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REGULATION OF MITOCHONDRIAL GENE EXPRESSION IN METAZOA

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And in the end, it's not the years in your life that count. It's the life in your years.

--Abraham Lincoln
ABSTRACT

Mitochondria are essential organelles of eukaryotic cells and their main function is to provide the cell with the ubiquitously used energy currency ATP. Impaired energy conversion caused by mitochondrial dysfunction is a direct cause of several human diseases. Mitochondria evolved from eubacteria-like endosymbionts and they contain their own genome as well as their own machinery for gene expression. Nevertheless, most mitochondrial proteins are nuclear encoded and mitochondrial energy conversion depends on the coordinated expression of the nuclear and the mitochondrial genome. Mitochondrial gene expression is regulated at different levels, including transcription, RNA maturation, RNA stability and translation. However, it is still largely unknown how these processes are coordinated and the involved molecular mechanisms that regulate mitochondrial gene expression are incompletely understood. In this thesis, the role of two important factors, the leucine-rich-pentatricopeptide repeat containing protein (LRPPRC) and the mitochondrial transcription termination factor 3 (MTERF3), in regulation of mitochondrial gene expression were investigated in the fruit fly *Drosophila melanogaster* and in the mouse. RNAi-mediated knockdown was used to study the *in vivo* function of the *Drosophila* LRPPRC homologue called bicoid stability factor (BSF). The results demonstrated that BSF is an essential mitochondrial protein involved in the control of mRNA stability and polyadenylation. In addition, BSF also functions as a coordinator of mitochondrial translation because the lack of BSF resulted in misregulation of mitochondrial translation. While *de novo* translation of some mitochondrial proteins was not changed, the synthesis of others was increased and a specific subset of mitochondrial polypeptides showed increased degradation in BSF mutant flies. To test whether BSF function is specific to flies or conserved among metazoans, we analyzed LRPPRC function in mammals using whole-body and tissue-specific *Lrpuc* knockout mice. Whole body knockout of *Lrpuc* resulted in embryonic lethality, demonstrating that LRPPRC is essential for mouse development. Heart-specific ablation of *Lrpuc* caused severe respiratory chain dysfunction, cardiomyopathy and premature death at 16 weeks of age. On the molecular level, we found that LRPPRC is involved in mitochondrial mRNA maturation and stability. Furthermore, we showed that LRPPRC together with the RNA mitochondrial stem-loop-interacting protein (SLIRP) forms an RNA-dependent complex that stabilizes an
extra-ribosomal pool of non-translated mRNAs. The LRPPRC-SLIRP complex is important for coordinating mRNA binding to the active ribosome. Transcription termination is the second molecular checkpoint in the regulation of mitochondrial gene expression. MTERF3 has been suggested to act as a repressor of mitochondrial transcription in mammals. To determine the underlying mechanisms by which MTERF3 represses mitochondrial transcription and to investigate whether its function is evolutionarily conserved between flies and mammals, we generated MTERF3 knockout flies and performed MTERF3 RNAi knockdown studies. Lack of MTERF3 in *Drosophila* caused severe respiratory chain dysfunction and lethality at the pupal stage. At the molecular level, MTERF3 deficiency caused increased initiation of mitochondrial transcription, suggesting that the role of MTERF3 as a repressor of mitochondrial transcription is evolutionarily conserved between flies and mice. By analyzing mitochondrial translation during different developmental stages, we identified a novel function for MTERF3 in ribosomal biogenesis in *Drosophila*. Lack of MTERF3 caused aberrant ribosomal biogenesis, due to an impaired assembly of the large ribosomal subunit. Interestingly, this function of MTERF3 in the control of ribosomal biogenesis is evolutionarily conserved in mice, validating the fly as a useful model organism to study mitochondrial gene expression.

**Keywords:** *Drosophila melanogaster, Mus musculus, mitochondrial gene expression, mitochondria, transcription, RNA maturation, translation, human diseases*

LIST OF PUBLICATIONS

I. The Bicoid Stability Factor Controls Polyadenylation and Expression of Specific Mitochondrial mRNAs in Drosophila melanogaster

**Ana Bratic**, Anna Wredenberg, Sebastian Grönke, James B Stewart, Arnaud Mourier, Benedetta Ruzzeneente, Christian Kukat, Rolf Wibom, Bianca Habermann, Linda Partridge, and Nils-Göran Larsson


*A.B and A.W contributed equally to this work.*

II. LRPPRC is Necessary for Polyadenylation and Coordination of Translation of Mitochondrial mRNAs

Benedetta Ruzzeneente, Metodi D Metodiev, Anna Wredenberg, **Ana Bratic**, Chan Bae Park, Yolanda Câmara, Dusanka Milenkovic, Volker Zickermann, Rolf Wibom, Kjell Hultenby, Hediye Erdjument-Bromage, Paul Tempst, Ulrich Brandt, James B Stewart, Claes M Gustafsson, and Nils-Göran Larsson

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III. MTERF3 Regulates Mitochondrial Ribosome Biogenesis in Invertebrates and Mammals


*A.W, M.L and A.B contributed equally to this work.*
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LIST OF ABBREVIATIONS

A+T Adenosine plus thymidine
A354V Alanine 354 to Valine
ADP Adenosine-di-phosphate
ATP Adenosine-tri-phosphate
ATP8/6 Mitochondrial DNA encoded subunit 8 and 6 of complex V
BN-PAGE Blue native polyacrylamide gels
bp base pair
BSF Bicoid stability factor
Bsfi Bicoid stability factor gene
C-terminal Carboxy terminal
Chip Chromatin immuno-precipitation
Cl-V Complex I-V of the oxidative phosphorylation system
COX1-3 Cytochrome oxidase subunit 1-3
CSBII Conserved sequence block II
Cyt b Cytochrome b of complex III
D-loop Displacement loop
Dm Drosophila melanogaster
DmTTF Drosophila mitochondrial transcription termination factor
DNA Deoxyribonucleic acid
EDTA Ethylen-di-amoeto-tetra-acetic acid
ELAC2 Zinc phosphodiesterase ELAC protein 2
ETC Electron transport chain
FADH2 Flavin adenine dinucleotide (reduced)
fMet-tRNA Formyl methionine tRNA
G Guanosine
GPX Gluthathione peroxidase
GTP Guanosine tri-phosphate
H Heavy
H+ proton
H2O2 Hydrogen peroxide
HeLA cells Cervix carcinoma cells derived from Henrietta Lacks
HMG High mobility group
HSP Heavy strand promoter
ICT1 Codon-independent peptidyl-tRNA hydrolase
IMM Inner mitochondrial membrane
kb Kilo base
kDa Kilo dalton
L Light
Lprrc LRPPRC gene
LRPPRC Leucine-rich-pentatricopeptide repeat containing protein
LSFC French Canadian variant of Leigh syndrome
LSP Light strand promoter
LSU Large ribosomal subunit
MELAS Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
MERRF Myoclonus epilepsy associated with ragged-red fibers
MnSOD Manganese superoxide dismutase
mRNA Messenger RNA
MRPS27 Mitochondrial ribosomal protein 27 of the small ribosomal subunit
MRPPR3  Mitochondrial RNAase P protein 3
MRPS16  Mitochondrial protein 16 of small ribosomal subunit
MRPS22  Mitochondrial protein 22 of small ribosomal subunit
mtDNA  Mitochondrial deoxyribonucleic acid
mEF  Mitochondrial elongation factor
MTERF 1-4  Mitochondrial transcription termination factor 1, 2, 3 and 4
mIF  Mitochondrial translation initiation factor
mPAP  Mitochondrial PolyA polymerase
mRRF  Mitochondrial ribosome recycling factor
N-terminal  Amino terminal
NADH  Nicotinamide adenine dinucleotide (reduced)
ND1,2,3,5,6  Subunits 1,2,3,5 and 6 of NADH dehydrogenase
ND4-4L  Subunits 4 and 4L of NADH Dehydrogenase
nDNA  Nuclear DNA
NSUN4  A 5-methylcytosine RNA methyltransferase
nt  Nucleotides
O₂⁻  Superoxide radical
OH  Hydroxyl radical
O₂H  Origin of replication of heavy strand
O₂L  Origin of replication of light strand
OMM  Outer mitochondrial membrane
ONO'O²⁻  Peroxynitrite anion
OxPHOS  Oxidative phosphorylation
P  Putative promoter
PGC1 α  Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
POLRMT  Mitochondrial RNA polymerase
PPR  Pentatricopeptide repeat motif
PTCD1-3  Pentatricopeptide repeat domain protein 1, 2 and 3
RF  Release factor
RNA  Ribonucleic acid
RNAi  Ribonucleic acid interference
RNase P  Ribonuclease P
RNase Z  Ribonuclease Z
ROS  Reactive oxygen species
rRNA  Ribosomal RNA
SLIRP  Stem-loop-interacting protein
sORFs  Small-open-reading-frames
SSU  Small ribosomal subunit
TFAM  Mitochondrial transcription factor A
TFB1M  Mitochondrial transcription factor B1
TFB2M  Mitochondrial transcription factor B2
tRNA  Transfer RNA
tRNAAla  Transfer RNA for Alanine
tRNAAsp  Transfer RNA for Aspartic acid
tRNALeu  Transfer RNA for Leucine
tRNAMet  Transfer RNA for Methionine
tRNAPhe  Transfer RNA for Phenylalanine
UCP  Uncoupling proteins
UTR  Untranslated region
WANCY  Tryptophan, Alanine, Asparagine, Cysteine and Tyrosine
1. INTRODUCTION

1.1. Mitochondrial origin

Mitochondria are organelles present in almost all eukaryotic cells. Their main function is to supply the cell with energy currency in the form of ATP by oxidative phosphorylation. However, mitochondria are also involved in many other vital processes, such as apoptosis, iron-sulfur cluster synthesis, ion homeostasis, and production of reactive oxygen species (ROS).

