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CHARACTERIZATION OF GTPBPs INVOLVED IN RIBOSOME ASSEMBLY AND FUNCTION IN HUMAN MITOCHONDRIA

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Karolinska Institutet

Stockholm 2023

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ISBN 978-91-8016-879-3

Characterization of GTPBPs involved in ribosome assembly and function in human mitochondria

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Nils Ringertz Biomedicum, Karolinska Institutet, Solnavägen 9, February 20 2023 at 13:00

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Abstract

Mitochondria are organelles often referred to as the powerhouses of the cell as they provide most of the chemical energy via aerobic respiration. However, in the last half a century, it has become clear that their function extends to other fundamental metabolic tasks. These fascinating organelles have their own DNA and protein synthesis machinery, the mitochondrial ribosome (mitoribosome), the latter made of a set of mitoribosomal proteins (MRPs) and rRNA that all together build a 2.7 MDa complex. The mitoribosome translates for 13 polypeptides which are later incorporated in the respiratory chain. Therefore, it is not surprising that mutations in MRPs or auxiliary factors involved in its assembly can lead to multisystemic human disorders. Here lies the importance of studying the molecular mechanisms of the mitoribosomal assembly process, which has been the focus of my PhD studies. Guanosine triphosphate binding proteins (GTPBPs) involved in ribosome biogenesis in bacteria have extensively been studied and have provided key knowledge for the understanding of the role of their human mitochondrial homologues identified so far. In my work, I have addressed the role of human mitochondrial proteins GTPBP5, GTPBP10 and GTPBP8 and their possible involvement in mitochondrial ribosome biogenesis. In **paper I**, we have biochemically characterised GTPBP5 by assessing its interactome and the effects of its depletion on mitochondrial functionality, showing the importance of this protein as an assembly factor. These data have later been confirmed in **paper II**, where we have structurally determined in more details the function of GTPBP5 as well as several other late-stage mitoribosomal assembly factors. GTPBP10 involvement in the assembly process has been determined biochemically in **paper III**, where we also co-immunoprecipitated GTPBP10 with the mitoribosome *in vivo* using a knock-in mouse model. Additionally, preliminary results discussed in this thesis regard the investigation of GTPBP8 function in mitochondrial gene expression, demonstrating its significance for mitochondrial viability.

List of scientific papers

I. **Miriam Cipullo**, Sarah F. Pearce, Isabel G. Lopez Sanchez, Shreekara Gopalakrishna, Annika Krüger, Florian Schober, Jakob D. Busch, Xinping Li, Anna Wredenberg, Ilian Atanassov, Joanna Rorbach[†] (2021). Human GTPBP5 is involved in the late stage of mitoribosome large subunit assembly. *Nucleic Acids Research*, 49(1):354-370.

II. **Miriam Cipullo**[*], Genís Valentín Gesé[*], Anas Khawaja, B. Martin Hällberg[†], Joanna Rorbach[†] (2021). Structural basis for late maturation steps of the human mitoribosomal large subunit. *Nature Communications*, 12(1):3673.

III. Jakob D. Busch, **Miriam Cipullo**, Ilian Atanassov, Ana Bratic, Eduardo Silva Ramos, Thomas Schöndorf, Xinping Li, Sarah F Pearce, Dusanka Milenkovic, Joanna Rorbach[†], Nils-Göran Larsson[†] (2019). MitoRibo-Tag Mice Provide a Tool for In Vivo Studies of Mitoribosome Composition. *Cell Reports*, 29(6):1728-1738.

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List of scientific papers not included in the thesis

I. Shreekara Gopalakrishna[*], Sarah F. Pearce[*], Adam M. Dinan, Florian A. Schober, **Miriam Cipullo**, Henrik Spåhr, Anas Khawaja, Camilla Maffezzini, Christoph Freyer, Anna Wredenberg, Ilian Atanassov, Andrew E. Firth, Joanna Rorbach[†] (2019). C6orf203 is an RNA-binding protein involved in mitochondrial protein synthesis. *Nucleic Acids Research*, 47(17):9386-9399.

II. Yao Shi, Juan Yuan, Vilma Rraklli, Eva Maxymovitz, **Miriam Cipullo**, Mingzhi Liu, Shuijie Li, Isabelle Westerlund, Oscar C. Bedoya-Reina, Petra Bullova, Joanna Rorbach, C. Christofer Juhlin, Adam Stenman, Catharina Larsson, Per Kogner, Maureen J. O'Sullivan, Susanne Schlisio and Johan Holmberg[†] (2021). Aberrant splicing in neuroblastoma generates RNA-fusion transcripts and provides vulnerability to spliceosome inhibitors. *Nucleic Acids Research*, 49(5):2509-2521.

III. Yuzuru Itoh[*], Anas Khawaja[*], Ivan Laptev, **Miriam Cipullo**, Ilian Atanassov, Petr Sergiev, Joanna Rorbach†, Alexey Amunts[†] (2022). Mechanism of mitoribosomal small subunit biogenesis and preinitiation. *Nature*, 606(7914):603-608.

IV. Cristina Remes[*], Anas Khawaja[*], Sarah F. Pearce, Adam M. Dinan, Shreekara Gopalakrishna, **Miriam Cipullo**, Vasileios Kyriakidis, Jingdian Zhang, Xaquin Castro Dopico, Olessya Yukhnovets, Ilian Atanassov, Andrew E. Firth, Barry Cooperman, Joanna Rorbach[†] (2023). Translation initiation of leaderless and polycistronic transcripts in mammalian mitochondria. *Nucleic Acids Research*, Online ahead of print.

Review and book chapter:

I. Isabel G. Lopez Sanchez[†], **Miriam Cipullo**, Shreekara Gopalakrishna, Anas Khawaja, Joanna Rorbach[†] (2020). Methylation of Ribosomal RNA: A Mitochondrial Perspective. *Frontiers in Genetics*, 17;11:761.

II. Sarah F. Pearce, **Miriam Cipullo**, Betty Chung, Ian Brierley, Joanna Rorbach[†] (2021). Mitoribosome Profiling from Human Cell Culture: A High Resolution View of Mitochondrial Translation. *Methods in Molecular Biology*, 2192:183-196.

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List of abbreviations

aa-tRNA	Aminoacyl tRNA
ATP	Adenosine triphosphate
BioID	Proximity-dependent biotin identification
CP	Central protuberance
cryo-EM	Cryogenic electron microscopy
cryoET	Cryoelectron tomography
CTF	Contrast transfer function
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
FRT	Flp Recombination Target
GAC	GTPase-associated centre
GTPBP	Guanosine triphosphate binding protein
HEK293T	Human embryonic kidney 293
HOS	143B human osteosarcoma
IMM	Inner mitochondrial membrane
MALSU1	Mitochondrial assembly of ribosomal large subunit 1
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MRGs	Mitochondrial RNA granules
MRM1	Mitochondrial rRNA methyltransferase 1
MRM2	Mitochondrial rRNA methyltransferase 2
MRM3	Mitochondrial rRNA methyltransferase 3
MRP	Mitoribosomal protein
mt-ACP	Mitochondrial acyl carrier protein
mtEFG1	Mitochondrial elongation factor G1
mtEFG2	Mitochondrial elongation factor G2
mtIC	Mitochondrial translation initiation complex
mtDNA	Mitochondrial DNA
MTERF4	Mitochondrial transcription termination factor 4
mtLSU	Mitochondrial large subunit

MTPAP	Mitochondrial poly(A) polymerase
mtPIC1	Pre-initiation complex 1
mtPIC2	Pre-initiation complex 2
mtRRF	Mitochondrial ribosome recycling factor
mtSSU	Mitochondrial small subunit
NHEJ	Non-homologous end joining
OxPhos	Oxidative phosphorylation system
PET	Polypeptide exit tunnel
POLRMT	DNA-dependent RNA polymerase
PTC	Peptidyl transferase centre
PUSL1	Protein pseudouridine synthase-like 1
ROS	Reactive oxygen species
SILAC	Stable isotope labeling by amino acids in cell culture
$TetO_2$	*tet* operator 2
TFAM	Mitochondrial transcription factor A
TFB1M	Human mitochondrial transcription factor B1

