on the termination site responsible for mTERF binding. These assays showed that mTERFD3, unlike mTERF, was not able to bind the termination site situated at the boundary between 16S rRNA and tRNA<sub>Leu(UUR)</sub> (figure 2.4, panel B). However, mTERFD3 produced a shift when 200 ng of recombinant protein was incubated with a 50 bp DNA probe
Figure 2.4. mTERFD3 binds the mtDNA at the transcription initiation site. A. Heparin affinity chromatography showed elution of mTERFD3 between 0.3M and 0.7 M KCl. B. EMSA using mTERM DNA probe. This experiment showed mTERF binding at the termination site (TERM); mTERFD3 was not able to bind to this site (left panel). First lane corresponds to DNA only, next three lanes in each gel corresponds to increasing amounts of protein. C. EMSA using HSP DNA. Recombinant mTERFD3 but not mTERF showed binding to a D-loop fragment that corresponds to the heavy strand promoter. Increasing amount of mTERFD3 shifts HSP probe (lanes 1,2). The binding was specific as a 10 fold excess of cold unspecific DNA was not able to compete the shift (lane 3) whereas excess of cold specific DNA was able to compete with labeled probe (lane 4).
corresponding to the transcription initiation site of the heavy strand promoter (HSP) (figure 2.4, panel C).

The specificity of binding was shown by the competition assays. When a 10 fold excess of cold unspecific DNA was incubated with the recombinant protein, mTERFD3 was still able to shift the HSP DNA, whereas the specific unlabeled DNA competed out the labeled probe.

We have also tested the binding of mTERFD3 to the mtDNA regulatory region using oligo duplexes designed to cover the entire D-loop region. However no specific binding was detected in these assays (data not shown). These findings indicate that mTERFD3 is a possible regulator of transcriptional initiation of the mitochondrial DNA together with other transcription factors.

2.4 DISCUSSION

With the goal to better define the mechanisms involved in the mitochondrial transcription regulation we pursued the functional characterization of the mouse mTERFD3. mTERFD3 is member of mTERF family of transcription factors and is conserved in vertebrates (Linder et al., 2005). We have previously identified this homologue in mouse and pursued biochemical characterization. Immunostaining together with mitochondrial fractionation experiments found that mTERFD3 is a matrix mitochondrial protein.

Differential gradient centrifugation experiments corroborated to size exclusion chromatography indicated that mTERFD3 is able to form dimers. The interaction is most likely facilitated by the leucine zipper domains present in the protein. At this point we
don’t know the functional significance of dimers formation. Interestingly, mTERF was shown to be able to form trimers, but only the monomer form is able to bind mtDNA.

Using DNA mobility shift assays we showed that mTERFD3 is able to bind mtDNA in the heavy strand promoter. The DNA binding activity in the promoter region makes mTERFD3 a good candidate for regulating transcriptional initiation from the heavy strand promoter. However, at this point we do not know if the observed binding is related to mTERFD3’s main physiological function. Attempts to footprint the region were not successful, suggesting that the binding is weak.

mTERFD3 is not necessary for initiation of the transcription since in vitro assays showed that mitochondrial RNA polymerase (POLRMT) can initiate transcription with transcription factor A (TFAM) and either TFB1M or TFB2M (Falkenberg et al., 2002). These factors are sufficient for the initiation of transcription from the HSP in vitro and our findings that mTERFD3 binds HSP suggest that it may act as a modulator of transcription directly or through interaction with other proteins. Recently mTERF has been involved in formation of a loop in the mtDNA that brings together the initiation and the termination site (Martin et al., 2005). mTERF extracted from cell lysates, but not from recombinant mTERF, binds HSP in vitro, therefore suggesting that other factors are necessary for the creation of this loop. Since mTERFD3 binds mtDNA in the initiation region, it is plausible to envision this protein as part of a complex of proteins helping mTERF interact with the initiation site and modulating initiation of transcription from the HSP.

Unlike mTERF, mTERFD3 does not bind the termination region situated at the 3’end of 16SrDNA. We also tested another possible interaction with the termination site
present in the D-loop and thought to be responsible for the termination of transcription of a long polycistronic transcript transcribed from the heavy chain promoter (Camamasudram et al., 2003). However we could not detected binding at this site (not shown). mTERFD1 the other mTERF homologue was recently characterized and was also shown to bind the promoter region (Park et al., 2007).