Mitochondria have several features characteristic for prokaryotic cells and this circumstance has led to the proposal of an endosymbiotic origin. According to this theory, mitochondria evolved from eubacteria-like endosymbionts around two billion years ago [1]. In favor of the endosymbiotic hypothesis, recent phylogenetic analyses suggest a common origin of mitochondria and the SAR11 clade of Alphaproteobacteria [2].

Mitochondria have several unique morphological, biochemical and genetic features:

1. Mitochondria are composed of two lipid bi-layers: the inner mitochondrial membrane and the outer mitochondrial membrane, that together enclose two aqueous compartments, the intermembrane space and the mitochondrial matrix [3] (Fig. 1). The outer and inner mitochondrial membranes differ in their composition. While the outer mitochondrial membrane contains many proteins that enable equilibrium of ions and solutes between the cytosol and the intermembrane space, the protein and cardiolipin rich inner mitochondrial membrane is in essence ion-impermeable [4]. In addition, the inner mitochondrial membrane contains the complexes of the oxidative phosphorylation (OXPHOS) system [5].

2. Mitochondria cannot be made de novo. All of the mitochondria in an embryo are derived from the maternally transmitted mitochondria in the oocyte. Thus, mitochondrial biogenesis in somatic tissues requires that new mitochondria are formed from the existing ones.

3. Mitochondria contain their own genome (mtDNA). In humans, mitochondria contain around 1500 different proteins, of which only about 1% are synthetized by mitochondrial ribosomes located in the mitochondrial matrix. The vast majority of mitochondrial proteins is synthetized on cytosolic ribosomes and post-translationally
imported into mitochondria [6]. All of the mitochondrially encoded proteins are essential components of the OXPHOS system, while nuclear-encoded proteins fulfill very distinct functions in mitochondria, such as regulation of mitochondrial gene expression, ion-sulfur cluster synthesis, protein synthesis and other metabolic processes [7-9]. An important feature of mitochondrial transcription and translation is that both take place in the same compartment, the mitochondrial matrix, suggesting that these processes are directly coupled to each other, similar to the situation in prokaryotes.

4. The mtDNA is tightly packed into DNA-protein complexes, termed mitochondrial nucleoids (Fig. 1). Each cell contains thousands of nucleoids, which often form clusters distributed throughout the mitochondrial network [10,11]. Nucleoids are considered to be the fundamental unit of mtDNA inheritance and segregation [12].

![Diagram of mitochondria showing mitochondrial ribosomes, inner membrane, outer membrane, nucleoids, intermembrane space, and cristae.]

Figure 1: Mitochondria are unique cell organelles with their own genome (mtDNA) and translation apparatus. Mitochondria are surrounded by two lipid bi-layers, the inner mitochondrial and the outer mitochondrial membrane that together enclose two aqueous compartments: the intermembrane space and the mitochondrial matrix.

5. There are multiple mtDNA copies in the mammalian cell. A human somatic cell contains up to a few thousand copies of mtDNA, whereas an oocyte contains up to $10^5$ mtDNA molecules. In mammals, the replication of mtDNA occurs independently of the cell cycle and nuclear replication [13]. This means that particular mtDNA molecules replicate several times or not at all during a single cell cycle [14].

6. The mitochondrial genome is maternally inherited. In contrast to the nuclear genome, mtDNA is exclusively asexually transmitted from the mother to the offspring in mammals and many other Metazoa. There are interesting exceptions, e.g Bivalvia, which have paternal transmission of mtDNA [15]. Interestingly, in
mammals and many other Metazoa sperm-derived mitochondria are rapidly destroyed after fertilization [16-18].

1.2. Biology of mtDNA

1.2.1. Evolution of the Metazoan mitochondrial genome

The good conservation of the genes and gene order in the small mitochondrial genome has made it a useful genetic marker to reconstruct phylogenetic relationships and a good model for genome evolution. The mitochondrial genome size has gone through substantial changes during evolution of Metazoa. For instance, the size of mtDNA is similar among Chordata, Arthropoda, Echinodermata and Platyhelminthes, whereas there is substantial heterogeneity of mtDNA size among Nematodes and Porifera. The large differences in mtDNA size are explained by gene duplications, gene rearrangements and increased size of non-coding sequences [19]. One striking example is the mitochondrial genome size of different Drosophila species that varies up to 3 kb, due to variability in the length of non-coding regions [20,21]. Although mitochondrial gene content is relatively stable throughout evolution, some metazoa, like the Arthropod Metaseiulus occidentalis, show a severe reduction in gene number [19]. Reduced mitochondrial gene number is often caused by the loss of tRNA genes, and more rarely by the loss of protein-coding genes. In some extreme cases, like for Cnidaria and arrow worms (Chaetognatha), an almost complete loss of mitochondrial tRNA genes has been observed. In this case the mitochondrial tRNAs have been functionally replaced by import of nuclear-encoded tRNAs [22,23]. In contrast to tRNA genes, the number of protein-coding genes is relatively stable in metazoa. One of the rare exceptions is the ATP8 gene that has been lost in 5 different taxa, including Nematodes [24]. In contrast to variation in gene content [25], gene order is more variable between metazoa [26]. Remarkably, metazoan organisms without mtDNA have recently been described, showing that mtDNA is not a prerequisite for metazoan life [27].

1.2.2. Organization of the mitochondrial genome

In contrast to the large linear nuclear genome that codes for thousands of genes, mtDNA is a rather small and compact circular molecule. Compared to other eukaryotes, the mitochondrial genome of metazoa is relatively small. For instance,
the mammalian mtDNA is a circular, double-stranded molecule of around 16.5 kb, and has a very dense gene structure with only few non-coding regions. The strands of the DNA duplex can be distinguished as the heavy (H) and the light (L) strand based on their respective purine content causing different sedimentation in alkaline cesium chloride gradients [28]. The mammalian mtDNA contains 11 mRNAs, coding for 13 proteins of the OXPHOS system, as well as 22 tRNAs and 2 rRNAs, that are part of the mitochondrial translation machinery. In addition, several recent studies suggest the presence of small open-reading-frames (sORFs) in the mitochondrial genome that supposedly code for peptides of 11-32 amino acids, which may be involved in retrograde signaling between the mitochondria and the remaining cytoplasm [29,30]. These mitochondrially encoded small peptides have been identified in several metazoans, including humans, Cnidaria and Porifera, suggesting that their function may be evolutionarily conserved, although their biological relevance, if any, is intensely debated [15,19,31].

The gene distribution among different metazoans shows a high level of divergence (Fig. 2). In mammals all protein-coding genes, except for ND6, are located on the H strand. In contrast, in the fruit fly Drosophila the 13 protein-coding genes are rather evenly distributed on both strands (Fig. 2). Another important difference between mitochondrial genomes of metazoans is the arrangement of the non-coding regions. The major non-coding region regulates replication and transcription of mtDNA [25] and this region has therefore been designated as the control region. In vertebrates, the control region is also called the displacement loop (D-loop), due to a characteristic triple-stranded structure generated by prematurely terminated replication of nascent H-strands [14]. The D-loop harbors important regulatory elements necessary to initiate replication and transcription of mtDNA [32]. A second non-coding region is located in a cluster of five tRNA genes, termed the WANCY region, and contains the origin of replication for the L-strand (Ol) of mammalian mtDNA (Fig. 2). Some other metazoas, e.g. insects and nematodes, have large non-coding regions, that are A+T rich and contain the origins of replication for both strands [20] (Fig. 2). For most metazoan clades there is only very limited information available about the structure of the mtDNA control region involved in the regulation of mitochondrial gene expression.
Figure 2. Metazoan mitochondrial genome organization. The mitochondrial genome is a double-stranded circular molecule that usually contains 37 genes encoding: 11 mRNAs (blue), 2 rRNAs (green), and 22 tRNAs (red). The gene distribution of mtDNA varies among different metazoan phyla. In contrast to mammalian mtDNA where the majority of genes is present on the heavy (H) strand, the Drosophila’s mtDNA genes are distributed on both strands. Another major difference between metazoan mitochondrial genomes is the arrangement of the non-coding regions (yellow). Mammalian mtDNA has two main non-coding regions containing the origins for replication of the H and L strand (O_h and O_l), respectively. In contrast, Drosophila mtDNA has only one major non-coding region (yellow) comprising both origins of replications (O_h and O_l).

1.3. Regulation of mitochondrial gene expression

Even though mitochondria contain more than 1500 proteins, numerous studies indicate high diversity in protein expression levels across different tissues, suggesting that oxidative phosphorylation capacity may change in a tissue-specific manner [33,34]. OXPHOS capacity can depend on the mitochondrial mass, the amount of respiratory chain complexes per mitochondrial mass and the regulation of the activity of individual complexes [35,36]. For instance, heart and skeletal muscle are the tissues with very high OXPHOS capacity, due to their substantial energy demands. In contrast, liver and kidney are characterized by lower OXPHOS capacity and are consequently less sensitive to OXPHOS defects [35]. This implies that mitochondrial OXPHOS capacity is set to meet organ-specific demands [35] and that changes in energy demands directly modulate the expression of mitochondrial proteins in order to assure a quick adaptation of the cell to the current metabolic state. The regulation
of mitochondrial function is thus a very complex process regulated by the coordinated expression of the nuclear and the mitochondrial genomes. Mitochondrial gene expression is controlled simultaneously at several different levels, including transcription, RNA processing and maturation, RNA stability, translation and assembly and turnover of mitochondrial OXPHOS complexes \([7,36-43]\). While the most of the studies have been focused on the regulation of mitochondrial transcription, very little is known about mitochondrial gene expression regulation at the level of translation and assembly of mitochondrial OXPHOS complexes.

