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PRINCIPAL INVESTIGATOR: Flavia Fontanesi

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14. ABSTRACT Mitochondria function requires the expression of structural subunits of the oxidative phosphorylation system encoded by the mitochondrial genome. Thus, mtDNA expression is essential for cellular energy production and its alteration leads to major energetic deficits in cells and tissues, which result in severe human diseases, namely mitochondrial disorders, such as encephalomyopathies and cardiomyopathies. Mitochondrial gene expression is predominantly regulated at the post-transcriptional level by the activity of nuclear-encoded mitochondrial RNA-binding proteins (mt-RBPs). A systematic study of mammalian mt-RBPs is missing and key questions regarding the mitochondrial gene expression process and the factors involved remain unanswered. The goal of our project is the development of a novel methodology to identify mt-mRNA-specific RBPs through the combination of single mt-mRNA immunoprecipitation and mass-spectrometry-based protein identification approaches. For this purpose, we aim to engineer mitochondrion-targeted RNA-binding domain (PUM1-HD) acting as selective mt-mRNA “handles” that can be used to isolate and characterize ribonucleoprotein complexes in human mitochondria. With the support of this DoD discovery award, we have generated a collection of 18 PUM1-HDs designed to target specific mt-mRNAs, purify the recombinant proteins and demonstrated the specific RNA-binding capacity in vitro of six of them. Moreover, we have subcloned these domains in a mammalian inducible expression system and obtained one HeLa cell lines expressing in an inducible manner the collection of PUM1-HDs targeted to mitochondria.					
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INTRODUCTION

Mitochondrial diseases are clinically and genetically heterogeneous multisystemic disorders mainly affecting high energy-demanding organs as heart, brain and skeletal muscles. They are associated with alterations in the mitochondrial oxidative phosphorylation (OXPHOS) system, leading to major cellular energetic deficits. Biogenesis of the OXPHOS system requires the expression of the 13 structural OXPHOS subunits encoded by the mitochondrial genome (mtDNA). Human mitochondria have evolved a small and compact genome from which a remarkably complex transcriptome is generated. Its physiological relevance stems from the fact that mitochondrial transcriptome composition and regulation play fundamental roles in modulating mitochondrial gene expression in response to the cell's energetic requirements. The mtDNA expression system has several features that distinguish it from its prokaryotic and eukaryotic cytoplasmic counterparts. In mitochondria, gene expression is predominantly regulated at the posttranscriptional level by the activity of nuclear-encoded mitochondrial RNA-binding proteins (mt-RBPs). Both the mt-RBP repertoire and their activities govern basal mRNA life from synthesis to expression and degradation. Owing to their pivotal role in mitochondrial mRNA biology, mutations in several mt-RBPs have been associated with severe forms of encephalo- and cardiomyopathy. However, despite the recent remarkable progress in our understanding of mammalian mitochondrial gene expression, key unanswered questions remain regarding the molecular mechanisms underlying this process, its regulation and the factors involved. For example, the available information concerning how mt-RBPs control mt-mRNA stability, translation and localization is very limited. This knowledge is necessary to deepen our understanding of the pathophysiology of primary mitochondrial disorders. Moreover, the major advances in the field remain restricted to the stepwise characterization of single mt-RBPs and a systematic study of mammalian mt-RBPs is missing, mainly due to the impossibility of editing human mtDNA at will. The goal of our project is the development of a novel methodology to identify mt-mRNA specific RBPs through the combination of single mt-mRNA immunoprecipitation and mass spectrometry-based protein identification approaches. Application of this methodology will allow us to systematically and comprehensively characterize the mt-mRNA protein interactome.

KEYWORDS

Mitochondria, mt-mRNAs, mt-RNA-binding proteins (mt-RBPs), PUM1-HD, posttranscriptional regulation, mitochondrial gene expression, CLIP assay, mitochondrial disorders.

ACCOMPLISHMENTS

What were the major goals of the project?

Our long-term goal is to determine the repertoire of proteins that directly bind to mitochondrial mRNAs. The main goal of this project is to develop a novel approach to identify mitochondrial RNA-specific binding proteins. The approach is based on the use of specific RNA-binding modules (PUM1-HD) that can be engineered to target specific mRNA of interest and can be used as “handles” to isolate ribonucleoprotein complexes in human mitochondria. The main goal can be break down in the following aims:

Aim 1: Establishing in vivo cellular models for specific mitochondrial mRNA isolation.

- 1.1 Generating a collection of PUM1-HDs designed to target specific mitochondrial mRNAs. Completed.
- 1.2 Test the RNA-binding properties of the engineered PUM1-HDs in vitro by EMSA analysis. Completed.
- 1.3 Clone the selected PUM1-HDs that successfully recognized the target mRNA for in vivo expression. Completed.
- 1.4 Establish HeLa cell lines expressing in an inducible manner the engineered PUM1-HDs targeted to mitochondria. Completed.
- 1.5 Test engineered PUM1-HDs induction of expression and mitochondrial localization. Completed.
- 1.6 Test engineered PUM1-HDs efficiency of immunoprecipitation. On going (20%).

Aim 2: Development of a PUMILIO-Based Mitochondrial PAR-CLIP/CLIP (PBM-CLIP) assay for the identification of mRNA-specific mt-RBPs. Ongoing. Even if no longer supported by this DoD award, we are currently working on optimizing the cross-linking and PUM1-HD-mediated immunoprecipitation of mitochondrial mRNAs and RNA-binding proteins. We believe that the more challenging portion of developing this new methodology consisted in the engineering of specific PUM1-HDs able to recognize individual mitochondrial

mRNAs, which we have successfully achieved. We are confident that we will be able to complete this remaining portion of the project in a short amount of time.

What was accomplished under these goals?

To identify proteins that directly bind to mt-mRNAs, our first goal is to construct mitochondrion-targeted RNA-binding polypeptides acting as selective mt-mRNA “handles” that can be used to isolate and characterize ribonucleoprotein complexes in human mitochondria. For the development of these tools, we have chosen to use as a scaffold to engineer sequence-specific RNA-binding modules, the human PUMILIO1 protein RNA-binding domain or PUM1-homology domain (PUM1-HD), which RNA recognition code has been established. We have identified by *in silico* analysis unique degenerated PUM1-HD binding sequences present in human mt-mRNAs and designed a collection of PUM1-HD repeat amino acid substitutions aimed to selectively modified PUM1-HD RNA sequence specificity, as described in our proposal preliminary data. The specific missense mutations were introduced in PUM1-HD DNA coding sequence, corresponding to amino acids 828-1176 of the full-length protein, by site-directed mutagenesis. In total we generated 27 modified PUM1-HDs, at least one for each mt-mRNA, including the two bicistronic mRNAs. These 27 mutant PUM1-HDs include the 13 originally selected and proposed in our application, 10 targeting alternative mt-mRNA degenerated PUM1-HD sequences and 4 generated by PyMol *in silico* protein modelling, as follow. It is known that three amino acids are required for the recognition and binding of a single nucleotide in the target RNA. One of these amino acids intercalates between nucleotide and is referred to as stacking residue. In a subset of modified PUM1-HDs, we identified one position in which the bulk of the staking residue does not properly occupy the space between adjacent nucleotides, which could compromise the PUM1-HD-RNA binding strength, and that we have modified accordingly.

The engineered PUM1-HD collection was generated using the plasmid for heterologous protein expression pTYB3 (Addgene), carrying the PUM1-HD polypeptide fused to an intein and chitin-binding domain. The intein fragment is a modified yeast protein-splicing element, which can undergo self-cleavage upon induction with a reducing agent. We expressed the wild-type and mutant PUM1-HD recombinant proteins in the *E. coli* BL21(DE3) strain and tested protein solubility by Western-blot detection of the fraction of PUM1-HD protein presents in the supernatant upon cell lysis and centrifugation. Overall, 21 engineered PUM1-HDs were soluble, and we could proceed to purify the protein by absorption onto a chitin-agarose column. Protein elution by intein cleavage was induced by overnight incubation of the column with 50 mM dithiothreitol (DTT) in a tightly closed tube, to obtain the tag-free PUM1-HD polypeptides. The efficiency of protein purification was evaluated by SDS-PAGE and Coomassie blue staining (the WT-PUM1-HD is reported as example in [Figure 1](#)). Overall, we were able to recover consistently about 60-80% of the PUM1-HD protein input and obtain 10-15 mg of each high-purity recombinant PUM1-HD proteins.

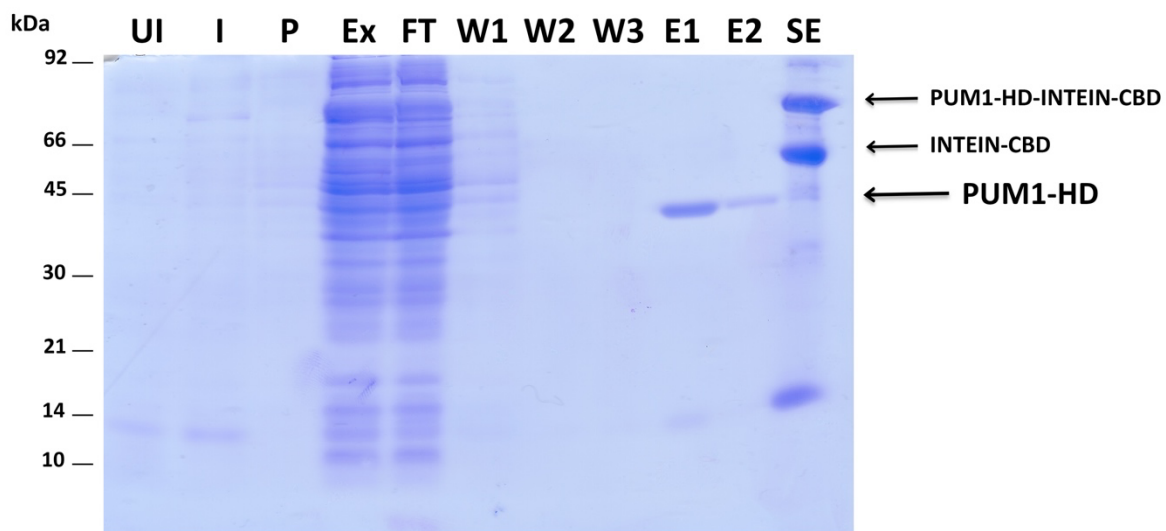


Figure 1: PUM1-HD recombinant protein purification. The PUM1-HD proteins wild-type (WT) is shown as example. Similar results were obtained for 21 engineered PUM1-HDs. Proteins were purified from the bacterial soluble extract (Ex) by absorption onto a chitin-agarose column. Material that did not bound to the column was collected as flow through (FT). Column was washed multiple times to reduced unspecific protein binding (W1-3) and eluted two times (E1-2) with DTT-containing buffer. Last, column was eluted with SDS-containing buffer (SE), which denatured and removed all remaining proteins from the column, in order to estimate the efficiency and intein cleavage.

The purified proteins were used to test the ability of engineered PUM1-HDs to specifically bind their target RNA sequence *in vitro* by electrophoretic mobility shift analysis (EMSA). Briefly, 20 bp biotin-labelled RNA oligonucleotides (10-15 pM), comprising the PUM1 recognition sequence or its degenerated variants were incubated with increasing concentrations (0.1 – 30 μ M) of recombinant protein for 1 hour at room temperature. RNA-protein complexes were resolved electrophoretically in a 6% non-denaturing polyacrylamide gel, transfer to nitrocellulose membrane and the biotinylated RNA was detected by chemiluminescence. The fraction of protein bound to the RNA was estimated from the proportion of RNA shifting to a higher mass. We obtained the following 8 engineered PUM1-HDs that effectively bind their targeted RNA: PUM1-HD-ND6-1, -ND6-2, -ND5-1, -ND5-2, -ND4, -ND3, -ND1, and -COX3 (Figure 2).

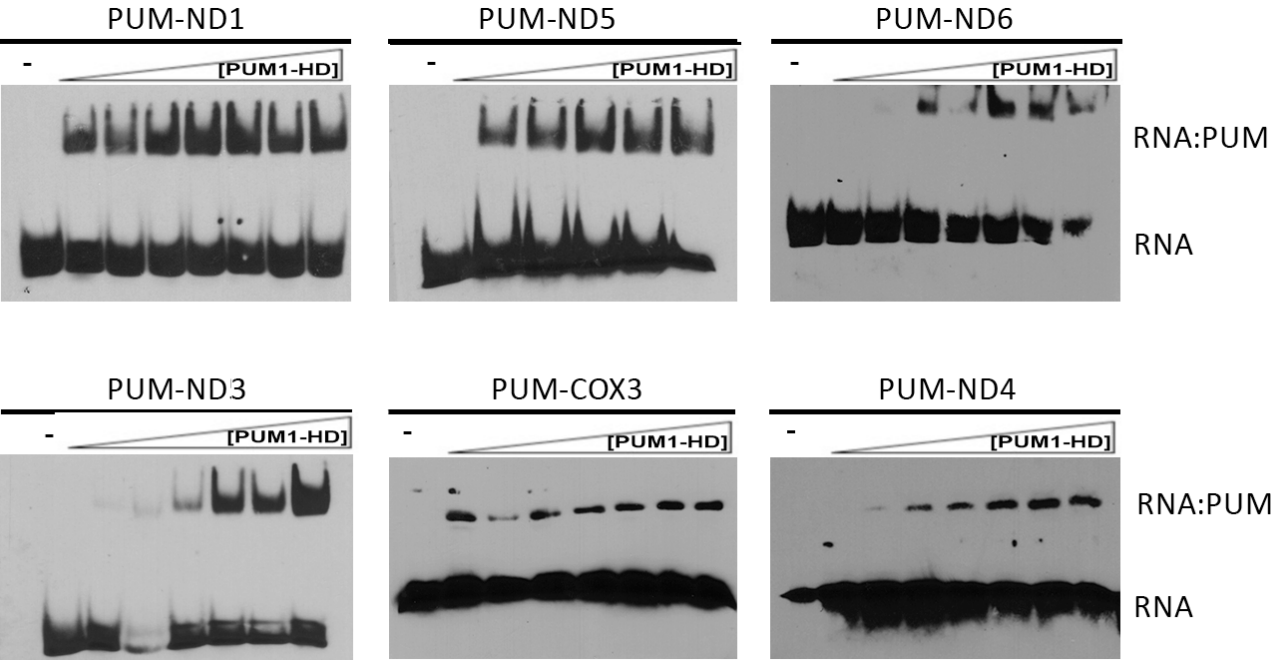
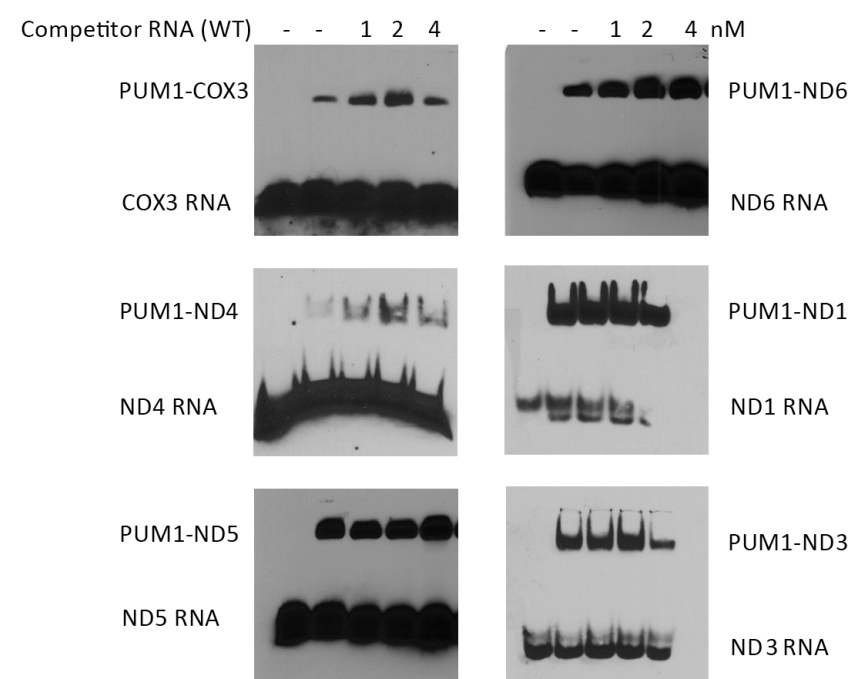


Figure 2: Electrophoretic Mobility Shift Assay (EMSA) analysis. Recombinant PUM1-HD proteins engineered to target the indicated mt-mRNAs, were incubated with the corresponding target RNA oligo (only one of the two successful PUM1-HDs targeting *ND5* and *ND6* mRNAs are shown). To detect possible RNA-protein interactions, the RNA-protein complexes were resolved in a 6%-acrylamide gel, transferred to membrane and detected by chemiluminescence. The image is representative of three independent biological replicas.



All observed PUM1-HD-RNA bindings were specific since they could not be outcompeted by equimolar amounts of WT target RNA (Figure 3). Moreover, no RNA-binding was detected between the PUM1-HDs and RNAs other than the targeted one (not shown), suggesting a high degree of RNA-binding specificity.

Figure 3: RNA-binding competition assay. Recombinant PUM1-HD proteins engineered to target the indicated mt-mRNAs, were incubated with the corresponding target RNA oligo and increasing concentrations of WT competitor unbiotinylated RNA (only one of the two successful PUM1-HDs targeting *ND5* and *ND6* mRNAs are shown). The image is representative of three independent biological replicas.

For the generation of the *in vivo* cellular models, we have elected to express the engineered PUM1-HD proteins in HeLa cells in an inducible manner. For this purpose, we selected the mammalian inducible pERV3/pEGSH expression system (Agilent Technologies), which allows for the in-frame fusion of the protein C-terminus with a FLAG tag. Additionally, we modified the pEGSH plasmid (now pEGSH-MTS) by incorporating the mitochondrial targeting sequence (MTS) Su9/COX8, so that we can easily sub-clone our engineered PUM1-HD proteins in frame with both the MTS and the FLAG tag. We generated pEGSH-MTS constructs expressing PUM1-HD proteins targeting ND6 (2 PUM1-HDs), ND5 (2 PUM1-HDs), ND4, ND3, ND1, and COX3 mt-mRNAs. As control, we also constructed a pEGSH-MTS plasmid expressing WT PUM1-HD. The pERV3/pEGSH system consents the expression of target genes in response to the insect hormone ecdysone or its analog ponasterone A (ponA), in mammalian cells expressing the *D. melanogaster* ecdysone receptor. We first generated HeLa CCL-2 cells constitutively expressing the ecdysone receptor. We then transfected these cells with the mitochondrion-targeted PUM1-HD-FLAG constructs and select stable transformants with hygromycin. We tested PUM1-HD protein expression and mitochondrial localization upon 24 h induction with 2 μ M ponA, using the HeLa mt-PUM1-HD-ND5-FLAG cell line as tester and determine that the engineered PUM1-HD protein is expressed and properly targeted to mitochondria (Figure 4).



Figure 3. Induction and mitochondrial localization of the mt-PUM1-HD-ND5-FLAG protein. Western blot analysis of mitochondria isolated from HeLa cells carrying the pEGSH-MTS-PUM1-HD-ND5-FLAG construct either uninduced (-) or induced (+) with 2 μ M ponA for 24 hr. Act1 was used as loading control.

Overall Key Research Accomplishments

- Generation of a collection of 27 engineered PUM1-HDs designed to target specific mitochondrial mRNAs.
- Analysis of protein solubility of the 27 engineered PUM1-HDs.
- Expression and purification of the 21 PUM1-HD soluble proteins for *in vitro* analysis.
- Determination of the mRNA binding capacity of the 21 soluble PUM1-HDs by EMSA analysis and identification of 8 engineered PUM1-HD binding ND6, ND5, ND4, ND3, ND1, and COX3 mt-mRNAs.
- Determination of mRNA binding specificity of the 8 engineered PUM1-HDs identified as RNA-binders by EMSA-based competition and selectivity assays.
- Generation of a pEGSH mammalian inducible expression vector carrying the mitochondrial targeting sequence (MTS) Su9/COX8 for rapid sub-cloning of our engineered PUM1-HD collection.
- Cloning of PUM1-HD-ND6-1, -ND6-2, -ND5-1, -ND5-2, -ND4, -ND3, -ND1, and -COX3 genes in the pEGSH-MTS vector.
- Generation of a HeLa cell line stably expressing the PonA receptor (HeLa-pERV3).
- Generation of a HeLa cell line stably expressing the PonA receptor and the engineered PUM1-HD-ND6-1, -ND6-2, -ND5-1, -ND5-2, -ND4, -ND3, -ND1, and -COX3 protein.
- Determination of PUM1-HD-ND5-1 expression and mitochondrial localization upon PonA induction.

What opportunities for training and professional development has the project provided?

Participation of PI and graduate student to the American Society for Biochemistry and Molecular Biology (ASBMB) Virtual Annual Meeting. April 26, 2021.

How were the results disseminated to communities of interest?

- Invited presentation: Invited speaker for the Mitochondrial Interest Group at ASBMB. American Society for Biochemistry and Molecular Biology (ASBMB) Virtual Annual Meeting. April 26, 2021. Talk title "Translational regulation of yeast mitoribosome biogenesis."
- Development of a novel course "Basis of mitochondrial medicine" for post-graduate education.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We successfully developed a collection of engineered RNA-binding protein domains able to selectively recognize individual mitochondrial mRNAs. These novel tools allow to overcome the limitations imposed by the impossibility to edit the mitochondrial genome and will allow to comprehensively identify mt-mRNA specific RBPs.. This novel methodology has the potential not only to assign new functions to known mitochondrial proteins but also to identify new mt-mRBPs and generate the first exhaustive atlas of the mitochondrial mRNA interactome. In consequence, we anticipate impacting our basic knowledge of mitochondrial function and biogenesis by gaining insight into the pathways that govern mRNA transactions in mitochondria. Moreover, from a biomedical perspective, the newly identified mt-RBPs would represent potential candidates when screening for disease-causing mutations in patients with mitochondrial disorders for which no variations have been detected in known disease-associated genes. Lastly, the novel PUM1-HD that we have constructed, targeted to mitochondria and engineered to selectively bind mt-mRNAs, will be made available to the scientific community. We and others will be able to use them to characterize mt-mRBPs in different cell types and in cells grown under different conditions. Moreover, we envision that the future integration of this mt-mRNA interactome-capture approach with the SILAC (Stable Isotope Labeling with Amino acids in Cell culture) methodology could be broadly applied to investigate quantitative changes in mitochondrial mRNA interactome composition in response to environmental cues relevant to disease status or pharmacological interventions.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS

Changes in approach and reasons for change

To determine the repertoire of proteins that directly bind to mt-mRNAs, our goal is to generate cell lines expressing mitochondrion-targeted RNA-binding polypeptides acting as selective mt-mRNA “handles” that can be used to isolate and characterize ribonucleoprotein complexes in human mitochondria. The more challenging aspect of our project is to successfully engineered PUM1-HD-derived sequence-specific RNA-binding modules. Since testing of RNA-binding properties directly in culture cells can be time and resources consuming, we decided to first test *in vitro* the ability of engineered PUM1-HDs to specifically bind their target RNA sequence by electrophoretic mobility shift analysis (EMSA). In this way, we pre-screened for PUM1-HD sequence specific RNA-binding capacity and only PUM1-HDs that efficiently bind to their target RNA sequences are selected for the generation of the *in vivo* cellular models. In case of *in vitro* lack of RNA binding, alternative mtPUM1-HDs were constructed. Indeed at least one alternative targeting sequence has been identified for each mt-mRNA. Overall, we have generated 27 engineered PUM1-HDs, of these 21 were expressed as soluble proteins and their RNA-binding properties could be tested *in vitro*. This effort resulted in 8 PUM1-HD proteins specifically binding their target mRNAs. The eight selected RNA-binding PUM1-HDs were then expressed in human cells in culture. We believe that the evaluation of the recombinant PUM1-HD RNA-binding properties *in vitro* has ultimately increase our overall project efficiency, which justify the inclusion of this additional step in the experimental plan.