We can conclude that mTERFD3 is a highly conserved mitochondrial protein with leucine zipper domains able to form dimers \textit{in vitro} and binding specifically the HSP of mtDNA. All these data suggest strongly that mTERFD3 is a possible player in regulation of mitochondrial DNA transcription regulation.
CHAPTER 3

MTERFD3 IS A MITOCHONDRIAL PROTEIN THAT MODULATES OXIDATIVE PHOSPHORYLATION IN VIVO

3.1 BACKGROUND

We showed that mTERFD3 is a bona fide mitochondrial protein, which was conserved throughout evolution. It is able to bind DNA in the promoter region and hence it is a potential factor involved in transcriptional regulation. In order to characterize the function of this protein in vivo, we created a mouse deficient in mTERFD3 using a gene trapping strategy. The recently characterized mTERFD1, member of mTERF protein family, is essential for survival since the disruption of mTERFD1 leads to embryonic lethality. Tissue specific inactivation of mTERFD1 in heart was associated with decreased OXPHOS activity, cardiomyopathy and unexpectedly increased mtDNA transcription (Park et al., 2007). These results suggest that mTERFD1 may act as a negative regulator of mtDNA transcription. In order to detect the consequences of mTERFD3 deficiency on mitochondrial transcription we analyzed in this work the function of this protein in different tissues. mTERFD3, unlike mTERFD1 is expressed only in higher eukaryotes and inactivation of its function in mouse would be expected to produce mitochondrial dysfunction.
3.2 MATERIAL AND METHODS

Production of mTERFD3 deficient mice. Embryonic stem cells containing gene trap with an insertion in the intron 2 of mTERFD3 gene were purchased from Lexicon Genetics Incorporated (129SVJ background). The ES clone (OST 453159) was injected in blastocysts and implanted in pseudopregnant mice at the University of Miami Transgenic Facility. Genotyping of the mice was performed using primers designed to amplify the wild type and knockout alleles.

Mitochondria preparation Standard methods were used for the preparation mitochondrial and postmitochondrial fractions in cultured cells and tissue homogenates. Mitochondria were obtained by homogenization and differential centrifugation of muscle tissue taken from the hind limbs of different aged animals. Muscle homogenates were prepared in 10 mM Hepes, 0.5 mM EDTA, 0.5 mM EGTA and 250 mM sucrose (pH 7.4) that contained a complete protease inhibitor cocktail (Roche Diagnostics), using a motor-driven teflon-pestle homogenizer. Samples were centrifuged at 2,000 g for 3 min. The supernatant was centrifuged at 12,000 g for 10 min.. The mitochondrial fraction was frozen in liquid nitrogen and stored at –80°C until needed.

Cell culture and growth curves. Two primary cell lines from WT fibroblasts and two from KO animals were obtained from lung tissues. Cells were grown in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1 mM pyruvate, 50 μg/ml uridine at 37°C in an atmosphere of 5% CO2. and immortalized by infection with E6 retrovirus. To determine the growth rate WT and KO fibroblasts cells were grown in DMEM with no glucose and 5mM of galactose. 10³ cells were plated in
triplicates in 6 well plates, trypsinized and counted every 24 h on a Coulter Cell Counter (Beckman Coulter, Fullerton, CA) over 6 days.

**Treadmill test.** Eight WT mice and eight KO females were run on a treadmill (Columbus Instruments, Columbus, OH, USA) set at 10 m/min for 15 min. The back of the treadmill was equipped with an electric grid that motivates the animal to keep running on the treadmill to avoid the noxious stimuli. Performance was measured by the number of times a mouse failed to stay on the running belt and fell into the stimulus grid.

**Electron Microscopy.** Tissues were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were subsequently dehydrated and embedded in plastic and analysed by electron microscopy for morphological abnormalities at the EM core facility of the University of Miami Miller School of Medicine.

**Body fat composition analysis.** Body fat composition was assessed by dual-energy X-ray absorptiometry (MEC LunarCorp).

**Blue Native Electrophoresis.** Blue native polyacrylamide gel electrophoresis (BN-PAGE) for the identification of individual complexes was performed as described (Nijtmans et al., 2002). Individual OXPHOS complexes were extracted using dodecylmaltoside in 0.75 M aminocaproic acid, 50 mM Bis-Tris, pH 7.0, and Serva G). 20 to 40 µg of mitochondrial protein were loaded in a 4 to 13% blue native gradient gel. Electrophoresis was performed with blue cathode buffer at 100 V for 1 h and then switched to constant current (16 mA). The blue cathode was changed to colorless buffer after the dye front reached half of the gel, and electrophoresis was stopped when the dye front left the gel. After native electrophoresis or SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane for immunoblot analyses. For in gel
complex I activity, the gel was incubated in 100 mM Tris-HCl, 0.14 mM NADH, 1 mg/ml nitroblue tetrazolium, pH 7.4, for 1 to 3 h at room temperature. For complex IV activity stain, gels were incubated as described previously for 2 to 4 h at 37°C. Proteins were transferred overnight to PVDF membranes as described above.