### 1.3.2. Transcription of mtDNA

#### 1.3.2.1. Promoters for transcription

In mammals, mitochondrial transcription is initiated from two main promoters, designated HSP and LSP, located in the D-loop region around 150 bp from each other. Transcription of the L-strand is initiated from LSP, and generates a primary transcript encoding one mRNA (ND6) and 8 tRNAs. Transcription of the H-strand has been reported to be initiated from two promoters denoted HSP1, located 16 bp upstream of the tRNA\(^1\) gene, and HSP2, located close to the 5’ end of the 12S rRNA gene (Fig. 3). The primary transcript generated from the HSP2 promoter is proposed to cover almost the whole length of the H-strand to generate 2 rRNAs, 12 tRNAs and 10 mRNAs. In contrast, transcription initiated from the HSP1 promoter is proposed to terminate prematurely at the 3’ end of the 16S rRNA gene and will consequently result in a shorter polycistronic primary transcript encoding only two rRNAs and two tRNAs (Fig. 3). Even though processed from the same polycistronic transcripts, rRNAs are more abundant in the mitochondrial matrix than the corresponding mRNAs. It has been proposed that this difference directly depends on the higher rate of transcription initiation from HSP1 in compared to HSP2 promoter. However, recent \textit{in vitro} transcription studies failed to show any significant transcription initiation driven by the HSP2 promoter, suggesting that transcription is driven only by the HSP1 and LSP promoters \([44]\). The higher abundance of rRNAs has also been attributed to a specific transcription termination step \([45]\), where mitochondrial transcription termination factor 1 (MTERF1) binds downstream of the 16s rRNA gene. However, this has been recently contradicted in a recent mouse knockout study, which shows that the MTERF1 knockout mouse have normal levels of rRNAs. It is
thus possible that higher abundance of rRNAs in comparison with mRNAs is simply due to differences in their stability [38]. In contrast to the situation in mammals, *Drosophila* mtDNA is suggested to be transcribed in four transcription units starting from the 5’ end of each gene block distributed on both strands [46,47] (Fig. 3).

**Figure 3. The location of cis-regulatory elements for transcription of fly and mammalian mtDNA.** A) Mammalian mtDNA transcription is initiated from two main promoters: HSP (HSP1, HSP2) initiates transcription of the heavy (H) strand, whereas LSP initiates transcription of the light (L) strand. While HSP2 and LSP promoters have been reported to drive synthesis of nascent transcripts that covers almost entire length of corresponding strand, the primary transcript from HSP1 has been reported to contain only 2 rRNAs and 2 tRNAs. B) In contrast to mammalian transcription, little is known about cis-regulatory elements for transcription of *Drosophila* mtDNA. The scheme indicates the location of putative promoters (P1-P4) and the two putative transcription termination sites, where the *Drosophila* transcription termination factor 1 (DmTTF) has been reported to bind.

### 1.3.2.2. Transcription initiation

**The basic components of transcription initiation machinery**

The human basic mitochondrial transcription machinery consists of mitochondrial RNA polymerase (POLRMT) and the two transcription factors, mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M) [32,48]. These three core components interact in order to assure specific promoter recognition and promoter melting, thereby allowing initiation of transcription. TFAM binds specifically to the cis-regulatory elements upstream of the LSP and HSP (HSP1) promoter to recruit the POLRMT/TFB2M complex to the initiation site via direct interaction with the C-terminal tail of TFAM [49] (Fig. 3). Subsequently,
TFB2M helps melting the promoter region and primes POLRMT to initiate the transcription [50].

Mitochondrial RNA polymerase
In metazoans, POLRMT is the only known RNA polymerase for mitochondrial transcription [51]. POLRMT is a single-subunit protein of 140kDa with sequence homology to bacteriophage RNA polymerases. POLRMT contains two functional domains. The N-terminal domain consists of the mitochondrial-targeting sequence, an AT-rich promoter-recognition loop and two pentatricopeptide motifs (PPR), whereas the C-terminal domain harbors the catalytic function of the RNA polymerase [52]. The main function of POLRMT is to initiate synthesis of new RNAs upon specific promoter binding at the sequence localized -1 to -2 relative to the LSP transcription initiation site [53]. The function of the two PPR motifs is not understood and POLRMT cannot initiate promoter-specific transcription on dsDNA on its own. Instead, it requires the presence of two additional transcription factors, TFAM and TFB2M, to accomplish transcription initiation [44].

Mitochondrial transcription factor A
TFAM was the first transcription factor assigned as a core component of the mitochondrial transcription initiation machinery in vertebrates. TFAM has the ability to bind, bend and wrap DNA [48,54]. It belongs to the high mobility group (HMG) family of proteins that can bind DNA. It contains two HMG domains, which can bind DNA, separated by linker region and the C-terminal tail is essential for specific activation of transcription [54,55]. TFAM specifically recognizes a short mildly conserved DNA sequence, located upstream of the LSP and HSP1 promoter regions [55,56]. Binding of TFAM induces a characteristic ‘U-turn’ in the mtDNA within the LSP promoter region, which enables direct interactions between the C-terminal tail of TFAM and the rest of the transcription machinery, thereby stimulating transcription [57,58]. Noteworthy, TFAM not only plays an essential role in mitochondrial transcription but is also a key-regulator of mtDNA copy number and mtDNA maintenance [42,59]. TFAM is the main factor packaging mtDNA into nucleoids [10,11,57]. TFAM has been reported to be phosphorylated and acetylated and these modifications are maybe important to regulate distinct functions of TFAM [60].
Mitochondrial transcription factor B1 and B2

In most metazoans, mitochondrial transcription factor B has two isoforms: TFB1M and TFB2M. Both isoforms have high homology to bacterial rRNA dimethyltransferases [49,61,62]. Even though both proteins interact with TFAM and POLRMT in vitro, the two TFBM isoforms have distinct functions. Recent studies in Drosophila have shown that down-regulation of DmTFB2M reduces the abundance of mitochondrial transcripts and mtDNA copy number, whereas down-regulation of DmTFB1M leads to reduced mitochondrial translation [39,63]. These findings have been supported by studies in mammals, where TFB1M has been shown to play an important role in ribosomal biogenesis by dimethylating two highly conserved adenine residues present in a conserved stem-loop at the 3’ end of the 12S rRNA [64]. In contrast, TFB2M is required for promoter melting and NTP binding, which will assure correct positioning of the priming nucleotide at the catalytic active site of POLRMT [50].

1.3.2.3. Transcription termination

Transcription termination is the second major checkpoint in the regulation of mtDNA gene expression. The mitochondrial transcription termination factor (MTERF) family members were suggested to play an important role in regulating mitochondrial transcription at the level of termination [65]. Mammals have four MTERF proteins, termed MTERF1 through MTERF4. MTERF3 and MTERF4 are highly evolutionarily conserved and shared among metazoans and plants, including homologues in C. elegans and Drosophila. In contrast, MTERF1 and MTERF2 are only present in vertebrates [65]. All four MTERF proteins are nuclear-encoded and targeted to mitochondria [7,37,38,66]. The common structural feature of MTERF proteins is the presence of repeated MTERF motifs, each consisting of three α-helices that form a triangle. Several MTERF motifs together form a right-handed superhelix, which gives MTERF proteins the shape of the half-doughnut [47,67,68].

In vivo studies on MTERF knockout mouse models indicate that the biochemical function of the MTERF proteins may not be limited to the regulation of mitochondrial transcription termination. For instance, MTERF4 regulates ribosomal biogenesis and mitochondrial translation by targeting the rRNA methyltransferase NSUN4 to the large mitochondrial subunit [7]. MTERF1, MTERF2 and MTERF3 have been suggested to control mtDNA expression at the transcriptional level.
MTERF1 has originally been described as a key-regulator of mitochondrial ribosomal biogenesis in mammals, due to its opposing functions to activate and terminate mitochondrial transcription. Termination of H-strand transcription in mammalian mitochondria occurs at two specific sites within the mitochondrial genome. MTERF1 binds two specific sites in the mitochondrial genome. The first site is located just after the 3’end of 16S rRNA gene, within a tridecamer sequence in the tRNA^L1^ gene, whereas the second site is located in the D-loop region [45]. Simultaneous binding of MTERF1 to both sites has been reported to result in the formation of a mtDNA loop containing just the rRNA genes. In vitro studies suggested that this loop formation facilitates reinitiation of transcription from the HSP1 promoter, thereby explaining why the steady-state levels of rRNA transcripts are more abundant than the levels of downstream mRNAs [45]. Surprisingly, recent in vivo studies have showed that MTERF1 is dispensable for rRNA synthesis and ribosomal biogenesis [38].

The binding of MTERF1 to a tridecamer sequence in tRNA^L1^ [69] prevents elongation of L-strand transcription to form antisense rRNA transcripts. This L strand transcription termination has also been reported to be important to prevent transcription interference at the LSP promoter [38,70] (Fig.3). A large proportion of LSP transcripts are prematurely terminated at the conserved sequence block II (CSBII) of D-loop. During transcription the nascent RNA strand and the non-template DNA strand forms an RNA-DNA hybrid, which adopts specific G-quadruplex structure at CSBII to stimulate premature termination of LSP transcription [71]. These G-quadruplex structures are possibly resolved by still unknown proteins to generate the RNA primers needed for H-strand replication initiation [71]. However, it still remains to be determined how the transition between LSP transcription and replication of H-strand is regulated.