Actual or anticipated problems or delays and actions or plans to resolve them

In term of delays, from June 2020 to May 2021 due to the COVID pandemic, the University imposed a restriction in the number of people working at any given time in lab spaces. This partially limited our access to the common cell culture room.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

PRODUCTS

Publications, conference papers, and presentations

Journal publications

- Seshadri S., Banarjee C., Barros M.H. and Fontanesi F. The translational activator Sov1 coordinates mitochondrial gene expression with mitoribosome biogenesis. *Nucleic Acid Research*. 48(12):6759-6774. (2020).

Books or other non-periodical, one-time publications

- Fontanesi F., Tigano M., Fu Y., Sfeir A. and Barrientos A. Human mitochondrial transcription and translation. In *The human mitochondrial genome. From basic biology to disease*. Elsevier (2020).

Other publications, conference papers and presentations

- * National presentation: Translational regulation of yeast mitoribosome biogenesis. Invited speaker for the Mitochondrial Interest Group at ASBMB. American Society for Biochemistry and Molecular Biology (ASBMB) Virtual Annual Meeting. Apr. 26, 2021.
- * International presentation: Coordination of mitochondrial gene expression with mitoribosome biogenesis. Invited speaker at the Gordon Research Conference in Mitochondria and Chloroplasts. Mount Snow (VT). Jul. 17-22, 2022. (The conference scheduled for the previous year, was postponed due to the COVID pandemic).
- Abstract: A novel approach to establish an atlas of human mitochondrial mRNA binding proteins. Gordon Research Conference in Mitochondria and Chloroplasts. Mount Snow (VT). Jul. 17-22, 2022. (The conference scheduled for the previous year, was postponed due to the COVID pandemic).
- Abstract: Mitochondrial mRNA structure as a driver of Leigh Syndrome. Gordon Research Conference in Mitochondria and Chloroplasts. Mount Snow (VT). Jul. 17-22, 2022. (The conference scheduled for the previous year, was postponed due to the COVID pandemic).

Website or other internet site

Nothing to report

Technologies

Nothing to report

Inventions, patent applications and licenses

Nothing to report

Other products

Educational aids/curricula

New course BMB616/MBS616/BMB726 - Basis of mitochondrial medicine". The course was offered to students in the Biomedical Sciences and, Biochemistry and Molecular Biology master programs, Miller School of Medicine, University of Miami, for the academic year 2020/2021. Additionally, the course was also offered to graduate students in the Biochemistry and Molecular Biology graduate program for the academic year 2021/2022.

Although neither my teaching time effort nor didactic material are supported by this award, the development of this new course is strictly connected to my research program. The course aims to provide a core introduction to the major aspects of mitochondrial biology and to integrate basic science knowledge with our current understanding of the role that mitochondrial dysfunction plays in human diseases. The course allows me to disseminate the findings of my research work and, more importantly, to expose students early in their biomedical career to the mitochondria field.

Research material

Genes encoding the PUM1-HD engineered to bind specific mitochondrial mRNAs cloned in plasmids for either protein purification or expression in mammalian cells. The constructs will be made available to the scientific community through the Addgene repository.

Others

Thanks to the support of this DoD grant, the PI, Dr. Flavia Fontanesi has obtained the transfer to the tenure-earning track at the rank of Assistant Professor of Biochemistry and Molecular Biology.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Flavia Fontanesi, PI, no changes

Barbara Santos de Oliveira, graduate student, no changes

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

Not applicable

APPENDICES

- Notification of Dr. Flavia Fontanesi transfer to the tenure-earning track.
- Manuscript Seshadri S., Banarjee C., Barros M.H. and Fontanesi F. The translational activator Sov1 coordinates mitochondrial gene expression with mitoribosome biogenesis. *Nucleic Acid Research*. 48(12):6759-6774. (2020).
- Book chapter Fontanesi F., Tigano M., Fu Y., Sfeir A. and Barrientos A. Human mitochondrial transcription and translation. In *The human mitochondrial genome. From basic biology to disease*. Elsevier (2020).
- Syllabus of the course BMB616/726 "Basis of mitochondrial medicine"



May 1, 2020

Dr. Flavia Fontanesi
665 NE 25th Street, Apt #2203
Miami, FL 33137

Dear Dr. Fontanesi,

I am pleased to formally notify you that your request to transfer to the tenure-earning track at the rank of Assistant Professor of Biochemistry and Molecular Biology has been approved, effective June 1, 2020. This approval is a reflection of the value we place in your service to the University and our desire to have you continue your productive academic career as a member of our faculty.

As a tenure earning faculty member, you will be expected to make normal progress toward tenure. Your progress will be evaluated annually, with your in-depth reviews scheduled to take place in the spring of 2023 and again in the fall of 2025.

During the fifth year of this appointment, you will receive your second in-depth review and evaluation of your progress toward promotion and tenure. At the conclusion of this review, if you achieve the rank of Associate Professor, but do not achieve tenure, your appointment on the tenure track may be extended for an additional three years, through May 31, 2028. Your tenure review is scheduled for the fall of 2027, with the award of tenure due by no later than June 1, 2028.

If you require any clarification about the terms of your appointment or this transfer, please contact Preston Pugh in the Office of Faculty Affairs at (305) 243-6551, or via email at preston.pugh@miami.edu.

I wish you the very best for continued success, and for a safe and productive year. If I can be of assistance to you in the future, please let me know.

Sincerely,

A handwritten signature in blue ink, appearing to read 'D. J. Birnbach'.

David J. Birnbach, MD, MPH
Executive Vice Provost

DJB/ap

cc: Dean Henri Ford

The translational activator Sov1 coordinates mitochondrial gene expression with mitoribosome biogenesis

Suhas R. Seshadri¹, Chitra Banarjee¹, Mario H. Barros² and Flavia Fontanesi^{1,*}

¹Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, Miami, FL 33136, USA and ²Departamento de Microbiologia, Instituto de Ciencias Biomedicas, Universidade de Sao Paulo, Sao Paulo 05508-900, Brazil

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ABSTRACT

Mitoribosome biogenesis is an expensive metabolic process that is essential to maintain cellular respiratory capacity and requires the stoichiometric accumulation of rRNAs and proteins encoded in two distinct genomes. In yeast, the ribosomal protein Var1, alias uS3m, is mitochondrion-encoded. uS3m is a protein universally present in all ribosomes, where it forms part of the small subunit (SSU) mRNA entry channel and plays a pivotal role in ribosome loading onto the mRNA. However, despite its critical functional role, very little is known concerning *VAR1* gene expression. Here, we demonstrate that the protein Sov1 is an *in bona fide* *VAR1* mRNA translational activator and additionally interacts with newly synthesized Var1 polypeptide. Moreover, we show that Sov1 assists the late steps of mtSSU biogenesis involving the incorporation of Var1, an event necessary for uS14 and mS46 assembly. Notably, we have uncovered a translational regulatory mechanism by which Sov1 fine-tunes Var1 synthesis with its assembly into the mitoribosome.

INTRODUCTION

Ribosome biogenesis is an energetically expensive process that needs to be regulated to facilitate the stoichiometric accumulation of ribosomal RNAs (rRNAs) and proteins, and to respond to the nutritional status of the cell (1). In *Escherichia coli*, among the several mechanisms in place, synthesis of most ribosomal proteins is regulated by negative feedback loops in which specific ribosomal proteins, when in excess, act as translational repressors by binding to their own mRNAs (2). Translational autoregulation control also operates in mitochondria from the yeast *Saccharomyces cerevisiae* to coordinate the synthesis of oxidative phosphorylation (OXPHOS) complex subunits with their

assembly into functional enzymes. In yeast mitochondria, however, regulation involves a ternary element known as translational activator that binds to both a specific mRNA to promote translation and to the newly synthesized protein to stabilize it and chaperone its incorporation into the specific OXPHOS complex (3). The yeast mitochondrial genome (mtDNA) encodes for eight polypeptides, of which seven are catalytic subunits of the OXPHOS system complexes and one, Var1 (Variant protein 1), is a structural component of the mitochondrial ribosome small subunit (mtSSU) (4,5). Whether Var1 synthesis and mitoribosome assembly are translationally regulated remains to be demonstrated.

Var1, recently renamed as uS3m according to the standard nomenclature for ribosomal proteins (6), is a positively charged soluble protein universally present in all ribosomes (bacterial, cytosolic and mitochondrial), where it localizes in the mtSSU head and forms part of the mRNA entry channel (7–10). Yeast Var1 owes its name to the high degree of variability it has amongst different strains (11), which results from insertions of GC clusters and changes in the length of AT-rich stretches, mainly coding for asparagine residues (12,13). The structure and location of the *VAR1* gene are also heterogeneous across eukaryotes (14). Whereas in metazoan Var1/uS3m is always encoded in the nuclear genome, it is expressed from their mitochondrial genome in several plants and fungi. The retention of the *VAR1* gene in the mtDNA is intriguing, considering that Var1 is a soluble protein, and therefore, hydrophobicity, which could prevent mitochondrial import, should not represent a barrier to nuclear transfer of the *VAR1* gene. Indeed, it has been shown that a functional Var1 protein can be allotopically expressed from a recoded gene relocated to the nucleus (15). Alternatively, *VAR1* retention in the mtDNA could be linked to the control of gene expression by the mitochondrial functional state, as previously proposed for mitochondrion-encoded OXPHOS subunits (16).

In mitochondria, gene expression is controlled mostly at the post-transcriptional level. Yeast mitochondrial (mt)

*To whom correspondence should be addressed. Tel: +1 305 243 7215; Fax: +1 305 243 7404; Email: ffontanesi@med.miami.edu

mRNAs contain long 5'-untranslated regions (5'-UTR), where translational activators bind to promote translational initiation. Numerous translational activators have been identified in yeast mitochondria, and their role in the biogenesis of the mitochondrion-encoded OXPHOS subunits has been extensively characterized (17). Their functions comprise multiple levels of regulation, including the coordinated synthesis of subunits from the same OXPHOS complex (18), and the assembly dependent translational control of gene expression (3). However, despite the wealth of information regarding the factors involved in the translation of mitochondrion-encoded OXPHOS subunits, very little is known concerning *VAR1* gene expression.

The open reading frame YMR066W was first identified in a random mutagenesis-based screen to encode for a mitochondrial protein required for the expression of the mitochondrial reporter gene *ARG8^m* placed under the control of *VAR1* 5'-UTR (19). The encoded polypeptide was renamed Sov1 for Synthesis of Var1 and proposed to promote *VAR1* mRNA translation (19). However, both *VAR1* and *VAR1::ARG8^m* transcripts were undetectable in *sov1* null mutant strains, and Sov1 involvement in Var1 synthesis was only based on the observation that the Arg8^m reporter protein level was increased when *SOV1* was over-expressed (19). More recently, the deletion of *sov1* gene, as well as several mitochondrial translational activators, have been linked to Sir2-dependent life span extension, suggesting a possible regulatory function of Sov1 in nucleus-mitochondrion communication (20).

In the present study, we have been able to stabilize *VAR1* mRNA in the absence of Sov1 and demonstrate that Sov1 plays a role as a *bona fide* *VAR1* translational activator. Furthermore, we have uncovered a negative feedback loop mechanism by which Sov1 regulates not only Var1 expression but also its assembly during mitoribosome biogenesis. Sov1 promotes *VAR1* mRNA translation and, additionally, interacts with newly synthesized Var1 and mediates its assembly into the mtSSU. In the absence of Sov1, mtSSU biogenesis is impaired at the late stages that involve the incorporation of Var1/uS3m, and also its interacting partners uS14 and mS46. Based on the dual role of Sov1 in Var1 synthesis and assembly, we propose a working model according to which mitochondrial *VAR1* gene expression is coordinated with mitoribosome assembly. From an evolutionary point of view, the existence of this regulatory negative feedback loop could contribute to explain the retention of the *VAR1* gene in the yeast mitochondrial genome.

MATERIALS AND METHODS

Yeast strains and media

All *S. cerevisiae* strains used are listed in Supplementary Table S4. The composition of the standard culture medium used is defined in the Supplementary Material.

Mitochondrial protein synthesis

Mitochondrial gene products were labeled with [³⁵S]-methionine (7 mCi/mmol, Perkin Elmer) in whole cells at room temperature in the presence of 0.2 mg/ml cyclohex-

imide to inhibit cytoplasmic protein synthesis (21). Equivalent amounts of total cellular proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane and exposed to X-ray film.

In organello mitochondrial translation and co-immunoprecipitation of newly synthesized polypeptides was performed as previously reported (22).

Sucrose gradient analysis

The sedimentation properties in sucrose gradients of Sov1-HA and the mitoribosomes were analyzed essentially as described (23). Mitochondria were prepared by the method of Herrmann *et al.* (24). Four mg of protein were solubilized in 400 µl of extraction buffer (20 mM HEPES, pH 7.4, 0.5 mM PMSF, 0.8% digitonin, 0.5 mM MgCl₂ or 5 mM ethylenediaminetetraacetic acid (EDTA) and 25 mM KCl) on ice for 5 min. EDTA was used to analyze the 54S and 37S subunits separately, while Mg²⁺ was added to preserve their interaction into the 74S monosome. The clarified extract obtained by centrifugation at 50 000 × g_{av} for 15 min at 4°C was applied to a 5 ml linear 0.3 M–1.0 M sucrose gradient containing 20 mM HEPES, 0.5 mM PMSF, 0.1% digitonin, 0.5 mM MgCl₂ or 5 mM EDTA and 25 mM KCl. Following centrifugation for 3 h and 10 min at 40 000 r.p.m. at 4°C using a Beckman 55Ti rotor, the gradients were collected in 14 equal fractions. Twenty µl from each fraction was used to determine the distributions of the proteins of interest by immunoblot analysis. For some experiments, the mitochondrial extracts were resolved in a 10 ml linear 10–30% sucrose gradient centrifuged for 24 h at 24 000 r.p.m. at 4°C using a Beckman 41Ti rotor. These gradients were collected in 26 equal fractions.

For protein identification, proteins from gradient fractions were methanol/chloroform precipitated as reported (25), and mass-spectrometry analysis was performed by SPARC BioCenter Molecular Analysis, The Hospital for Sick Children, Toronto, Canada.

Sov1-HA immunoprecipitation

A functional Sov1 fused in frame to a hemagglutinin (HA) tag was expressed from an integrative vector in strains carrying a null mutant allele of *sov1*. Proteins were extracted from isolated mitochondria as for sucrose gradient analysis (see above). Clarified extracts were incubated with Anti-HA-conjugated agarose beads (Sigma) or protein A-agarose beads (Thermo) as a negative control for 4 h at 4°C with gentle rocking. After centrifugation at 500 × g_{av} for 5 min, supernatants were recovered, and beads washed 3 times with cold phosphate-buffered saline + 0.4% digitonin. The Sov1-HA fusion protein was eluted with Laemmli buffer, and samples were analyzed by Western blotting. All antibodies used in the study are listed in Supplementary Table S5. For Sov1-HA immunoprecipitation analysis from sucrose gradient, fractions were diluted with an equal volume of extraction buffer without digitonin prior to incubation with either Anti-HA-conjugated or protein A-conjugated agarose beads.

Statistical analysis

All the experiments were done at least in duplicate. Quantitative data are presented as the means $\pm$ S.D. of absolute values or percentages of control from triplicate experiments. The values for the several parameters studied were compared by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Generation of $\Delta sov1$ strains with stable mtDNA

To determine the exact role that the Sov1 protein plays in yeast mitochondrial gene expression, we started by generating a modified *sov1* null mutant strain. In yeast, pharmacological treatments or genetic defects that abolish mitochondrial translation are known to affect mtDNA maintenance (26). However, retention of the mitochondrial genome in translation mutants can be achieved by overexpression of the *RNR1* gene (25), encoding the catalytic subunit of the ribonucleotide-diphosphate reductase, or the *YMC2* gene (23), encoding a mitochondrial glutamate transporter (27). We reasoned that if lack of Sov1 compromises *mt-VAR1* gene expression, as previously reported (19), it would, in turn, affect mitoribosome formation and overall mitochondrial protein synthesis, leading to mtDNA depletion. To prevent mtDNA loss, we replace the entire *SOV1* ORF with a KAN-MX4 cassette conferring geneticin resistance in a diploid W303 wild-type (WT) strain overexpressing either the *RNR1* or the *YMC2* genes. Additionally, the strain carries a mtDNA devoid of introns (I^0) and the *pRS316-VAR1^u* construct (15), which allows for the allotopic expression of a fully functional Var1 protein fused to the Cox4 mitochondrial targeting sequence, hereinafter referred to as universal Var1 (Var1^u).

Following sporulation of the heterozygous $\Delta sov1$ diploid and tetrad dissection, haploid *sov1* null mutant spores carrying either no plasmid, *VAR1^u*, *RNR1* or *YMC2*-expressing plasmids were selected. Only $\Delta sov1$ spores expressing Var1^u retained mtDNA, while all others resulted in being ρ^0 (Supplementary Table S1). The mtDNA instability phenotype appears to be associated with the complete absence of the Var1 protein since overexpression of the *RNR1*, or *YMC2* gene alone was not sufficient to promote mtDNA retention in a *var1* null mutant strain (Supplementary Table S1). Additionally, when cells were grown in glucose-containing media for 2 days (~ 15 generations), the $\Delta sov1/VAR1^u$ strain formed *petite* colonies with a slightly increased frequency compared to control ($\sim 10\%$ versus 2.5% , Supplementary Table S1) and neither concomitant overexpression of *RNR1* or *YMC2* genes decreased the formation of *petite* colonies to WT level (Supplementary Table S1). Thus, for all the following experiments, we utilized strains expressing Var1^u, unless otherwise indicated. Moreover, to limit ρ^0 accumulation, cells were routinely grown from fresh ρ^+ cultures and for short periods of time.

It has been previously reported that Var1^u expression in a WT strain increases *petite* accumulation (28). However, the *VAR1^u-HA* construct that was used for the study was not able to complement the respiratory deficient phenotype of a

mitochondrial *var1* mutant (19), suggesting that the tagged Var1 protein is not functional and may be more prone to aggregation. In our hands, the W303 I^0 strain expressing the *pRS316-VAR1^u* construct accumulated *petite* colonies (Supplementary Table S1) at levels comparable to the values reported for S288c derivative WT strains (29).

Lack of Sov1 impairs mitochondrial translation

With the modified $\Delta sov1$ strain available, we could ask phenotypic questions meaningfully. First, we analyzed the respiratory growth of the *sov1* null mutant strain expressing Var1^u by serial dilution growth test in media containing either the respiratory substrates ethanol and glycerol (YP-EG) or the fermentable carbon source glucose (YPD) as control. The growth of the $\Delta sov1/VAR1^u$ mutant was dramatically attenuated in non-fermentable YP-EG media (Figure 1A), due to the decrease of endogenous cell respiration to $\sim 20\%$ of WT value (Figure 1B). The $\Delta sov1$ respiratory defect was not due merely to the absence of *mt-VAR1* gene expression since it occurs in the presence of the Var1^u protein synthesized from the nucleus, which is sufficient to fully support respiratory growth of a mitochondrial *var1* null mutant strain (Figure 1A).

To better dissect the cause of the respiratory deficient phenotype of the $\Delta sov1/VAR1^u$ strain, we then analyzed the enzymatic activities and steady-state levels of the mitochondrial OXPHOS complexes. NADH-cytochrome *c* reductase (NCCR), cytochrome *c* oxidase (COX) and ATP synthase (ATPase) enzymatic activities, measured spectrophotometrically, were all largely reduced in the $\Delta sov1/VAR1^u$ mutant compared to control (Figure 1B). Accordingly, the levels of fully assembled *bc1* complex (or CIII), COX (or CIV) and ATP synthase (or CV), analyzed by Blue-Native PAGE, as well as the amount of mitochondrion-encoded CIII and CIV subunits *Cytb*, *Cox1*, *Cox2* and *Cox3* detected by SDS-PAGE, were severely decreased in $\Delta sov1/VAR1^u$ mitochondria (Figure 1C and D). However, succinate dehydrogenase (or CII), the only OXPHOS complex entirely formed by nucleus-encoded subunits, accumulated in the $\Delta sov1/VAR1^u$ mutant as in the WT strain (Figure 2C). Overall, this phenotype is suggestive of a mitochondrial genome expression defect.

The gene expression defect was not related to mtDNA levels, which were similar in the $\Delta sov1/VAR1^u$ mutant and WT strains (Figure 1E). It was not related either to compromised transcription or mRNA instability, since the steady-state levels of mitochondrial *COX1*, *COX2* and *CYTB* mRNAs were actually elevated in the null *sov1* mutant versus control (Figure 1F). This could represent a compensatory effect associated with the respiratory deficiency, similar to what was reported for another mtSSU mutation associated with a partial translational defect (30). On the contrary, analysis of mitochondrial translation by following the rate of incorporation of S³⁵-radiolabeled methionine into mitochondrion-encoded polypeptides showed that the newly synthesized mt-Var1 protein was undetectable in the $\Delta sov1/VAR1^u$ strain for pulses up to 1 h (Figure 1G). Additionally, overall mitochondrial protein synthesis, although not abolished, was markedly decreased in the *sov1* null mutant compared to WT despite the presence of allotopically

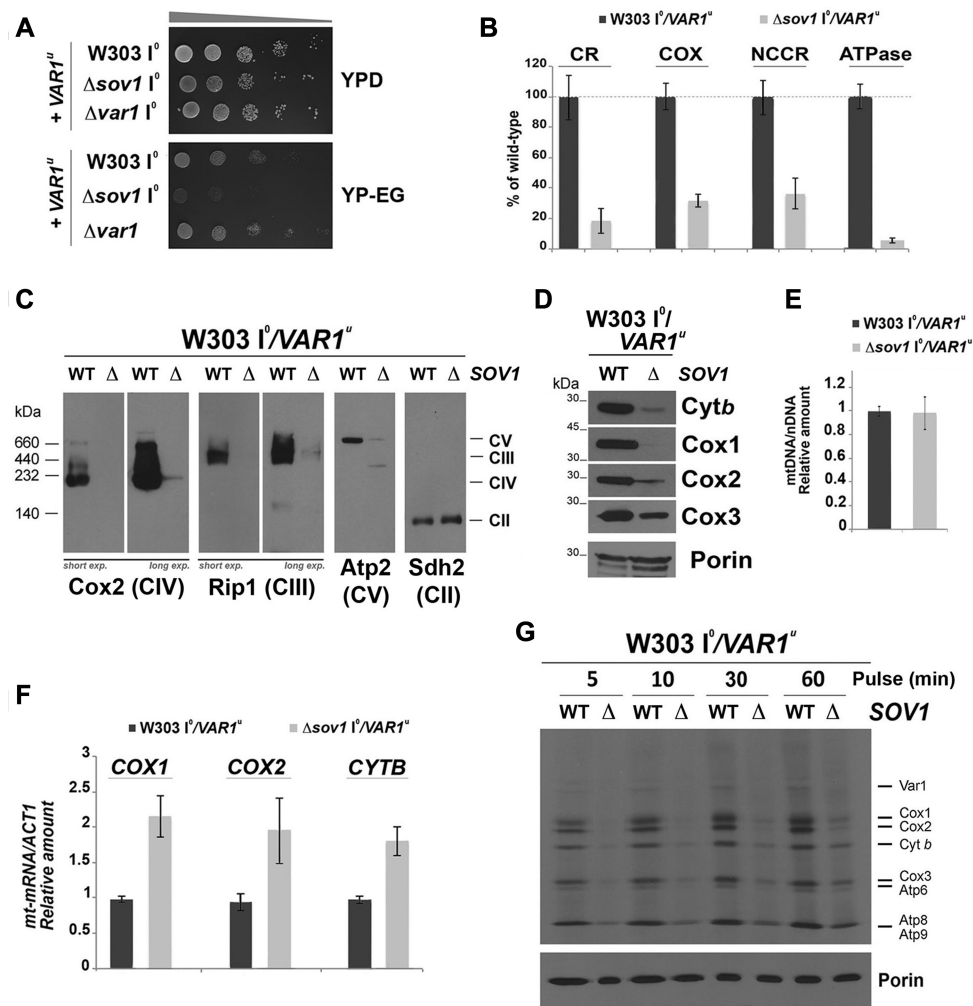


Figure 1. Characterization of the respiratory capacity and OXPHOS system in the absence of Sov1. (A) Serial dilution growth analysis of the indicated strains in media containing fermentable (YPD) or respiratory (YP-EG) carbon sources. Pictures were taken after 2 (YPD) or 3 (YP-EG) days of incubation at 30°C. (B) Measurement of OXPHOS parameters in the indicated strains. The graph shows endogenous cell respiration rate (CR) measured polarographically, and the enzymatic activities of cytochrome *c* oxidase (COX), NADH-cytochrome *c* reductase (NCCR) and ATPase, measured spectrophotometrically and expressed as the percentage of WT. Bars represent the mean $\pm$ SD of three independent repetitions. (C) Steady-state levels of OXPHOS complexes extracted with lauryl maltoside from isolated mitochondria, analyzed by BN-PAGE and detected by immunoblotting with the indicated antibodies. (D) Steady-state levels of the indicated mitochondrion-encoded subunits of complex III (CIII) and complex IV (CIV) analyzed by SDS-PAGE and immunoblotting. Porin was used as loading control. (E) Mitochondrial DNA content (mtDNA) measured in whole cells by qPCR amplification of *COX1* and *ACT1* genes, markers of mtDNA and nuclear DNA (nDNA) levels, respectively. Bars represent the mean $\pm$ SD of three independent repetitions. (F) Steady-state levels of the indicated mitochondrion-encoded mRNAs, measured by qPCR and normalized by the level of *ACT1* mRNA. Bars represent the mean $\pm$ SD of three independent repetitions. (G) Metabolic labeling with ^{35}S -methionine of newly synthesized mitochondrial products in whole cells either WT or lacking *sov1* (Δ) and expressing *Var1*^u during increasing pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right.

expressed *Var1*^u (Figure 1G). Taken together, these data suggest that *Sov1* is required for *mt-VAR1* gene expression and plays an additional role in overall mitochondrial protein synthesis beyond promoting *mt-VAR1* mRNA stability and/or translation.