**Enzyme activity.** Mitochondria were isolated from muscle using standard procedures. The activity of complex IV was assessed by reading the oxidation of cytochrome c at 550nm. Complex II +III was assessed following the reduction at cytochrome c at the same wave length (Barrientos et al.,1998)

**Mitochondrial protein synthesis.** Mitochondrial protein synthesis was performed in lung fibroblasts isolated from control and KO mice as described (Chomyn, 1996). Briefly, fibroblasts were plated until they reached 70% confluence, washed and incubated for 1 hour in methionine free media. After 4 min treatment with emetine to inhibit cytoplasmic ribosomes, cells were incubated for 30 min with 60 µCi/ml S\(^{35}\) methionine. Cells were washed, trypsinized and 100 µg protein were loaded in a SDS PAGE. After fixation and fluorography with EN\(^3\)HANCE the gel was dried, exposed for 3 days and analyzed using a phosphorimaging system.

**Northern Blots.** RNA was isolated from different tissues using Trizol reagent (Gibco-BRL). RNA samples were tested by ultraviolet absorption ratio \(A_{260}/A_{280}\) for purity and concentration. Values for \(A_{260}/A_{280}\) were >1.8 for all RNA extraction. 10 µg of RNA for each sample were denatured, electrophoresed into a 1.5% formaldehyde agarose gel, transferred to a nitrocellulose membrane, and cross-linked to the membrane with ultraviolet light. Prehybridization and hybridization were done using a commercial solution (Ambion). cDNA probes were labeled with \(\gamma\) dCTP\(^{32}\) by random primer
extension (Roche) and added to the hybridizing solution. Hybridization was performed at 42°C for 18 h. The blots were then washed several times with a final wash in 0.1× standard sodium citrate (SSC) and 0.1% SDS at 65°C. The relative content of mRNAs was evaluated by scanning densitometry using a Phosphorimager model 400S and ImageQuant quantitation software (Molecular Dynamics, Sunny Valley, CA).

**Statistical analysis.** Statistical significance was determined for differences between a control group and an experimental group with two-tailed, unpaired Student $t$-test. * indicates $P < 0.05$. The error bars in each figure represent standard deviations.

### 3.3 RESULTS

#### 3.3.1 Generation of mTERFD3 KO mice.

In order to analyze the functional significance of mTERFD3 in *vivo* a mouse deficient in this protein was produced using a gene trapping strategy. An embryonic stem cell clone (OST453159) that contains a viral insert in the mTERFD3 locus was purchased from Lexicon Pharmaceutical (The Woodlands, TX). As a result of the trap insertion in the intron 2 of the mTERFD3 gene, a fusion transcript containing exon 2 and viral trap is formed (figure 3.1, panel A). Since the trap contains stop codons in all reading frames no protein is being produced. The ES clone was injected in blastocysts and chimera mice have been produced. We have obtained germline transmission and after successive crossings heterozygous and homozygous animals were produced. As shown in figure 3.1, panel B, the gene was successfully disrupted and no mTERFD3 RNA was detected in KO tissues analyzed. Also western blots using the anti-mTERFD3 polyclonal antibody did show no detectable mTERFD3 protein in several tissues analyzed (figure 3.1, panel C).
Figure 3.1. Gene trapping strategy for the creation of mTERFD3 deficient mice. A. Genotyping of mice was done using primers hybridizing to the viral trap and flanking the insertion region. Mice homozygous for the wt (+/+) or the trapped allele (-/-) as well as heterozygous animals (+/-) were analyzed. B. RT PCR detected no mTERFD3 cDNA in the heart, kidney and brain. C. No mTERFD3 protein was detected in the several tissues analyzed by western blot using a polyclonal antibody.
3.3.2 mTERFD3 deficient mice display decreased exercise tolerance and grip strength.

The mTERFD3 deficient mice obtained through insertional mutagenesis have a normal life span and do not show gross abnormalities. There was a significant decrease in the body weight of KO females (p=0.02), evident particularly in older age groups (figure 3.2, panel A). However, in the males, even though there was a trend toward decreased weight in KO animals compared with WT, no statistical difference (p=0.07) was noticed (figure 3.2, panel B).

In order to evaluate the effect of mTERFD3 deficiency on body fat content we have used DEXA scanning. Interestingly a tendency for decreased body fat content was observed in mTERFD3 deficient females (figure 3.2, panel C). The most significant decreased in total body fat was found in the 9 month old group (p=0.012). Interestingly, the same trend was found in males without reaching statistical significance. Together these data suggest a gender and age dependent effect that will necessitate further investigation using more animals and a colony with homogeneous background.
Figure 3.2 Phenotype analysis of mTERFD3 KO mice. A and B: Weight curves of females and males mice show significant weight loss in mTERFD3 KO females. *=p<0.05. C Total body fat content was measured using DEXA scanning in mice of different ages. Significant differences were observed in the 9 mo old female group (*=p<0.05).
The findings that fat content is reduced in the KO females suggested the presence of an altered metabolic pathway in the mTERFD3 deficient mice. In order to identify a possible phenotype in the KO animals, we have performed detailed investigation of metabolical parameters such as heat production, oxygen consumption and locomotor activity. Extensive metabolic monitoring studies were conducted in a Comprehensive Lab Animal Monitoring System from Jackson Laboratory. In this set of experiments we used six control females and six mTERFD3 KO females.