Introduction

The number of studies related to mitochondrial biology has increased exponentially in the last decades. In the sixties and seventies, the role of mitochondria as the main providers of cellular energy under the form of adenosine triphosphate (ATP) has been established together with the chemiosmotic theory explaining the oxidative phosphorylation system (OxPhos) (Taanman, 1999). Concurrently, growing knowledge stressed the importance of mitochondria in the generation of metabolites for the synthesis of macromolecules, shaping further the function of these organelles in bioenergetics and biosynthesis pathways (Brand et al., 2013). However, the scientists were only beginning to understand the multiple tasks that these organelles carry out not as mere, independent workers within the cell, but as active signalling players, influencing what occurs in the cytosol. Mitochondria are now known to be important regulators of Ca^{2+} homeostasis (Bravo-Sagua et al., 2017) as well as reactive oxygen species (ROS) (Brieger et al., 2012), which also serve as signalling molecules. They are involved in the apoptosis process via the release of cytochrome c, a caspase activator, in the cytosol (Bock and Tait, 2020), and in thermogenesis, by generating heat as a by-product of oxidative respiration (Ricquier and Bouillaud, 2000). Additionally, more recent research has highlighted the central role of mitochondria in the innate immune responses against viral and bacterial pathogens as well as cellular damage (West et al., 2011). The plethora of processes in which these organelles are involved explains their implication in several diseases which affect multiple organs and makes them the new focal point for the development of novel therapeutic approaches for conditions like cancer, neurodegenerative disorders, and diabetes. For these reasons, the investigation of the basic molecular processes underlying mitochondrial gene expression is of high significance. Therefore, the study of mitoribosome biogenesis and assembly factors involved in this process piqued my interest during my PhD studies. As frequently happens, I have encountered many unexpected findings during this journey, which helped to shed light on the role of several proteins implicated in these pathways. I hope the reader will enjoy learning about these discoveries as much as I enjoyed uncovering them.

1 Literature review

1.1 Introduction to mitochondrial gene expression

Nearly all eukaryotic cells strictly rely on small membrane-bound subcellular compartments known as mitochondria as their energy suppliers. How these organelles have evolved is quite an interesting story; in the early 20th century many had suggested that mitochondria originate from endosymbiotic bacteria, and several studies later, also thanks to the advent of high-throughput sequencing technologies, it was shown that they are the descendants of alpha-proteobacteria (Gray, 2012). Along with a circular double-stranded DNA (mtDNA) of approximately 16.5 kb, mitochondria developed their own replication, transcription, and translation systems, with more than 100 proteins being imported from the cytoplasm to take part in these processes (Figure 1). Mitochondria contain several copies of mtDNA, and the number varies depending on the cell type (Kelly et al., 2012). The mtDNA is an intron-less molecule, highly condensed, packed with proteins in membrane-less compartments termed nucleoids (Bonekamp and Larsson, 2018). The most abundant protein present in nucleoids is the mitochondrial transcription factor A (TFAM), which regulates the level of compaction of the mtDNA, rendering it more or less accessible for the transcription or replication machinery (Rubio-Cosials et al., 2011; Farge et al., 2014). The mtDNA is transcribed into 11 mRNAs (two of which are bicistronic), 2 rRNAs and 22 tRNAs. The mRNAs encode for 13 polypeptides, which constitute Complex I, Complex III and Complex IV of the OxPhos system. The mtDNA is transcribed by the DNA-dependent RNA polymerase (POLRMT) and other accessory factors into long polycistronic transcripts, resembling what occurs in prokaryotes (D'Souza and Minczuk, 2018). The majority of the mitochondrial polycistronic precursors are later processed by endonucleolytic cleavage at the tRNA junctions by RNase P and RNase Z (ELAC2) nucleases, in what is known as the tRNA punctuation model (Ojala et al., 1981). However, not all transcripts are flanked by tRNAs, and, although recent studies suggest the involvement of FASTK proteins in non-canonical RNA processing mechanisms, this still needs further examination (Wolf and Mootha, 2014; Antonicka and Shoubridge, 2015; Boehm et al., 2017; Ohkubo et al., 2021; Clemente et al., 2022).

It is believed that RNA processing occurs co-transcriptionally in membrane-less structures distinct from nucleoids, known as mitochondrial RNA granules (MRGs). The MRGs were first reported via the visualization of 5-BromoUridine labelled discrete punctae within the mitochondrial matrix (Iborra et al., 2004). Several years later, they were found to be inhabited by various proteins responsible for RNA processing and

mitoribosome assembly (Antonicka et al., 2013; Jourdain et al., 2013, 2016) (Figure 1).

Mitochondrial RNAs are subjected to different modifications before they reach full maturity. Mitochondrial transcripts, as cytosolic transcripts, exhibit polyadenylated tails at the 3'-end, albeit they are shorter (~30-70 nucleotides) than the cytosolic ones (~250 nucleotides). Polyadenylation is needed to generate stop codons of 7 out of 11 transcripts that do not possess one; nevertheless, the exact function of the long poly(A) tails is still not fully understood (Bratic et al., 2016). The enzyme responsible for polyadenylation is the mitochondrial poly(A) polymerase (MTPAP) and a mutation in MTPAP is associated with spastic ataxia with optic atrophy, implying the important role of polyadenylation in mitochondria (Crosby et al., 2010). Interestingly, mt-tRNAs undergo extensive post-transcriptional processing as more than 100 modification sites have been identified, with up to 15 different kinds of modifications (Suzuki and Suzuki, 2014; Suzuki et al., 2020). Mutations in mt-tRNAs are responsible for numerous clinical manifestations as they are implicated in mitochondrial diseases, cancer and neurodegenerative disorders (Suzuki, 2021). To a lesser extent as compared to mt-tRNAs, mt-rRNAs carry 10 (so far identified) modifications, which include methylations and pseudouridylations inserted by nuclear-encoded enzymes whose role is essential for correct mitoribosome assembly and stability (Rebelo-Guiomar et al., 2019; Lopez Sanchez et al., 2020). As for tRNAs, mt-rRNAs mutations are also associated with multisystemic disorders (Lopez Sanchez et al., 2021). The assembly of the mt-rRNAs into the two ribosomal subunits starts co-transcriptionally in the MRGs and, in coordination with the cytosolic synthesis and import of all ribosomal proteins and assembly factors, ultimately concludes in mitoribosome formation (Figure 1).