In flies, a mitochondrial transcription termination factor (DmTTF) has been shown to regulate transcription termination by binding to two homologous non-coding sequences located at the 3’ ends of two gene clusters transcribed in opposite directions [72-74] (Fig. 3). DmTTF knockdown in Drosophila tissue culture cells led to increased levels of RNAs mapping downstream of the DmTTF binding sites (Fig. 3). Interestingly, the levels of RNAs mapping between the promoter in the A+T rich non-coding region and the DmTTF binding site were also depleted, suggesting that DmTTF might also have the role of the transcriptional activator, similar to mammalian MTERF1 [75]. In contrast to MTERF1, DmTTF exclusively binds a
sequence containing only A-T base pairs [69,76].
MTERF2 associates with mitochondrial nucleoids [77] and has a role in the regulation of mtDNA transcription under certain stress conditions [66]. Furthermore, it has recently been suggested that MTERF2 is involved in the regulation of replication fork progression, as overexpression of MTERF2 results in mtDNA depletion and accumulation of replication intermediates in the tissue culture cells [78,79]. Even though the effect of MTERF2 on mtDNA copy number has been confirmed in vivo by knockout studies [66], further studies are needed to understand the molecular role of MTERF2 in the regulation of mitochondrial transcription and replication.

MTERF3 plays a role in regulation of mtDNA transcription and allows fine-tuning of the oxidative phosphorylation capacity by acting as a transcriptional repressor [39]. MTERF3 knockout mice die during embryogenesis, while tissue-specific ablation of MTERF3 in heart and skeletal muscle causes mitochondrial cardiomyopathy resulting in premature death at 18 weeks of age. Lack of MTERF3 in heart leads to aberrant transcription profile, with increased steady-state levels of all mRNAs and tRNAs encoded by promoter proximal to genes. In contrast, the steady state levels of ND6 and distal tRNAs were decreased. Furthermore, chromatin immuno-precipitation (Chip) studies showed that MTERF3 binds the D-loop region containing the two promoters for transcription. This suggests that MTERF3 negatively regulates mitochondrial transcription by acting as a repressor to prevent collision of transcription complexes on opposite mtDNA strands [37]. Still, the precise mechanism underlying repression of mitochondrial transcription by MTERF3 remains unknown.

In contrast to mammalian MTERF3, Drosophila MTERF3 (DmMTERF3) has been suggested to play a role in the regulation of mitochondrial protein synthesis in cultured Drosophila cells. Depletion of DmMTERF3 in cultured insect cells did not have an effect on several mitochondrial transcripts, while de novo synthesis of mitochondrial polypeptides was overall decreased, with ND1 being the most affected [80]. Thus, it is of fundamental importance to further investigate the in vivo role of DmMTERF3 in flies and its putative conserved role in repression of mitochondrial transcription in metazoa. Molecular understanding of suggested role of MTERF3 in transcription and translation may provide novel insights how these two processes are coordinated in metazoa.
1.3.3. RNA processing and maturation

1.3.3.1. Mitochondrial transcripts

Mitochondrial transcription generates polycistronic transcripts that undergo extensive processing to generate mature mRNAs, rRNAs, and tRNAs. Importantly, almost all protein and rRNAs-coding genes are directly flanked by a tRNA gene and tRNA excision from the polycistronic transcript plays an important role in mRNA maturation. According to the ‘tRNA punctuation model’, the folded tRNA structures in polycistronic molecules are recognized by various mitochondrial RNases (Fig. 4). Excision of tRNAs is therefore required to produce most, but not all, mature rRNAs and mRNAs [81,82]. Since most mature RNAs correspond to a single gene (with the exception of ND4/ND4L and ATP8/ATP6 that are bicistronic transcripts with two overlapping open reading frames), accurate excision of tRNAs from primary polycistronic transcripts is of crucial importance for mitochondrial gene expression. Accurate tRNA processing is regulated by specific endonucleolytic RNases, i.e. RNase P and RNase Z that are responsible for cleavage of polycistronic molecules at the 5’ and the 3’ end of tRNAs, respectively [83-85] (Fig. 4). In contrast to tRNA molecules, mitochondrial rRNAs and mRNAs have some peculiar structural features compared to their cytoplasmic counterparts. Mitochondrial rRNAs are smaller than the cytoplasmic rRNAs. Mitochondrial mRNAs usually start immediately at the start codon or in rare cases have very short 5’ UTRs. The mRNAs lack a 5’ 7-methylguanosine cap at the 5’ end, and many of them also do not have 3’ UTR regions. Furthermore, mRNAs do not contain introns and base modifications [81]. Importantly, all released mitochondrial transcripts undergo some type of post-transcriptional modifications, e.g. polyadenylation, methylation, pseudouridylation, that are important for their functionality [86] (Fig. 4).

Maturation of mitochondrial tRNAs is accomplished by post-transcriptional addition of a CCA triplet to their 3’ end catalyzed by an ATP(CTP):tRNA nucleotidyltransferase (Fig. 4). The CCA addition and the modification of specific bases assure a proper folding of tRNAs, base pairing and interaction with aminoacyl-tRNA synthetases [82,87,88].
Figure 4. Post-transcriptional regulation of mtDNA expression. Primary transcripts undergo extensive processing in order to generate mature RNAs. The folded tRNA structures are recognized by mitochondrial RNAse P and Z, which cleave the polycistronic RNA at the 5’ or 3’ end of the tRNAs to release tRNAs, mRNAs and rRNAs. Maturation of released RNA species is accomplished via specific post-transcriptional modifications, which include specific nucleotide modification in rRNA and tRNA species, and polyadenylation of mRNAs. There are also mRNA decay pathways to control the stability of transcripts.

In contrast to tRNAs, maturation of mRNAs requires polyadenylation at their 3’ terminus by mitochondrial poly-A polymerase (mtPAP) [86] (Fig. 4). Mitochondrial mRNAs have long 3’ polyA tails containing around 50 adenines immediately after the stop codon [81]. It has been proposed that polyadenylation is a two-step process.
In the first step, a short adenine tail is added to the 3’ end of the mature transcript by a still unidentified enzyme. In the second step, the short adenine tail is extended by the action of a mitochondrial isoform of mtPAP, resulting in an about 50 adenines long polyA tail [89]. Even though the precise role of the poly A tail is still unclear, it is widely accepted that polyadenylation at the 3’ termini is required to create stop codons of 7 mRNAs in human mitochondria and 4 mRNAs in Drosophila mitochondria [90]. Polyadenylation may have an additional role in providing mRNA stability [87,91,92]. However, further studies are required to reveal the precise role that 3’ polyA tails play in the regulation of mitochondrial transcript stability and translation.

1.3.3.2. Post-transcriptional regulation

Despite their common polycistronic origin, the steady-state levels of mature mRNAs, tRNAs and rRNAs vary widely [93]. For instance, mitochondrial rRNAs are 10-20 times more abundant than the most abundant mRNAs (COX1 and COX2), and significant differences have also been observed in the relative abundance of 16S and 12S rRNA. Thus, the levels of mitochondrial transcripts must be controlled post-transcriptionally, indicating the importance of RNA-binding proteins in the regulation of mitochondrial gene expression.

Post-transcriptional regulation of rRNAs stability

Post-transcriptional nucleotide modifications are required for the proper functioning of non-messenger RNAs (Fig. 4). The rRNAs undergo several modifications, some of which are co-transcriptional while others occur once the rRNAs are assembled into pre-ribosomes [86]. The vast majority of modifications are performed by site-specific mitochondrial RNA methyltransferases. Five specific nucleotides of 12S rRNA are known to be modified by methylation, but most of the responsible methyltransferases have not been yet identified [86]. Recently, it has been reported that mammalian TFB1M modifies 2 adenine residues within the stem loop near the 3’ end of 12S rRNA. In the absence of this specific modification, the stability/assembly of the small ribosomal subunits is impaired leading to loss of mitochondrial translation [64]. How these modifications impair the stability of small ribosomal subunits remains to be explained. Dimethylation of both adenines might be a checkpoint for the assembly of the ribosomal subunits into a monosome. Thus, in the absence of the 12S rRNA stem
loop methylation, small ribosomal subunits cannot assemble and become unstable. In contrast, lack of ribosomal targeting of NSUN4, a 12S rRNA cytosine methyltransferase, results in impaired assembly of monosomes with persistence of the two unassembled ribosomal subunits [7]. This further implies that methylation of 12S rRNA is a step-wise process, which has to be coordinated and tightly controlled to ensure proper mitochondrial ribosomal biogenesis and mitochondrial translation. In contrast to 12S rRNA, 16S rRNA nucleotide modifications occur primarily at the 2-O ribose. However, the presence of 16S RNA methyltransferases has not been yet identified in metazoan mitochondria [86].