Sov1 is an *mt-VAR1* mRNA translational activator

The *mt-VAR1* gene is transcribed as part of a polycistronic RNA that also contains the *ATP9* and *tRNA*^{Ser} genes (31). Endoribonucleolytic cleavage of the primary transcript releases a 2.4 kb precursor *mt-VAR1* mRNA, which is further processed to generate the mature 1.9 kb *mt-VAR1* mRNA,

including a 162 bp-long 5'-UTR and a 550 bp-long 3'-UTR (Figure 2A and (15)). It has been previously reported that while the accumulation of the precursor mRNA is independent of *Sov1*, the mature *mt-VAR1* mRNA is undetectable in a *sov1* null mutant (19), making it challenging to prove that *Sov1* functions as an *mt-VAR1* mRNA translational activator. Likewise, in our *W303 I°* genetic background, the steady-state level of the precursor *mt-VAR1* mRNA was not affected by the lack of *Sov1*, whereas the accumulation of the mature *mt-VAR1* mRNA was considerably decreased in the $\Delta\text{sov1 } I^\circ/VAR1^\circ$ mutant (Figure 2B). These data support a model in which *Sov1* is not required for the precursor *mt-VAR1* mRNA processing, but for the stability of the mature

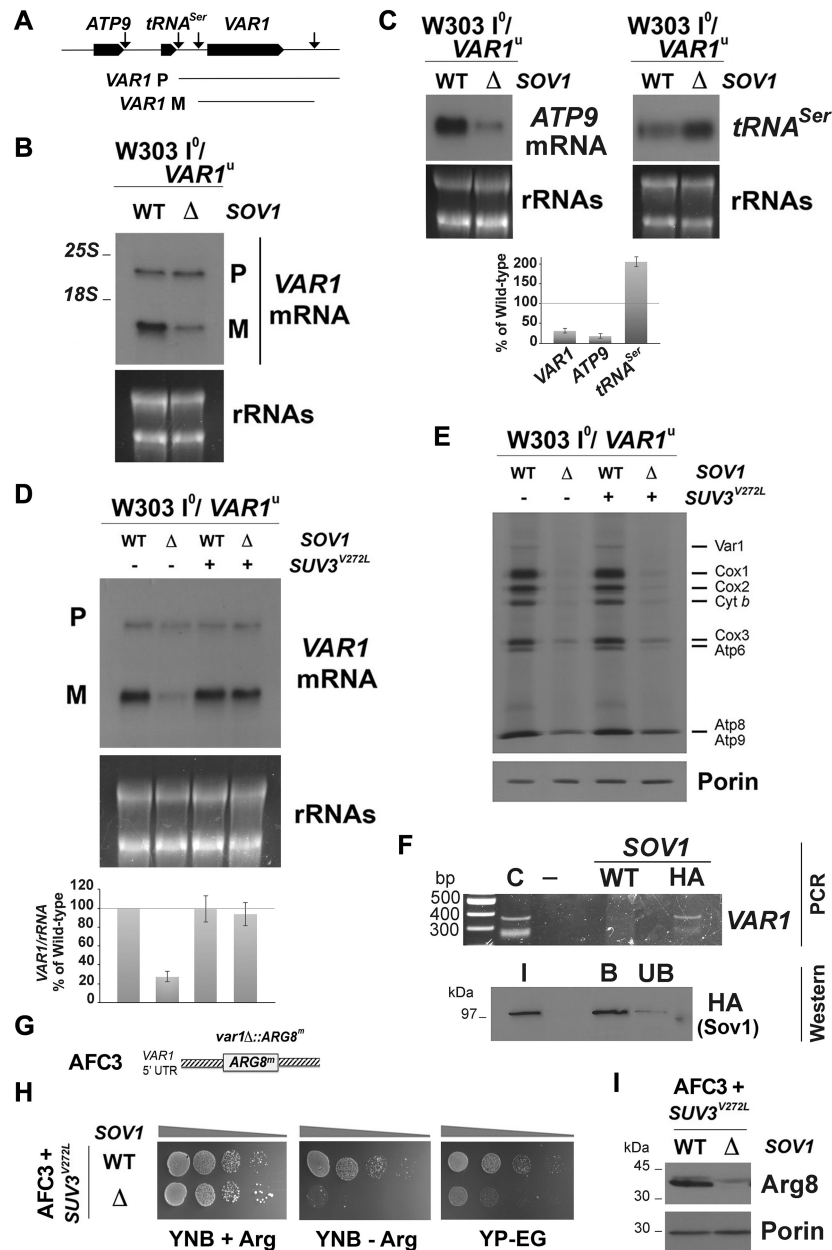


Figure 2. Role of Sov1 in *VAR1* mRNA stability and expression. (A) Schematic representation of the mitochondrial primary polycistronic transcript encompassing *ATP9*, *tRNA^{Ser}* and *VAR1* genes (in thicker bars). The sites of endoribonucleolytic cleavage are indicated by arrows. The position of the 2.4 kb precursor (P) and 1.9 kb mature (M) *mt-VAR1* mRNA are indicated by black lines. (B) Northern-blot analysis of total cellular RNAs to detect mature (M) and precursor (P) *mt-VAR1* mRNA steady-state levels in WT and *sov1* null mutant (Δ) strains expressing *Var1^u*. Cytosolic rRNAs are used as loading control. The position of cytosolic 28S- and 18S-rRNAs (3392 and 1798 nt, respectively) relative to *mt-VAR1* mRNAs is indicated. (C) Northern-blot analysis of total cellular RNAs as in panel (B) to detect *ATP9* mRNA and *tRNA^{Ser}*. Cytosolic rRNAs are used as loading control. Lower panel: relative amounts of mature *mt-VAR1* and *ATP9* mRNAs and *tRNA^{Ser}* in the *sov1* null mutant strain. Signal from panels B and C were quantified and expressed as percentage of WT. Bars represent the mean $\pm$ SD of three independent repetitions. (D) *mt-VAR1* mRNA steady-state levels in the indicated strains, as in A. Quantification of the signal for the mature *mt-VAR1* mRNA is reported below the image. Bars represent the mean $\pm$ SD of three independent repetitions. (E) Metabolic labeling with ³⁵S-methionine of newly synthesized mitochondrial products in whole cells for 10-min pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Strains included were the WT or *sov1* null mutant (Δ), expressing (+) or not (–) a second copy of the *SUV3* gene carrying the V272L mutation and *Var1^u*. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right. (F) Co-immunoprecipitation of Sov1-HA with *mt-VAR1* mRNA, using anti-HA-conjugated agarose beads. In the upper panel the presence of *mt-VAR1* mRNA in the bound material was analyzed by cDNA synthesis and PCR amplification. A strain carrying a non-tagged Sov1 protein (WT) was used as negative control. Purified mtDNA was used to detect the 376 bp expected amplicon in a control PCR (C). No template DNA PCR was performed as negative control (–). The lower panel shows the analysis of immunoprecipitated fractions by immunoblotting with an anti-HA antibody. I: input. B: bound. UB: unbound. Equivalent amounts of unbound and bound samples were loaded in the gel. (G) Scheme depicting the mitochondrial genotype of strains carrying mitochondrial *ARG8* (*ARG8^m*) as a reporter of mRNA translation. (H) Serial dilution growth analysis of the indicated strains in yeast nitrogen base minimum media (YNB) supplemented or not with arginine and complete respiratory (YP-EG) media. Pictures were taken after 3 days of incubation at 30°C. AFC3 = *mt-var1 Δ ::ARG8^m* + *pRS316-VAR1^u*. (I) Steady-state levels of the Arg8 protein in the indicated strains. Porin was used as loading control.

mt-VAR1 mRNA. However, we failed to detect the polycistronic transcript, which is most likely a very low abundant molecule, in either the WT or *sov1* null mutant strains. Moreover, the accumulation of the mature *ATP9* mRNA and tRNA^{Ser}, belonging to the same primary RNA that *mt-VAR1*, resulted greatly altered in the absence of Sov1. In particular, the *ATP9* mRNA level was severely decreased, while the amount of tRNA^{Ser} was approximately double in the $\Delta sov1/VAR1^u$ strain compared to control (Figure 2C). Taken together, these data suggest that Sov1 affects multiple aspects of the metabolism of the *mt-VAR1*-containing polycistronic transcript.

In yeast, mitochondrial translation and mRNA splicing are tightly connected since maturases required for mRNA processing are encoded in *COX1* and *CYTB* gene introns (32,33). To test whether the presence of introns in the mtDNA could affect the *sov1* null mutant phenotype, we analyzed the respiratory growth and mitochondrial translation efficiency in $\Delta sov1/VAR1^u$ strains carrying an intron-containing (I^+) or I^0 mtDNA. No differences were observed between *sov1* null mutants irrespectively of their mtDNA intron composition in terms of growth (Supplementary Figure S1A), indicating that Sov1 is not involved in mitochondrial mRNA splicing. Although expression of *COX1* and *CYTB* in the strain harboring introns were negatively impacted by the absence of Sov1 (Supplementary Figure S1B), most likely as a consequence of decreased maturase expression owing to the general defect in mitochondrial translation. Incidentally, despite the severe decrease in *ATP9* mRNA levels in the *sov1* null mutant, the rate of Atp9 synthesis was not further affected when compared to the other mitochondrion-encoded OXPHOS subunits (Supplementary Figure S1B).

The observed instability of the *mt-VAR1* transcript could explain why no *mt-VAR1* translation is detected in the *sov1* null mutant strain. To further investigate this aspect of Sov1 function, we looked for strategies that could help to stabilize the mature *mt-VAR1* mRNA in cells lacking Sov1. In yeast, the mitochondrial genome is transcribed in polycistronic RNAs, which are then processed to generate the mature transcripts. In the case of protein-coding genes, the mRNA site of 3' cleavage is determined by a conserved stretch of 12 nt, the dodecamer sequence (34,35). In addition to defining the site of 3' processing, the dodecamer sequence has been proposed to play a role in mt-mRNA stability and translation (34). The truncation of a fragment of the *VAR1* mRNA 3'-UTR comprising the dodecamer sequence dramatically decreases transcript stability and is associated with respiratory deficiency (36). A spontaneous suppressor of this respiratory growth defect has been isolated and shown to carry a dominant missense mutation in the DEXH/D-box helicase Suv3 (36). Together with the exoribonuclease Dss1, Suv3 forms the mitochondrial RNA degradosome (37). The Suv3 suppressor missense mutation V272L occurs in the helicase motif Ia, which is involved in RNA binding (38), and is associated with alterations in mRNA steady-state levels and accumulation of excised group I introns (39). Since Suv3^{V272L} has been shown to enhance the expression of a 3'-UTR truncated *mt-VAR1* mRNA (36), we introduced a single copy of the dominant *SUV3*^{V272L} allele in WT and *sov1* null mutant cells. Expression of the Suv3^{V272L} variant in the

$\Delta sov1/VAR1^u$ strain restored the accumulation of mature *mt-VAR1* mRNA to WT levels (Figure 2D). Notably, newly synthesized mt-Var1 polypeptide remained undetectable in $\Delta sov1/VAR1^u/SUV3^{V272L}$ cells (Figure 2E), suggesting that Sov1 is required for *mt-VAR1* mRNA translation.

Sov1 is a pentatricopeptide (PPR) domain-containing protein (40). PPR motifs are degenerate 35 amino acid repeats, which mediate RNA-binding; although in a small number of cases, they have also been shown to bind to DNA or proteins (41). To test whether Sov1 role in promoting *mt-VAR1* gene expression involves its direct interaction with the *mt-VAR1* mRNA, we next performed an immunoprecipitation assay. For this purpose, we generated a Sov1-HA-tagged polypeptide (Sov1-HA)-expressing construct, which in single copy was sufficient to fully complement the respiratory deficient phenotype of a *sov1* null mutant strain (Supplementary Figure S2A). Moreover, Sov1-HA accumulated in mitochondria as a peripherally associated membrane protein (Supplementary Figure S2B-C). Mitochondrial extracts from a *SOV1-HA* I^0 strain were incubated with anti-HA-conjugated agarose beads to immunoprecipitate Sov1-HA (Figure 2F, lower panel). The presence of *mt-VAR1* mRNA in the precipitate fractions was analyzed by cDNA synthesis and polymerase chain reaction (PCR) amplification of a fragment, including the *VAR1* 5'-UTR and the initial 216 bp of the coding sequence. Following PCR, we detected the *VAR1* amplicon specifically in the Sov-HA bound fraction (Figure 2F, upper panel), indicating that Sov1-HA interacts with the *mt-VAR1* mRNA. Specific RNA binding is a common feature of canonical yeast mitochondrial translational activators, including the PPR-protein Pet309 (3). Sov1 binding to the *mt-VAR1* mRNA could directly promote *mt-VAR1* mRNA stability and translational initiation. Moreover, Sov1 could interact with the unprocessed *mt-VAR1*-containing RNA, affecting its secondary structure and/or its accessibility to processing enzymes.

Lastly, to further confirm the Sov1 requirement for *mt-VAR1* mRNA translational initiation, we deleted the *SOV1* gene in a strain carrying the mitochondrial reporter *ARG8^m* (42) under the control of *VAR1* 5'- and 3'-UTR (Figure 2G) in the presence of the *SUV3*^{V272L} allele. The reporter gene is a recoded version of the nuclear *ARG8* gene that encodes for a matrix-localized arginine biosynthetic enzyme. In this way, growth in media lacking arginine represents a direct analysis of mitochondrial translation. The absence of growth on minimal medium lacking arginine and the decreased accumulation of the Arg8 protein in the AFC3- $\Delta sov1$ mutant (Figure 2H-I) indicate that *ARG8^m* expression also depends on Sov1 and strengthens the specificity of Sov1 in *VAR1* expression.

Sov1 is required for the late stages of mitoribosome SSU assembly

Sov1 acts by promoting *mt-VAR1* mRNA stability and translation. However, its function cannot be fully bypassed by allotopic Var1^u expression, suggesting that Sov1 plays an additional role/s in mitochondrial protein synthesis. It has been previously shown that complementation of mutants in the mitochondrial translation factors Pet111 and Aep3 by allotopic expression of *COX2* and *ATP8* respectively de-

depends on a high level of expression achieved by the use of multicopy vectors (43). Therefore, we tested whether overexpression of *VAR1^u* from the multicopy plasmid YEp351 would improve the respiratory growth of the *sov1* null mutant. Only a modest improvement in respiratory growth was observed when *VAR1^u* was overexpressed (Supplementary Figure S1C), which strengthens the hypothesis that Sov1 is required for mitochondrial protein synthesis in function/s other than *mt-VAR1* translation.

To fully understand Sov1 function and the reason why overall mitochondrial translation is compromised in the Δ *sov1*/*VAR1^u* mutant strain, we turned our attention to the mitochondrial ribosomes. First, we investigated the mitoribosome sedimentation properties by sucrose gradient analysis using mitochondrial protein extracts prepared in the presence of either EDTA, to separate mtSSU and mtLSU, or Mg^{2+} , to preserve monosome interactions. mtSSU and mtLSU were detected by immunoblot analysis with antibodies against proteins mS37 and uL3, respectively. The sedimentation profiles of both the mtSSU and the mtLSU were similar when using WT and *sov1* mutant mitochondrial extracts (Figure 3A and B). However, when EDTA was used, we observed broader peaks in the *sov1* null mutant compared to control (Figure 3A), which could indicate ribosomal particle heterogeneity or instability. Moreover, in the *sov1* null mutant, we could detect a lower proportion of the mS37 protein signal sedimenting in the monosome fractions (Figure 3B). This observation was further confirmed by the direct comparison of mS37 and uL3 signals from the relevant sucrose gradient fractions ran in parallel in a single gel (Supplementary Figure S3). The levels of the mtSSU protein mS37 were decreased in the monosome-corresponding fractions in the *sov1* null mutant compared to WT, suggestive of an impaired association between the mtSSU and mtLSU. Additionally, we detected an increase of the mtLSU marker uL3 in the absence of Sov1 in both the fractions where the mtLSU and the monosome peak (Supplementary Figure S3). We then further explored mitoribosome subunits accumulation in WT and Δ *sov1*/*VAR1^u* mitochondria, by detecting the mt-rRNAs by qPCR, as a marker of mitoribosome abundance. We observed a 3-fold increase in the levels of mtLSU *21S-rRNA* in cells lacking Sov1 (Figure 3C). Additionally, the steady-state levels of representative mtLSU mitoribosome proteins were also increased in *sov1* null mutant mitochondria (Figure 3D). Conversely, the mtSSU *15S-rRNA* and representative protein steady-state levels were unchanged in the Δ *sov1*/*VAR1^u* strain compared to WT (Figure 3C and D). We have previously reported that the accumulation of the mtLSU is increased in mitochondria lacking the mtSSU protein mS38 (30). Thus, it appears that this phenotype is not strictly dependent on the absence of Sov1, since it is shared with at least another strain characterized by partially compromised mitochondrial translation. Increased mtLSU accumulation could result from a cellular response attempting to compensate for the inefficient mitoribosome performance. However, we can not exclude the possibility that the biogenesis of the two mitoribosome subunits is subject to co-regulation, a scenario that warrants future investigations.

Since Sov1 is required for Var1 expression in mitochondria, we generated an antibody against the Var1 protein.

The polyclonal antibody allowed us to detect both mitochondrially and allotopically expressed Var1 polypeptides, which can be separated by size under certain electrophoretic conditions (Supplementary Figure S4). Similar to the other mtSSU proteins, Var1 accumulated in equal amounts in WT and *sov1* null mutant mitochondria (Figure 3D), suggesting that Var1^u is stable in the absence of Sov1. However, while in WT mitochondria all detected Var1 protein appeared to incorporate into the mtSSU, in the Δ *sov1*/*VAR1^u* strain, only a small portion of Var1^u sedimented in the fractions corresponding to the mtSSU (Figure 3E), suggesting that Var1 protein assembly into the mitoribosome is impaired.

It has been previously reported that the 37S mtSSU shifts to a slower sedimenting 30S particle in either cells treated with the mitochondrial translation inhibitor erythromycin, which leads to the lack of Var1 protein (44), or a strain carrying a thermosensitive mutation in *mt-VAR1* 5'-UTR, which also results in the complete absence of the Var1 protein at the non-permissive temperature (45). Furthermore, it has been roughly estimated by 2D electrophoresis that the 30S mtSSU particle lacks 3 to 5 proteins (44,46), one of which has been identified as uS14 (46).

Using several conditions for sucrose gradient sedimentation analysis, we were not able to resolve the 37S and 30S mtSSU particles in our Δ *sov1*/*VAR1^u* strain (Figure 3A, B and E). One possibility is that both molecules are present in the *sov1* null mutant mitochondria: the fully assembled 37S mtSSU, which would support the detected residual mitochondrial translation, and the Var1-free 30S incomplete mtSSU particle, making it arduous to discriminate them. In order to better characterize the mtSSU molecules accumulating in the Δ *sov1*/*VAR1^u* strain, we subjected the corresponding sucrose gradient fractions (fractions 7-8-9 from Figure 3A and fractions 12-13-14 from Figure 3E) to mass-spectrometry analysis. In WT and *sov1* mutant samples, we detected all mtSSU proteins with the exception of mS38, mS47 and mS48 (Supplementary Table S2). Most mtSSU proteins, including Var1, were present at lower levels in the *sov1* mutant fractions compared to control (1.5- to 2.5-fold decrease). Additionally, the abundance of uS14 and mS46 was 7- to 10-fold reduced in the *sov1* mutant samples in comparison to WT (Figure 3F and G; Supplementary Table S2). According to the recently reported high-resolution structure of the yeast mitoribosome, Var1/uS3m, uS14 and mS46 interact with each other's to form part of the mitoribosome head and mRNA-entry channel (Figure 3F (7)). We observed that in the absence of Sov1, the incorporation of Var1, uS14 and mS46 into the mtSSU is compromised. Taken together, our data suggest that Sov1 is required for the late stages of mtSSU biogenesis. Specifically, Sov1 could act by mediating the incorporation of an assembly module formed by Var1, uS14 and mS46 into the mtSSU. Alternatively, Sov1 may be required for the proper incorporation of the Var1 protein, which in turn is a pre-requisite for the assembly of uS14 and mS46 into the mtSSU. We predicted that, if Var1, uS14 and mS46 are sequentially assembled, Var1 incorporation into the mtSSU late-assembly intermediate would not be affected by the absence of uS14 or mS46. To better understand the interdependence in the assembly of these three mtSSU proteins, we analyzed mitoribosome