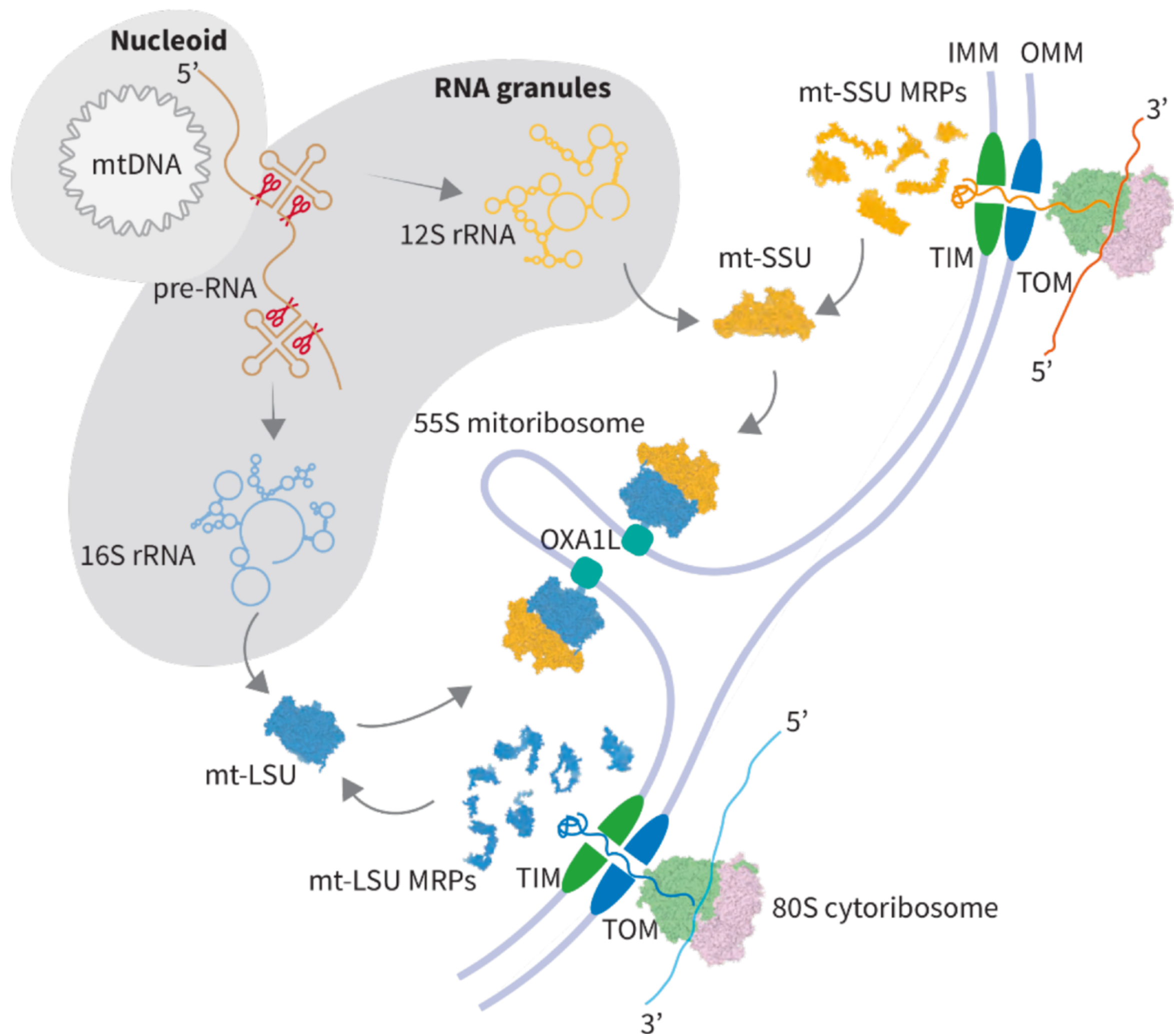

Figure 1: Overview of mitochondrial gene expression with a special focus on mitoribosome biogenesis. The mtDNA, which resides in nucleoids, is transcribed into polycistronic transcripts that are processed in the MRGs. The small subunit 12S mt-rRNA and the large subunit 16S mt-rRNA are modified and engaged in the mitoribosome assembly process. In parallel, MRPs translated by the 80S cytosolic ribosome are imported via the TIM-TOM import system and incorporated into the mitoribosome, which then tethers to the IMM via Oxa1L. Reprinted from Lopez Sanchez & Kruger (Lopez Sanchez et al., 2021) under the terms of the Creative Commons Attribution Licence 4.0 (CC BY).

1.2 Mammalian mitoribosome

The mammalian mitoribosome is a macromolecular structure made of RNA and protein, that performs protein synthesis within mitochondria. Like all ribosomes, it consists of two subunits: the 39S mitochondrial large subunit (mtLSU), which includes the 16S mt-rRNA, 52 MRPs and a structural mt-tRNA, and the 28S mitochondrial small subunit (mtSSU), which includes the 12S mt-rRNA and 30 MRPs. With the advances in cryogenic electron microscopy (cryo-EM), structures of the mitoribosome and its

two subunits at near-atomic resolution have been determined, giving an unparalleled understanding of the architecture of this complex machinery (Brown et al., 2014; Greber et al., 2014, 2015; Amunts et al., 2015). Interestingly, this has demonstrated that, despite its evolutionary origins, the mitoribosome has developed unique features and has diverged significantly from bacterial and other eukaryotic ribosomes.

The 5S rRNA is an integral part of prokaryotic and eukaryotic cytoplasmic ribosomes, located in the central protuberance (CP), and it is important for the enhancement of translation in bacteria (Ammons et al., 1999). However, in mammalian mitochondrial ribosomes, the 5S rRNA has been replaced by a mitochondrially-encoded tRNA ($tRNA^{Val}$ or $tRNA^{Phe}$) with a yet-undiscovered function (Brown et al., 2014; Greber et al., 2014; Rorbach et al., 2016). In addition, the mitoribosome experienced a reduction in RNA content in favour of protein mass, leading to a shift in the RNA: protein ratio from 2:1 in bacteria to 1:2 in mammals. Specifically, the mitoribosome has acquired 36 mitochondrial-specific proteins that create a network surrounding the inner catalytic core, while conserved MRPs have developed N- and C- terminal extensions. The function of these extensions is still poorly understood, although it is thought that many of them are important to stabilize the overall architecture of the mitoribosome.

As the mitochondrial translation goes on, nascent polypeptides, which are highly hydrophobic, emerge from the mitochondrial polypeptide exit tunnel (PET) and are subsequently inserted in the IMM as part of the membrane-embedded subunits of the respiratory chain complexes. The mitoribosome PET remodelling is designed to accommodate these polypeptides by exhibiting more hydrophobic walls compared to the bacterial ribosome (Brown et al., 2014; Greber et al., 2014). Mitochondrial-specific protein mL45, located near the exit tunnel, hinders the PET when the mitoribosome is not actively translating via its N-terminal domain, rendering it available as a result of the interaction with Oxa1L translocase, once translation starts (Itoh et al., 2021).

Intriguingly, mitochondrial transcripts lack the Shine-Dalgarno sequence (Anderson et al., 1981), which facilitates the recognition of mRNAs in bacteria. Consequently, the mtSSU has developed a novel mRNA entry site which includes the mitochondrial specific pentatricopeptide repeat domain protein mS39 as the main docking point for leaderless mRNAs (Amunts et al., 2015; Greber et al., 2015).

Despite the evolutionary divergence, the mRNA decoding centre and the peptidyl transferase centre (PTC), the most primordial ribosomal regions, remain highly conserved (Amunts et al., 2015; Greber et al., 2015).

1.3 Mitoribosome assembly

As previously mentioned, the mitoribosome assembly process is the result of the coordination between the nucleus and mitochondria; it involves the translation and import of all MRPs from the cytosol and the transcription and processing of the 12S and 16S mt-rRNA in mitochondria.

Although several studies have shown the hierarchical incorporation of ribosomal proteins in bacteria in the past years (Kaczanowska and Rydén-Aulin, 2007; Shajani et al., 2011), only recently, in 2018, work by Bogenhagen et al. revealed important insights into the mechanisms of mitoribosome assembly (Bogenhagen et al., 2018). Using pulse labelling with stable isotopes (SILAC) experiment, they were able to track the kinetics of newly synthesized polypeptides via the detection of ^{13}C-labelled MRPs at different time intervals. It was found that the entire assembly process occurs in 2-3 hours and that MRPs are imported in excess, with the ones not being engaged in the assembly being rapidly degraded. Additionally, Bogenhagen et al. dissected the pathway by identifying early, intermediate and late binding clusters of MRPs for the mtLSU, and early and late binding clusters of MRPs for the mtSSU (Figure 2) (Bogenhagen et al., 2018). How and when $tRNA^{Val}$ is incorporated into the mtLSU structure remains to be clarified.

The research on structural characterization of the mtLSU and mtSSU biogenesis has given a further understanding on the arrival of MRPs. Specifically, the earliest mtLSU intermediate analysed so far lacks proteins of the CP (mL40, mL46, mL48), which were reported to be early MRPs by Bogenhagen et al., and bL33, bL35 and bL36, which were reported to be late-stage MRPs (Bogenhagen et al., 2018; Cheng et al., 2021). Based on other studies, bL33, and bL35 are incorporated before CP reaches full maturity, whereas bL36 is the last mtLSU MRP to be recruited (Brown et al., 2017; Cheng et al., 2021; Chandrasekaran et al., 2021; Cipullo et al., 2021; Hillen et al., 2021; Lenarčič et al., 2021; Cheng et al., 2021; Rebelo-Guiomar et al., 2022). Notably, the MRPs recruited to the ribosome in the latest stages of assembly locate in the intersubunit interface region (Bogenhagen et al., 2018). This is in agreement with previous work on bacterial and cytosolic ribosomes, as the PTC is amongst the last ribosome regions to reach full maturity, ensuring that only fully functional ribosomes become translationally active (Jomaa et al., 2014; Kargas et al., 2019; Li et al., 2013a; Zhang et al., 2014). The recent structural work on mtLSU biogenesis has further indicated that the same appears to be true for mammalian mitoribosomes (Brown et al., 2017; Chandrasekaran et al., 2021; Cipullo et al., 2021; Hillen et al., 2021; Lenarčič et al., 2021; Cheng et al., 2021; Rebelo-Guiomar et al., 2022).