**Post-transcriptional regulation of mRNA stability**

It is widely accepted that the two most important processes determining steady-state levels of mRNAs are transcription initiation and stability. While the basic machinery for transcription initiation has been described, RNA-binding proteins involved in post-transcriptional modifications of the mitochondrial transcriptome and the mechanisms by which they govern mitochondrial gene expression are largely unknown. To date, several members of the pentatripeptide-repeate (PPR)-domain containing family of proteins have been proposed to play a pivotal role in promoting stability or translation of individual mitochondrial transcripts in mammals. The PPR family of proteins was originally identified in Arabidopsis thaliana, as a large family comprising 400-600 members. PPR proteins are characterized by the presence of an 35-amino-acid motif repeated in tandem up to 30 times per protein [94]. Plant PPR-domain-containing proteins are involved in RNA editing, RNA processing, RNA splicing and translation of mitochondrial and chloroplast transcripts, most likely due to their ability to bind RNA [94]. Interestingly, in mammals only 7 PPR proteins have been identified to date: pentatripeptide repeat domain protein 1, 2, and 3 (PTCD1-3), mitochondrial RNA polymerase (POLRMT), mitochondrial RNAase P protein 3 (MRPPR3), mitochondrial ribosomal protein of the small ribosomal subunit (MRPS27), and the leucine-rich-pentatripeptide repeat containing protein (LRPPRC). All of these proteins are predicted to be localized to mitochondria. Despite their common RNA binding ability, the seven PPR-containing proteins have different functions in the regulation of mitochondrial gene expression. For instance, PTCD1 is involved in the processing of 3’ ends of primary polycistronic transcripts by interacting with the RNAase Z, also known as ELAC2 [84]. In contrast, PTCD2 is
specifically involved in processing of the Cyt b primary transcript [95]. PTCD3 and MRPS27 play an important role in mitochondrial translation, most likely due to direct interactions with the 12S rRNA of the small ribosomal subunit [96,97]. LRPPRC is the first identified and most studied human PPR protein. Numerous reports suggest different functions of LRPPRC in regulating mitochondrial RNA metabolism and mitochondrial gene expression [98-103]. Additionally, LRPPRC has been suggested to have a function in regulation of nuclear gene expression, suggesting that the function of LRPPRC may not be limited to mitochondria [104].

A mitochondrial function for LRPPRC was revealed when it became clear that the French-Canadian variant of Leigh syndrome (LSFC) is caused by missense mutation A354V in LRPPRC. Patients with LSFC suffer from mental retardation, lesions in the basal ganglia, and severe episodes of lactic acidosis. Patient-derived cell lines showed a reduction in LRPPRC levels, causing a severe reduction in steady-state levels of many mitochondrial mRNAs [98]. In particular, the levels of COX1 and COX3 transcripts were reduced, resulting in reduction of COX1 and COX3 protein levels and severe cytochrome c oxidase deficiency [100]. These findings suggest that LRPPRC does not only play an important role in regulating RNA stability, but that it also regulates translation of specific mRNAs. LRPPRC interacts with the mitochondrial stem-loop-interacting protein (SLIRP) to form protein complex [98]. However, the role of LRPPRC-SLIRP protein complex in regulation of mRNA metabolism remains unclear. In addition to SLIRP, many additional proteins have been identified to interact with LRPPRC. It has been reported that LRPPRC may have a function in cytoplasmic RNA export and nuclear transcription by interacting with the translation initiation factor 4E and peroxisome-proliferative-cofactor 1a (PGC1α), respectively [104-108]. This proposed nuclear function of LRPPRC may suggest that LRPPRC coordinates expression of the nuclear and the mitochondrial genome. However, a recent report suggests that LRPPRC is exclusively localized to mitochondria [109]. Thus, some of the proposed extra-mitochondrial functions of LRPPRC may have to be revisited. Interestingly, phylogenetic analyses suggest that the LRPPRC protein is only present in metazoans. In Caenorhabditis elegans LRPPRC seems to be absent, whereas Drosophila melanogaster has two LRPPRC homologues. Of the two homologues, the bicoid stability factor (BSF) is more closely related to the mammalian LRPPRC [109]. In Drosophila, BSF has been suggested to control the stability of bicoid mRNA by binding to the 3’ untranslated region. The
Bicoid protein act as a morphogen in the anterior-posterior body patterning of the fly embryo [110]. However, even though the ablation of bsf causes lethality in flies, the function or distribution of bicoid mRNA is not affected in bsf null mutants. This implies that BSF function may not be restricted to embryogenesis and it may have additional functions. Thus, the further understanding of the role that BSF plays in *Drosophila melanogaster* can provide important insights into the function of LRPPRC family of proteins and the molecular mechanisms by which those proteins regulate mitochondrial gene expression.

### 1.3.4. Translation

Mitochondrial translation and subsequent post-translational modifications are the last steps in mitochondrial gene expression. The mitochondrial translation machinery is distinct from its cytoplasmic counterpart and resembles prokaryotic translation systems. Mitoribosomes are protein-rich structures that sediment as particles of 55S. The mitoribosome is composed of 2 subunits: the small ribosomal subunit (SSU, 28S), and the large ribosomal subunit (LSU, 39S). Each subunit contains one rRNA, the 12S rRNA in the SSU and the 16S rRNA in the LSU. Both rRNAs are shorter than the corresponding cytoplasmic rRNAs and consequently make up only 30% of the mitoribosome mass. In contrast, rRNAs are much more prominent constituents in bacterial and cytoplasmic ribosomes, reaching 70% and 50-60% of the mass, respectively. Therefore, the lack of rRNAs in mitoribosomes is complemented by a higher protein content, half of which are specific for mitochondrial ribosomes and do not have homologues in bacterial or cytoplasmic ribosomes [111,112]. The high protein content of mitochondrial ribosomes might provide a protection to the shorter rRNAs from the highly oxidative environment present in mitochondria [113]. Despite some similarities with the prokaryotic 70S ribosomes, mitochondrial ribosomes have some unique features. First, mitochondria use a different genetic code compared to the universal genetic code [114]. AGA and AGG that normally encode arginine are stop codons in mitochondria. Furthermore, UGA, which is universally used as a stop codon for tryptophan in mitochondria. Second, in mammals mitochondria use a simplified decoding system for translation. The mitochondrion contains only 22 tRNAs, all of which are encoded by mtDNA. Third, mammalian mitochondrial mRNAs lack a Shine-Delgarno sequence in their 5’ UTR. Furthermore, the mRNAs do not contain a polyadenylation signal, therefore the polyA tail often starts
immediately after the stop codon. Finally, in contrast to bacterial and cytoplasmic ribosomes, the mitochondrial SSU binds mRNAs in a sequence independent manner and in the absence of translation initiation factors and aminoacylated tRNAs [111].

1.3.4.1. The mitochondrial translation apparatus

Protein synthesis in mitochondria consists of four steps: initiation, elongation, termination and ribosomal recycling. All of these processes are dependent on nuclear-encoded proteins. To date, the basic mechanisms of mitochondrial translation are largely known and the majority of the involved proteins have been identified. Protein synthesis is initiated by two mitochondrial initiation factors: mtIF2 and mtIF3. In the first step of initiation, the 55S monosome actively dissociates by binding of mtIF3 to the SSU. Subsequently, mtIF2 binds to the formed SSU:mtIF3 complex allowing mRNA to feed into 28S small ribosomal subunit. After the pass of the first 17 nucleotides, sliding of mRNA through the ribosome is paused to verify whether the start codon is positioned at the P site of the ribosome. Upon correct codon:anticodon interaction, mtIF3 promotes binding of formyl-Methionine-tRNA (fMet-tRNA) to the start codon. Thereafter, the large subunit joins and initiation factors are released, resulting in a completed 55S initiation complex that is ready to enter the elongation phase of protein synthesis [111]. In contrast, if the start codon is not recognized by the SSU or if fMet-tRNA is absent, the mRNA slides further through the SSU and eventually dissociates [111]. In contrast to bacteria and eukaryotic ribosomes, mitoribosomes have a single species of tRNA\(^{M}\) that is dedicated to both, initiation of protein synthesis and peptide-chain elongation. Thus, if tRNA\(^{M}\) is formylated it interacts with mtIF2, while non-formylated tRNA\(^{M}\) interacts with elongation factors [115].

In contrast to the initiation and termination phase, the elongation phase occurs in a similar fashion as in bacteria, and highly conserved elongation factors are involved, mtEF Tu, mtEF Ts and mtEF G [114]. During the elongation phase, mtEF Tu promotes binding of aminoacylated tRNAs to the A site of the ribosome. Upon binding, hydrolysis of GTP triggers the release of mtEF Tu-GDP resulting in the conformational change of the ribosome allowing peptide-bond formation, thereby leaving a deacylated tRNA at the P site and a peptidyl-tRNA at the A site of the ribosome. Subsequently, mtEF G induces translocation of the peptidyl-tRNA from the A to the P site of the ribosome enabling entry of the next acylated tRNA. The
mitochondrial ribosome continues the protein synthesis until the stop codon reaches the P-site of the SSU [111].

In bacteria there are two factors involved in termination of the protein synthesis, denoted release factor 1 and 2 (RF1 and RF2). In mammals, bioinformatic analyses indicated the presence of four mitochondrial release factors: RF1, RF1a, ICT1, and C12orf56. However subsequent biochemical analyses could only confirm release activity and specific binding to the UAA and UAG stop codons of mtRF1a. Binding of mtRF1a to the stop codon in the presence of GTP triggers hydrolysis of the peptide bond at the peptidyl-transferase center on the LSU resulting in release of polypeptide chain [8,116,117]. Subsequent binding of the mitochondrial ribosome recycling factor 1 and 2 (mRRF1 and mRRF2) promotes ribosomal dissociation, release of mRNAs and deacylated tRNAs to allow the another round of protein synthesis [114].

1.3.4.2. The functional OXPHOS system

The OXPHOS system consists of 13 mtDNA-encoded proteins and around 80 nuclear-encoded proteins that are imported from the cytosol to the mitochondria. The nucleus-encoded OXPHOS subunits have to be properly processed before assembly with the mtDNA-encoded subunits in the inner mitochondrial membrane to form a functional OXPHOS system. To facilitate the assembly of the respiratory chain, mitochondrial ribosomes are present in close proximity to the IMM enabling a direct coupling between protein synthesis and efficient formation of the OXPHOS system [115] (Fig. 1).