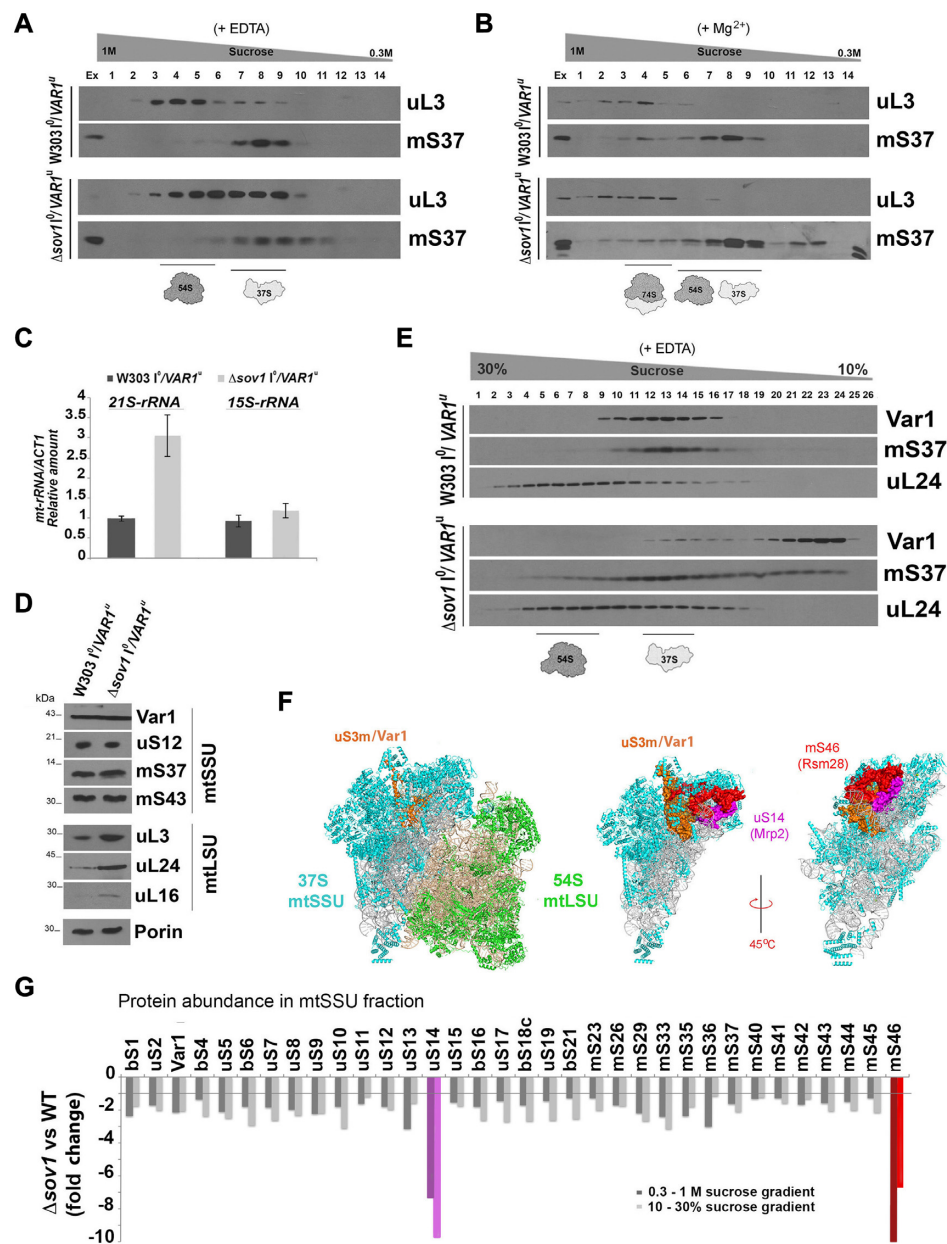


Figure 3. Characterization of mitochondrial ribosomes in cells lacking Sov1. (A and B) Sucrose gradient sedimentation analyses of mitoribosomal protein markers on mitochondrial extracts prepared from the indicated strains in the presence of 0.8% digitonin and either EDTA (A) or Mg²⁺ (B). (C) Steady-state levels of mitochondrial rRNAs 21S and 15S, measured by qPCR and normalized by the level of ACT1 mRNA. Bars represent the mean $\pm$ SD of three independent repetitions. (D) Steady-state levels of the indicated mitoribosome subunits analyzed by SDS-PAGE and immunoblotting. Porin was used as loading control. (E) Sucrose gradient sedimentation analyses of mitoribosomal protein markers on mitochondrial extracts prepared as in A. (F) Location of the mtSSU proteins Var1 (uS3m), uS14 and mS46 in the yeast mitoribosome structure (PDB ID: 5MRC) (7). (G) Mitoribosome SSU protein relative abundance determined by mass-spectrometry analysis of mtSSU fractions from the 0.3–1 M sucrose gradient in A and the 10–30% sucrose gradient in E.

function and sedimentation properties in strains lacking either uS14 or mS46. Mutant strains expressed Var1^u and Rnr1 to prevent mtDNA loss. We observed that, whereas the deletion of uS14 abolished respiratory growth and mitochondrial translation, the absence of mS46 did not compromise either of them (Supplementary Figure S5A and B), suggesting that mS46 is not essential for mitoribosome function. In line with this observation, the mtSSU sedimentation profile in a strain lacking mS46 closely resembled the WT control (Supplementary Figure S5C). Notably, in the absence of mS46, Var1 protein entirely co-sedimented with

the mtSSU marker uS12 (Supplementary Figure S5C), indicating that Var1 incorporation into the mtSSU is not dependent on mS46, but occurs prior to mS46 assembly. Conversely, when we analyzed the Var1^u sedimentation profile in a strain lacking uS14, we could not detect any Var1 signal in the fractions corresponding to the mtSSU (Supplementary Figure S5C), suggesting that Var1 and uS14 assembly into the mtSSU are interdependent. Overall, our data favor a model in which Var1/uS3m and uS14 are co-inserted into the mtSSU, while mS46 is incorporated in a subsequent step.

Sov1 interacts with newly synthesized Var1 and the mitoribosome SSU

We have established that Sov1 is required for the late stages of mtSSU biogenesis. To better understand whether Sov1 performs its function in mitoribosome assembly indirectly or through a direct physical interaction, we aimed to characterize the network of Sov1 interacting partners. For this purpose, we utilized the respiratory competent Sov1-HA-expressing strain, described above (Supplementary Figure S2A). Under different sucrose gradient conditions, Sov1-HA was detected by immunoblotting mainly in a single complex in fractions corresponding to ~200 kDa (Figure 4A and B). However, when the X-ray films were exposed for longer times, we were able to detect a small portion of Sov1-HA co-sedimenting with the mtSSU, especially when the analysis was performed in the presence of Mg^{2+} (Figure 4A and B). No Sov1-HA signal was present in the mtLSU and monosome fractions (Figure 4A and B). Lastly, a small fraction of the total Var1 signal detected in the sucrose gradients sedimented with the Sov1-HA lighter complex (Figure 4A and B). Based on the observed sedimentation profiles, we tested whether Sov1 interacts with the mtSSU and Var1 by immunoprecipitation of the Sov1-HA protein with anti-HA antibody-conjugated beads. We first performed the experiment in strains with or without expression of Var1^u. Since allotopically expressed Var1^u protein migrates slightly faster than mitochondrion-encoded Var1 polypeptide in 10%-SDS-PAGE (Supplementary Figure S4B), we were able to detect both in the bound material fractions (Figure 4C). Moreover, the signal of the mtSSU protein mS37 was also enriched in the Sov1-HA immunoprecipitated samples (Figure 4C), suggesting that Sov1 interacts with Var1 and the mtSSU. However, the Sov1-Var1 interaction was not preserved when the immunoprecipitation was performed in the presence of EDTA (Supplementary Figure S6A), which could be indicative of a relatively labile bond. Since Var1 is a structural component of the mtSSU, the interaction of Sov1 with Var1 could be indirect and occur only in the context of the mtSSU. To explore this possibility, we repeated the Sov1-HA immunoprecipitation experiment in a strain expressing Var1^u and devoid of mtDNA, in consequence, devoid of mt-rRNAs and ultimately mitoribosomes. In this context, we were still able to immunoprecipitate Var1^u protein together with Sov1-HA (Figure 4D), indicating that the interaction between Sov1 and Var1 is independent of mitoribosome integrity. Moreover, we analyzed the interaction of Sov1-HA with newly synthesized mitochondrial polypeptides by *in organello* translation in presence of S³⁵-methionine and immunoprecipitation with an anti-HA antibody. We observed that newly synthesized Var1 was specifically co-immunoprecipitated together with the Sov1-HA protein (Figure 4E). Taken together, our data support a model in which Sov1 interacts with Var1/uS3m and mediates its incorporation in the mtSSU.

Sov1 cooperates with the mitochondrial chaperones Tcm62 and Ssc1 to prevent newly synthesized Var1 aggregation

Although, a small fraction of Sov1-HA co-sediments with the mtSSU, most Sov1-HA protein is present in a smaller complex of ~200 kDa (Figures 4B and 5A). To better

dissect the network of Sov1 interactions, we performed Sov1-HA immunoprecipitation from the gradient fractions (fractions 11–12 in Figure 4B) corresponding to the 200 kDa Sov1-HA-containing complex and subjected the bound material to mass-spectrometry analysis. Together with Sov1-HA, we identified the putative mitochondrial chaperone Tcm62, the mitochondrial DEAD-box helicase Mss116 and Var1 (Supplementary Table S3). However, we did not detect either uS14 or mS46 polypeptides. The Tcm62 protein shares 38% sequence similarity with the mitochondrial chaperone Hsp60 and is required for the assembly of MRC CII (47). Additionally, Tcm62 was shown to prevent aggregation of mitochondrial Var1 protein at high temperatures (48). Next in order, Mss116 plays multiple roles in mitochondrial transcript metabolism (49). Specifically, we have recently reported that Mss116 interacts with the PPR-translational activator Pet309 and is required for optimal mitochondrion-encoded COX subunit 1 (Cox1) expression (50). However, no specific defect in mitochondrial *VAR1* translation was observed in an *mss116* null mutant under standard conditions (50).

Subsequently, to confirm the mass-spectrometry results, we obtained specific antibodies against Tcm62 (48) and uS14 (Supplementary Figure S4C) and first assessed their sedimentation properties by sucrose gradient analysis. It has been established before that Tcm62 forms a high molecular mass protein complex of ~850 kDa (48). Under our analysis conditions, Tcm62 sedimented in two distinct peaks, the smaller of which largely overlapped with the 200 kDa Sov1-HA-containing complex (Figure 5A). Additionally, while a minor fraction of Var1 protein also co-sedimented with the small Sov1-HA complex, no extra-mitoribosomal pool of uS14 was detected (Figure 5A). We then repeated the Sov1-HA immunoprecipitation from the gradient fractions in which Sov1-HA sediments, corresponding specifically to the mtSSU and the 200 kDa Sov1-HA complex peaks. Bound and unbound material were analyzed by immunoblotting and allowed us to confirm that Sov1-HA interacts with Tcm62 and Var1 in a complex that does not contain uS14 (Figure 5B). These results indicate that Var1 and uS14 do not form a pre-assembly module, whose incorporation into the mtSSU is mediated by Sov1. Moreover, our data suggest that Tcm62 dissociates from Sov1-HA once that the latest engages with the mtSSU to promote Var1 assembly (Figure 5A and B).

Var1 is the only soluble mitochondrion-encoded protein. It is positively charged and prone to aggregation especially at high temperatures (24). It has been previously reported that Tcm62 exerts a chaperone-like function and prevent heat-induced aggregation of mitochondrial Var1 protein (48). Since Sov1 physically interacts with Tcm62 and the extra-mitoribosomal pool of Var1, we tested whether Var1 is prone to heat-induced aggregation in the absence of Sov1. Isolated mitochondria were incubated at 24 or 37°C for up to 40 min, after which soluble and aggregated molecules were separated by centrifugation and analyzed by immunoblotting, as reported (48). Exposure of WT mitochondria to the higher temperature of 37°C induced a moderate accumulation of Var1 aggregates only after 40 min of incubation and independently on Var1^u expression (Figure 5C). Otherwise, the lack of Sov1 was associated with a de-

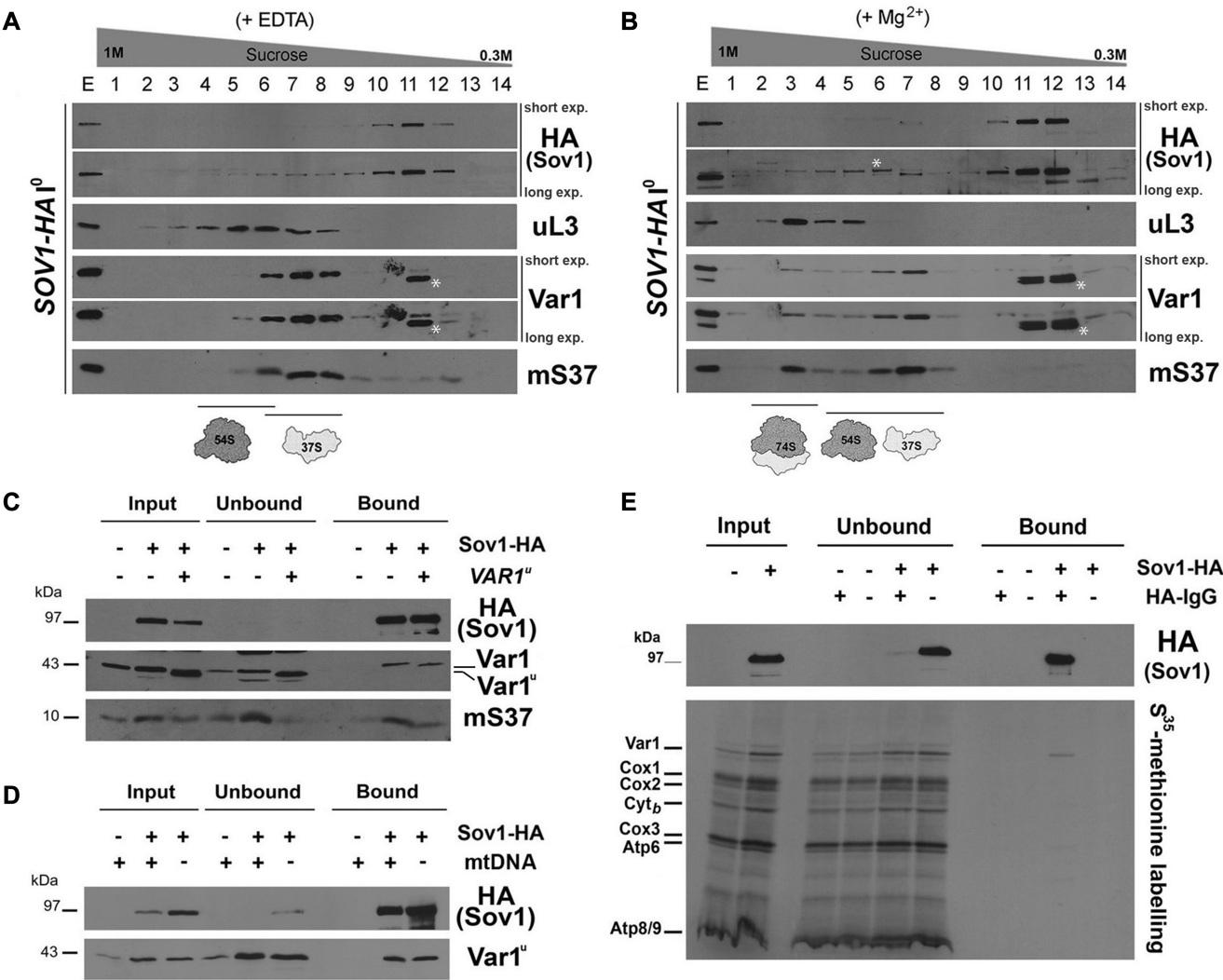


Figure 4. Identification of Sov1 interacting partners. (A and B) Sucrose gradient sedimentation analyses of mitoribosomal protein markers on mitochondrial extracts prepared from a strain expressing a Sov1–HA-tagged protein in the presence of 0.8% digitonin and either EDTA (A) or Mg²⁺ (B). * indicates Var1 and HA Abs unspecific bands. (C) Co-immunoprecipitation of Sov1–HA with Var1 and the mtSSU marker mS37, using anti-HA-conjugated agarose beads. *SOV1*–HA strains expressing (+) or not (–) Var1^u were used. A strain carrying a non-tagged Sov1 protein (W303 I⁰) was used as negative control (–). (D) Co-immunoprecipitation of Sov1–HA with Var1 in a strain devoid of mtDNA (–). The Sov1–HA I⁰ strain and a strain carrying a non-tagged Sov1 protein (W303 I⁰) were used as positive (+) and negative (–) controls respectively. In panel C and D, double amounts of bound relative to unbound samples were loaded in the gel. (E) Co-immunoprecipitation of Sov1–HA with mitochondrial newly synthesized Var1 protein, using anti-HA-conjugated agarose beads. A strain carrying a non-tagged Sov1 protein (W303 I⁰) and protein A-conjugated beads were used as negative controls (–). Equivalent amounts of unbound and bound samples were loaded in the gel.

tectable Var1 signal in the pellet fraction already at 24°C, which became the predominant signal at the 37°C latest time point (Figure 5C). Var1 aggregation in the *sov1* null mutant was not exclusively dependent on the lack of mitoribosome assembly, since it was largely more pronounced than in a ρ^0 /VAR1^u strain (Figure 5C), suggesting that Sov1 is required to prevent Var1 protein aggregation.

In addition to Tcm62, it has also been reported that the mitochondrial Hsp70 chaperone Ssc1 interacts with newly synthesized Var1 and maintains it in a soluble assembly competent state (24). Since Sov1 also interacts with newly synthesized Var1, we asked whether Sov1 and Ssc1 form a ‘chaperone-complex’ to mediate Var1 folding and assembly into the mtSSU. We first tested a possible Sov1–Ssc1 inter-

action by co-immunoprecipitation analysis. However, under all the conditions tested, we were unable to detect any Ssc1 signal in the Sov1–HA bound fractions (Supplementary Figure S6B). Neither, we detected Ssc1 in our Sov1–HA bound fraction mass-spectrometry analysis (Supplementary Table S3). If Sov1 and Ssc1 do not physically interact, they could act on Var1 sequentially. More specifically, Sov1 may be able to bind newly synthesized Var1 only once it has acquired its native conformation by Ssc1-mediated folding. In consequence, we would expect that a compromised Ssc1 chaperoning function should prevent the Sov1–Var1 interaction. To test this hypothesis, we performed a Sov1–HA immunoprecipitation analysis of *in organello* newly synthesized polypeptides using mitochondria

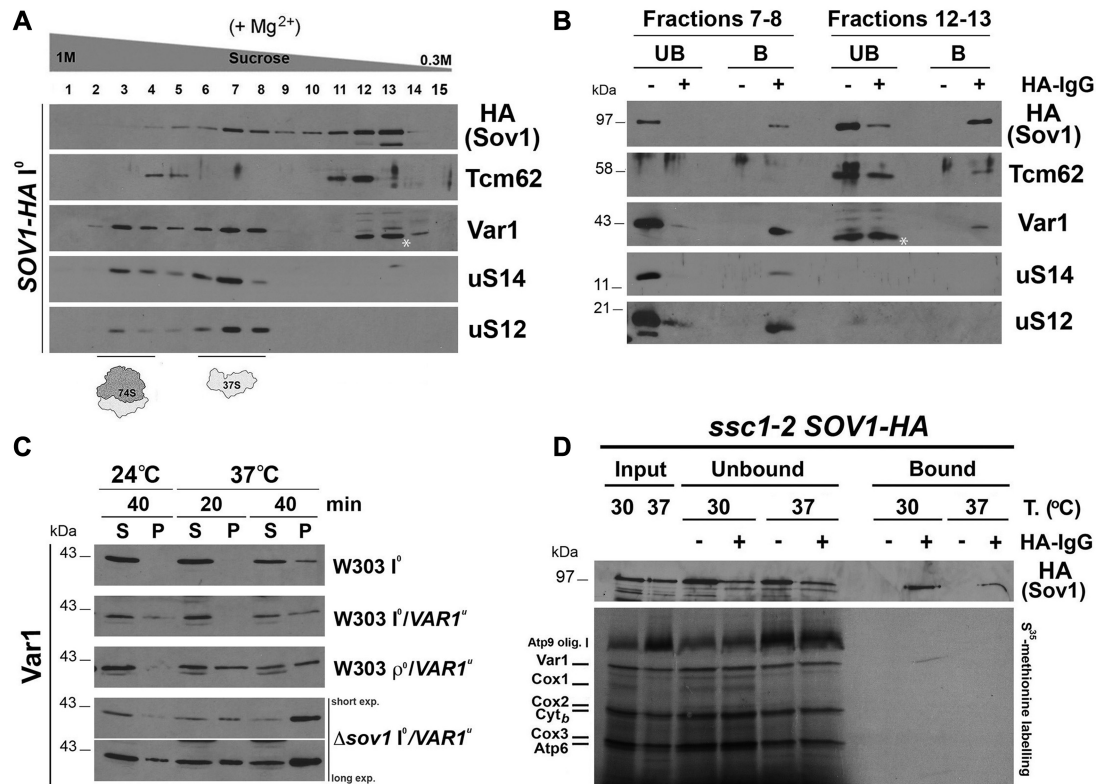


Figure 5. Role of Sov1 and the mitochondrial chaperones Tcm62 and Ssc1 in Var1 biogenesis. (A) Sucrose gradient sedimentation analyses of mitochondrial extracts prepared in the presence of Mg^{2+} from a strain expressing a Sov1-HA tagged protein. * indicates Var1 Ab unspecific bands. (B) Co-immunoprecipitation of Sov1-HA protein with the mitochondrial chaperone Tcm62 and the indicated mtSSU proteins from sucrose gradient fractions. Fractions 7-8 and 12-13 from the gradient reported in panel (A) were used for immunoprecipitation with either Anti-HA-conjugated (+) or protein A-conjugated (-) agarose beads. Equivalent amounts of unbound (UB) and bound (B) samples were loaded in the gel. (C) Analysis of Var1 protein aggregation in the indicated strains. Isolated mitochondria were incubated at either 24°C or 37°C for the indicated amount of time. Soluble (S) and insoluble (pellet, P) material were separated by centrifugation and analyzed by immunoblotting. (D) Interaction of Sov1-HA with mitochondrial newly synthesized Var1 protein in a *ssc1-2* thermosensitive mutant. *In organello* mitochondrial translation was performed at the permissive (30°C) or not permissive (37°C) temperature for 30 min. Anti-HA-conjugated (+) and protein A-conjugated (-) agarose beads were used for immunoprecipitation. Equivalent amounts of unbound and bound samples were loaded in the gel.

isolated from the thermosensitive mutant *ssc1.2*. The mt-Hsp70 chaperone plays multiple key roles in mitochondrial biogenesis, including mitochondrial import, translation and protein folding. The *ssc1.2* mutation was originally described to compromise mitochondrial import at the non-permissive temperature of 37°C (51). Additionally, we reported that already at the permissive temperature (30°C), the *ssc1.2* mutant strain is partially respiratory deficient, mainly due to a decrease in mitochondrial Cox1 translation (52). The Cox1 synthesis defect and the accumulation of a newly synthesized Atp9 oligomers were further exacerbated at 37°C, while the rate of Var1 synthesis was not affected (Figure 5D and (24)). Moreover, it has been shown that the *ssc1.2* mutation does not affect the capacity of the chaperone to bind newly synthesized Var1, but compromises its release, leading to Var1 sequestration (24). Immunoprecipitation of the Sov1-HA tagged protein in the *ssc1.2* genetic background led to the recovery of newly synthesized Var1, when *in organello* translation was performed at 30°C. This interaction was completely lost at the non-permissive temperature of 37°C (Figure 5D), suggesting that Sov1 binds to Var1 only after it is released from Ssc1.