Regarding the mtSSU, the most immature intermediate reported so far lacks mS38, uS11m, bS21m, uS14m and mS37 (Harper et al., 2022), the majority of which belong

to the late binding clusters of MRPs previously identified (Bogenhagen et al., 2018). These MRPs are now reported to associate in the following order: mS38 binds first, followed by uS11 and uS21m, followed by uS14m and in later stages by mS37 (Harper et al., 2022; Itoh et al., 2022). Notably, the arrival of MRPs is often linked to the action of assembly factors with whom they would sterically clash on the ribosome, emphasizing the importance of their interdependence (Harper et al., 2022; Itoh et al., 2022).

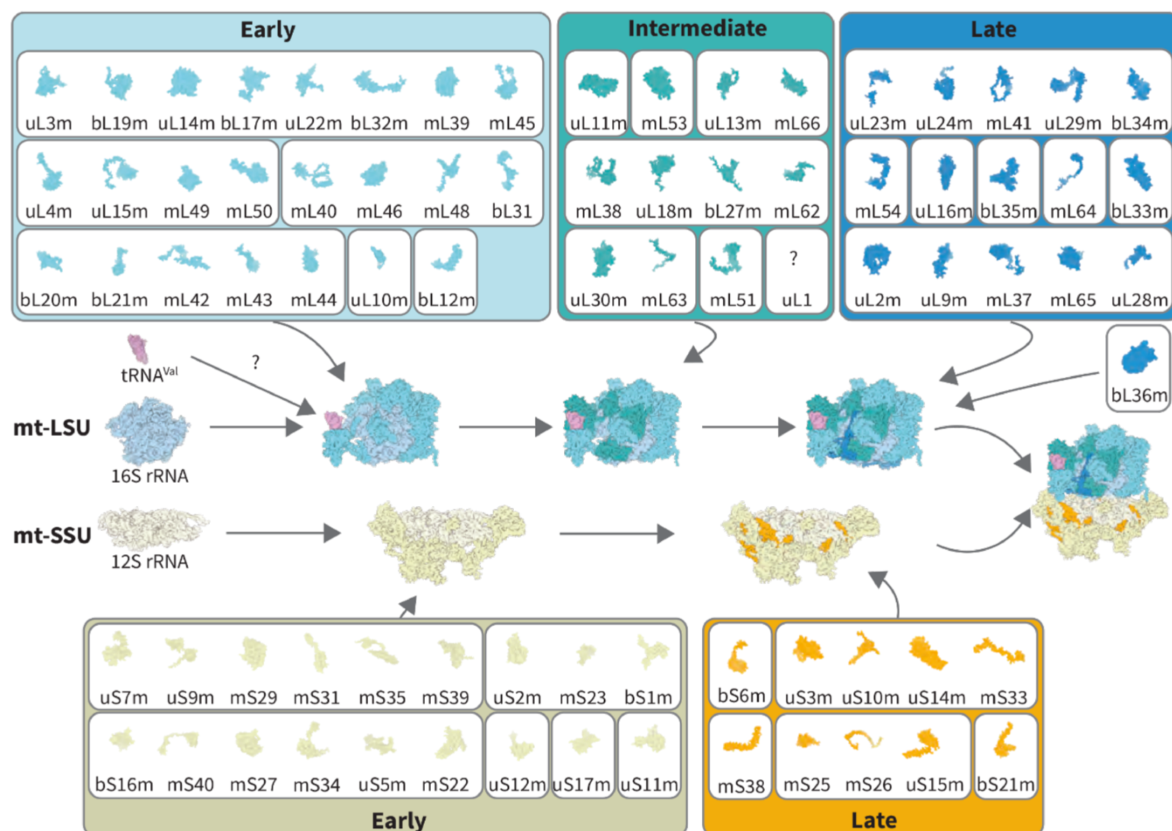

Figure 2: Hierarchical incorporation of MRPs. Schematics based on Bogenhagen et al., 2018 illustrates the hierarchical incorporation of MRPs**.** Reprinted from Lopez Sanchez & Kruger (Lopez Sanchez et al., 2021) under the terms of the Creative Commons Attribution Licence 4.0 (CC BY).

Based on the intricacy of the assembly process, it is apparent that, as observed for other ribosomal systems, it requires the participation of auxiliary factors at different stages of maturation. GTPBPs, DEAD-box RNA helicases, rRNA modifying enzymes such as methyltransferases and pseudouridine synthases, and other proteins participate in the assembly of either the mtLSU or the mtSSU.

1.3.1 GTPBPs in mitoribosome assembly

GTPBPs play a central role in ribosome assembly as they represent one of the largest classes of assembly factors both in bacteria and eukaryotes, suggesting their function is evolutionary conserved. GTPBPs belong to the family of P-loop NTPases and are involved in multiple molecular processes within the cell such as signalling pathways, translation, differentiation and proliferation, transport and cytoskeleton organization (Leipe et al., 2002). Their catalytic activity lies in the G-domain, which is responsible for the hydrolysis of GTP and is made of five motifs, G1-G5. The G1 motif (GXXXXGK[TS]), better known as the Walker A motif, is the most conserved and it allows the binding to the alpha and beta phosphates. The G2 and G3 motifs, also known as Switch I and Switch II domains because of their conformational change between GDP and GTP-bound states, regulate Mg^{2+} coordination and binding to the gamma phosphate. The G4 motif is responsible for the specific binding to the guanine base via hydrogen bond and the G5 motif, the least conserved, interacts with the guanine via water-mediated hydrogen bonds. Overall, the G-domain is conserved among GTPBPs across different species (Verstraeten et al., 2011). During the assembly process, GTPBPs have multiple roles: they can provide the energy for the binding of ribosomal proteins and binding or the release of other assembly factors. They can also accelerate conformational changes of the rRNA or stabilize the ribosomal components. In this way, they can work as quality control checkpoints to prevent subunit joining during maturation and they can couple ribosome assembly with other cellular pathways, acting as sensors of the GTP/GDP ratio (Karbstein, 2007).

Several human GTPBPs have bacterial counterparts with whom they share similar functions in the mitoribosome biogenesis process; further details regarding their role are discussed in the following subparagraphs.

1.3.1.1 GTPBPs in mtSSU assembly

Two GTPBPs are found to contribute to the assembly of the mtSSU: **MTG3** (Noa1/C4orf14) and **ERAL1** (Figure 3). MTG3 is the human homologue of bacterial YqeH and yeast Mtg3, both proteins involved in the regulation of the small subunit assembly (Loh et al., 2007; Uicker et al., 2007; Anand et al., 2009; Paul et al., 2012). In human mitochondria, MTG3 depletion causes a defect in mitochondrial translation by mainly affecting the mtSSU. It was suggested to associate with the mtSSU in a GTP-dependent manner and that GTP hydrolysis is used as a release mechanism (Kolanczyk et al., 2011; He et al., 2012). ERAL1, as its bacterial homologue Era, binds helix 45 at the 3’-end of the 12S mt-rRNA and its loss also causes mtSSU impairment.

(Sharma et al., 2005; Dennerlein et al., 2010; Uchiumi et al., 2010; Tu et al., 2009, 2011). Intriguingly, in helix 45 reside two adjacent adenines that are methylated by rRNA methyltransferase TFB1M (Seidel-Rogol et al., 2003), a reaction which is eased by the intervention of an RNA-binding protein involved in mtSSU biogenesis, namely RBFA (Itoh et al., 2022; Rozanska et al., 2017). Recent structural data have shown that MTG3, ERAL1, TFB1M and RBFA work in concert for the maturation of the mtSSU decoding centre, with MTG3 and ERAL1 being involved in platform formation and stabilization of the 12S mt-rRNA 3'-end (Harper et al., 2022; Itoh et al., 2022).