Our data showed that mTERFD3 KO females have similar oxygen consumption and carbon dioxide production when compared to controls (figure 3.3, panel A). The food and water intake were also measured but no significant differences were noted (figure 3.3, panel B). The respiratory exchange ratio calculated as the ratio between the CO2 production and oxygen consumption was not altered in the KO compared to WT mice (figure 3.3, panel C). Another measure of basal metabolism namely heat production was measured in both groups of animals but no difference was seen (figure 3.3, panel D). Finally, the overall locomotor activity of mice in cages was assessed by measuring the cumulative XY ambulation and no significant difference was noted (figure 3.3, panel E). Also, measurement of blood pressure, heart rate, blood glucose levels and lipid levels were not changed in KO animals (not shown).

All these data suggest that in captivity condition the metabolic rate of mice deficient in mTERFD3 is not different when compared with wild type. We can not exclude, however, the possibility of significant changes in conditions of stress.
Figure 3.3. Basal metabolism in mTERFD3 deficient mice. Six control females and six mTERFD3 KO of 9 month of age were analyzed for basic metabolic parameters using the Comprehensive Cage Monitoring System (The Jackson Laboratory). A. Oxygen consumption and carbon dioxide production B. Total food and water intake C. Respiratory exchange ratio (RER) D. Heat production. E. Cumulative ambulation. No significant differences in the metabolic parameters were observed in the KO animals (red bars) when compared with littermates controls (blue bars).
Because mTERFD3 deficient mice did not show obvious abnormalities, we focused on exploring a potential defect in tissues with high energy demand such as muscle. Muscle requires a fine control of mitochondrial gene expression in response to complex metabolic demands. A tendency for decreased grip strength was observed in KO animals. (figure 3.4, panel A) suggesting that muscle strength may be affected in mTERFD3 KO animals.

To delve more into the presence of a subtle muscular phenotype the animals were submitted to exercise tolerance test by running on a motivational treadmill. Control and KO animals were trained to run on the treadmill at a speed of 10 m/min in order to avoid an unpleasant stimulation. The number of falls on the motivational grid was counted and an evident difference was observed between the groups. The KO animals performed worse as shown by the increased number of falls on the motivational grid (figure 3.4, panel B).

The significant decrease in muscle endurance and the tendency for decreased grip strength in mTERFD3 KO mice suggest an important role of mTERFD3 in muscle mitochondria.
Figure 3.4. mTERFD3 deficient mice display decreased grip strength and decreased exercise endurance

A. Grip strength test was performed to evaluate the muscle function in 6 control and 5 KO of 9 month old females. B. The performance on the treadmill was quantified using age and sex matched animals by counting the number of falls in different animal groups. Mice were trained to run on treadmill in order to avoid falls on motivational grid. * indicates significant changes p<0.05.
3.3.3 Fibroblasts that lack mTERFD3 show decreased growth in galactose and decreased cytochrome oxidase activity.

As a first step in analyzing the effect of mTERFD3 deletion we focused on fibroblasts isolated from lung of WT and KO animals. We have produced two cell lines for each group and grown in DMEM media. We noticed a decreased growth of the fibroblasts deficient in mTERFD3. The growth in media supplemented with 5mM galactose and with no glucose (figure 3.5, panel A) was decreased for both WT and KO cells. In this media, cells that are OXPHOS deficient grow slower since they rely only on OXPHOS to produce ATP. These findings suggest that mTERD3 KO fibroblast have a defect in OXPHOS.

To confirm a possible OXPHOS defect in the mTERFD3 KO cell fibroblasts we measured the enzymatic activity of cytochrome oxidase. We detected a significant deficiency in one of the KO fibroblasts clones - KO2 when compared with control. The other cell line KO1 showed only a mild decrease without reaching statistical significance (figure 3.5, panel B). Other complexes analyzed did not show differences in the enzymatic activities.

To further analyze the respiratory defect we performed *in gel* enzymatic activities for complexes I and IV. These assays showed reduced activities in one of the KO lines (figure 3.5, panel C). The OXPHOS complexes were also analyzed by blue native immunobloting and revealed decreased amounts of fully assembled complex I and complex IV. However the steady state levels of some of the OXPHOS subunits were not affected as shown by immunobloting (figure 3.5, panel D). All these results suggest the presence of an OXPHOS defect in fibroblasts deficient in mTERFD3.
Figure 3.5. mTERFD3 deficient fibroblasts have OXPHOS deficiency. A. Growth curves of control and KO2 fibroblast were determined in glucose and in media where glucose was substituted with galactose. B. cytochrome oxidase activity was determined in the two control and two KO cell lines. * indicates statistical significant changes (p<0.05). C. The in gel activity of complex I and complex IV were determined in the more deficient KO clone (KO2, left panel). Western blots for the blue native gel electrophoresis for the complex I and IV are shown in the right part of panel C. D. Western blots of selected individual subunits of OXPHOS (SDS-PAGE).
To determine if the decreased activity was related to mitochondrial protein synthesis, we labeled mitochondrial translation products in fibroblasts using $^{35}$S methionine after inhibiting the cytosolic ribosomes with emetine. When compared to WT fibroblasts, a decrease in mitochondrial protein synthesis was observed in the mTERFD3 deficient fibroblasts (figure 3.6, panel A). The most affected mitochondrial proteins were COX2, COX3, ATP6 and ND6 protein levels.