Sov1 C-terminus is required to promote *mtVAR1* mRNA translation

The Sov1 protein contains eleven PPR domains (Figure 6A, (40)). PPR proteins are present exclusively in eukaryotes and function mainly in organellar post-transcriptional RNA metabolism, including RNA processing, stability, and expression (41). While in yeast mitochondria only 15 PPR proteins have been so far identified (40), in terrestrial plants, they represent a large family with more than 500 known polypeptides (41). Domain analysis of known plant PPR proteins has highlighted a common organization with any additional no-PPR functional domain localized in the C-terminus portion of the polypeptide (53). Following the last PPR domain, Sov1 also contains a 109 amino acid long C-terminus stretch (Figure 6A). To investigate the functional relevance of this portion of the Sov1 protein, we introduced by site-directed mutagenesis 2 stop codons after nucleotide 2376 of the *SOV1-HA* gene in order to generate a Sov1 truncated polypeptide lacking amino acids 793-898. Single copy expression of the *SOV1¹⁻⁷⁹²* construct in the Δ *sov1/VAR1^u* strain restored the respiratory capacity

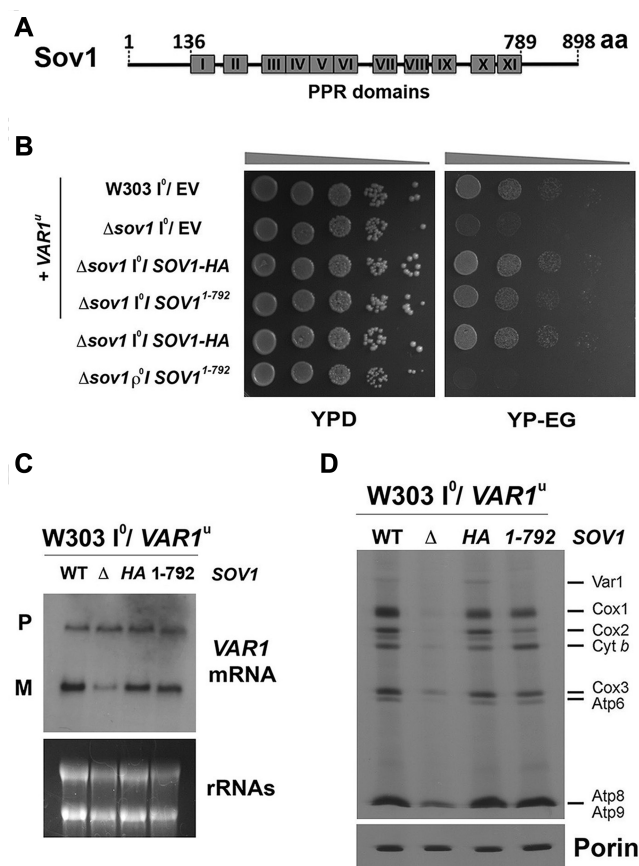


Figure 6. Analysis of Sov1 protein domains. (A) Schematic representation of Sov1 protein PPR domains. (B) Serial dilution growth analysis of the indicated strains in media containing fermentable (YPD) or respiratory (YP-EG) carbon sources. Pictures were taken after 2 (YPD) or 3 (YP-EG) days of incubation at 30°C. (C) Steady-state levels of mature (M) and precursor (P) *mt-VAR1* mRNA in the indicated strains as in Figure 2A. rRNAs are used as loading control. (D) Metabolic labeling with ³⁵S-methionine of newly synthesized mitochondrial products in whole cells for 10-min pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right. WT Sov1, Δ: *soy1* null mutant, HA: Sov1-HA tagged, 1-792: C-terminus truncated Sov1 lacking aminoacids 790–898.

of the mutant almost to WT level, as seen by the growth in respiratory substrate-containing media (Figure 6B). Moreover, the C-terminus truncated Sov1 protein was sufficient to stabilize the mature *VARI* mRNA (Figure 6C) and to support overall mitochondrial translation in the presence of allotypically expressed Var1^u (Figure 6D). However, cells expressing the *SOV1*¹⁻⁷⁹² variant were unable to synthesize mitochondrion-encoded Var1 protein (Figure 6D), suggesting that the C-terminus domain of Sov1 is required to promote *VARI* mRNA translation. Indeed, when the Δ*soy1*/*VARI*^u/*SOV1*¹⁻⁷⁹² strain was plated in non-selective media, all cells that lost the *VARI*^u construct also lost mtDNA (Figure 6B), due to their incapacity to synthesize the mt-Var1 protein. In this way, we were able to separate the function of Sov1 in promoting *VARI* mRNA translation from its role in mediating Var1 protein assembly into the mtSSU.

Sov1 regulates the rate of *mtVARI* mRNA translation

In yeast mitochondria, gene expression is regulated by negative feedback loops, which couple the rate of translation of key structural subunits with their assembly into OXPHOS complexes (3). So far, assembly dependent translational regulatory systems have been described to control the expression of CIV subunit Cox1, CIII subunit Cytb and CV subunits Atp6 and Atp8 (3). These regulatory loops rely on the dual function of a tertiary factor that acts on the mRNA 5'-UTR to promote translation and binds to the newly synthesized polypeptide to mediate assembly. Since the two activities are mutually exclusive, the distribution of the tertiary factor between two functionally distinct pools determines its availability as a translational activator. In other words, if the assembly of the target subunit is compromised, the tertiary factor remains bound to it and is prevented from binding to the mRNA and promoting translational initiation (3).

The dual capacity of Sov1 to promote *mt-VARI* mRNA translation and to mediate newly synthesized Var1 protein assembly into the mtSSU reminded us of the property of a translational regulation tertiary factor. To investigate whether Sov1 plays a role in the regulation of Var1 expression, we analyzed the rate of *mt-VARI* translation in relation to the amount of Sov1 protein. Specifically, we over-expressed Sov1 using an episomal plasmid in a WT strain, independently or in combination with allotypically expressed Var1^u. The increase in the amount of Sov1 was associated with a higher rate of mt-Var1 synthesis (Figure 7A). On the contrary, the increase in Var1^u protein levels resulted in a decrease in *mt-VARI* mRNA translation (Figure 7A). Whereas, co-expression of both Sov1 and Var1^u, restored mt-Var1 synthesis to levels comparable to control (Figure 7A). The observed changes in *mt-VARI* translation were reflected in the Var1 protein steady-state level, while other mtSSU proteins were unaffected (Supplementary Figure S5A). Moreover, the different rates of Var1 expression in the strains analyzed were not due to alterations in the accumulation of *mt-VARI* mRNA (Supplementary Figure S7B).

Lastly, similar tendencies in the rate of *mt-VARI* translation were observed upon Sov1 and/or Var1^u expression in mtLSU mutants, in which mitochondrial translation is only partially compromised (Figure 7B). An increase in Var1 expression in these mutants, however, was not sufficient to restore respiratory growth (Supplementary Figure S5C).

Taken together, our data support a working model (Figure 7C) in which the amount of Sov1 not-bound to Var1 protein determines the rate of mt-Var1 expression. In this way, Sov1 regulates Var1 synthesis in an mtSSU assembly dependent manner.

DISCUSSION

Constructing mitochondrial ribosomes is an expensive metabolic process that is essential to maintain cellular aerobic bioenergetics. In *S. cerevisiae*, it involves two mtDNA-encoded rRNAs and 73 proteins, only one of which, Var1 or uS3m is encoded in the mitochondrial genome and synthesized in mitoribosomes. In this manuscript, we have uncovered a regulatory mechanism that coordinates the synthesis

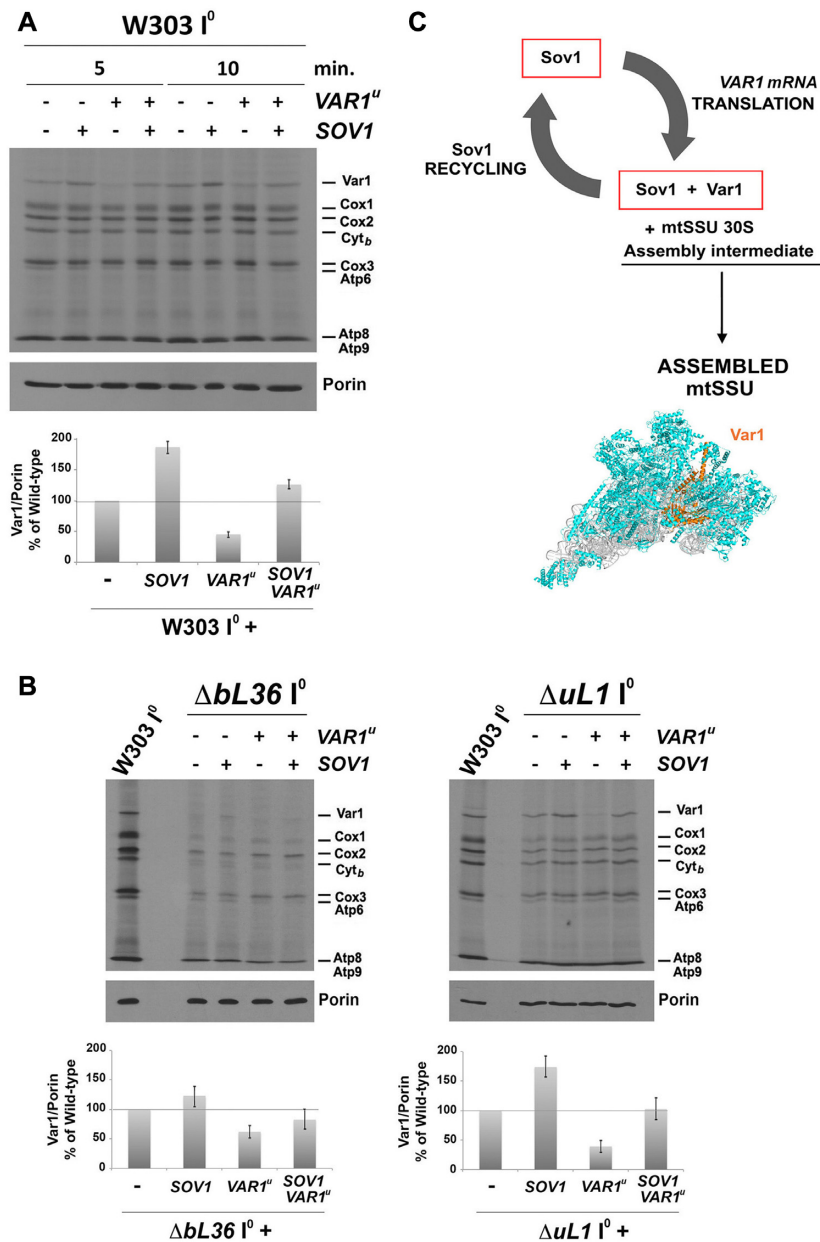


Figure 7. Sov1-dependent regulation of Var1 translation. (A) Metabolic labeling with ³⁵S-methionine of newly synthesized mitochondrial products of the indicated strains in whole cells for 5- and 10-min pulses in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Newly synthesized polypeptides are identified on the right. Quantification of newly synthesized Var1 for 10-min pulse is reported as % of WT in the lower panel. Bars represent the mean ± SD of three independent repetitions. (B). Metabolic labeling with ³⁵S-methionine of newly synthesized mitochondrial products of the indicated strains as in A for 10-min pulses. (C) Model of Sov1 role in Var1 translational regulation. See text for explanation.

and assembly of Var1 into the maturing mtSSU, have gained insight into the actual Var1 assembly process, and have provided a potential explanation for the retention of the *VAR1* gene in the mitochondrial genome.

Var1/uS3m is a component of the mitoribosome mRNA entry channel. In all living organisms, ribosomes are responsible for translating the information encoded by the mRNAs into proteins. Despite fundamental differences in the mechanisms of transcript-ribosome recognition, initiation of translation universally involves the loading of the

mtSSU onto the mRNA. In yeast mitochondrial ribosomes, similarly to bacteria, the mRNA entry channel is located between the head and the shoulder of the mtSSU and is formed by Var1/uS3m, and also proteins uS4 and uS5 (7). Although more extensive remodelings of the mRNA entry channel have occurred in cytosolic and mammalian mitochondrial ribosomes; nevertheless, the uS3m protein remains a conserved structural component (8,10). Moreover, with the exception of trypanosomal mitochondrial ribosomes (54), uS3m also plays a key functional role by directly

interacting with the mRNA. For example, it has been shown that in human cytosolic ribosomes, uS3m can interact with abasic sites in the mRNA and play a role in translation quality control (55). In prokaryotes, the helicase activity of uS3 and uS4 proteins guarantees that mRNA secondary structures are resolved in order for single-strand transcripts to enter the narrow mRNA tunnel (56). A function that in yeast mitochondria could be performed by 5'-UTR RNA-binding proteins, as we recently proposed for the RNA helicase Mss116 (50).

Although recent high-resolution cryo-electron microscopic structures of mitochondrial ribosomes have provided a notable amount of information regarding their composition and function (7–8,54), mitoribosome assembly pathways and factors involved have just started to emerge (25,57–58). Here, we show that in yeast mitochondria Var1/uS3m protein is incorporated into the mtSSU in the late-assembly stages, which is in line with earlier observations reporting the accumulation of a 30S particle in yeast strains lacking Var1 (44,45). Additionally, the process appears to be conserved in bacteria, where uS3 was classified as a member of the fourth and last ribosomal protein assembly cluster (59). Our data indicate that Var1/uS3m biogenesis requires the function of the mitochondrial protein Sov1, which interacts with newly synthesized Var1 and mediates its incorporation into the mtSSU. In a similar manner, the specific chaperone Yar1 binds to the yeast cytosolic ribosomal protein uS3m to prevent its aggregation and promotes its assembly into the immature pre-40S particle to generate the functional ribosomal small subunit (60). Otherwise, in *Trypanosoma brucei* mitoribosome uS3m is part of the early assemblosome, although a cluster of nine assembly factors prevents it from interacting with the other mitoribosome proteins until the late stages of maturation (58). Mitochondrial Var1/uS3m protein is prone to aggregation, which here we have shown to be prevented by three mitochondrial proteins acting in concert, the mt-Hsp70 chaperone Ssc1, the putative chaperone Tcm62 and Sov1. These chaperones also cooperates to promote Var1 post-translational biogenetic steps. Sov1 and Tcm62 form a chaperonin complex that interacts with Var1 protein after its mt-Hsp70-dependent folding. The molecular mass of Sov1+Var1+Tcm62 roughly correspond to the 200 kDa size of the complex. Moreover, Tcm62 is released before Sov1-mediated incorporation of Var1 into the mtSSU. In this way, two mitochondrial multifunctional chaperones and one Var1-specific assembly factor coordinate Var1 folding and assembly.

Our mass-spectrometry analysis of mitochondrial fractions isolated from a strain lacking Sov1 suggests that uS14 and mS46 protein incorporation into mitoribosomes is also compromised in the absence of Sov1. Our data are in agreement with previous observations regarding uS14 assembly (46). However, a fraction of Var1/uS3m can still be incorporated in the mtSSU in a Sov1-independent manner. A possibility is that the Var1/uS3m protein assembled in the mtSSU in the absence of Sov1 does not fully acquired the proper native conformation, which is, in turn, required for efficient incorporation of uS14 and mS46. Indeed, we determined that Var1/uS3m, uS14 and mS46 do not interact to form a late assembly module prior to their incorporation

into the mtSSU. However, Var1/uS3m assembly into the mtSSU is uS14-dependent and we cannot exclude that Sov1 recruits uS14 after interacting with the mtSSU. On the contrary, mS46 incorporation into the mtSSU occurs in a Sov1-independent manner, after Var1 and uS14 assembly. Moreover, the assembly of the mtSSU protein mS38, which is not detected by mass-spectrometry, also occurs after Var1, uS14 and mS46 have been incorporated, since these three proteins are present in the mtSSU that accumulates in a $\Delta mS38$ strain (30). In the case of the mammalian mitoribosome, uS3m and uS14 are part of the same late-binding assembly group, together with uS10m and mS33 (57).

Ultimately, the late formation of a complete mRNA entry channel during assembly appears to be a conserved feature in ribosome biogenesis. Since the mRNA entry channel grants the mtSSU the capacity to interact with transcripts and to potentially initiate translation, it is plausible that its formation could represent a target for regulation. Notably, the yeast cytosolic ribosomal protein uS14 is subject to a feedback translational control system, in which by binding to its own mRNA, uS14 represses its expression in the absence of assembly partners (61).

In this study, we provide experimental evidence demonstrating that Sov1 acts as *in bona fide* *mt-VAR1* mRNA translational activator. These include: (i) The complete absence of Var1 synthesis in a *sov1* null mutant strain in which mature *mt-VAR1* mRNA is stable (Figure 2D and E). (ii) The lack of expression in a *sov1* null mutant strain of the mitochondrial *ARG8^m* reporter gene under the control of *mt-VAR1* 5'-UTR and the consequent lack of growth in the absence of arginine (Figure 2G–I). (iii) The binding of Sov1 protein to *mt-VAR1* mRNA (Figure 2F). (iv) The exclusive requirement of the Sov1 C-terminus domain for *mt-VAR1* translation, despite the presence of mature *mt-VAR1* mRNA and Var1^u-containing mitoribosomes (Figure 6). (v) Lastly, the direct correlation between Sov1 protein levels and the rate of Var1 synthesis (Figure 7). Furthermore, we suggest that *mt-VAR1* expression is controlled by the amount of Var1-unbound Sov1 protein. In this way, Sov1 establishes a negative feedback regulatory loop that coordinates mitochondrial Var1 synthesis and mtSSU assembly.

In the yeast *S. cerevisiae*, it has been well established that mitochondrion-encoded key subunits of the OXPHOS complexes are the subject of assembly dependent control of gene expression. This type of translational regulation plays a pivotal role in the biogenesis of the yeast mitochondrial OXPHOS system and is achieved through a distinctive mechanism that involves dual function tertiary factors binding to the mRNA and the newly synthesized protein in a mutually exclusive manner (3). Now, with the characterization of Sov1 function in *mt-VAR1* gene expression, we extend assembly dependent translation regulation to yeast mitoribosome biogenesis.

Which is then the possible physiological significance of this regulatory system? Since Var1 is prone to aggregation, coupling its expression to the amount of protein that can be assembled into the mitoribosomes could contribute to preventing the deleterious effects associated with an excess of free protein. Moreover, whereas the protein stoichiometry in the assembled and functional mtSSU has to be respected, an exceptional imbalanced in gene copy number

exists between nuclear and mitochondrion-encoded components. Translational regulation could contribute to counteracting this imbalance during the biogenetic process. In general, the complexity and abundance of ribosomes entails several levels of regulation to avoid unnecessary waste of cellular energy in the production of excess components. In this frame, to pace the rate of Var1 synthesis with its assembly makes the mitoribosome biogenesis a more efficient process. Alternatively, it has been shown for mammalian mitoribosomes that an excess of nuclear-encoded ribosomal proteins is synthesized and imported into mitochondria (57). It is tempting to speculate that by controlling the rate of Var1 synthesis and incorporation into available mitoribosome late assembly intermediates, the amount of functional mtSSU could more rapidly vary in response to changes in cellular metabolism. The gain in system flexibility would ultimately contribute to explain the retention of the *VAR1* gene in the mtDNA.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Human mitochondrial transcription and translation

Flavia Fontanesi¹, Marco Tigano², Yi Fu², Agnel Sfeir² and Antoni Barrientos^{1,3}

¹Department of Biochemistry and Molecular Biology, University of Miami, Miller School of Medicine, Miami, FL, United States, ²Skirball Institute of Biomolecular Medicine, Department of Cell Biology, NYU School of Medicine, New York, NY, United States, ³Department of Neurology, University of Miami, Miller School of Medicine, Miami, FL, United States

2.1 Introduction

Mitochondria are semiautonomous eukaryotic organelles of endosymbiotic bacterial origin that have retained a vestige of their ancestral genome: the mitochondrial DNA (mtDNA). Along with evolution, most genes required to sustain the transcription of the mtDNA, as well as the translation of the resulting mRNAs, have been transferred to the nucleus. Therefore mitochondrial gene expression requires the cooperation and coordination of two physically separated genomes.

The human mtDNA is a 16.6 kbp circular DNA that is present in 100–1000 copies per cell and is critical for proper cellular function. Purification of mtDNA using high sucrose density gradients revealed two strands, heavy and light (H and L), with different sedimentation properties owing to a slight skew in purine versus pyrimidine composition. The human mtDNA encodes for a total of 37 genes (13 proteins, 22 transfer RNAs, and 2 ribosomal RNAs) and is transcribed using two major promoters, the H-strand (HSP) and L-strand (LSP) promoters. Transcription yields large precursor polycistronic RNA molecules [1,2] that are later processed into discrete transcripts by specific enzymes [3]. The HSP and LSP promoters are located in a noncoding region, which also contains a displacement loop (D-loop) that is critical for mtDNA replication and coordinates the switch between replication and transcription. The 13 polypeptides expressed from the mtDNA are all essential components of the oxidative phosphorylation (OXPHOS) system enzymes. These proteins are synthesized in mitochondrial ribosomes, which differ from their bacterial and cytoplasmic counterparts, and are specialized in the synthesis of hydrophobic proteins. Mitochondrial ribosomes are anchored to the matrix face of the mitochondrial inner membrane to facilitate the

cotranslational membrane insertion of the newly synthesized polypeptides and coordinate their assembly with nucleus-encoded protein partners to form the OXPHOS enzymatic complexes (reviewed in Refs. [4,5]). With a proteome of ~ 1500 factors that are predominantly encoded by the nucleus, proper mitochondrial gene expression and function is largely dependent on the nuclear genome.

In this chapter, we will describe and discuss the fundamental processes of mitochondrial gene expression: transcription and translation.

2.2 Coordination of mitochondrial DNA replication and transcription

2.2.1 Overview of mitochondrial DNA replication

The human mtDNA is replicated by DNA polymerase γ (Pol γ) in concert with critical components of the replication machinery, as extensively discussed in Chapter 1, MtDNA replication, maintenance, and nucleoid organization. Briefly, the minimal mitochondrial replisome has been reconstituted *in vitro*. It is composed of the mitochondrial replicative DNA polymerase Pol γ subunit A (Pol γ A, coded by the gene *POLG*) complexed with the dimeric Pol γ subunit B (Pol γ B, coded by the gene *POLG2*), the hexameric helicase Twinkle (coded by the gene *TWNK* or *PEO1*) and mtSSB (coded by the gene *SSBP1*). In addition to the minimal replisome components, several proteins play instrumental roles in mtDNA replication and maintenance *in vivo*. These include the mitochondrial transcription factor A (TFAM), mitochondrial genome maintenance exonuclease 1 (MGME1), DNA ligase 3 (LIG3), Ribonuclease H1 (RNASEH1), two topoisomerases (TOP1mt and TOP3A), and the mitochondrial RNA polymerase (POLRMT). POLRMT is a single-subunit DNA-directed RNA polymerase. It plays central roles in both mitochondrial transcription (see Section 2.3) and mtDNA replication. During replication, POLRMT serves as a primase for mtDNA replication on both strands. POLRMT initiates H-strand transcription ~ 200 nt upstream of O_H, and the transcript is used by POLG as a primer for H-strand synthesis [6]. L-strand replication starts when leading-strand synthesis copies past the O_L. *In vitro* assays allowed identifying a stem-loop structure within the O_L that allows POLRMT binding to synthesize primer for lagging strand replication [7,8].

Given its dual function in transcription and replication, the regulation of POLRMT is key to allow the coordination of the two processes. The switch between transcription and replication was recently characterized in depth and shown to be mediated by the transcription elongation factor (TEFM) [9]. In the absence of TEFM, POLRMT commits to replication by generating primers for H-strand and then L-strand replication. On the other hand, the interaction with TEFM boosts the processivity of POLRMT to produce full-length transcripts from HSP and LSP.

2.2.2 The mitochondrial DNA control region

The noncoding portion of mtDNA (NCR) is a 1.1 kbp region spanning from *tRNA^{Phe}* to *tRNA^{Pro}*. Being the only noncoding portion of mtDNA, it has been reported to accumulate the highest number of base substitutions, both inter and intraspecies [10,11]. Despite being prone to mutations, the NCR harbors constrained sequences that are important for mtDNA replication and transcription, including the LSP and HSP promoters and the origin of replication of the H-strand—O_H. Extended sequence analysis of the NCR across different organisms identified three conserved regions—CSBs 1–3—located between LSP and O_H [12]. CSB1 (25 bp) is present in all organisms, CSB2 (17 bp) is partially conserved, and CSB3 (18 bp) is absent in certain branches of evolution [13]. The NCR often exists in D-loop configuration, whereby a fragment of DNA that is 650 nt in length and termed 7S DNA displaces the L- and H-strands [14]. The proportion of mtDNA molecules in this conformation can vary from 10% to 90% in a cell-type and species-specific fashion (reviewed in Ref. [10]).

It is well-established that mitochondrial transcription and replication are mutually exclusive, a step that is necessary to avoid replication fork collapses due to collision with the transcription machinery. Although, the initial steps of both processes are highly overlapping, and both processes share a common RNA primer that can either prime transcription or replication (see below). Accordingly, the mtDNA NCR is a key regulator that dictates the choice between the two DNA transactions.

2.2.2.1 The mitochondrial displacement loop

The 7S DNA is an abortive replication event that initiated from an RNA primer generated at the LSP by POLRMT [7,15]. This RNA primer is about 200 nt long [16], and while the transition to DNA has not been mapped to a single nucleotide level, it can take place between CSB1–CSB2 and the O_H [6]. This suggests that the RNA primer is subject to significant processing by RNaseH1 or MGME1 before replication. The total levels of 7S DNA are controlled by Pol γ and other replisome proteins. They include Pol γ B and mtSSB, as well as mitochondrial transcriptional factors, such as TFAM [17,18]. It is estimated that 95% of the replication events fired from RNA primers are used to generate 7S DNA molecules instead of full-size mtDNA, reinforcing the critical role of this structure in mtDNA metabolism.