1.3.1.2 GTPBPs in mtLSU assembly

The characterization of the latest steps of mtLSU assembly has revealed the function of GTPBPs previously shown to be entailed in this pathway: GTPBP5, GTPBP10, GTPBP7 and GTPBP6 (Figure 3). In **papers I, II** and **III**, we specifically studied the roles of **GTPBP5** and **GTPBP10**. These two GTPases share the same bacterial homologue, ObgE, which has been implicated both biochemically and structurally in the latest stages of 50S subunit maturation (Feng et al., 2014; Gkekas et al., 2017; Jiang et al., 2006). Obg proteins encompass a highly conserved glycine-rich N-terminal domain (Obg domain), the G-domain and a C-terminal domain. Comparison of the primary sequence indicates an overall high level of conservation of both the Obg domain and the G-domain between these proteins; however, the predicted and experimental tertiary structures reveal distinct differences consistent with deletions and expansions found in these two domains, which may reflect on functional divergences between GTPBP10, GTPBP5 and ObgE. GTPBP10 and GTPBP5 interact with the mtLSU and their knock-out has deleterious effects on mitochondrial functionality (Lavdovskaia et al., 2018; Busch et al., 2019; Cipullo et al., 2020; Maiti et al., 2018, 2020). Interestingly, several pieces of information have evidenced the non-redundancy between these proteins: GTPBP10 overexpression does not rescue the GTPBP5 knock-out phenotype (Maiti et al., 2020), and no reciprocal pull-down has been observed in each protein interactome analysis (Lavdovskaia et al., 2018; Busch et al., 2019; Cipullo et al., 2020; Maiti et al., 2018, 2020). In addition to biochemical data, recent cryo-EM structures revealed that they bind the mtLSU in an analogous position but at different stages of assembly (Brown et al., 2017; Cheng et al., 2021; Chandrasekaran et al., 2021; Cipullo et al., 2021; Hillen et al., 2021; Lenarčič et al., 2021; Rebelo-Guiomar et al., 2022).
The human mitochondrial equivalent of previously described bacterial RbgA and yeast Mtg1p is **GTPBP7**. RbgA is involved in the late steps of 50S subunit assembly and its depletion causes accumulation of 45S intermediate particles (Jomaa et al., 2014;

Seffouh et al., 2019). In *Saccharomyces cerevisiae,* Mtg1p appears to have a role in the stabilization of the 21S rRNA (Barrientos et al., 2003). Similarly, GTPBP7 interacts with the mtLSU and its loss causes a reduction in mitochondrial protein synthesis (Kotani et al., 2013; Kim and Barrientos, 2018). It was observed that its enzymatic activity is triggered by the mtLSU, as reported in bacteria for several GTPBPs (Kotani et al., 2013). More recently, it was shown to act as a quality control checkpoint by preventing subunit joining and subsequently enabling intersubunit bridge formation (Kim and Barrientos, 2018). In *Trypanosoma brucei*, Mtg1 binds the 54S large subunit at the PTC (Jaskolowski et al., 2020; Tobiasson et al., 2021); in human, it binds in two different regions at two separate mtLSU maturation stages. In one of these configurations, it is suggested to work as a quality control checkpoint to ensure correct folding of the 16S mt-rRNA (Cipullo et al., 2021), whereas, in the structure where it directly contacts the PTC, it is suggested to work as a checkpoint for methylation (Chandrasekaran et al., 2021). Nevertheless, its exact role and the interchange between these two conformations need further clarification.

GTPBP6 is a GTPase of dual function as it has been reported to be involved in mtLSU biogenesis and the recycling process (Lavdovskaia et al., 2020; Hillen et al., 2021). Interestingly, its bacterial homologue, Obg protein HflX, belongs to a family of well-established recycling factors with no additional functions (Coatham et al., 2015; Zhang et al., 2015). GTPBP6 knock-out causes a decrease in monosome formation with consequent accumulation of various sub-assembly intermediates (Lavdovskaia et al., 2020). Intriguingly, cryo-EM analysis showed that GTPBP6 interacts with the mtLSU in one of the last steps of mtLSU assembly and it possibly induces the release of other assembly factors (Hillen et al., 2021).

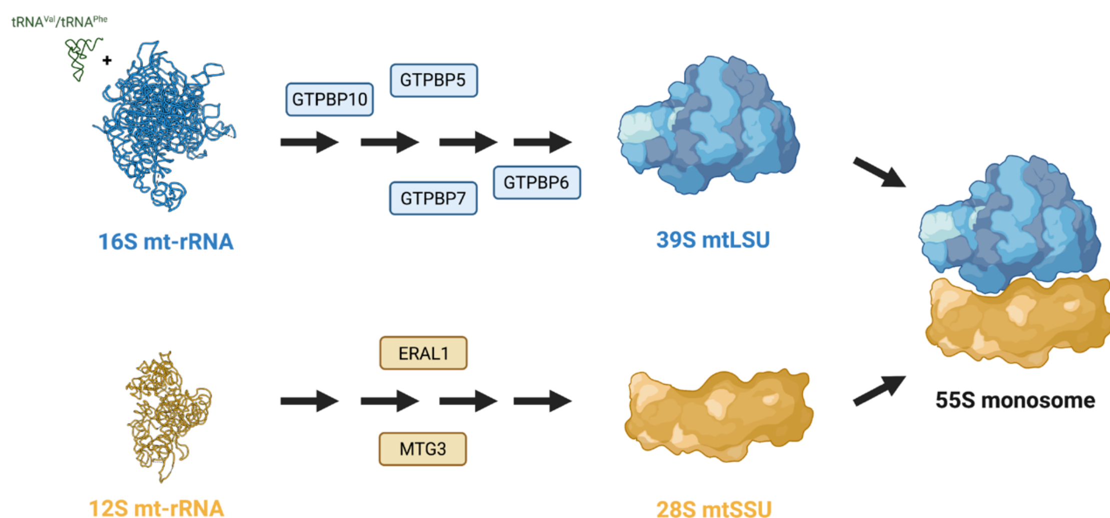

Figure 3: GTPBPs involved in the biogenesis of the mtLSU and mtSSU. Schematics of the GTPBPs involved in 55S monosome assembly. Structures of the 16S mt-rRNA and the 12S mt-

rRNA have been inferred from the model PDB 6SZG (Aibara et al., 2020). Created with BioRender.com.

1.3.2 Other relevant mtLSU assembly factors

As mentioned above, several accessory factors contribute to the assembly of the mitoribosome. In the following, I will introduce some that contribute to the mtLSU assembly as they accompanied me during my studies.

The rRNA methyltransferase NSUN4 is a protein of dual function: it is responsible for methylation of the human 12S mt-rRNA C1488 and it forms a stable heterodimeric complex with mitochondrial transcription termination factor 4 (MTERF4) (Cámara et al., 2011; Metodiev et al., 2014) The nature of their bond has been first described by two crystal structures which showed that the C-terminal part of MTERF4 contacts the N-terminal part of NSUN4, forming a positively charged path on the surface, which might facilitate RNA recognition (Spåhr et al., 2012; Yakubovskaya et al., 2012). The NSUN4-MTERF4 complex was first reported to bind to the mtLSU and prevent the formation of the 55S monosome in a premature manner, and its importance was substantiated by the fact that depletion of either of them leads to decreased monosome formation and defects in translation (Busch et al., 2019; Metodiev et al., 2014). Recent studies (**paper II**) confirmed that they participate in the last steps of mtLSU biogenesis by contributing to the final folding of the PTC (Chandrasekaran et al., 2021; Cipullo et al., 2021; Hillen et al., 2021; Lenarčič et al., 2021; Cheng et al., 2021; Rebelo-Guiomar et al., 2022).

In human mitochondria, the 16S mt-rRNA exhibits three 2'-O-ribose methylations: Gm2811, Um3039 and Gm3040. Gm2811 and Um3039 are highly conserved modifications located in the PTC and are inserted by mitochondrial rRNA methyltransferase 1 (MRM1) and mitochondrial rRNA methyltransferase 2 (MRM2), respectively (Lee and Bogenhagen, 2014; Rorbach et al., 2014). Near Um3039 resides Gm3040, which undergoes methylation by mitochondrial rRNA methyltransferase 3 (MRM3). The association of MRM2 and MRM3 in mtLSU maturation has had recent structural evidence (**paper II**) (Cipullo et al., 2021; Lenarčič et al., 2021; Cheng et al., 2021). Um3039 and Gm3040 play essential roles since the lack of MRM2 and MRM3 lead to alteration of the assembly process and consequently translation (Rorbach et al., 2014). This has been further corroborated by the finding that patients carrying mutations in MRM2 develop a cellular respiratory deficiency and mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome (Garone et al., 2017).