The OXPHOS system consists of 5 complexes embedded in the inner mitochondrial membrane. The 13 mtDNA-encoded proteins are essential subunits of Complexes I (NADH:ubiquinone oxidoreductase), III (Ubiquinol-cytochrome c oxidoreductase), IV (cytochrome c oxidase) and V. Complex II (succinate oxidoreductase) is the only complex consisting exclusively of nuclear-encoded subunits (Fig. 5). Complex I, II, III and IV form the electron transport chain (ETC), which together with complex V form the OXPHOS system. Complex I contains 7 mtDNA-encoded subunits and is the largest complex of the ETC. Electron carriers, NADH and FADH2, feed electrons to Complex I and Complex II, respectively. Electrons are further transferred to Complex III via the small water-soluble molecule coenzyme Q (ubiquinone). From Complex III electrons are passed to cytochrome c, a small inter-membrane space protein loosely associated with the intermembrane space at the periphery of inner-
mitochondrial membrane. Complex IV further catalyzes the electron transfer from cytochrome c to molecular oxygen, thereby reducing it to water [118,119] (Fig. 5). The electron transfer via the ETC results in a proton motive force, that translocates protons from the matrix to the inter-membrane space (Fig. 5). In contrast to Complex I, III and IV, electron transfer from Complex II fuels the electron to the respiratory chain without translocating protons across the inner-mitochondrial membrane [118]. The return of protons to the mitochondrial matrix is energetically favored and directly coupled to ATP synthases by Complex V (ATP synthase), which, in turn, generates ATP from ADP and phosphate ions (Fig 5).

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**Fig. 5. Schematic model of the oxidative phosphorylation system and the production of reactive oxygen species.**

**A)** ATP is generated by oxidative phosphorylation conducted by the four respiratory chain complexes (CI-CIV) and ATP synthase (CV) located in the inner mitochondrial membrane. Energy released by the electron transfer from NADH and FADH$_2$ to O$_2$ is used to pump protons (H$^+$) via CI, CI$\text{I}$, and CIV. The proton gradient across the inner mitochondrial membrane drives ATP production via ATP synthase (CV). The proton gradient can be dissipated by re-entry of protons to the mitochondrial matrix through uncoupling proteins (UCP), which leads to uncoupling of respiration and ATP synthesis. **B)** As a by-product of oxidative phosphorylation, reactive oxygen species (ROS) are formed. Superoxide (O$_2^-$) is an abundant ROS in the cell and is generated by CI and CI$\text{I}$. Cells protect themselves from oxidative damage by expressing a variety of antioxidant enzymes that convert ROS into less harmful by-products. Superoxide anion is converted to hydrogen peroxide (H$_2$O$_2$) by manganese-superoxide dismutase (MnSOD). Hydrogen peroxide is then converted to water by glutathione peroxidase (GPX), the most abundant peroxidase in the cytosol and mitochondria. Even though hydrogen peroxide is not as harmful for the cell, it can be converted to the highly reactive hydroxyl radical (OH$^-$) in the presence of transition metals via the Fenton reaction [120].
Most of the ATP within the cell is generated by mitochondria. In order to provide the cell with enough ATP, synthesis of the respiratory chain complexes requires tightly controlled expression of both nuclear and mitochondrial genome to prevent not only electron leakage and subsequent accumulation of toxic reactive oxygen species (Fig.5), but also malfunctioning of OXPHOS system, which has been linked to numerous mitochondrial diseases.

1.4. Mitochondrial dysfunction and human diseases

Mitochondrial diseases are characterized by pleiotropic phenotypes and very variable ages of onset. To date, many different pathogenic mutations in the nuclear or mitochondrial genome have been linked to human mitochondrial diseases [121]. Mutations in nuclear genes cause mitochondrial diseases by affecting mtDNA expression or maintenance or by affecting the function or assembly of OXPHOS subunits. Most mutations of mtDNA cause mitochondrial dysfunction by impairing mitochondrial translation or by affecting the function of specific subunits of the OXPHOS system (Fig.6).

1. Mitochondrial diseases caused by mtDNA mutations

In contrast to pathogenic mutations in nuclear genes, mtDNA mutations are not transmitted according to mendelian principles [121]. Furthermore, mutations in mtDNA do not necessarily cause a phenotype unless present at a high proportion of the total mtDNA. Due to the high mtDNA copy number per cell, mitochondrial dysfunction is only caused when pathogenic mtDNA mutations reach a certain threshold level. This threshold depends on the type of the mutation and on the tissue [28]. Within a given tissue, the threshold may not be reached in all cells often resulting in a mosaic-pattern of respiratory chain dysfunction. Thus, the amount of affected cells per affected tissue or organ will directly determine whether a mitochondrial disease will become apparent or not [28,120]. In addition to the mutation pattern that varies between different mitochondrial diseases, clinical phenotypes of mitochondrial disorders also show high heterogeneity, from mild ocular myopathy, age-related hearing loss, diabetes mellitus to severe cardiomyopathy and fatal infantile disorders, such as Leigh syndrome [122,123]. It is often difficult to determine a direct link between a specific genotype and the clinical phenotype of a mitochondrial disease. This is because one pathogenic mutation can
cause different pathogenic features and because the same phenotype can be caused by different pathogenic mutations [122,124-126] (Fig. 6).

![Mitochondrial Diseases Diagram](https://via.placeholder.com/150)

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<th>Mitochondrial Diseases</th>
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**Respiratory chain dysfunction**

To date, two types of mtDNA mutations, mtDNA rearrangements (deletions, duplications) [127] and point mutations [128] have been linked to different human diseases. The size of mtDNA deletions can vary from a single base up to several kb. The most common deletion in mtDNA spans a 5 kb region between the Cyb and COX2 genes and encompasses several tRNAs and protein-coding genes [127]. The
Regulation of Mitochondrial Gene Expression in Metazoa

Ana Bratic

A proportion of mtDNA with a single large deletion has to exceed 60% in a single cell to cause respiratory chain dysfunction [129]. Single large deletions of mtDNA are usually sporadic and rarely maternally inherited, whereas multiple deletions of mtDNA typically are caused by mutations in nuclear genes regulating mtDNA replication [130,131] (Fig. 6).

In addition to large deletions, more than 100 different pathogenic point mutations in tRNA and rRNA genes of mtDNA have been found to cause mitochondrial disease [125,126,132]. Mutations in tRNA genes typically lead to impaired translational dysfunction, by affecting RNA processing [133], tRNA stability [134], tRNA aminoacylation [132], post-transcriptional modifications [135], and/or interaction with translation elongation factor EF Tu [111]. Some pathogenic tRNA mutations only cause respiratory chain dysfunction if present at a level above 95% [136,137]. One of the most common causes of mitochondrial encephalopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) is the A3243G point mutation within the tRNA\(^{L1}\) gene. This mutation alters the structure of the wobble base of tRNA\(^{L1}\) and causes specific translational errors by affecting anticodon modification. A second major site of point mutations is in the tRNA\(^{K}\) gene. For example, the A8344G point mutation completely abolishes aminoacylation and specific anticodon-codon interactions, thereby causing translational defect that leads to myoclonus epilepsy associated with ragged-red fibers syndrome (MERRF) [111,132]. It is unclear how tRNA mutations that all have the effect to impair mitochondrial translation can cause so different mitochondrial disease phenotypes.

2. Mitochondrial diseases caused by mutations in nuclear-encoded proteins

Mutations in nuclear genes encoding mitochondrial proteins cause a variety of mitochondrial diseases with mendelian inheritance. Regulation of mtDNA replication and/or mtDNA maintenance is affected in many of these disorders [138]. Furthermore, mutations in genes encoding mitochondrial proteins necessary for regulation of mitochondrial translation are also common causes of human mitochondrial disease, e.g. genes encoding the translation elongation factors, e.g. mtEF Tu, mtEF Ts, and mtEF G1, [139] or ribosomal subunits, e.g. MRPS16 and MRPS22 [140,141]. In addition, mutations affecting amino-acyl tRNA synthetases, rRNA and tRNA modifying enzymes and ribosomal assembly factors have been identified in patients with mitochondrial disease [111,123,141-146]. There are also examples of nuclear mutations that affect genes controlling mRNA [147]. A well
known examples is the French-Canadian Leigh syndrome caused by mutations in the \textit{Lrp3} gene [148], and the autosomal-recessive spastic ataxia present in Ohio Amish families, which is associated with defective mRNA maturation, caused by mutation in a highly conserved residue of mtPAP [149]. However, RNA metabolism is still poorly understood and new animal model systems are needed to better understand the underlying molecular mechanisms and to identify new proteins involved in these processes.
2. SPECIFIC AIMS

Mitochondria are key organelles of eukaryotic cells and their main function is to provide the cell with the energy currency ATP produced by the OXPHOS system. Importantly, mitochondria contain their own genome and tight regulation of mitochondrial gene expression is essential to match cellular energy demands. Mitochondrial gene expression is regulated on different levels, including regulation of mitochondrial transcription, RNA maturation and translation. Misregulation of mitochondrial gene expression results in mitochondrial dysfunction and is the cause for several fatal human diseases. However, it is still largely unknown what are the molecular mechanisms that regulate mitochondrial gene expression, which factors are involved and whether these factors are evolutionarily conserved.

The specific aims of my study were:

**Paper I.** To establish the fruit fly *Drosophila melanogaster* as a model system to study mitochondrial gene expression. In particular, to analyze the *in vivo* function of the Bicoid stability factor (BSF), an orthologue of the mammalian leucine-rich pentatricopeptide protein (LRPPRC), in the regulation of mitochondrial gene expression and physiology in Drosophila.

**Paper II.** To analyze the *in vivo* function of the LRPPRC protein in the control of mitochondrial gene expression at the post-transcriptional level in mice and to identify interaction partners of LRPPRC.