The 7S DNA extends until a termination region known as TAS, which is in close proximity to *tRNA^{Pro}* [19]. The process of replication termination at TAS remains uncharacterized [20], but might be influenced by the levels of Pol γ and Twinkle. Specifically, under physiological conditions, Pol γ occupancy within the TAS region is high while Twinkle binding is low, leading to a stalled/paused replication fork. However, when mtDNA copy number decreases and synthesis is needed, the levels of Twinkle at TAS increase to promote the

restart of replication and allowing the replisome to bypass the 7S DNA [21]. Accordingly, the regulation of mtDNA replication appears to be dictated by pretermination as opposed to replication initiation, and this is in strike contrast to the mechanisms that govern the control of replication firing in the nuclear genome.

Transcriptional regulators can also influence replication. For example, MTERF1, a mitochondrial transcription terminator, has been suggested to influence the pausing of replication at different sites throughout the mitochondrial genome [22]. Whether MTERF1 or another unknown factor regulates the pausing at TAS is yet to be determined. Notably, footprint analysis of bovine TAS showed a protected region compatible with a molecularly unknown protein of 48 kDa [23]. Lastly, a new idea has emerged, suggesting that the regulation of mtDNA replication could also be governed spatially by contact sites between mitochondria and the ER [24].

2.2.2.2 *The switch between replication and transcription*

As aforementioned, mtDNA molecules undergo replication or transcription in a mutually exclusive manner, and the switch between the two processes is influenced by the sequence of the NCR in the CSB region (Fig. 2.1). The initiation of transcription is common to both LSP and HSP promoters and requires TFAM [25]. TFAM binds upstream of promoter sequences prompting the bending of DNA in a typical U-shape that is then able to recruit POLRMT [26]. The preinitiation complex transiently recruits TFB2M to assist in promoter melting. In contrast to T7 RNA polymerase, POLRMT does not make contact with downstream DNA and therefore proceeds very slowly, often leading to pausing [27,28]. The recruitment of the elongation factor TEFM stimulates the processivity of POLRMT [9,29]. Following initiation, transcription proceeds from the LSP and HSP promoters to generate genome size polycistronic RNAs that are processed in RNA granules (see sections below). In the case of LSP, the sequence composition of CSB2 causes POLRMT to stall. Specifically, the polymerase stalls within a G-rich stretch of 16 nt that have the propensity to generate a bulky secondary G-quadruplex (G4) structure that can terminate transcription [30]. The presence of TEFM destabilizes the G-quadruplex and thus acts as a transcriptional antiterminator that allows POLRMT to bypass CSB2 and transcribe the entire strand. In contrast, when TEFM is not loaded, transcription by POLRMT terminates at CSB2, generating a replicative primer for Pol γ [9,31,32].

The commitment to either replication or transcription can be envisioned as a multistep process that is subject to tight regulation by several factors, including TFAM, POLRMT, and TEFM. Moreover, the switch is influenced by the sequence of the control region (Fig. 2.1). Additional *in vivo* and *in vitro* experiments are required to define better the molecular steps that regulate this critical mtDNA metabolic choice. Nevertheless, the existence of a tight mechanism that regulates the balance between transcription and replication might have necessitated high conservation of the underlying D-loop region.

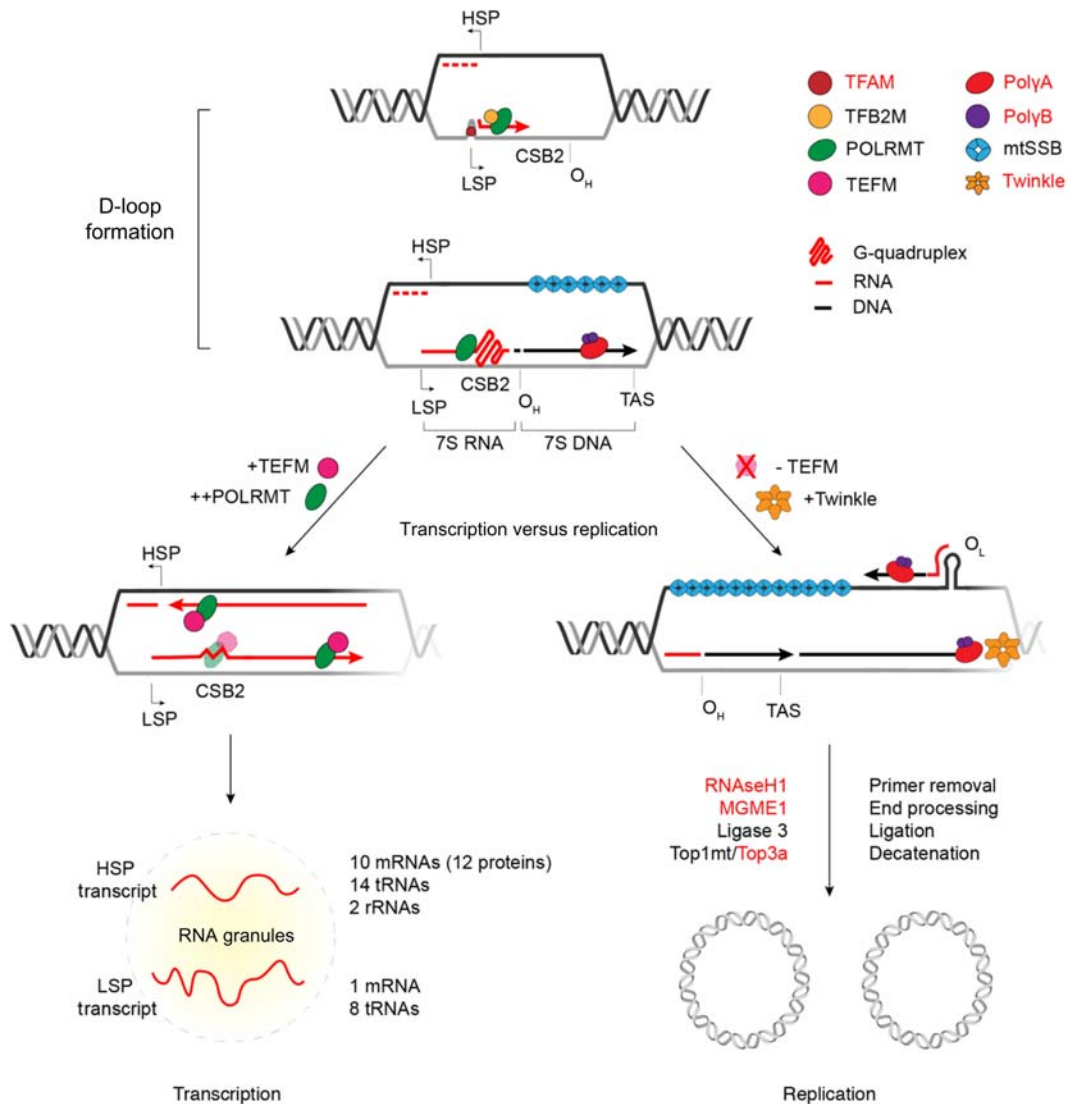


Figure 2.1: D-loop formation and transcription-replication switch.

(Top) The D-loop is generated from a transcriptional event promoted by TFAM at the light strand promoter LSP. POLRMT and TFB2M are recruited to LSP and transcribe an RNA molecule of 200 nt until a G-quadruplex sequence blocks the process. This primer can be used by Poly γ to generate the 7S DNA that displaces the hybridization between heavy- and light-strand while mtSSB stabilizes the single stranded DNA. (Lower Left) Higher levels of POLRMT and recruitment of TEFM allow to bypass the G-quadruplex block and start genome size transcriptions from both LSP and HSP. The two large polycistronic RNA molecules are further processed in the RNA granules. (Lower Right) Lack of TEFM and recruitment of Twinkle at the TAS allow a fully functional replisome to be assembled and replicate the mtDNA accordingly to the strand-displacement model. Additional factors will complete the process by removing RNA primers, processing and ligating the ends and decatenating the two newly synthesized mtDNA molecules. Mutations in proteins indicated in *red* (black in print version) are associated with human mitochondrial pathologies.

2.3 Mitochondrial transcription and mitochondrial RNA transactions

2.3.1 Mitochondrial DNA transcription

Mitochondrial transcription starts from strand-specific promoters located in the D-loop and is carried out by the single-subunit POLRMT that is related to the RNA polymerase of T7 bacteriophage, both at the level of sequence homology and structurally. The X-ray structure of human POLRMT revealed that the homology is especially evident for both the C-terminal catalytic domain and the N-terminal promoter-binding domain [33]. The POLRMT catalytic domain resembles a hand composed of palm, fingers, and thumb domains [33]. Within the palm domain localizes the active site of the enzyme that catalyzes nucleic acid synthesis by a canonical two-metal-dependent reaction. Promoter recognition and binding are mediated by the AT-rich loop located in the N-terminal domain. A second element of the N-terminus domain, the intercalating hairpin, and the specificity loop in the C-terminus domain are involved in the formation of the transcription bubble at the promoter site [33]. However, despite POLRMT being able to bind to the promoter region in a sequence-specific manner, it is incapable of initiating transcription on its own. The presence of unique domains, including the flexible N-terminal extension and the pentatricopeptide repeat (PPR) domain, could potentially explain the requirement for transcription factors during transcription initiation.

Mitochondrial transcription consists of three steps: initiation, elongation, and termination. As for mtDNA replication, mitochondrial transcription entirely depends on nucleus-encoded transcription factors. In the last decade, numerous structural and biochemical studies have shed light onto this process, although still uncharacterized aspects remain as well as controversies mainly regarding the role of transcriptional factors [34]. According to the prevailing model (Fig. 2.2), the transcription factor A (TFAM) binds to the promoter upstream of the transcription start site and recruits POLRMT. Additional binding of the transcription factor B2 (TFB2M) to the initiation complex favors promoter melting and initiation of RNA synthesis. A transcription factor exchange leads to the incorporation of the elongation factor (TEFM), which allows POLRMT to synthesize complete transcripts. Transcription termination is mediated by the termination factor 1 (MTERF1) and, most likely additional factors yet to be identified.

Transcription of the L-strand starts at the LSP in the D-loop and generates a large polycistronic transcript encompassing eight tRNAs, and the *MTND6* mRNA. Additionally, transcription from LSP is responsible for the synthesis of the H-strand replication primer. Contrary to the well-defined LSP, transcription of the H-strand has been proposed to involve two H-strand promoters, HSP1 and HSP2, located 19 bp upstream of the *tRNA^{Phe}* gene, and 2 bp upstream of the *12S-rRNA* gene, respectively [1]. Whereas HSP1 drives the synthesis of a primary transcript encompassing the rRNAs, *tRNA^{Phe}* and *tRNA^{Val}*,

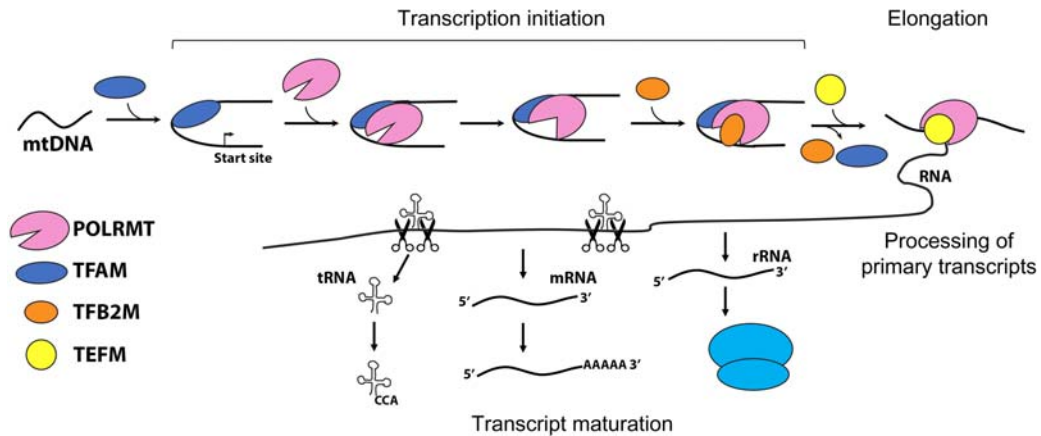


Figure 2.2: Mitochondrial transcription and transcript maturation.

(Top) Mitochondrial transcription initiates with the binding of TFAM to the promoter region upstream of the transcription start site. POLRMT is recruited by TFAM to the promoter, where it binds to a specific DNA sequence. POLRMT undergoes a conformational change accompanied by the binding of TFB2M to form the initiation complex. Exchange of initiation factors TFAM and TFB2M with the elongation factor TEFM drives the transition from transcription initiation to elongation. (Bottom) Mitochondrial transcription generates long polycistronic transcripts in which mRNA and rRNA sequences are intercalated by tRNAs. Processing of the primary transcripts at the tRNA sites liberates the messenger and ribosomal RNAs that are further modified to generate mature RNAs.

transcription from HSP2 generates a genome sized polycistronic transcript. The existence of two H-strand promoters was initially proposed to explain the higher abundance in rRNAs and how differential regulation in mRNA and rRNA expression could be achieved [35]. However, the functionality of HSP2 has been recently questioned by the observation that in *MTERF1* knockout mice no changes in RNA relative abundances were observed [36], which would be expected if *MTERF1* stimulates transcription from the HSP1, as it does *in vitro*, through DNA looping between the promoter and the *tRNA^{Leu}* LSP/HSP1 common termination site [37].

Some experimental approaches commonly used to study mitochondrial transcription are listed in [Box 2.1](#).

2.3.1.1 Transcription initiation

TFAM belongs to the high mobility group (HMG-box) domain protein family and can bind, unwind, and bend mtDNA without sequence specificity. Based on these properties, TFAM plays a crucial role in mtDNA maintenance and organization into nucleoids [38–40] (see [Chapter 1: MtDNA replication, maintenance, and nucleoid organization](#)). Additionally, TFAM is necessary for mitochondrial transcription initiation [41], an activity that requires

BOX 2.1 RNA-seq-based approaches for the study of the mitochondrial transcriptome

In *RNA-seq* or *RNA deep-sequencing* analysis, transcripts are isolated from biological samples (cultured cells or tissues) and converted into cDNA by reverse transcription. cDNAs are then shredded into fragments, which are ligated to sequencing adapters for next-generation sequencing (NGS). NGS generates a sequencing library, which is composed of a large number of reads. These reads are aligned with the genome to identify novel transcripts and quantified to determine differential gene expression. Modifications of this approach, such as *Parallel Analysis of RNA ends* (PARE), have been developed to sequence the 3' and 5' transcript ends and map RNA processing sites. Moreover, increased sequencing error can be used as a marker for nucleotide modification. RNA-seq-based approaches are also used to study RNA–protein interactions. In the case of *RNA footprinting*, all transcript regions protected from ribonuclease digestion are detected, although this approach does not identify the polypeptide bound to them. Protein-specific binding sites can be characterized by RNA-seq upon protein–RNA *UV-cross-linking and immunoprecipitation* (CLIP) of the polypeptide of interest.

its C-terminal tail [42] and binds with high affinity and in a sequence-specific manner to the D-loop region, 10–35 nt upstream of the transcription initiation site. TFAM binding to the promoter induces a stable DNA U-turn, and it recruits POLRMT to form the preinitiation or closed initiation complex [26,27,32,43,44], which has an identical topology at both L- and H-strand promoters [25]. The POLRMT N-terminal extension domain mediates its interaction with TFAM and positions the enzyme active site near to the point of transcription initiation. In particular, the POLRMT intercalating hairpin is located 4 nt upstream of the initiation site, where it separates the DNA strands [45]. Recruitment of TFB2M to POLRMT stabilizes this melting-competent conformation to form the open initiation complex [46]. TFB2M and its paralog TFB1M were initially identified based on their sequence homology with the yeast mitochondrial transcriptional activator Mtf1. The three proteins have structural similarities with rRNA methyltransferase domains [47]. However, whereas TFB1M has retained an rRNA methyltransferase function, TFB2M is an *in bona fide* transcriptional activator that facilitates promoter melting and is also required for the formation of the first RNA phosphodiester bond [48]. Recent *in vitro* and *in vivo* studies have suggested that under certain conditions, which favor DNA strand spontaneous separation, POLRMT and TFB2M could initiate transcription in the absence of TFAM [49,50]. However, the biological relevance of these observations remains to be fully elucidated.

Certainly, TFAM plays complex and diverse roles in mitochondrial biogenesis, which include regulation of mitochondrial gene expression by TFAM levels, as explained in the replication section. A high intra-mitochondrial TFAM concentration inhibits mitochondrial transcription and replication, thus favoring nucleoids packaging. On the contrary, a low

concentration of this factor stimulates transcription differentially for the different promoters, with HSP2 as the most active [51]. Thus, whereas in the presence of low TFAM amounts, expression of OXPHOS subunits is favored, at intermediate concentrations, transcription of rRNA and primers for mtDNA replication is preferred.

Of the several genes involved in mitochondrial transcription, to date, only *TFAM* has been associated with human diseases. *TFAM* pathogenic mutations have been reported in patients with progressive, fatal liver failure accompanied by mtDNA depletion [52] (see Chapter 14: Mitochondrial DNA-related diseases associated with single large-scale deletions and point mutations). Variations in TFAM steady-state levels and mtDNA content have also been reported in several neurodegenerative diseases [53].

2.3.1.2 Transcription elongation

The initiation-to-elongation transition involves the exchange of the transcription factors interacting with POLRMT. After promoter escape by POLRMT, the initiation factors TFAM and TFB2M are released, and the elongation factor TEFM is recruited to form the elongation or antitermination complex [54]. TEFM forms a homodimer through the interaction of its C-terminal domains and occupies a POLRMT-binding site overlapping with TFB2M, which explains the necessity of TFB2M release from the initiation complex for TEFM binding. The N-terminus portion of the protein contains a resolvase-like domain, homolog to bacterial Holliday-junction resolvases, which has been repurposed for mitochondrial transcription [32]. Recent structural analysis has shown that TEFM stabilizes the intercalating hairpin domain of POLRMT, separating the nascent RNA strand from the template DNA and contributes to the formation of a narrow RNA exit channel [32]. In this way, TEFM strongly promotes POLRMT processivity and allows the synthesis of near-genome-length transcripts by preventing the formation of secondary structure in the nascent RNA [29,32]. Ultimately, TEFM helps POLRMT bypassing RNA regions prone to form complex secondary structures as well as oxidative lesions, such as 8-oxo-guanosine, which may cause premature transcription termination [29]. In the absence of TEFM, transcription from the LSP is often terminated around the strong G-quadruplex-forming region CSB2, which is believed to favor mtDNA replication [9,30,55], as discussed above.

2.3.1.3 Transcription termination

At the end of each transcription cycle, POLRMT dissociates from the mtDNA. The termination site for transcription initiated at the LSP is a 22-nucleotide sequence located within the *tRNA^{Leu}* gene and recognized by the termination factor MTERF1 [56]. Indeed, the L-strand does not encode any genes beyond this point. The crystal structure of MTERF1 revealed repeated two- α -helices motifs resembling domains present in other nucleic acid-binding proteins [57,58]. The helical fold allows MTERF1 an extensive surface interaction with the major groove of the substrate DNA duplex. MTERF1 binding induces a DNA 25° bend,

duplex melting, and flipping of three nucleotides [57,58]. Base-flipping is essential for binding stability and transcription termination. It has been proposed that MTERF1 acts as a roadblock interfering with the transcription elongation complex [57,58]. This would imply that MTERF1 could arrest transcription bi-directionally [59], as it does *in vitro* at a close to 1:1 protein—DNA molar ratio [60]. However, a distinct polarity has been observed for MTERF1 activity *in vivo*: whereas MTERF1 is required to terminate L-strand transcription, it only partially terminates transcription of the H-strand [36,60]. In contrast to the well-characterized process of L-strand transcription termination, insights into the mechanism of H-strand transcription termination are currently limited.

From a physiological perspective, a role of MTERF1 in the termination of transcripts initiated at the HSP1 was proposed to explain the higher steady-state levels of rRNA compared to mRNAs. Alternatively, MTERF1 could have an indirect effect on rRNA levels since the termination of LSP transcription at the *tRNA^{Leu}* site may prevent the synthesis of antisense rRNA transcripts that could interfere with mitoribosome biogenesis [36,61]. Interestingly, several mitochondrial diseases associated mutations in the *tRNA^{Leu}* gene occur in the MTERF1 binding site. Among them, the most common mtDNA mutation, the A3243G transition, which accounts for about 80% of MELAS cases [62], and it has been shown to affect transcription *in vitro* [63]. Although the pathogenic effects of tRNA mutations are typically attributed to defective mitochondrial translation, it cannot be ruled out the possibility that alterations in transcriptional termination could contribute to the disease phenotype.

Lastly, three human MTERF1 paralogs have been identified, MTERF2–4 [64]. Their structure is similar to MTERF1, suggesting a role in nucleic acid binding [58,65–67]. However, none of them seems to play a role in transcription termination. Recent studies in mouse models indicate a role for MTERF3 and 4 in mitoribosome biogenesis [68,69], although their functions remain to be fully characterized.

2.3.2 The mitochondrial transcriptome

Although human mitochondria have evolved a small and compact genome, the corresponding transcriptome is remarkably complex. Indeed, despite their common polycistronic origin, the documented wide variation in the abundance of mature mitochondrial transcripts [70] suggests the existence of extensive posttranscriptional regulatory processes. It has been estimated that the rate of synthesis of mitochondrial mRNAs in HeLa cells is 50–100 times lower to the rate of rRNA transcription leading to a disparity in their steady-state levels ranging from 50- to 300-fold [71]. However, more recent studies based on RNA deep-sequencing approaches (see Box 2.1) in 143B cells reported an rRNA to mRNA ratio of 10:1 and substantial differences among individual mRNA abundance, with the lower levels detected for *MTND6* mRNA [70]. The observed differences may be attributed to the

technology used for the analysis. However, cell line- or tissue-specificity may also contribute to the apparent discrepancy.

Mitochondrial transcription produces three primary transcripts, which are processed into the mature coding and noncoding RNAs that constitute the mitochondrial transcriptome. Mitochondrial mRNAs lack conventional 5'- and 3'-untranslated regions and introns. Exceptions are the *MTCO1*, *MTND1*, and *MTATP8* mRNAs, which have 3 nt, 2 nt, and 1 nt, respectively, preceding the start codon, and the bicistronic transcripts *MTATP8/ATP6*, and *MTND4L/ND4*, in which the ORFs in the second position have a significant 5'- leader sequence. Mitochondrial noncoding RNAs include the ribosomal 12S and 16S-rRNA and a complete set of 22 transfer RNAs (tRNAs). Recently, long and small noncoding RNAs (lncRNAs and sRNAs) have been also identified [70]. Three lncRNAs (*lncND5*, *lncND6*, and *lncCYTB*) have been detected in mammalian mitochondria with multiple approaches [70,72]. In cell cultures, their abundance was reported to be comparable to their complementary mRNA. However, different ratios of lncRNA/mRNA were observed in human tissues, indicating that the expression of complementary coding and noncoding RNAs can be regulated independently. Moreover, lncRNAs are resistant to cleavage by single strand-specific ribo-endonucleases suggesting that they form duplexes with their complementary mRNAs and that they could play a regulatory function in mammalian mitochondrial gene expression [73]. Regarding the mitochondrial sRNAs, whereas two main classes of 21 and 26 nt respectively, mainly derived from tRNA genes, have been identified, their physiological roles remain unclear [70].