Mitochondrial assembly of ribosomal large subunit 1 (MALSU1) is a mtLSU assembly factor whose downregulation causes a reduction in mitochondrial translation (Rorbach et al., 2012; Wanschers et al., 2012). Its first structural report revealed that it binds the mtLSU in complex with the LYRM protein L0R8F8 and the mitochondrial acyl carrier protein (mt-ACP), preventing premature subunit joining (Brown et al., 2017). Interestingly, it remains associated throughout the last steps of mtLSU assembly (Chandrasekaran et al., 2021; Cipullo et al., 2021; Hillen et al., 2021; Lenarčič et al., 2021; Cheng et al., 2021; Rebelo-Guiomar et al., 2022). In addition to its role in assembly, it was recently suggested to work as an anti-association factor also during the rescue of stalled ribosomes, an aspect that needs further elucidation (Desai et al., 2020).

1.4 Mitochondrial translation

The mitochondrial translation cycle can be divided into three phases: initiation, elongation and termination. In mitochondria, mtIF3 and mtIF2 drive mitochondrial initiation whereas in bacteria IF1, IF2 and IF3 are required. Interestingly, mtIF2 encompasses a 37 aminoacidic mitochondrial specific domain extension which functionally compensates for the lack of IF1 (Gaur et al., 2008; Yassin et al., 2011; Kummer et al., 2018). During the initiation process, several intermediates are formed: pre-initiation complex 1 (mtPIC1), pre-initiation complex 2 (mtPIC2) and the mitochondrial translation initiation complex (mtIC). mtPIC1 consists of a fully mature mtSSU associated with mtIF3. mtIF3 establishes tight interactions with the mtSSU, preventing the association of the mtLSU and fMet-tRNA^{fMet} (Christian and Spremulli, 2009; Koripella et al., 2019; Khawaja et al., 2020). Subsequently, the peripherally mitochondrial-specific ribosomal protein mS37 contacts the N-terminal domain of mtIF3, facilitating the arrival of mtIF2 and the formation of mtPIC2 (Khawaja et al., 2020). In mtPIC2, mtIF2 induces the association of the mtLSU, together with the replacement of mtIF3 with the fMet-tRNA^{fMet} and the arrival of the mRNA, resulting in the formation of the final initiation complex (mtIC), competent for translation elongation (Khawaja et al., 2020).

Mitochondrial translation elongation is the most conserved phase of the translation cycle with regard to the bacterial and cytosolic systems (Ott et al., 2016; Rodnina, 2018; Kummer and Ban, 2021), and it proceeds with the ribosome scanning of the mRNA codon by codon while generating the corresponding polypeptide. During this process, each codon is matched by an aminoacyl tRNA (aa-tRNA) and peptide bond formation is catalysed, followed by translocation of the mRNA-tRNA pair. Mitochondrial elongation factor EF-Tu (mtEF-Tu) bound to GTP delivers the aa-tRNAs

to the mitoribosomal A site, near the GTPase-associated centre (GAC), in a similar manner to its bacterial counterpart (Desai et al., 2020). The GAC is responsible for the anchoring and activation of translational factors, thus it triggers GTP hydrolysis by mtEF-Tu further inducing its release from the ribosome (Moazed et al., 1988; Wintermeyer et al., 2004; Schmeing and Ramakrishnan, 2009; Schmeing et al., 2009; Schuette et al., 2009). At this stage, the peptidyl transferase reaction can occur, followed by the transfer of the polypeptide from the A-site to the P-site and high fidelity in-frame translocation of the mRNA bound to the tRNA by mitochondrial elongation factor G1 (mtEFG1) (Koripella et al., 2020; Kummer and Ban, 2020).
Once the mitoribosome encounters a stop codon, the latter is recognised by release factors which lead to the hydrolysis of the polypeptide chain from the tRNA (Nadler et al., 2022). Subsequently, the two ribosomal subunits are recycled by the action of two splitting factors, mitochondrial ribosome recycling factor (mtRRF) and mitochondrial elongation factor G2 (mtEFG2) (Rorbach et al., 2008; Tsuboi et al., 2009). As previously discussed, GTPBP6 is a novel putative recycling factor, which may provide alternative recycling pathways, specifically during stress conditions (Lavdovskaia et al., 2020).

1.5 Preliminary results: characterisation of the role of GTPBP8 in mitochondrial gene expression

Another GTPBP of interest for this thesis is **GTPBP8**, a protein which localises in the MRGs but whose function has not yet been determined (Tu and Barrientos, 2015; Maiti et al., 2018). YsxC, GTPBP8 bacterial homologue, participates in 50S subunit maturation (Cooper et al., 2009; Chan and Wong, 2011; Wicker-Planquart et al., 2015; Ni et al., 2016). A recent structural study shows that GTPBP8 homologue from *Trypanosoma brucei,* EngB, also binds to the interface of the large ribosomal subunit (Jaskolowski et al., 2020). Interestingly, in yeast, Mrx8 is required for Cox1 translation initiation and human GTPBP8 is capable of rescuing the mitochondrial defective phenotype of Mrx8 depleted strains (Verma et al., 2021). Nevertheless, GTPBP8 function is far from being understood and in this thesis, I report some initial characterization of its role in mammalian mitochondrial gene expression.

Firstly, we performed immunocytochemistry along with subcellular fractionation experiments using C-terminal Flag-tagged GTPBP8 (GTPBP8::FLAG) and confirmed its localisation within the mitochondrial matrix. To further investigate whether GTPBP8 binds the mitoribosome, we next sought to determine its interactome by performing Flag-immunoprecipitation followed by mass spectrometry of HEK293T cells

overexpressing GTPBP8::FLAG, using mitochondrially-targeted luciferase (mtLuc::FLAG) as control. Interestingly, no enrichment of either the mtSSU or the mtLSU was observed. A pool of different mitochondrial proteins that belong to the MRGs was found: some assembly factors from the mtLSU (GTPBP10, MRM1, DDX28, MRM3, DHX30, RPSUD3, RPSUD4, TRUB2, NGRN) and the mtSSU (NOA1 (MTG3), METTL17, TFB1M, ERAL1, MTERF3) were detected, along with some mitochondrial tRNA synthases and factors involved in mitochondrial transcription. Thus, no specific interactor was found associated with GTPBP8 but only a group of proteins of the MRGs belonging to different pathways.

Because GTPBPs are reported to bind to the ribosome in a GTP-bound state, we designed the S124A mutation in the Walker A motif of GTPBP8 G-domain (GTPBP8^{S124A}::FLAG) to check whether this would impede GTP-hydrolysis and help capture GTPBP8 transient interactions. After checking that wild-type and mutant GTPBP8 overexpression does not affect MRPs steady-state levels and monosome formation, we repeated FLAG-immunoprecipitation analyses. However, GTPBP8^{S124A}::FLAG did not provide any new insights into GTPBP8 interactome compared to wild-type GTPBP8 as MRPs were not enriched as well as factors previously detected in proteomic examination (uL3m, uS15m, MRM3, TRMT10C).

Overall, we were not able to identify GTPBP8 specific interactors using the Flag-immunoprecipitation experiment. Therefore, further studies are required, and the utilization of different approaches may help elucidate the GTPBP8 interactome network.

To complement our interactome analysis and study the effects on mitochondrial functionality, we used two GTPBP8 knock-out cell lines generated via the CRISPR/Cas9 technology. Growth analyses were carried out in galactose media, which requires cells to rely on the OxPhos system. GTPBP8 knock-out cells displayed strong proliferative incapability compared to HEK293T control, suggesting they have an intrinsic OxPhos defect. This phenotype was confirmed by BN-PAGE and in-gel activity analyses where both knock-outs revealed a reduction in Complex I and Complex IV activity. Additionally, proteomic analysis of GTPBP8 knock-out mitolysates exhibited a substantial decrease in steady-state levels of OxPhos proteins from Complex I, III and IV, as well as of MRPs from mtLSU and mtSSU, compared to wild-type control, further suggesting that GTPBP8 depletion causes alterations in the mitochondrial gene expression overall. As expected, steady-state levels of proteins belonging to Complex II and Complex V were stable. Interestingly, also several assembly factors of both subunits were downregulated.

xBecause of the decrease in MRPs and OxPhos proteins in GTPBP8 knock-outs, we were intrigued to analyse the ribosome profile of these cells via sucrose gradient centrifugation experiments. Interestingly, both knock-outs showed reduced 55S monosome formation, which was further reflected in reduced mitochondrial translational rates, as assessed by [^{35}S]-metabolic labelling of mitochondrial proteins. To confirm that the mitochondrial defect is determined by GTPBP8 ablation rather than by unspecific effects, we generated two rescue cell lines, one using wild-type GTPBP8::FLAG construct (GTPBP8^{RESCUE_wt}) and one using GTPBP8^{S124A}::FLAG construct (GTPBP8^{RESCUE_S124A}), and transfected them in one of the two knock-out cell lines. A sucrose gradient centrifugation experiment together with a mitochondrial translation assay were performed. The sucrose gradients profile showed a partial recovery of monosome formation in GTPBP8^{RESCUE_wt} compared to control, whereas GTPBP8^{RESCUE_S124A} exhibited a similar phenotype as the two knock-outs. Mitochondrial translation assay showed that GTPBP8^{RESCUE_wt} cells fully rescue the translation defect as much as control and HEK293T overexpressing GTPBP8 cell lines, as opposed to GTPBP8^{RESCUE_S124A}. Overall, this suggests that wild-type GTPBP8 is able to retrieve the mitochondrial impairment observed in the absence of GTPBP8, whereas S124A mutation alters GTPBP8 function, probably by interfering with GTP-hydrolysis.