To investigate if the altered mitochondrial protein synthesis correlates at the transcriptional level we extracted mRNA from WT and KO fibroblasts and performed northern blots. The majority of the mitochondrial transcripts analyzed showed a decrease in KO fibroblasts compared to control (figure 3.6, panel B). The transcripts that were markedly altered were COX1, COX2, ND6 and ATP6. We did not see a direct correlation between the decrease in mRNA and their corresponding protein. However we can conclude from these results that fibroblasts deficient in mTERFD3 have an OXPHOS defect that is associated with decreased mtDNA transcription and decreased protein synthesis.
Figure 3.6. mTERFD3 deficient fibroblast have altered mitochondrial protein synthesis and decreased mitochondrial transcripts. A. In vivo mitochondrial protein labeling of mTERFD3 deficient fibroblast with $^{35}$S methionine in the presence of emetine. The graph shows quantification of different mitochondrial proteins normalized by cytochrome b amount. B mitochondrial transcripts from the KO fibroblast were analyzed by northern blot quantified and expressed as percentage of control after normalization with beta actin. In the table is shown the relative amount of KO mRNA expressed as percentage from control.
### 3.3.4 Muscle deficient in mTERFD3 has decreased complex IV activity and increased citrate synthase activity.

Since the weak performance on the treadmill could be explained by a muscle defect in OXPHOS, we have analyzed by histochemistry the activity of cytochrome oxidase in muscle sections. As we can see in the figure 3.7, a decreased staining for COX activity was seen in the muscle of the mTERFD3 KO mice, in concordance with the findings in fibroblasts. Also an increase in succinate dehydrogenase (SDH) staining in the mTERFD3 deficient mice was noticed, indicating mitochondrial proliferation.

To further explore possible OXPHOS defects, mitochondria from muscle of the KO and WT animals were analyzed by spectrometry enzyme assays. We observed a significant decrease in cytochrome oxidase activity in the muscle mitochondria from KO animals (figure 3.8, panel A), in concordance with our findings in fibroblasts. In addition, complex II+III activity was significantly reduced (figure 3.8, panel B). Interestingly this decrease was associated with an increased activity of citrate synthase in the muscle homogenates. Increased citrate synthase suggests mitochondrial proliferation, indicating that compensatory mechanisms are activated in the muscle of mTERFD3 KO animals as a response to a partial OXPHOS defect.

Similar results were obtained in our laboratory recently analyzing mTERFD3 ko mice obtained from a congenic strain (Wenz and Moraes personal communications) confirming that indeed, mTERFD3 mice display decreased OXPHOS activity in muscle mitochondria. All these findings are in concordance with our previous results in mouse fibroblasts suggesting that mTERFD3 deficiency is responsible for OXPHOS defect.
Figure 3.7. Muscle deficient in mTERFD3 has decreased COX activity. Histochemistry for cytochrome oxidase and succinate dehydrogenase activities was performed in muscle sections of WT and KO animals. We observed increased cytochrome oxidase staining together with increased succinate dehydrogenase staining in mTERFD3 deficient muscle sections.
Figure 3.8. Muscle deficient in mTERFD3 has a significant OXPHOS deficiency
A. COX activity was measured in the mitochondria extracted from the muscle of 20 mo
old animals. B Complex II +III activity was measured in the same samples. C citrate
synthase activity as measured in muscle homogenates. All activities are normalized by
mitochondrial proteins *=p<0.05.
We have also analyzed mitochondrial extracted from heart, liver and brain, tissues with high energy demands and in which mTERFD3 is expressed abundantly. However we could not detect so far a defect in the OXPHOS in these tissues (figure 3.9).

**Figure 3.9. Complex IV enzymatic activity in heart brain and liver.** The enzymatic activity of citochrome oxidase was measured spectrophotometrically in mitochondria isolated from heart (A), brain (B) and liver (C). No significant differences between the WT and KO were identified.
To confirm the defects in the oxidative phosphorylation system identified using enzymatic activity measurements we performed *in gel* activities assays using mildly extracted complexes in a blue native electrophoresis. Consistent with the previous findings the levels of complex I and IV were indeed reduced in muscle of KO mice (figure 3.10, panel A). Next, we wanted to determine if the defect in the enzymatic OXPHOS activities was associated with altered complex assembly, we analyzed the respiratory complexes separated by blue native electrophoresis using immunoblots. The steady state levels of OXPHOS complexes did not show major changes with this method (figure 3.10, panel B).