2.3.3 Mitochondrial RNA-binding proteins and RNA biology

Posttranscriptional regulation of mitochondrial gene expression is mediated by an array of RNA-binding proteins (RBPs), which are involved in all aspects of RNA biology, including transcript processing, maturation, stability, and degradation. Global mitochondrial RNA–protein interactions have been mapped by UV-cross-linking and immunoprecipitation (CLIP). An array of polypeptides, albeit unidentified, was detected, ranging from 15 to 120 kDa [74]. More recent studies based on RNase footprinting identified 88 distinct protein-binding sites, 33 of which were within mRNAs, 8 in rRNAs, 7 in tRNAs, and 40 in transcription regulatory sites and noncoding transcripts [75]. Furthermore, the RNA interactome is known to be highly dynamic and change under different physiological conditions. For example, inhibition of mitochondrial translation with chloramphenicol revealed 270 RNA sites protected from endonuclease cleavage [75]. From these, 124 protein footprints located within mitochondrial mRNAs, only 22 of which were recognized as mitochondrial ribosome stalling sites [75].

The known mitochondrial RBPs represent a diverse group of proteins, which includes members of the PPR and Fas-activated serine/threonine kinase (FASTK) families and other

unique RBPs. PPR proteins were first identified in plants, where they represent a large family of sequence-specific RBPs involved in all aspects of organellar gene expression [76]. They are defined by the presence of degenerated 35-amino acid sequences, the PPR motifs, repeated in tandem 2–30 times [77,78]. Each PPR motif folds in two antiparallel α -helices and contacts one RNA base. In tandem, multiple PPR motifs are predicted to form a solenoid structure, where the central groove is involved in the RNA binding [33,79]. To date, seven PPR proteins were identified in mammalian mitochondria: POLRMT, the leucine-rich PPR cassette (LRPPRC), the PPR domain-containing proteins 1 and 2 (PTCD1 and 2), the mitoribosome SSU proteins mS27 and mS39, and the mitochondrial RNase P protein 3 (MRPP3) [80]. Additionally, the mitochondrial FASTK family comprises six members, FASTK and its homologs FASTKD1–5 [81]. All the members of the family contain three conserved C-terminal domains, namely FAST1, FAST2, and RAP. While RAP is believed to bind RNA [82], the precise function of the FAST domains remains unknown. However, the lack of conservation in key enzymatic residues has questioned the initially proposed FASTK kinase activity [83]. Interestingly, structural modeling has suggested that FASTK proteins share a repeated α -helix motif architecture with PPR proteins [81].

Mitochondrial RBPs play fundamental roles in the regulation of mitochondrial gene expression, which can occur at the transcriptional and posttranscriptional levels, including translational and posttranslational regulation, by an array of regulatory mechanisms whose relative contributions remain to be fully elucidated.

2.3.3.1 Mitochondrial RNA processing

Transcription of the H- and L-strands generates long polycistronic transcripts that require extensive processing to produce mature RNAs. Processing involves cleavage of primary transcripts, polyadenylation, and base modification (Fig. 2.2).

In the mitochondrial genome, the rRNA and most coding sequences are separated by tRNAs. According to the tRNA punctuation model, endonucleolytic excision of the tRNAs releases ribosomal and messenger RNAs [84]. Processing of tRNAs from the primary transcripts is performed by RNaseP and the RNaseZ enzyme ELAC2 at the 5'- and 3'-end, respectively. The mitochondrial RNaseP is an entirely proteinaceous endonuclease formed by three subunits MRPP1–3. It lacks the catalytic RNA component typical of bacterial RNaseP enzymes. MRPP3 is the catalytic subunit of the complex and contains three PPR motifs and a putative metallonuclease domain. MRPP1 and 2 subunits form a subcomplex that remains bound to the RNA after MRPP3-mediate processing and could play a role in promoting 3'-end maturation by ELAC2 and/or tRNA stability. This is in line with recent findings indicating that primary transcript processing follows a hierarchical order according to which 5'-end cleavage of tRNAs precedes the 3'-end cleavage [85–87]. The existence of an order of processing also suggests that the evolutionary conserved ELAC2 enzyme could only act on smaller RNA substrates, whose complex secondary structure has been resolved by the mitochondria-specific

RNaseP complex [88]. Lastly, ELAC2 interacts with the PPR protein PTDC1, which is also involved in tRNA processing and can specifically act as a *tRNA^{Leu}* negative regulator, although its mechanism of action remains to be elucidated [3].

The tRNA punctuation model does not explain all primary transcript cleavage events, as four sites exist in which mRNAs are not flanked by tRNAs. These are the 3'-end of *MTND6* mRNA, the 5'-ends of *MTCO1* and *MTCYB* mRNAs, and the junction between *MTATP6* and *MTCO3* mRNAs. RNaseP performs the cleavage *MTCO1* mRNA 5'-end [3,89]. However, neither RNaseP nor ELAC2 are involved in the processing of the three remaining non-tRNA containing junctions. A role in the cleavage of the *MTCYB-ND5* precursor mRNA has been proposed for the PPR protein PTCD2 [90] and FASTK proteins D4 and D5 [91,92]. Moreover, several FASTK proteins, including FASTK and FASTKD1–5 participate in RNA processing at noncanonical sites and affect mRNA steady-state levels [81], although their exact mechanism of action remains to be fully understood.

Defects in mitochondrial primary transcript processing due to mutations in MRPP1 and 2, ELAC2, and FASTKD2 have been identified in patients with infantile multisystemic mitochondrial disorders (reviewed in Ref. [93]) (see Chapter 14: Mitochondrial DNA-related diseases associated with single large-scale deletions and point mutations).

2.3.3.2 Mitochondrial RNA maturation

After processing of the primary transcripts, all mRNAs, except *MTND6*, are polyadenylated by the poly(A) polymerase MTPAP, which adds an average of 45 nt at the 3'-end of the transcripts [94]. Seven mitochondrial mRNAs do not contain a stop codon, and polyadenylation is required to complete their coding sequence correctly. For these mRNAs, polyadenylation has been shown to increase their stability [95]. However, the opposite effect has been observed for some other mRNAs as a consequence of attenuated polyadenylation. Whereas this has suggested that the role of polyadenylation is mRNA-dependent, distinct pools of adenylated and nonadenylated transcripts exist in mitochondria [96,97]. MTPAP forms a homodimer with a high affinity for ATP and UTP [98,99]. However, it lacks a canonical RNA-binding domain, and it does not require RNA-binding cofactors. Thus, it is unclear how MTPAP recognizes its substrates. Spurious MTPAP polyadenylation activity on rRNA and mature tRNAs has been reported, and it is believed to be mitigated by the exonuclease PDE12 [100]. Polyadenylation is essential for mitochondrial gene expression, and mutations in MTPAP have been associated with progressive spastic ataxia and optic atrophy [101] (see Chapter 14: Mitochondrial DNA-related diseases associated with single large-scale deletions and point mutations).

Differently from mRNA maturation, mitochondrial noncoding RNAs undergo chemical nucleotide modification. Base modifications of 12S and 16S-rRNAs include 2'-*O*-ribose-methylation and pseudouridylation. A single pseudouridylated site has been identified in

position 1397 of human *16S*-rRNA. This modification is most likely catalyzed by the RPU4 Ψ synthase and is essential for mitoribosome assembly and stability [102,103]. More numerous are the known sites of rRNA methylation. Two adenines (human A936 and A937) in the *12S*-rRNA are methylated by the transcription factor TFB2M paralog, TFB1M that has retained its methyltransferase activity [104,105]. Additionally, methylation of the *12S*-rRNA cysteine 841 has been shown to be catalyzed by the NSUN4 enzyme [69]. Regarding the *16S*-rRNA, three nucleotides located in the peptidyl transferase center (PTC) are methylated by a group of closely related methyltransferase enzymes, MRM1–3 [106,107]. Lastly, the recently reported methylation of the *16S*-rRNA adenine 947 is catalyzed by the tRNA methyltransferase TRMT61B [108]. Although the exact role of rRNA methylation in mitoribosome assembly, stability, or function has not been dissected in all cases, the rRNA base modifications are ultimately necessary for efficient and accurate mitochondrial translation.

Even more extensive modifications occur during tRNA maturation. The first step in mitochondrial tRNA maturation is the addition of a CCA sequence at their 3'-end, which is not encoded in the mtDNA but is posttranscriptionally synthesized by the tRNA nucleotidyltransferase 1 enzyme TRNT1 [109]. CCA addition is required for aminoacyl-tRNA synthetase binding and amino acid loading, as well as for tRNA interaction with the translational elongation factor Tu [110]. CCA addition is followed by tRNA base modifications at numerous specific positions, which are also necessary for accurate tRNA folding and function (reviewed in Ref. [111]). Several mitochondrion-specific enzymes responsible for tRNA modification have been identified in humans and shown to be frequently associated with severe infantile mitochondrial disorders (reviewed in Refs. [93,111]). The most common base modification is pseudouridylation, which provide structural rigidity to the tRNA molecules [112]. Of particular functional relevance are the modifications of the first position of the tRNA anticodon, which generate unusual bases, also referred to as wobble bases, known to facilitate non-Watson–Crick base pairing and then the recognition of multiple codons by the same tRNA [113]. This gain in anticodon pairing ability plays a key role in mitochondrial protein synthesis, which relies on the minimal number of tRNAs for translation (the 22 tRNAs encoded by the mtDNA).

2.3.3.3 Mitochondrial RNA chaperones and mRNA stability

The stability of the mitochondrial mRNAs generated by H-strand transcription is regulated by LRPPRC, a leucine-rich PPR protein. LRPPRC interacts with the RBP SLIRP to form a complex [114], which extensively binds mRNAs throughout the transcriptome. The LRPPRC–SLIRP complex acts as an RNA chaperone [115]. By resolving mRNA secondary structures, LRPPRC–SLIRP enables transcript polyadenylation and facilitates translation [116]. Indeed, LRPPRC amounts in mitochondria correlate with mRNA

polyadenylation and steady-state levels [117,118]. Moreover, LRPPRC knockout in mice leads to a severe mitochondrial translation defect [116].

Whereas the mRNA stabilization function of LPPRC is associated with polyadenylation, the FASTK protein mediates the processing and stability of the only nonpolyadenylated transcript, the *MTND6* mRNA [91]. Both LPPRC and FASTK functions are closely associated with the RNA degradosome, which mediates RNA decay in mitochondria. The human RNA degradosome is a heterodimer formed by the 3′–5′ exoribonuclease PNPase and the NTP-dependent helicase SUV3 [119]. Lack of either degradosome complex component leads to the accumulation of RNA degradation intermediates and aberrant and polyadenylated RNA species [119,120]. Moreover, the dinucleotidase REXO2 has been shown in human mitochondria to degrade short oligonucleotides with a preference for RNA substrates [121,122]. Notably, the accumulation of dinucleotides in REXO2 mutant mice favors aberrant translation initiation from noncanonical sites [122].

A homozygous founder mutation in *LRPPRC* gene was identified in the Quebec region as one of the first nuclear gene mutations associated with mitochondrial disease. LRPPRC mutations result in the French-Canadian variant of Leigh syndrome associated with respiratory chain complex IV (or cytochrome *c* oxidase) deficiency [123] (see Chapter 14: Mitochondrial DNA-related diseases associated with single large-scale deletions and point mutations).

2.3.3.4 Mitochondrial RNA translation activators

The characteristics of mitochondrial mRNAs condition their recognition by mitoribosomes for translation initiation, a process that remains poorly understood (see section 2.4). Human mitochondrial mRNAs lack Shine/Dalgarno sequences, canonical 5′-untranslated region (5′-UTR), and a 5′-methylguanosine cap [80]. Thus mammalian mitochondrial ribosomes do not recognize the start codon using the Shine/Dalgarno interaction between the mRNA and the 16S-rRNA as in prokaryotes. Further, this system does not use a cap-binding and scanning mechanism as in the eukaryotic cytoplasm. In yeast, mRNAs do not contain Shine–Dalgarno sequences either, but they have long 5′-UTRs where mRNA-specific translational activators bind to facilitate translation initiation [124]. The existence of translation factors in human mitochondria has been a lasting object of debate. A single translational activator, TACO1 has been so far identified [125]. TACO1 has an N-terminus positively charged domain that binds to *MTCO1* mRNA specifically to promote its association with the mitochondrial small ribosomal subunit and subsequent translation [125,126]. In humans, mutations in *TACO1* have been associated with complex IV deficiency and late-onset Leigh syndrome [125,127] (see Chapter 14: Mitochondrial DNA-related diseases associated with single large-scale deletions and point mutations). The identification of TACO1 suggests that mitochondrial RBPs can act on specific mRNAs to selectively regulate their translation. In

this line, FASTK3 is also required for efficient *MTCO1* mRNA translation without affecting mRNA steady-state levels [128]. Beyond the potential existence of ternary factors, a general role in mRNA recognition has been proposed for the mitoribosome-specific protein mS39, which locates in close proximity to the mtSSU mRNA entry channel and contains nine PPR domains [129]. In a recent structural analysis of the translating mitoribosome by cryoelectron microscopy (cryo-EM), mS39 has been shown to directly interact with *MTCO3* mRNA and proposed to mediate the initial interaction between mRNAs and the mitoribosome initiation complex [130].

2.4 Mitochondrial translation

Protein synthesis is universally catalyzed by the ribosome. Within mitochondria, the mitoribosomes synthesize a small set of proteins encoded in mtDNA. The assembly and function of the mitochondrial translation machinery require the participation of both the nuclear and mitochondrial genetic systems. While the two mitochondrial ribosomal RNAs (*12S* and *16S*-rRNAs) and a full set of 22 mitochondrial tRNAs are transcribed from mtDNA, all ribosomal proteins (MRPs), mitoribosome assembly factors as well as aminoacyl-tRNA synthetases and translation factors are synthesized in the cytosol and imported into mitochondria.

The mitochondrial translation system evolved from that of the bacterial ancestor of mitochondria. As a consequence, the catalytic properties of mitochondrial and bacterial ribosomes are similar. This is evident from the fact that the proteins and RNA domains of mitochondria and bacteria that contribute to decoding and peptide bond formation share a high degree of similarity. Also, whereas mitoribosomes are not sensitive to some inhibitors of cytoplasmic ribosomes, such as cycloheximide, they are sensitive to antibiotics such as chloramphenicol. Translation factors are conserved, and several mitochondrial factors can functionally replace their homologs in bacteria [131]. Nevertheless, evolution of the mitochondrial system resulted in (1) deviations in the genetic code [132,133], (2) significant differences in the actual process of translation [134], and (3) the formation of mitochondrial ribosomes that differ significantly in structure and composition, not only in comparison to their bacterial relatives but also among different species [135,136]. A recent study has examined the composition and evolutionary history of mitoribosomes across the phylogenetic tree by combining three-dimensional structural information with a comparative analysis of the secondary structures of rRNAs and published proteomic data. This has allowed for the generation of a map of the acquisition of structural variation and reconstruction of the stages that shaped the evolution of at least the mtLSU and led to this diversity, allowing the authors to conclude that structural patching has fostered mitoribosome divergence [136].

The mitochondrial translation machinery is biomedically relevant because mitoribosomes share a sensitivity to antibiotics similar to common infectious bacteria. Also, they are

emerging as new targets for cancer therapeutics (reviewed in Ref. [137]). Additionally, mutations in mitoribosome proteins, rRNAs, and translation factors are responsible for a heterogeneous group of human multisystemic OXPHOS disorders, frequently involving sensorineural hearing loss, encephalomyopathy, and hypertrophic cardiomyopathy (reviewed in Ref. [5]).

In this section, we will briefly summarize the elements and functions of the mitochondrial translation machinery. Some experimental approaches commonly used to study mitochondrial translation are listed in [Box 2.2](#).

2.4.1 The mitochondrial translation machinery

2.4.1.1 Mitoribosome structure

The mammalian mitoribosomes were first isolated in the late 1960s/early 1970s [138–140]. They differ from bacterial (70S) and cytoplasmic ribosomes (80S) in their lower RNA:protein ratio, where significant amounts of RNA have been replaced by mitochondrion-specific proteins. The mammalian mitoribosome is a 55S RNA–protein complex, formed by a 39S large subunit (mtLSU) composed of 52 mitoribosome proteins (MRPs), a 16S-rRNA and a structural tRNA (Val in human cells), and a 28S small subunit (mtSSU) formed by 30 MRPs and a 12S-rRNA [141,142]. Approximately half of the mitochondrial ribosome proteins have bacterial homologs, although they frequently contain N- or C-terminal extensions of unclear function [135]. However, cryoelectron microscopic analyses of bovine mitochondrial ribosomes have shown that the catalytic region at the interface of both subunits is largely conserved [141,142]. The mitochondrion-specific subunits physically compensate for the loss of structural RNA, which is evident in the human and porcine mitoribosome [143–147]. These mitochondrion-specific proteins are mainly peripherally distributed over the solvent-accessible surface, forming clusters at the central protuberance, the L7/L12 stalk, and adjacent to the polypeptide exit site. Similar to the protein extensions, these proteins accommodate novel positions rather than compensate for the missing rRNA, and could have been recruited to stabilize the general structure of the ribosome [148]. Additionally, some mitochondria-specific proteins appear to be important for establishing intersubunit bridges [149], a feature that is different from cytoplasmic ribosomes that typically contain RNA–RNA intersubunit connections. A specific characteristic of the mitoribosome is the absence of a 5S-rRNA, as it had long been speculated, but the presence in the mtLSU central protuberance of *tRNA^{Val}*, which plays an integral structural role. In the mtDNA, the *tRNA^{Val}* gene is located in the middle of the genes encoding for the 12S and 16S-rRNAs. These three RNAs are transcribed together as a polycistronic transcript similar to that of the bacterial rRNA operon.

BOX 2.2 Experimental approaches for the study of mitochondrial translation

Despite multiple attempts to develop cell-free approaches to analyze mitochondrial protein synthesis, an *in vitro* mitochondrial translation system remains to be established. Methodologies currently used to study mitochondrial translation in isolated mitochondria (*in organello*) and in whole cultured cells include:

Metabolic labeling. This approach takes advantage of the different sensitivity of cytoplasmic and mitochondrial ribosomes to antibiotics to semiquantitatively study translation rates in isolated mitochondria (which always have cytoplasmic ribosomes bound to the outer membrane) or whole cells. Following inhibition of exclusively cytoplasmic protein synthesis with cycloheximide or emetine, a radiolabeled precursor, usually ^{35}S -methionine, is added and its incorporation into newly synthesized mitochondrial proteins can be assessed in a time-dependent manner.

Ribosome profiling. Originally developed for the analysis of cytosolic protein synthesis, ribosome profiling has been recently adapted for mitochondrial translation studies. The method is based on deep sequencing of mRNA fragments protected from nuclease digestion by the mitochondrial ribosomes during their translation. It provides a snapshot of all transcripts being actively translated in mitochondria at a specific time point. Additionally, it allows identifying ribosome pause sites within the transcriptome.

Cryogenic-electron microscopy (cryo-EM). For cryo-EM analysis, an aqueous solution of the molecule of interest is applied to a grid, snap-frozen, and imaged by electron microscopy. Recent advancements in EM and computer algorithms for the analysis of EM single-particle images have allowed the determination of the mitochondrial ribosome structure at near-atomic resolution. These studies have provided information regarding mitoribosome components and their arrangement as well as insights into the molecular mechanisms of mitochondrial translation.

Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC). SILAC is a metabolic labeling method that uses *in vivo* metabolic incorporation of “heavy” ^{13}C - or ^{15}N -labeled amino acids into proteins followed by mass spectrometry (MS) analysis for comprehensive identification, characterization, and quantitation of proteins. The SILAC approach has been used to study mitochondrial ribosome biogenesis. For this purpose, cells are initially cultured in the presence of “light” (regular) ^{12}C - or ^{14}N -containing amino acids, followed by transfer in media containing “heavy” amino acids for increasing time periods. Quantitative isotope incorporation into a polypeptide is detected by MS and provides a time-course of protein assembly into multimeric macro-molecules, such as the mitoribosome.

The recent reconstructions of the 55S human [141,150,151] and porcine [142,152,153] mitoribosome at high resolution by cryo-EM (see Box 2.2), have allowed distinguishing important features in the mammalian mtSSU. Similar to the mtLSU, several peripheral rRNA helices present in bacteria are either truncated or missing in the 12S-rRNA, but the tertiary structure of the 12S-rRNA core and the overall positions of the MRPs with bacteria

homologs are preserved in the structure. A significant structural remodeling is observed at the mRNA entrance of the mtSSU compared to the bacterial SSU. This remodeling serves to accommodate mammalian mitochondrial mRNAs, which have either null or very short 5'-UTRs [154], as mentioned earlier.

An additional major remodeling is observed at the A (aminoacyl) and P (peptidyl) tRNA binding sites, where some subunits present in bacterial ribosomes (e.g., uL5 at the P-site, bL25 and the A-site) have been lost in order to accommodate human tRNAs, which contain highly variable loops at the tRNA elbow. However, the P-site finger, unique to the mammalian ribosome, compensates for these missing interactions. A distinctive property of mitoribosomes is the acquisition of an intrinsic GTPase activity through the GTP-binding protein, mS29 of the 28S mtSSU subunit [141,142,155]. The GTPase activity is probably linked to subunit association since mS29 locates at the subunit interface and is involved in coordinating two mitochondria-specific bridges.

The polypeptide exit tunnel is adapted to the transit of hydrophobic nascent peptides [151,153]. The tunnel exit site consists of conserved proteins from bacteria, namely bL23, bL29, bL22, bL24, and bL17, which create a ring around the exit site. Moreover, this conserved core is surrounded by a second layer of protein density, consisting of bL33 and mL45, which promote anchoring of the mtLSU to the inner mitochondrial membrane [151,153]. The structure of the human mitoribosome with nascent polypeptide shows its extensive interactions with specific hydrophobic residues of the tunnel wall [141]. Membrane anchoring aligns the polypeptide exit site with the OXA1L translocon to facilitate cotranslational membrane insertion of newly synthesized proteins. The overall tunnel path is similar to that of bacterial and cytoplasmic ribosomes but different from yeast mitoribosomes.

Mitoribosomes are bound to the inner membrane, presumably as a result of their specialization on the synthesis of hydrophobic membrane proteins, which are cotranslationally inserted into the inner membrane [146,156]. Several inner membrane proteins mediate membrane binding of ribosomes such as the OXA1L machinery, which facilitates the insertion of nascent polypeptides into the inner membrane [157–163].

2.4.1.2 Mitoribosome biogenesis

The process of mitoribosome assembly requires the assistance of a growing number of non-ribosomal proteins. These proteins include RNA modification enzymes, guanosine triphosphatases (GTPases), DEAD-box RNA helicases, and kinases [164,165]. They act as assembly factors to guide the processing and modification of mitoribosomal components and their temporal association to form preribosomal particles during the assembly of individual subunits, and formation of the monosome. Mitoribosome biogenesis follows a maturation pathway that is just starting to emerge and involves the cooperative assembly of

protein sets, forming structural clusters and preassembled modules [166,167]. SILAC (Stable Isotope Labeling with Amino Acids in Cell Culture)-based metabolic labeling followed by proteomics studies (see Box 2.2) have shown that for each subunit, the protein components are synthesized in excess and imported into mitochondria, where their stoichiometric accumulation is regulated by degradation of the nonassembled free protein fractions [167]. The biogenesis of the two mitoribosome subunits is coordinated. It starts cotranscriptionally with mtLSU proteins forming a subcomplex on an unprocessed RNA containing the *16S rRNA*, whose formation is required for precursor RNA processing and liberation of the *12S-rRNA* as a condition for mtSSU protein incorporation [85]. Recent investigations have revealed quality control mechanisms that are in place to ensure that only the mature mtSSU and mtLSU are assembled into functional monosomes [69,165,168,169]. Although the number of mitoribosome assembly factors identified is increasing steadily, their specific functions and the molecular details of mitoribosomal biogenesis remain, in most cases, to be fully understood. A recent manuscript has reported the structures of two late-stage assembly intermediates of the human mtLSU isolated from a native pool within a human cell line and solved by cryo-EM. A comparison of the structures allowed for the identification of new assembly factors and to reveal insights into the timing of rRNA folding and protein incorporation during the final steps of mtLSU maturation [150].