**Paper III.** To analyze the *in vivo* function of the mitochondrial transcription termination factor 3 (MTERF3) in fruit flies and mice in order to identify novel evolutionarily conserved pathways involved in the control of mitochondrial gene expression.
3. RESULTS AND DISCUSSION

**Paper I: The Bicoid Stability Factor Controls Polyadenylation and Expression of Specific Mitochondrial mRNAs in *Drosophila melanogaster***

In this work, we identified the Bicoid stability factor (BSF) as a new factor involved in the regulation of mitochondrial gene expression in the fruit fly *Drosophila melanogaster*. We showed that BSF is an essential nucleus-encoded protein that regulates mitochondrial gene expression post-transcriptionally by controlling the stability, polyadenylation and translation of mitochondrial mRNAs. BSF was originally identified as a protein that controls the localization and stability of the cytoplasmic bicoid mRNA by binding to its 3’prime untranslated region [110]. The Bicoid protein functions as an anterior morphogen in the early fly embryo but has no function later in development or adulthood. In addition to its role as a regulator of bicoid function, BSF has also been suggested to regulate the expression of early zygotic genes during *Drosophila* embryogenesis by binding to a short consensus sequence in the 5’ prime untranslated region of these mRNAs [150].

By phylogenetic analyses we identified BSF as a homologue of the mammalian LRPPRC protein, a member of the family of pentatricopeptide proteins. As the mammalian LRPPRC protein is localized mainly in the mitochondrial matrix [109], we examined the subcellular localization of BSF by colocalization studies using a BSF-FLAG-GFP protein in Schneider and HeLa cells and subcellular fractionation experiments. In contrast to previous reports that suggest that BSF is localized to the cytoplasm or the nucleus [110,150], we showed that BSF is predominantly localized within mitochondria, suggesting that BSF may be involved in regulation of mitochondrial function.

To analyze the *in vivo* function of BSF in flies, we used RNAi-mediated knockdown. Ubiquitous RNAi expression resulted in up to 80% downregulation of the *bsf* transcript causing a delay in development, reduced pupae-to-adult hatching rates, reduced fecundity, and premature lethality, demonstrating that BSF function is essential for fly survival. Furthermore, adult BSF knockdown flies exhibited reduced climbing ability due to muscle weakness, which might be caused by mitochondrial dysfunction.
In order to test more directly for the function of BSF in mitochondria, we analyzed activity of the respiratory chain in BSF knockdown flies. We observed that BSF downregulation resulted in severe respiratory chain dysfunction. The function of Complex I was most strongly affected, likely due to its high number of mitochondrial encoded proteins. BSF knockdown flies also had a reduced ATP/ADP ratio in comparison to controls and increased lactate/pyruvate ratios, probably a compensatory consequence of compromised respiratory chain function in order to maintain redox homeostasis.

To identify the cause of the severe RC dysfunction, we performed a detailed study on steady-state levels of mitochondrial-encoded transcripts by northern blot and quantitative real time PCR. Lack of BSF caused reduction in the steady-state levels of all analyzed mitochondrial mRNAs at the larval and adult stages, except for ND6 that was reduced only at the adult stage. In contrast to mRNA levels, steady-state levels of 12S rRNA were significantly increased, while 16S rRNA levels were slightly decreased. Steady-state levels of all tested tRNAs were increased in BSF knockdown larvae and adult flies, suggesting that the decreased steady-state levels of mRNAs are not a consequence of a general reduction in mitochondrial transcription. In summary, these results suggest that BSF regulates mitochondrial gene expression posttranscriptionally, most likely by controlling the stability of mature mRNAs.

To strengthen this finding, we examined the profiles of de novo synthesized mRNAs by an in organello transcription assay. We observed a massive increase in de novo transcription suggesting that upon lack of BSF mature mitochondrial mRNAs undergo massive degradation. Surprisingly, reduced steady-state levels of mitochondrial mRNAs did not result in a reduced mitochondrial translation rate, but instead we observed that in the absence of BSF de novo translation profiles had unexpected aberrant patterns. For instance, we observed a selective increase in the de novo synthesis of Complex I (ND1-ND6) and Complex IV (COX2) subunits, while de novo synthesis of Complex V (ATP6) and Complex III (CYTB) subunits was not changed. Interestingly, in the knockdown flies we identified a translational product running at a molecular weight slightly higher than ND1. This product and the COX2 protein were subjected to an increased degradation soon after synthesis suggesting that they failed to assemble into the mature respiratory chain complex. Consistently, steady-state levels of the mature respiratory chain complexes were reduced and in-gel Blue-Native Polyacrylamide gels (BN-PAGE) experiments showed significant reduction in in-gel activity of Complex I and Complex IV in BSF knockdown flies. To link the reduced
steady-state levels of mitochondrial transcripts to the observed aberrant translation patterns, we analyzed the nature of mature mitochondrial mRNAs by sequencing their 5’ and 3’ ends. In BSF knockdown flies, all mRNAs, except for ND6, lacked a polyA tail at their 3’ end. In addition, we observed the presence of polycistronic mRNAs that retained tRNAs at their 3’ end (ND3 plus tRNAAs and COX2 plus tRNAAs) and COX3 that retained ATP8/6 at its 5’ end. Sequencing of these polycistronic molecules revealed the presence of shorter polyA tails, suggesting that polyadenylation can occur at any 3’ end and is not specific for mature transcripts. The presence of shorter polyA tails at the 3’ end of polycistronic transcripts also suggested that the polyadenylation defects observed in BSF knockdown larvae, are caused by the absence of BSF and are not an indirect consequence of defective translation. In summary, we show that BSF is a novel essential factor that regulates mitochondrial gene expression in fruit flies by controlling polyadenylation of specific mitochondrial mRNAs and by coordinating mitochondrial translation. The results obtained using Drosophila as a model organism, prompted us to ask whether the BSF homologue LRPPRC has a similar function in controlling polyadenylation and translation in mammalian mitochondria.

**Paper II. LRPPRC is Necessary for Polyadenylation and Coordination of Translation of Mitochondrial mRNAs**

In this work we show that the mammalian LRPPRC protein forms an RNA-dependent complex with the SLIRP protein and that this complex is important to maintain the stability of mitochondrial transcripts, to facilitate mRNA-polyadenylation and to coordinate translation of mitochondrial mRNAs. In order to address the *in vivo* function of LRPPRC in mammals we generated a conditional LRPPRC knockout allele. Full-body LRPPRC knockout mice died during development at embryonic day 8.5. In contrast, mice that lacked LRPPRC specifically in heart and skeletal muscles survived to adulthood but died at the age of 16 weeks. Western blot analyses confirmed the absence of LRPPRC protein from 4 weeks of age and onwards in the mutant heart. Heart-specific LRPPRC knockout mice died from severe mitochondrial cardiomyopathy, accompanied by increased mitochondrial mass and abnormal mitochondria with enlarged cristae.
To address the biochemical consequences caused by the lack of LRPPRC, we assessed activity of the respiratory chain in the heart-specific LRPPRC knockout mice. We observed profound reduction in the activity of Complex IV. In contrast, activities of the other respiratory chain complexes were mostly unaffected, consistent with reports in human LSFC patients [151].

To verify the role of LRPPRC in the stability of mitochondrial transcripts, we performed northern blot analyses to assess the steady-state levels of mRNAs. We found a severe reduction of steady-state levels of all mRNAs, except ND6. In contrast, levels of rRNAs were increased and tRNAs were not changed suggesting that LRPPRC function is specifically required to stabilize mitochondrial mRNAs. In agreement, pulse-chase in organello transcription labeling experiments confirmed that the stability of the newly synthesized mitochondrial transcripts was reduced in the absence of LRPPRC. To complement the lack-of-function studies we performed gain-of-function experiments to address whether LRPPRC is also sufficient to maintain stability of mitochondrial mRNA transcript. Overexpression of LRPPRC protein alone was sufficient to cause slightly increased levels of all mRNAs, except for ND6, while the levels of rRNAs remained unchanged. In conclusion, these results show that LRPPRC is a key-factor involved in the regulation of mitochondrial gene expression at the posttranscriptional level.

The reduced steady-state levels of mitochondrial transcripts in the LRPPRC mutants may affect synthesis of the 13 mitochondrial polypeptides. To test for this, we performed in organello translation assays and observed an aberrant translation pattern. Translation of ND1, ND2 and ND5 was significantly increased, while translation of ND3, ND6, COX1 and ATP6 was almost completely abolished in LRPPRC mutant animals. In order to analyze whether post-transcriptional changes of mitochondrial mRNAs could account for this aberrant translation pattern, we sequenced their 5’ and 3’ ends. All mRNAs, except for ND6, had a shorter polyA tail at their 3’ termini, while 5’ termini remained unchanged. We also checked for polyadenylation status and found no change in the length of polyA tails at the 3’ termini of both rRNAs. These results suggest that LRPPRC is specifically involved in the extension of polyA tails of oligoadenylated mature mRNAs and does not affect rRNAs.

LRPPRC interacts with the SLIRP protein to regulate posttranscriptional gene expression in mitochondria [98]. Interestingly, lack of LRPPRC in the tissue-specific knock out mice was accompanied by the absence of the SLIRP protein, despite normal
levels of SLIRP transcript. This finding suggests that the stability of the SLIRP protein depends on the presence of LRPPRC. In order to further characterize the nature of the LRPPRC-SLIRP complex we performed size-exclusion chromatography. We found that the stability of the LRPPRC-SLIRP complex was diminished by treatment with high salt, EDTA or RNAase suggesting that formation of the LRPPRC-SLIRP complex is dependent on the presence of RNA. We next performed gradient sedimentation analyzes to investigate the interaction between the LRPPRC-SLIRP complex, mitochondrial transcripts and mitochondrial ribosomes. We found that the LRPPRC-SLIRP complex is essential to stabilize the free-pool of mitochondrial transcripts that are not bound to the ribosome. Additionally, LRPPRC coordinates translation of different mitochondrial transcripts, since in its absence mature mRNAs randomly enter the ribosomes causing the observed aberrant translation pattern. In summary, we propose a novel role of LRPPRC in the regulation of mtDNA expression at the post-transcriptional level. LRPPRC is necessary for mRNA stability and polyadenylation, and as such has an important role to maintain the pool of untranslated transcripts and to coordinate mitochondrial translation.