We have also checked the steady state levels of some of the individual subunits and we did not detect major changes (figure 3.10, panel C). However, when tissue from mice with homogeneous background was used an evident decrease in ND 39, SDH, core1 and COX1 subunits was identified (personal communication of Dr.Wenz). All these results confirm the presence of OXPHOS defect in the muscle of mTERFD3 KO animals.
Figure 3.10. OXPHOS assembly is not affected in the mTERFD3 KO muscle. A. In gel activity for complex I and IV in muscle mitochondria. B Blue Native immunoblotting for the same samples as in panel A using anti ND 39 and anti-COX1 antibodies. C. Some of the individual subunits of respiratory complexes were analyzed by SDS PAGE.
3.3.5 Mt DNA transcripts are decreased in the deficient muscle.

In order to see if there is any correlation between the decrease OXPHOS activity in muscle and mitochondrial transcription, we sought to quantify the mitochondrial mRNAs using northern blots. Our findings in fibroblasts suggested that mTERFD3 deficiency is associated with decreased mtDNA transcription. We have extracted RNA from muscle of WT and KO mice and analyzed the levels of mitochondrial transcripts using northern blotting in order to see if muscle mitochondrial transcripts are affected. A decrease in the levels of transcripts relative to 12S RNA was seen in most of the analyzed transcripts (figure 3.11, panel A). The most significant alterations occurred in ATP6 (34%) and ND6 (32%) similar to what we found in fibroblasts (figure 3.11, panel B). Similar results were obtained when the amount of transcripts were normalized to actin mRNA. It is interesting that, while most of the mRNA are decreased in the mTERFD3 KO muscle, the 12S RNA is increased, suggesting the presence of a deregulation of mRNA/rRNA ratio as a result of mTERFD3 disruption.

These results are in concordance with our findings in fibroblasts and suggest that indeed, mTERFD3 is a positive regulator of mitochondrial transcription. Interestingly, mTERFD1, a recently characterized mTERF family member was shown to be a transcriptional repressor (Park et al., 2007).

All these results suggest that of mTERF family members are actively involved in mitochondrial transcription regulation.
Figure 3.11. Mitochondrial DNA transcripts are decreased in the KO muscle. A. Northern blots for different mitochondrial transcripts in the muscle. B. Quantification of northern blots is shown in graphical format expressing the ratio KO (light color bar in panel B) / WT (dark color bar) relative to 12S. The lower part of panel B shows the relative levels of mRNA expressed as percentage from WT.
3.3.6 Mitochondrial proliferation is seen in mice deficient in mTERFD3

We have shown that muscle mitochondria have decreased OXPHOS activity and that is associated with increased citrate synthase activity, a commonly used marker for mitochondrial proliferation. Also an increased succinate dehydrogenase staining was observed in the muscle sections of mTERFD3 deficient mice. All these findings suggest that muscle mitochondria undergo proliferation. To confirm this we performed electron microscopy to look for ultrastructural defects in the muscle of mTERFD3 KO mice (figure 3.12). We have noticed an obvious increase in the number of mitochondria of mTERFD3 KO mice, together with large and abnormal shaped organelles.

The citrate synthase increase, together with the extensive mitochondrial proliferation seen by EM suggests the activation of compensatory mechanisms in response to decreased OXPHOS activity. These morphological changes are characteristic of mitochondrial proliferation and often seen in human diseases associated with OXPHOS defects.

To identify a possible mechanism responsible for mitochondrial proliferation we have analyzed by western blot PPARγ coactivator (PGC1-α), a known regulator of mitochondrial biogenesis. As shown in figure 3.13, PGC1-α was significantly increased in the KO muscle when compared to WT. RTPCR experiments performed in our laboratory showed a 3 fold increase in the level of mRNA in the KO mice (personal communication of Dr. Wenz).

Taken together our results show that mTERFD3 deficiency is responsible for an OXPHOS defect and accompanied by compensatory mitochondrial proliferation.
Figure 3.12. Mitochondria proliferation in mTERD3 KO muscle. Electron micrographies of gastrocnemius muscle are shown. We observed obvious increase in the number of mitochondria in the KO muscle (middle panel). The right panel shows enlarged mitochondria in the KO muscle mitochondria.

Figure 3.13. PGC-1α is upregulated in mTERFD3 KO muscle. Western blot of muscle homogenates shows PGC-1α upregulation in the muscle of mTERFD3 KO animals. As controlled we used COX 1 and tubulin.
3.4 DISCUSSION

With the goal of better defining the mechanisms involved in mitochondrial transcription regulation we pursued the functional characterization of the mouse mTERFD3. mTERFD3 is member of mTERF family of transcription factors and is conserved in vertebrates (Linder et al., 2005).