In summary, GTPBP8 has an essential role in mitochondrial gene expression; its depletion leads to severe mitochondrial dysfunction as evidenced by alterations in the OxPhos system as well as a decrease in monosome formation and mitochondrial translation. Reduced steady-state levels of MRPs from both subunits and assembly factors are additional proof that mitochondrial ribosome assembly is also compromised. Nevertheless, our interactome analysis was inconclusive and we were not able to identify GTPBP8's main binding partners. Thus, more research is needed to ascertain the precise role of GTPBP8 in mitochondria.

2 Research aims

Cryo-EM brought innovative advances in obtaining atomic resolution structures of macromolecular complexes. The structural studies of the mammalian mitoribosome have given a lot of unprecedented knowledge about its complexity together with recent studies which elucidated the late assembly steps of both the mtLSU and mtSSU. Patients with mutations in assembly factors and MRPs manifest in various diseases with wide clinical spectra. It is expected that future whole exome sequencing will reveal more pathogenic mutations within genes implicated in mitoribosome biogenesis. Therefore, the main aim of the studies presented in this thesis was to determine the physiological basic mechanisms of mitoribosome assembly to counteract future pathological scenarios. For this purpose, we decided to target putative candidate GTPBPs hypothesized to be implicated in this pathway.

In **paper I** the aim was to elucidate the role of GTPBP5 in the mtLSU assembly pathway in human cells with the use of different biochemical tools.

In **paper II** the aim was to visualise native late-stage intermediates of mtLSU by employing GTPBP5 human cell models generated in **paper I**. To achieve this, we used two biochemical approaches in combination with cryo-EM.

In **paper III** the aim was to create a tool to study mitoribosome biogenesis *in vivo* via the generation of MitoRibo-Tag knock-in mice expressing MRP mL62. Specifically, I focused on the biochemical characterization of GTPBP10, which was found to co-purify with mitoribosomes.

3 Materials and methods

3.1 Generation of Flp-In T-Rex 293 stable mammalian cell lines

Human embryonic kidney 293 (HEK293T) cells are amongst the most frequently used cell models for research purposes. They were originally cultured in the 1970s by Alex Van der Eb and later immortalised by Frank Graham (Russell et al., 1977; Louis et al., 1997). The main advantage of using these cells is that they are very robust and fast-growing, they can double in approximately 34-36 hours, and they are easy to transfect, which makes them ideal for genetic manipulation.

To study the interactome of our proteins of interest (GTPBP5, GTPBP10 and GTPBP8), we utilized HEK293T cells in combination with the Flp-In T-Rex system. This method takes advantage of the *Saccharomyces cerevisiae*-derived DNA recombination system. It consists of the use of an Flp recombinase and the introduction of a Flp Recombination Target (FRT) site in a specific genomic region of the host cell line (Craig, 1988; Sauer, 1994). The gene of interest is later integrated at the FRT site via Flp recombinase-mediated DNA recombination (O'Gorman et al., 1991). The main advantage of this system is that the CMV promoter upstream of the gene of interest comprises two copies of the *E. coli tet* operator 2 ($TetO_2$) sequence, enabling tetracycline/doxycycline-inducible expression in a dose-dependent manner (Hillen et al., 1983; Hillen and Berens, 1994). In the absence of tetracycline/doxycycline, the Tet repressor protein, expressed from a designated vector, forms a homodimer and binds the $TetO_2$ region, inhibiting the expression of the gene of interest. However, upon tetracycline/doxycycline addition, tetracycline/doxycycline binds the Tet repressor homodimer causing a conformational change of the latter which consequently detaches and leads to transcription (Figure 4). In the studies described in **paper I**, **II** and **III** and in the preliminary results described in the "Literature review" section, we used this approach to overexpress a C-terminal flagged version of GTPBP5, GTPBP10 and GTPBP8 to facilitate the identification of their main interactors which would otherwise not be detected by simply relying on the endogenous expression levels.

HEK293T cells overexpressing the GTPBPs were grown at 37°C under 5% CO_2 atmosphere in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% tetracycline-free fetal bovine serum (FBS), glutamine, penicillin/streptomycin, uridine, and hygromycin and blasticidin, which are used for selection. Expression was induced by addition of doxycycline to the culture medium prior to the experimental procedure.

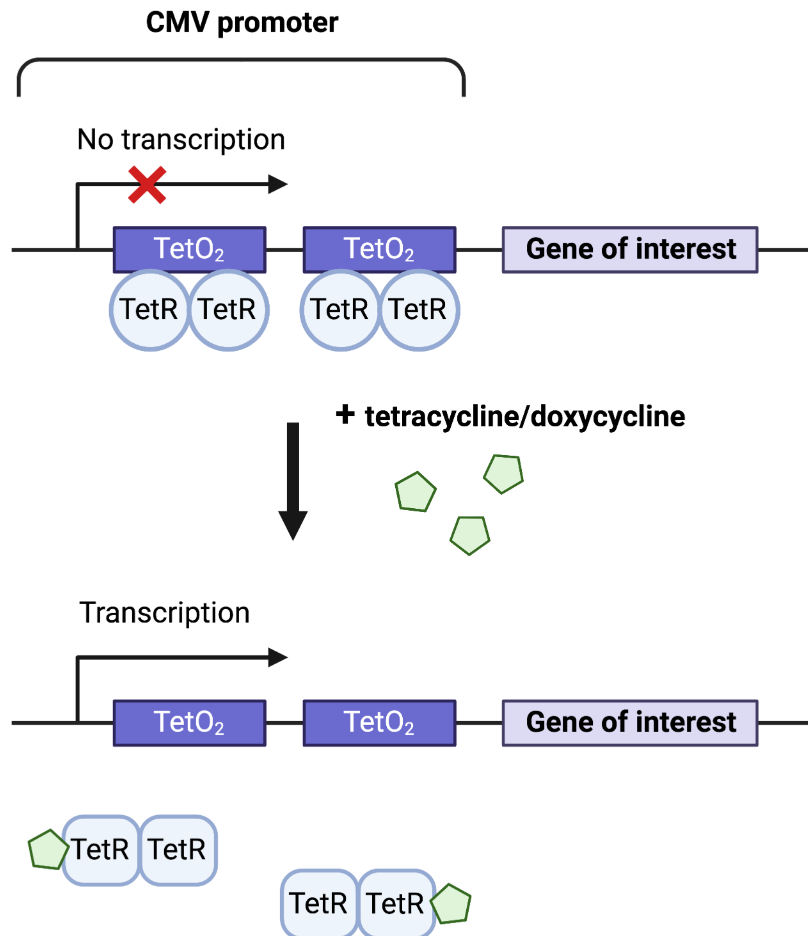

Figure 4: Overview of tetracycline/doxycycline regulation. The expression of the gene of interest is regulated by the absence or presence of tetracycline/doxycycline which causes the repression or the activation of transcription, respectively. Created with BioRender.com.

3.2 Generation of knock-out HEK293T cell lines

Generation of HEK293T cells knocked-out of GTPBP5, GTPBP10 and GTPBP8 was performed using CRISPR/Cas9. This method is an efficient gene-editing technology widely used to modify, delete or insert regions of DNA. CRISPR was first discovered in bacteria where it is used as an anti-viral defence mechanism (Hille and Charpentier, 2016). Based on this system, it was later found that small guide RNA molecules can direct the Cas9 nuclease and promote the cleavage of specific genomic loci (Cong et al., 2013). A single cut can lead to double-strand breaks which can be repaired by homologous recombination or by the error-prone non-homologous end joining (NHEJ), resulting in the deletion of short DNA stretches (Adli, 2018). The generation of knock-out cell lines in our studies was performed by employing two gRNAs targeting separate sites and causing deletion of larger DNA fragments (Ran et al., 2013).