**Paper III. MTERF3 Regulates Mitochondrial Ribosome Biogenesis in Invertebrates and Mammals**

Termination of transcription is a key step in the regulation of mitochondrial gene expression. MTERF3, a member of the mitochondrial transcription termination family of proteins, acts as a repressor of mitochondrial transcription in mammals that negatively regulates mitochondrial gene expression through binding to the mtDNA promoter region [37]. However, the exact molecular mechanism how MTERF3 regulates mitochondrial gene expression remains unclear. MtDNA gene content is highly conserved among metazoa, therefore we hypothesized that the functions of key-factors involved in the regulation of mitochondrial gene expression might also be conserved among metazoa. In order to test this hypothesis and to further elucidate the molecular function of MTERF3 we decided to use the fruit fly as a model organism. In this work, we show that the function of MTERF3, as a negative regulator of mitochondrial transcription is conserved among metazoa. In addition, we identified a novel role of MTERF3 in ribosomal biogenesis. Furthermore, our results suggest that initiation of mitochondrial transcription and ribosomal biogenesis have to be tightly
coordinated in order to control mtDNA expression and regulation of oxidative phosphorylation capacity.

By phylogenetic analysis we identified a single gene ortholog of the mammalian Mterf3 gene in the genome of the fruit fly Drosophila, termed DmMterf3. Co-localization studies confirmed that DmMTERF3 is a mitochondrial protein. In order to address the in vivo function of MTERF3 in Drosophila, we generated DmMterf3 knockout flies by ends-out homologous recombination. Homozygous DmMterf3 knockout larvae were significantly smaller than controls and they eventually died at the third-instar stage, demonstrating that DmMTERF3 function is essential for fly survival. To further analyze the molecular function of DmMTERF3, we assessed the steady-state levels of mitochondrial transcripts in DmMterf3 knockout flies. Steady-state levels of almost all tested mitochondrial mRNAs were severely increased in mutant larvae, similar to what has been observed in MTERF3 knockout mice, suggesting that the function of MTERF3 in the control of mtDNA expression is evolutionarily conserved between flies and mice.

As DmMterf3 null mutants die relatively early during development, we used RNAi-mediated knockdown of DmMterf3 to analyze DmMterf3 function in later stages. Ubiquitous expression of a DmMterf3 RNAi construct resulted in down-regulation of DmMterf3 expression up to 90% in comparison to controls. Similar as in the mutant flies, RNAi knock down of DmMTERF3 caused reduction in body size and lethality at the pre-pupa stage. Furthermore, knockdown of DmMterf3 resulted in respiratory chain dysfunction, whereas the activities of Complex I and Complex IV were the most affected. Next we assessed the steady-state levels of assembled mitochondrial complexes by BN-PAGE. Levels of assembled Complex I and IV were severely reduced in DmMterf3 knockdown larvae in comparison to controls. To ensure specificity of the DmMterf3 knockdown phenotype, we performed a genomic rescue experiment. Therefore, we co-expressed the Mterf3 gene from Drosophila pseudobscura, a close relative of Drosophila melanogaster, together with the DmMterf3 RNAi construct. The DmTerf3 knockdown phenotype was completely rescued indicating that the observed phenotypes are specifically caused by the downregulation of DmMTERF3 expression.

Similarly to the DmMterf3 knockout flies, forced expression of DmMterf3 RNAi also caused an increase in steady-state levels of almost all tested mRNAs and all tested tRNAs. Interestingly, only steady-state levels of 16S rRNA were severely down-
regulated. The increased steady-state levels of tRNAs led us to perform in organello transcription assay, as we previously observed that increased tRNAs levels correlate well with increased de novo transcription in flies [152] and mice [7,153]. We performed in organello transcription assay at different time points during development. Significant increase in de novo transcription was only observed at later time points, at 4 and 5 days after egglaying. Interestingly, in organello translation assays in contrast showed that de novo translation was already reduced 3 days after egglaying, at a time where we did not observe any changes in de novo transcription or mRNA steady-state levels. These results suggest that the primary cause for the respiratory chain dysfunction in DmMterf3 mutants may be a defect in mitochondrial translation and that changes in mitochondrial transcription are a secondary consequence as a result of a compensatory mechanism. Consistently, decreased levels of 16S rRNA in DmMterf3 mutant flies may also indicate a defect in translation. Downregulation of 16S rRNA was also observed in Mterf3 knockout mice [37]. Therefore, we performed in organello translation assays in Mterf3 knockout mice and confirmed that mitochondrial translation is impaired in the absence of MTERF3. To further strengthen this finding, we assessed the assembly of mitochondrial ribosomes by gradient sedimentation analyses of mitochondrial extracts from DmMterf3 knockdown flies and Mterf3 knockout mice. In both flies and mice, lack of MTERF3 caused impaired assembly of the large ribosomal subunit resulting in an impaired ribosomal biogenesis. To strengthen this finding, we performed RNA-immunoprecipitation experiments to analyze the RNA-binding specificity of MTERF3. We observed that MTERF3 binds 16S rRNA with high specificity, suggesting that MTERF3 contributes to the stabilization and/or modification of 16S rRNA and thereby controls the assembly of the large 39S ribosomal subunit. In summary, we show that MTERF3 promotes mitochondrial translation by regulating ribosomal biogenesis and that this process is tightly linked to the repression of mitochondrial transcription in order to optimize mtDNA expression. Since this dual function of MTERF3 is conserved between flies and mice, we proposed that MTERF3 is a key factor in the control of mtDNA expression in metazoans.
4. CONCLUDING REMARKS

Mitochondrial gene expression is a complex process that is tightly regulated. However, despite its importance very little is known about the underlying mechanisms that fine-tune mitochondrial gene expression in response to different metabolic demands and which proteins are involved in the control of mitochondrial gene expression.

In this thesis I used in vivo studies in flies and mice to study the function of two important proteins involved in mitochondrial gene expression, LRPPRC and MTERF3. We show that the fly LRPPRC orthologue termed BSF is an essential mitochondrial protein that regulates polyA tail length of mature mitochondrial mRNAs. Furthermore, BSF deficiency causes enrichment of unprocessed polycistronic transcripts and aberrant translation patterns. These findings are in contrast to previous reports that had suggested that BSF is a cytoplasmic protein involved in the regulation of the Bicoid morphogen during fly embryogenesis. Similar to BSF knockdown in flies, heart-specific ablation of mouse LRPPRC led to severe respiratory chain dysfunction, due to reduced stability of mitochondrial mRNAs, shortened polyA tails and misregulated translation, causing premature death. Furthermore, we demonstrated that LRPPRC and SLIRP form an RNA-dependent complex, which is necessary to maintain and stabilize the free pool of non-translated transcripts that are not bound to the ribosome, thereby preventing non-translated transcripts to randomly bind to the assembled ribosome. Altogether our findings suggest that LRPPRC is an evolutionarily conserved mitochondrial protein, which plays an essential role in regulating mitochondrial gene expression at the post-transcriptional level by controlling polyadenylation and coordination of translation of mitochondrial mRNAs. Noteworthy, a similar function has recently been described for the human LRPPRC protein, suggesting that the findings in flies and mice are directly relevant to understanding LRPPRC disorders in humans.

Transcription termination has been proposed to be an important checkpoint in the regulation of mtDNA gene expression, and MTERF3 has been suggested to act as a repressor of mitochondrial transcription in mammals. We show that MTERF3 negatively regulates mitochondrial transcription in flies. Additionally, we provide evidence that MTERF3 is a part of a molecular checkpoint, which coordinates the rate of transcription with the maturation of the ribosome. We demonstrated that ribosomal biogenesis is impaired and mitochondrial transcription is upregulated in the
absence of MTERF3 in flies and mammals. Thus, our findings point to the existence of a novel evolutionary conserved mechanism by which MTERF3 controls ribosomal biogenesis and coordinates mitochondrial transcription and translation. We further show that MTERF3 specifically binds to 16S rRNA, and by contributing to its stability and/or modification has a critical role in the biogenesis of the large ribosomal subunit (39S). In summary, we show that two essential pathways that regulate mitochondrial gene expression at the transcriptional and post-transcriptional levels are highly conserved between flies and mammals.

Still, many questions regarding the molecular functions of the mitochondrial transcription termination family and LRPPRC proteins remain to be answered. How are these factors regulated to optimize mtDNA expression and which additional factors are necessary to ensure coordination of mitochondrial gene expression. As a part of this thesis, we have developed a number of different methodologies, which enrich the toolbox of techniques to study mitochondrial transcription and translation in fruit flies. Furthermore, we generated novel fly and mouse knockout lines, which will be valuable tools for future studies concerning the molecular functions of LRPPRC and MTERF3 in flies and mammals. In addition, since the fruit fly is an excellent model system to carry out genetic screens, the new genetic tools can be used in genetic screens to identify novel interaction partners of LRPPRC and MTERF3. The evolutionary conserved function of both proteins suggests that this approach will likely result in novel insights about the control of mitochondrial gene expression and will eventually contribute to a better understanding of the pathogenesis of human mitochondrial diseases caused by misregulation of mitochondrial gene expression.
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