2.4.2 Mitochondrial protein synthesis

2.4.2.1 Mitochondrial translation initiation

The overall steps of protein synthesis in mammalian mitochondrial ribosomes are similar to those in bacteria. However, mitochondrial translation differs substantially from bacterial or cytosolic translation systems, particularly at the initiation step. Key differences are seen in the interaction of the mitoribosome with the mRNAs, the tRNAs, and the mitochondrial translational factors. An important difference involves the lack of 5' leader sequences by most mitochondrial mRNAs to promote their binding to the ribosome through the potential action of translation activators as it has been shown in yeast mitochondria [170]. Moreover, mitochondria contain a single *tRNA^{Met}* that fulfills the dual role of the initiator and elongator *tRNA^{Met}*. A fraction of met-*tRNA^{Met}* is formylated by mitochondrial methionyl-tRNA formyltransferase (MTFMT) to generate *N*-formylmethionine-*tRNA^{Met}* (fMet-*tRNA^{Met}*), which is used for translation initiation [171,172]. The differences are also contributed by the fact that mitochondrial translation initiation lacks initiation factor 1, which is essential in all other translation systems [173]. Whereas translation initiation in bacteria involves three essential canonical initiation factors IF1, IF2, and IF3, mammalian mitochondria have homologs of the latter two, namely mtIF2 and mtIF3 [174,175].

Mitochondrial translation starts on an mtIF3-bound mtSSU that would then recruit the mt-mRNA (Fig. 2.3). Mammalian mtIF3 retains only ~25% sequence homology with respect to its bacterial counterparts [175], although its basic domain organization, comprising N- and C-terminal domains that are connected through a flexible linker, is similar to bacterial IF3. However, mtIF3 has ~30-amino-acid-long N- and C-terminal extensions (NTE and CTE), which are important for the destabilization of incorrect initiation complexes (the CTE) or to control the affinity of IF3mt for the 39S subunit (the NTE) [175–178]. A cryo-EM structural characterization of the mtSSU–mtIF3 complex has shown that the N-terminal domain of mtIF3 interacts with more mtSSU elements than its bacterial counterpart, thus enhancing its affinity for the mtSSU [178]. The mtIF3-C-terminal domain sits on the mtSSU in the vicinity of the P-site, and it interacts with several 12S-rRNA helices. Its position would be expected to prevent the joining of the 39S subunit with the 28S subunit by directly interfering with the formation of two of the conserved intersubunit bridges [178]. It has been proposed that in this way, mtIF3 prevents the premature joining of the mtLSU until a proper preinitiation complex composed of IF2mt, mRNA, and initiator tRNA has been formed [178], a mechanism conserved in the bacterial system. How mtIF3 detects the presence of a bound mRNA and prevents the accommodation of the initiator tRNA in the absence of the mRNA remains to be fully understood. However, superimposition of the mtSSU–mtIF3 structure with the structure of the 55S-fMet-tRNA^{Met}-mtIF2 complex [130] have allowed to interpret the role of the C-terminal domain and its mitochondrion-specific

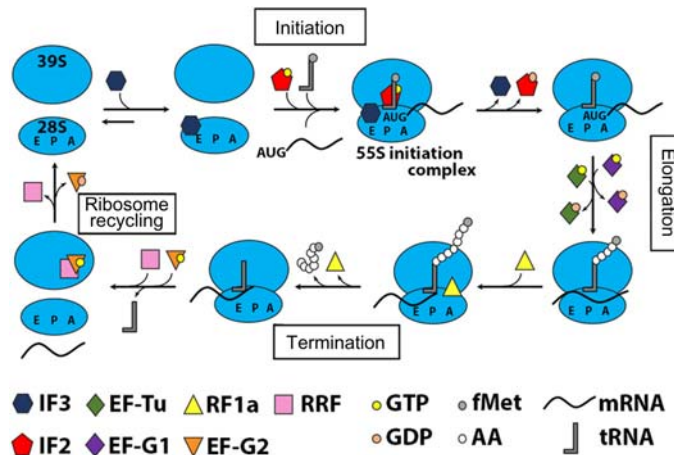


Figure 2.3: Mitochondrial translation in mitoribosomes.

Schematic representation of the four stages of protein synthesis, and the basic translation factors involved. Initiation involves mtIF2 and mtIF3; elongation is catalyzed by mtEF-Tu, mtEF-Ts, and mtEF-G1; termination is facilitated by the release factor mtRF1a, and ribosome recycling requires mtRRF and mtEF-G2. See a detailed explanation in the text.

CTE in destabilizing the initiator tRNA in the absence of mRNA, and to highlight the potential role of several lysine residues on the mtIF3-C-terminal domain [178].

Following the recruitment of the mt-mRNA to the mtIF3-bound mtSSU, GTP-bound IF2mt stimulates the binding of fMet-*tRNA*^{Met} to the mtSSU [174]. Although charged *tRNA*^{Met} and mtIF2 can bind the mtSSU in the absence of the mt-mRNA; the association is weak [172]. When a positive codon:anticodon interaction occurs, a stable complex is formed and triggers the recruitment of the mtLSU. A recent structure determination by cryo-EM of the complete translation initiation complex has revealed key features of mammalian mitochondrial translation initiation [130]. The study identified unique features of mtIF2 that are required for specific recognition of fMet-*tRNA*^{Met} and regulation of its GTPase activity. Mitochondrial IF2 contains a mitochondrion-specific 37-amino-acid-long domain insertion that has been proposed to mimic the function IF1 plays in bacteria by sterically blocking the binding of initiator tRNA to the ribosomal A site [130]. Although mtIF2 does not form specific interactions with mRNA, by closing the decoding center, it may help to stabilize the binding of leaderless mRNAs. The cryo-EM structure showed *MTCO3* mRNA to be engaged with the PPR protein mS39, which is found at the entrance of the mt-mRNA channel. As mentioned earlier, this interaction may facilitate subsequent threading of the mRNA into the mRNA channel for start codon–anticodon interaction. The mRNA-mS39 contacts are not expected to be sequence- or even structure-specific, but it has been noted that starting at codon #7 the mt-mRNAs frequently show U-rich sequences that may be the determinant for PPR association and promote initial binding of the mitochondrial mRNAs to the initiation complex [130]. The mRNA channel is lined by a positively charged mitochondrion-specific extension of uS5m positioned between the entrance and the A site and has been proposed to guide the mRNA toward the P-site, where the codon–anticodon interaction fixes the initiation codon and stabilizes the mRNA binding in frame [130].

Recruitment of the mtLSU triggers hydrolysis of the mtIF2-bound GTP to GDP concomitantly with the release of both mtIF2 and mtIF3 from the mtSSU. If f-Met-*tRNA*^{Met} is not available or if the start codon is not present in the P-site, the inspection by the initiation factors fails, the monosome is not formed, and the mRNA is released.

2.4.2.2 Mitochondrial translation elongation

Once the monosome is formed, elongation of the nascent chain proceeds by cycles of aminoacyl-tRNAs binding, peptide bond formation, and displacement of deacylated tRNAs (Fig. 2.3). The main factor involved in this step is the mitochondrial elongation factor Tu (mtEF-Tu), a highly conserved GTPase, which, in its GTP-bound activated form, delivers the aminoacyl-tRNAs to the mitoribosome A site through the formation of a ternary complex. This process uses the energy released from GTP hydrolysis, which yields an inactive GDP-bound mtEF-Tu that is then released from the ribosome and is regenerated by the

elongation factor Ts (mtEF-Ts), a nucleotide exchange factor that promotes the exchange of GDP with GTP [179].

The release of mtEF-Tu leads to the formation of the peptide bond, catalyzed at the mtLSU PTC. Once the bond is formed, the mitoribosome P-site is occupied by a deacylated mt-tRNA, and the dipeptidyl-tRNA is found in the A-site. Mitochondria possess two elongation factor Gs, one of which, mtEF-G1 acts during elongation by catalyzing both the translocation of dipeptidyl-tRNA from the A site to the P-site and the movement of the mRNA to expose the next codon in the A site. During translocation, the rate-limiting step for tRNA-mRNA movement is a rearrangement of the ribosome (so-called unlocking), which is induced by the binding of GTP-bound mtEF-G1 to the ribosome and accelerated by GTP hydrolysis and consequent release of GDP-bound mtEF-G1 from the mitoribosome [180]. The cryo-EM structures of the human and porcine mitoribosomes have recently confirmed the presence of the E-site [141,142], and despite its differences from the characteristic bacterial structure, the deacylated mt-tRNA moves to the E-site before exiting the mitoribosome. The translation elongation process is repeated by multiple cycles until a stop codon is positioned in the A-site.

2.4.2.3 Mitochondrial translation termination and mitoribosome recycling

When the translation machinery encounters a stop codon, protein synthesis is complete, and the termination codon is recognized by a ribosome release factor (mtRF1a) that in the presence of GTP induces the release of the newly formed polypeptide from the mtLSU [181] through a conserved mechanism [182] (Fig. 2.3). Although the mtRF1a is believed to be sufficient to terminate all 13 ORFs, this factor is a class I RF, which specifically recognizes the codons UAA and UAG. In human mitochondria, UAA and UAG are used as stop codons to terminate nine monocistronic and two bicistronic ORFs, respectively. However, although the triplets following the coding sequence of ORFs *MTCO1* and *MTND6* are AGA and AGG, respectively, the termination codons of transcripts positioned at the A-site of the human mitoribosome in intact cells were found to terminate with the classical UAG codon. The mechanism involves a -1 frameshift, potentially driven by structured RNA immediately downstream of the termination codons within the transcripts [154,183]. This mechanism is plausible for humans, but would not work in all vertebrates, which has suggested that other release factors might be involved in terminating the translation of these two mt-mRNAs, perhaps in other mammalian species [184,185].

Following the action of the release factor, two ribosomal recycling factors, mtRRF1 [186], and the second mitochondrial member of the elongation factor G family, mtEF-G2 [187], promote the disruption of the mitoribosome posttermination complex by enabling the dissociation of the ribosomal subunits and the release of mt-mRNA and deacylated mt-tRNA (Fig. 2.3). The binding of GTP-bound mtEF-G2 to the mtRRF1-bound mitoribosome post-termination complex dissociates the ribosome into its two subunits, and at least the release

of mtEF-G2 from the mtLSU requires the hydrolysis of GTP [187]. A recent cryo-EM structure of the human 55S mitoribosome—mtRRF1 complex has revealed that a mitochondrion-specific N-terminal extension of the recycling factor makes multiple mitochondrion-specific interactions with functionally critical regions of the mitoribosome, including rRNA segments that form the PTC and those that connect PTC with the mtLSU GTPase-associated center [188]. The interactions of mtRRF1 with the mitoribosome have been proposed to ensure complete inaccessibility to tRNAs and other ligands to both PTC and the entrance of the polypeptide exit tunnel of the mitoribosome [188].

Once the mtRRF1 and mtEF-G2 are finally released from the mtLSU, the translation cycle can reinitiate.

2.4.2.4 Cotranslational membrane insertion of newly synthesized polypeptides

Mitoribosomes are bound to the inner membrane, presumably as a result of their specialization on the synthesis of hydrophobic membrane proteins, which are cotranslationally inserted into the inner membrane. Visualization of human mitoribosomes studied *in situ* by cryoelectron tomography (cryo-ET) has revealed a single major contact site of the mtLSU with the inner mitochondrial membrane, mediated by the mitochondria-specific protein mL45 [189]. Further cryo-EM studies showed that mL45 sits at the exit of the polypeptide exit tunnel at the interface of the mitoribosome and the mitochondrial inner membrane [130,151,153]. A central domain in mL45 shows homology to the carboxy-terminal domain, membrane-binding segment, of TIM44, a subunit of the inner membrane translocase essential for protein import [190]. Because TIM44 is known to establish interactions with cardiolipin, the signature lipid of the mitochondrial inner membrane, it is possible that mL45 could also mediate cardiolipin-mediated tethering of the mitoribosome to the inner membrane in an orientation that facilitates cotranslational membrane insertion of newly synthesized polypeptides as they exit the tunnel. Although the precise mechanism involved remains to be understood, cryo-EM analyses have shown that the N-terminal tail of mL45 (amino acids K38–N64) inserts into the exit tunnel and occludes it during translation initiation [130]. Considering that the N-terminal extension of mL45 must be displaced from the tunnel during translation elongation, it has been hypothesized that the mL45 N-tail could facilitate membrane insertion of nascent chains by recruiting the OXA1L translocon machinery.

2.5 Compartmentalization of gene expression

An important aspect of mitochondrial gene expression relates to the compartmentalization of the processes involved. At least three distinct types of foci relevant to mtDNA expression have been identified and visualized within the mitochondrial matrix of human cells. Those are the mitochondrial nucleoids, RNA granules, and the RNA degradosome [119].

2.5.1 Mitochondrial DNA nucleoids

As explained in [Chapter 1](#), MtDNA replication, maintenance, and nucleoid organization, eukaryotic cells typically contain hundreds to thousands of mtDNA genomes assembled into hundreds of dense proteinaceous structures known as nucleoids [\[191,192\]](#). This organization in nucleoids provides stability to the mitochondrial genomes and is essential for their inheritance and segregation [\[193\]](#).

Early quantitative analyses of the size and mtDNA content of nucleoids in cultured mammalian cells suggested that an average nucleoid could contain 5–7 mtDNA genomes packed in a space with a diameter of only 70 nm [\[194\]](#). However, recent superresolution microscopy data have revealed that mammalian mitochondrial nucleoids have a uniform size of ~100 nm and contain only 1–2 copies of mtDNA [\[195\]](#). The packaging of mammalian mtDNA in nucleoids is accomplished with the assistance of DNA-binding proteins, including two particularly abundant proteins, essential for mtDNA replication: TFAM and mtSSB [\[38,192\]](#). Studies in *Xenopus* oocytes have shown that the ratio of TFAM per mtDNA is developmentally regulated and inversely correlates with the number of mtDNA molecules that are engaged in replication or transcription [\[196\]](#). Nucleoid purification approaches followed by mass spectrometry to analyze their protein composition, revealed a large variety of proteins involved in all aspects of mitochondrial gene expression [\[192,197,198\]](#). The use of formaldehyde cross-linking allowed determining the nucleoid proteins that are in close contact with the mtDNA, and proposing a model for a layered structure of mtDNA nucleoids. In this model, replication and transcription occur in the central core, whereas RNA processing, translation, and OXPHOS complex assembly may occur in the peripheral region [\[192\]](#), which transitions to a different compartment known as the RNA granule.

2.5.2 Mitochondrial RNA granules

As a breakthrough in the compartmentalization of mtDNA expression field, analysis of newly transcribed RNA in human mitochondria from cultured cells by bromouridine (BrU) labeling identified distinct BrU-positive RNA foci in close proximity to the nucleoids [\[194\]](#). These foci, termed RNA granules, contain the protein GRSF1 (G-rich sequence binding factor 1) and RNase P and accumulate mt-RNAs to regulate their processing, storage, sorting, or translation [\[199,200\]](#). These foci also contain ribosomal proteins [\[200\]](#) and ribosomal RNA modifying enzymes [\[201\]](#). Furthermore, GRSF1 depletion induces a defect in the assembly of the mitoribosomal SSU and a marked attenuation in mitochondrial protein synthesis [\[199,200\]](#).

This has raised the possibility that ribosome biogenesis could occur near or within the RNA granules. Affinity purification of tagged GRSF1 identified ribosomal proteins [\[200\]](#) and the 16S-rRNA methyltransferases MRM1, MRM2, and MRM3/RMTL1 [\[201\]](#). Affinity

purification of tagged MRM3 coeluted ribosomal proteins and ribosome assembly factors that colocalize to the RNA granules, such as GRSF1 and the DEAD-box helicase DDX28 [201,202]. Recent comprehensive affinity purification studies of GRSF1 and DDX28, followed by mass spectrometry analysis by two independent groups, have yielded similar results [92,203]. In the study performed by our group, the DDX28 interactome was analyzed in extracts prepared in the presence of increasing salt concentrations to assess the strength of the interactions. DDX28 was found associated with five major groups of relevant proteins [165,203]. (1) Mitochondrial proteins; (2) mitochondrial assembly factors; (3) mitochondrial translational factors; (4) RNA metabolism factors, including transcription factors, RNA processing and modifying enzymes, and RNA stability and degradosome proteins; and (5) proteins previously identified as mtDNA nucleoid components, such as the helicase DHX30 that acts as a transcriptional regulator.

The overlapping pool of proteins associated with either mitochondrial nucleoids or RNA granules reflects their proximity and the lack of membranous boundaries sealing these compartments [119]. Their functional and physical dynamics to attend the necessities of newly transcribed RNAs is highlighted by the observation by fluorescence microscopy that some 10%–15% of nucleoids and RNA granules do actually overlap in most studies, probably reflecting sites of active transcription [119,194,199,200]. A quantitative assessment of these dynamics showed that after an initial short pulse of 20 minutes, most BrU-positive RNA foci colocalize within 200 nm of mtDNA, but after more extended periods (or a pulse-chase) the Br-positive RNA foci become distributed randomly [200]. Thus mitochondrial assembly could initiate in granule-nucleoid overlapping foci [167,204], possibly in a cotranscriptional manner as it occurs in bacteria [205], to end within the granule environment [92,165,203,206], or even outside the granule, as it has been recently suggested [207].

As yet another connection between mtDNA nucleoids and RNA granules, two mtDNA replication factors, Twinkle and mtSSB, have been found to participate in granule formation and mtRNA processing/degradation in an interplay with GRSF1 [207], which highlights the role of at least these proteins in multiple aspects of mtDNA gene expression.

2.5.3 Mitochondrial RNA degradosome

The RNA granules occasionally colocalize with RNA breakdown complexes termed RNA degradosomes (the PNPase-SUV3 helicase complex) formed only in specific foci (the D-foci) [119].

RNA granules and D-foci are intimately connected not only physically, but also functionally. For example, a translational variant of FASTK colocalizes with mitochondrial RNA granules and is required to regulate specifically the expression of the

MTND6 mRNA, the only protein-coding sequence located on the L-strand transcript. Mechanistically, FASTK binds the L-strand precursor RNA at multiple sites both within and downstream of the *ND6*-coding sequence and, together with the mitochondrial degradosome, participates in generating the mature form of the *MTND6* mRNA [91]. In another example, GRSF1 has been shown to cooperate with the mitochondrial degradosome in the degradation of mitochondrial long noncoding lncRNAs that contain G-quadruplexes (G4s), which are prone to be formed due to the GC skew in the mtDNA. GRSF1 promotes G4 melting to facilitate degradosome-mediated decay [208]. Furthermore, whereas the lncRNAs are most prominently derived from the L-strand surrounding the *ND6* gene, it has been shown that the mt-RNA patterns for a double mtSSB/GRSF1 knockdown resemble those of degradosome-deficient mitochondria [207]. These observations have raised the hypothesis that mtSSB could participate in the degradation of G4-containing RNAs by binding unfolded RNAs following GRSF1 action and prevent them from reforming G-quadruplexes [207].

Research perspectives

Over the last 50 years, dramatic progress has been made in the understanding of mitochondrial replication, transcription, and translation systems, and the mechanisms and factors involved in each process. The recent development of high-resolution cryo-EM methods, in combination with novel proteomics and classical genetic and biochemical procedures, and mouse and human cell gene-editing approaches is speeding the understanding of mitochondrial gene expression at the molecular, cellular, and organismal level.

Several essential questions that are still open are anticipated to be the focus of future investigations over the next few years. Potential open questions, scattered through the text, and future aims for the field include: disclosing the determinants of mtDNA copy number control across different cell types; devising approaches to edit the mtDNA; fully understanding the switch between replication and transcription; dissect the mechanism of transcription termination; disclosing how are processed all the non-tRNA containing junctions in polycistronic transcripts; developing an *in vitro* mitochondrial translation system; performing mitoribosome structural studies capturing the simultaneous presence of mRNA and the two translation initiation factors to understand better the mechanism of mitochondrial translation initiation, or capturing the presence of the two factors involved in recycling to disclose their interplay in resetting the translation machinery; identifying all mitoribosome assembly factors and their specific roles; or better characterizing the functional overlaps among the components of the nucleoid the RNA granule and the degradosome.

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BMB 726
Basis of mitochondrial medicine

Basic Course Information

Instructor: Dr. Flavia Fontanesi

Department of Biochemistry and Molecular Biology

Office: UM Medical Campus 1420 NW 9th Avenue, NRB #103B

Phone: 305-2437215

E-mail: ffontanesi@med.miami.edu

Office hours: By appointment

Teaching Assistant: Dr. Alba Timon Gomez, Department of Neurology

E-mail: axt809@med.miami.edu

Dr. Eva Nyvltova, Department of Neurology

E-mail: eva.nyvltova@med.miami.edu

Class meeting time and location:

Lectures: Tuesday 2.30 – 4.00 pm. RMSB #6018.

Small group discussion: Thursday 2.30 – 4.00 pm (groups A and B alternate weeks).

Credits: 2 credits

Course Overview

Overall, the course aims to integrate basic knowledge in mitochondrial structure and function with our current understanding of the role mitochondria play in human health and disease. The first part of the course provides a core introduction to the major aspects of mitochondrial biology, including mitochondrial metabolism, genetics and biogenesis. The second part of the course focus on the role of mitochondrial and metabolic dysfunction in specific human disorders.

Course Learning Outcomes

Students should be able to:

- Summarize basic concepts in mitochondrial cell biology, genetics and biochemistry.
- Understand causes and effects of mitochondrial dysfunction in an array of human pathologies.
- Critically read scientific literature and discuss research findings.
- Explaining differential diagnosis in primary mitochondrial disorders and discuss mitochondrial case studies.
- Understand the principles and key steps of basic experimental protocols for mitochondria isolation cells and analysis of respiratory rates.

Course Format

13-week course including one 1.5-hour lecture and one 1.5-hour small group discussion per week. The small group classes will include primary literature and case study discussion.

Assessments and Evaluation

Grading will be based on two multiple choice tests, participation in the small group and paper discussions.

Tentative Course Schedule and Topics

Week 1 - January 25 - 27

Class 1: Introduction to mitochondria structure, dynamics and inter-organellar interactions.

Small group A: Research paper 1 discussion.

Week 2 – February 1 - 3

Class 2: Mitochondrial genome and human mitochondrial genetics.

Small group B: Research paper 1 discussion.

Week 3 – February 8 - 10

Class 3: Mitochondrial biogenesis and gene expression.

Small group A: Research paper 2 discussion

Week 4 – February 15 – 17

Class 4: Mitochondrial OXPHOS function and biogenesis.

Small group B: Research paper 2 discussion

Week 5 – February 22 - 24

Class 5: Mitochondrial OXPHOS function and biogenesis.

Small group A: Research paper 3 discussion

Week 6 – March 1 - 3

Class 6: The multiple functions of mitochondria, a metabolic hub.

Small group B: Research paper 3 discussion

Week 7 – March 8 - 10

Class 7: Primary mitochondrial disorders.

Small groups A and B: A physician perspective on primary mitochondrial disorders. Guest presenter: Dr. Kumarie Latchman, Assistant Clinical Professor of Clinical and Translational Genetics.

Spring recess March 12-20

Week 8 – March 22 – 24

Optional Lab experience – Mitochondria isolation and bioenergetics measurements
Test 1 – March 24

Week 9 – March 29 – 31

Class 8: Therapeutic approaches in mitochondrial medicine.

Small group A: Approaches to prevent maternal transmission of mitochondrial diseases, technical and ethical discussion.

Week 10 – April 5 – 7

Class 9: Mitochondrial dysfunction and its role in metabolic and cardiovascular diseases.

Small group B: Approaches to prevent maternal transmission of mitochondrial diseases, technical and ethical discussion.

Week 11 – April 12 – 14

Class 10: Mitochondria and cancer.

Small group A: Case study of a primary mitochondrial disorders.

Week 12 – April 19 – 21

Class 11: Mitochondrial dysfunction and neurological disorders. Guest lecturer: Dr. Antoni Barrientos, Professor of Neurology.

Small group B: Case study of a primary mitochondrial disorders.

Week 13 – April 26 – 28

Class 12: Mitochondria and aging. Guest lecturer: Dr. Antoni Barrientos, Professor of Neurology.

Test 2 – April 28