To better define its function, we created mice deficient in mTERFD3 using gene trapping strategy. The mTERFD3 KO have normal life span but have a lower weight than control littermates and tend to have less body fat. Comprehensive metabolic studies showed no major differences in the oxygen consumption, CO2 production or cumulative ambulation in the KO animals. Although we have not created a congenic strain, all comparisons were done among littermates. Therefore, we do not believe that the lack of a strong phenotype is related to the overall genetic background. Moreover, recent experiments conduct in Carlos Moraes’ laboratory, using mTERFD3 backcrossed 5 times to C57/Bl6 background confirmed our initial findings (Wenz and Moraes, personal communications). It is not clear why mice KO for mTERFD3 do not show a strong phenotype, but it is probably related to the partial nature of the defects. It is possible that such phenotype will be observed only during stressful situations, and the laboratory is currently testing this hypothesis.

We have analyzed fibroblasts deficient in mTERFD3 and found a slower growth curve when grown in media selective for OXPHOS function suggesting that fibroblasts have indeed a respiratory defect. To confirm this we measured the cytochrome oxidase activity in the fibroblasts and we found a significant decrease when compared to WT.
We showed that the mtDNA transcripts were altered in the mTERFD3 KO fibroblasts and this was associated with decreased mitochondrial protein synthesis suggesting that mTERFD3 is important in mitochondrial gene expression regulation. Recently a similar role was attributed to a member of mTERF2 family in Drosophila (Roberti et al., 2006). This protein, mTERF3 was knocked down and produced a decrease in mitochondrial proteins but no alteration in the transcripts. A regulatory mechanism for the mitochondrial gene expression at the translational level was proposed, possibly through interactions with other regulatory factors. The human homologue of mouse mTERFD3, mTERFL was found to be important in cell cycle regulation since addition of serum to serum-starved cells drastically reduced its levels (Chen et al., 2005).

We did observe a trend for decreased grip strength in the KO animals and lower performance in the treadmill running suggesting a possible muscular defect. Indeed, in the muscle of mTERFD3 deficient mice we observed a decreased cytochrome oxidase activity in concordance with our initial findings in fibroblasts. Besides complex IV activity muscle deficient in mTERFD3 shows decrease in complex II+III activity and increased citrate synthase activity. However no other histological or biochemical defects were detected in brain, heart, liver or kidney.

We also showed that mitochondrial transcription is reduced in mice deficient in mTERFD3 suggesting that mTERFD3 is upregulates of mitochondrial transcription. Interestingly, the decrease in mRNAs was associated with increase in the 12S rRNA, suggestion that mTERFD3 is involved in regulating the mRNA/rRNA ratio.

The OXPHOS defect in muscle is significant but did not reach the critical threshold necessary for producing a severe phenotype. In human mitochondrial disorders
tissues display distinct sensitivities to defective mitochondrial OXPHOS. These tissues can counteract OXPHOS defects by stimulating mitochondrial biogenesis; however, above a certain threshold the lack of ATP can not be counteracted and cell death ensues. Citrate synthase is commonly increased in tissues with OXPHOS defects and is associated with mitochondrial proliferation. Electron microscopy sections of muscle from mice deficient in mTERFD3 demonstrated the presence mitochondrial proliferation suggesting that lack of mTERFD3 induces a compensatory mechanism as a response to OXPHOS defect. The mitochondrial proliferation observed in the muscle is likely a compensatory mechanism responsible for adaptation to the OXPHOS defect. The KO mice may be able to compensate for the absence of mTERFD3 in the laboratory setting, but are likely to develop a disadvantageous phenotype when exposed to stress conditions.

The mitochondrial transcription is regulated in response to the complex metabolic needs of the mammalian cell but the mechanisms are largely unknown. Environmental signals induce the expression of PGC-1 family coactivators (PGC-1α, PGC-1β and PRC), which activates specific transcription factors (NRF-1, NRF-2, and ERRα) that turn on OXPHOS genes (Scarpulla, 2002). In this way the mitochondrial gene expression is directly linked to environmental conditions and it can be adjusted in functions of the demands. We have found that muscle of mTERFD3 deficient mice had a significant increase in the PGC1-α. PGC-1α upregulation is likely a compensatory mechanism activated to cope with a mild OXPHOS defect. PGC1α increase is in concordance with the increased citrate synthase and mtDNA proliferation.

PGC1α is activated by stimuli like exercise, cold or fasting and is responsible for increased lipolysis. Since mTERFD3 is also abundant in brown adipose tissue it may be
involved in the adaptation to cold. The decreased fat content in KO females could be explained by an increased lipolysis due to increased fatty acid oxidation.

The possibility that thyroid hormones could directly influence mitochondrial OXPHOS gene transcription has been proposed (Enriquez et al., 1999). Hormone responsive elements have been identified in the mtDNA D-loop regulatory region and in some of the OXPHOS genes. The presence of putative HREs at the borderline between 16S rRNA and Leu-tRNA overlapping the mtTERF binding site raises the possibility of control of other potential steps of the transcriptional process by way of receptor–mTERF interaction.

Since mTERFD3 binds mtDNA in the regulatory region and defects in mtDNA transcripts have been observed we suggest that mTERFD3 acts as a transcription factor modulating gene expression, probably together with other proteins. It is possible that mTERFD3 is responsible for mitochondrial heavy strand promoter selection favoring one promoter over another. This would explain the altered mRNA/rRNA ratio together with decreased mitochondrial protein synthesis. However, we can not exclude the involvement of mTERFD3 in other steps of gene expression regulation such as protein synthesis or mtDNA replication. It is interesting to note that its homologue, mTERFD1, was shown to act as repressor of mtDNA expression in mouse (Park et al., 2007). It is possible that both of these factors interact with mTERF and together modulate mtDNA expression. mTERFD3 is not essential for survival or development but may be important in metabolically challenging situations. Further metabolic challenges such as high fat diet or cold exposure may be necessary to make the functional significance of mTERFD3 in mice clear.
This work provides yet another example of modulatory mechanisms for the highly regulated OXPHOS process. By inactivating a gene coding for a mitochondrial protein homologue to mTERF, we produced a mouse model with deficiency in OXPHOS with evident phenotype in muscle. mTERFD3 is not embryonic lethal and is present only in higher eukaryotes suggesting that it was acquired during evolution in order to respond to specific demands of mammalian metabolism. Its function may be related to modulation of mitochondrial gene expression in response to environmental changes. The mTERFD3 KO mice displayed typical features of mitochondrial myopathy like decreased exercise endurance, decreased OXPHOS capacity and increased mitochondrial proliferation. Loss of mTERFD3 \textit{in vivo} is associated with mitochondrial proliferation in muscle, a mechanism that frequently is activated in mitochondrial disorders and believed to be mediated by PGC-1α.

We showed that mTERFD3 binds mtDNA promoter \textit{in vitro} and that is able to form dimers. However the significance of dimer formation needs further investigation. Is important to note that mTERF is also able to form oligomers, but only the monomeric form is able to bind DNA. Since mTERF is not able to bind the heavy strand promoter alone is possible to envision one of the mTERF homologues as the partner that mediates the mTERF-heavy strand promoter interaction. mTERF, mTERFD3 and mTERFD1 have coil-coils domains and they may interact and with each other forming hetero-oligomers and coregulating mitochondrial gene expression (figure 4.1).
Figure 4.1. Schematic representation of mTERF-like proteins in mitochondrial transcription regulation. mTERF depicted in green binds simultaneously the initiation (HSP) and the termination sites (TERM) and favors formation of rDNA loop. mTERFD3, depicted in red binds the heavy strand promoter and acts as a positive transcriptional regulation, whereas mTERFD1 (yellow) acts as a repressor. Mitochondrial RNA polymerase (POLRMT), transcription factor A (TFAM) and one of the transcription factors B isoform TFB1M or TFB2M are necessary for the initiation transcription.

Another possible role for mTERFD3 function is selection of promoter usage. We showed mTERFD3 deficiency is responsible for a decrease in mitochondrial transcripts. It is reasonable to suggest that mTERFD3 acts as a positive regulator of mitochondrial transcription at the level of HSP. Since the mTERFD3 KO displays decreased mRNA levels and increased 12S RNA, it is possible that mTERFD3 preferentially increases the usage of HSP2. In its absence, the transcription takes place mostly from HSP1, and an excess of 12S RNA is produced relative to the mRNA transcripts. This model would suggest that mTERFD3 is able to modulate promoter usage by interacting with mitochondrial RNA polymerase. Further experiments are necessary to investigate for
possible interaction with POLRMT, and to determine the precise mechanism of mTERFD3 involvement in mitochondrial transcription regulation.

Interestingly mTERFD1 was shown to be a repressor of mitochondrial transcription. However, unlike mTERFD1, mTERFD3 is not embryonic lethal. mTERFD3 acts as a modulator and is not essential for transcriptional initiation since the POLRMT, TFAM, and TFB1M/TFB2M were shown to be sufficient for transcriptional initiation.

Unlike mTERF, mTERFD3 and mTERFD1 do not bind the termination region. However, indirect binding through the mediation of other proteins is possible.

The observation that mTERF also modulates mtDNA replication pausing suggests that mTERF is involved in coregulation of both replication and transcription. An unregulated collision of oppositely moving transcription and replication complexes would dramatically inhibit DNA replication and provoke genomic instability. Hence, mTERF is probably important in maintaining genomic stability. mTERFD3 may also play a similar role in avoiding the collision of transcription machinery since mTERFD3 KO muscle is associated with altered mtDNA transcription. Future studies to determine the effect of mTERFD3 deletion on different mitochondrial transcripts are necessary in order to test this hypothesis.

It appears that the function of mTERF family members is far more complex than previously thought with implications not only in transcription but also in replication and possibly protein synthesis. Future studies of mTERF family members will likely shed light on mechanism of mitochondrial gene expression and help understand the pathogenesis of mitochondrial disorders.
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