3.3 Cryo-electron microscopy

A fundamental limit to the resolution of a microscope is the wavelength of the radiation used to form images. Once it became clear that electrons have wave-like properties and can be accelerated to reach wavelengths of orders of magnitude smaller than a single atom, the era of electron microscopes began. The number of published structures obtained by electron microscopy has exponentially increased in the last decade, the vast majority being the result of an increase in the number of cryo-EM microscopes globally (Chua et al., 2022).

The constraints of using an electron beam lie in the fact that high electron doses can damage the biological sample; moreover, electrons have low penetration capability, and thus they are unable to image samples thicker than roughly 0.5 μm. Cryo-EM introduced for the first time a vitrification step in the sample preparation pipeline which allows for rapid cooling of the sample, creating an ultrathin layer of vitrified water enclosing the specimen in native conditions; this additionally reduces radiation damage and protects from harsh vacuum conditions inside the microscope (Knapek and Dubochet, 1980; Dubochet and McDowall, 1981).

Benefits of cryo-EM include the requirement of small amounts of starting material and the potential of characterising the structure of small molecules (~ 60 kDa) as well as complexes up to several MDa big in size. In more recent years, the development of new electron detectors, better processing software and computer power, contributed to building structures of molecules at resolutions that were not thought possible (Kühlbrandt, 2014).

3.3.1 Sample preparation

The success of a cryo-EM experiment strongly depends on the quality of the purified biological sample. The negative staining method is often used after sample purification to assess this aspect (Figure 5). In this approach a thin layer of heavy salt solution embeds the specimen, creating a cast around it which scatters electrons, leaving the encapsulated particles lacking the stain for visualisation (Brenner and Horne, 1959). This provides useful low-resolution information on the morphology of the particles, their heterogeneity and concentration, and the presence of contaminants. The purified particles are subsequently loaded onto grids, vitrified and imaged. The micrographs obtained undergo data processing which culminates in the generation of a map that can be further modelled (Figure 5).

In **paper II**, the sucrose gradient centrifugation experiment of GTPBP5 knock-out cells and FLAG-immunoprecipitation experiment of GTPBP5 overexpressing cells were

employed for cryo-EM sample preparation and will be discussed shortly in the next paragraphs.

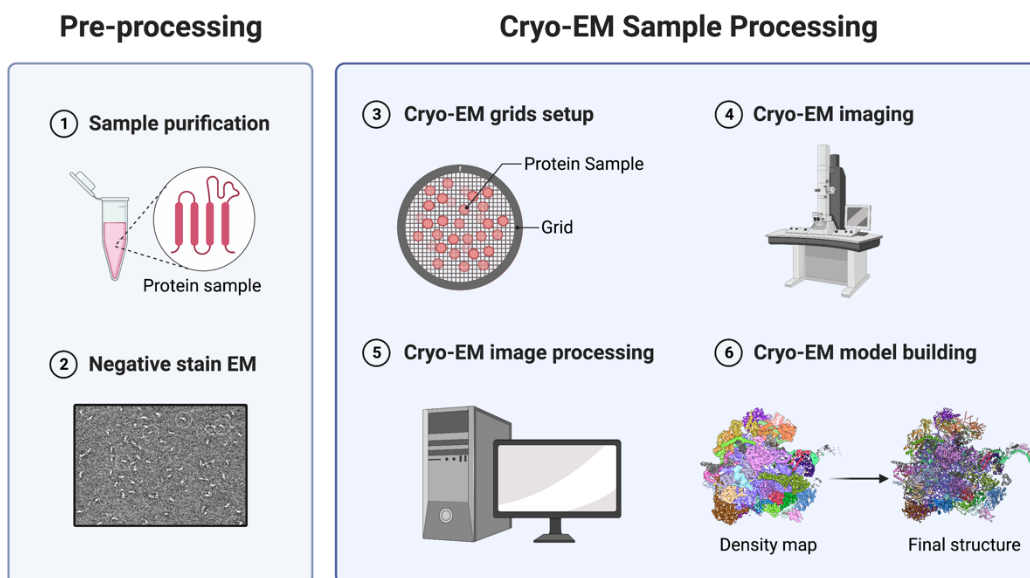

Figure 5: Pipeline of a cryo-EM experiment. The map and the model indicated in the schematics have been inferred from the PDB 7O9K (Cipullo et al., 2021). Adapted from "Cryogenic Electron Microscopy Overview", by BioRender.com. Retrieved from https://app.biorender.com/biorender-templates.

3.3.1.1 Sucrose gradient centrifugation experiment

Density gradient centrifugation is a common technique performed for the separation and purification of particles of different sizes or organelle purification. In ribosomal studies, it is used to separate the ribosome from the free subunits and inspect their assembly status. It consists of the preparation of a continuous sucrose gradient where the density increases from the top to the bottom of the tube. The sample containing a mixed population of macromolecules is loaded on the top. Sucrose gradients are based on rate-zonal separation: the particles have a higher density than the gradient and are separated according to their size and mass.

The analyses of assembly factors involved in ribosome biogenesis are often carried out via this methodology together with other techniques. It can show if the studied factor co-sediments with the ribosomal subunits or, in the case of its depletion, if there is an accumulation of sub-assembly intermediates or a reduction in monosome levels. In **paper I** and **paper III** and in the preliminary results described in the "Literature review" section we applied this technique for the study of the mitoribosome profile in GTPBP5, GTPBP10 and GTPBP8 knock-out cells. In **paper II**, we isolated mtLSU

fractions separated via a sucrose gradient centrifugation experiment from GTPBP5 knock-out cells and analysed them via cryo-EM.

3.3.1.2 Flag-immunoprecipitation experiment

Immunoprecipitation analyses are used for interactome studies as they promote high-yield isolation of a protein of interest with its interactors. In the work presented in this thesis, we generated C-terminal Flag-tagged GTPBP5, GTPBP10 and GTPBP8 and subjected them to pull-down experiments followed by mass spectrometry analyses. The immunoprecipitation was performed using highly specific monoclonal antibodies, which recognise the FLAG tag, that are covalently bound to an agarose resin. Elution was performed using 3X Flag peptides that compete with the protein of interest leading to its release from the beads. In **paper I** and **paper III** and in the preliminary results described in the "Literature review" section we used this method to study GTPBP5, GTPBP10 and GTPBP8 binding patterns. In **paper II** we utilised this method to isolate the GTPBP5-bound intermediate of assembly and image it via cryo-EM.

3.3.2 Data processing

Data processing in cryo-EM corrects for the limitations occurring during the imaging of a sample and it is carried out using Relion and CryoSparc softwares. For starters, the electron source represents a double-edged sword for this procedure: too high doses can cause sample damage whereas too low doses may lead to too little informative data. Therefore, a limited maximum electron dose for imaging is applied, which results in the acquisition of micrographs with a low signal-to-noise ratio. Furthermore, micrographs must be corrected for beam-induced motion and aberrations of the microscope.

The first step in the data processing workflow is motion correction: when the electron beam strikes the sample, it causes its motion. This results in blurred images, thus limiting the final resolution of the reconstructed 3D structure. Newly developed detectors enable the recording of "movies", a series of frames collected at a particular time, which helps to reduce the beam-induced motion per frame (Li et al., 2013). The motion is further corrected computationally by algorithms capable of reconstructing the motion trajectory, undoing it and averaging all the frames in one single image (Zheng et al., 2017). After motion correction, the next step is to fit the microscope contrast transfer function (CTF). The CTF is a mathematical function that describes how the aberrations in a transmission electron microscope affect the final image of a sample. It is used to correct these aberrations in order not to create artifacts (Zhang,

2016). Because the collected micrographs contain a mixture of various particles, each kind displaying different orientations, particles have to be classified. 2D classification is a tool that sorts projections of different particles along with projections of different views; these are further averaged and combined in one 2D-image. This function is very useful to estimate the amount of contamination of a sample and determine the molecules of interest. Starting from multiple 2D classes, it is later possible to extrapolate an initial 3D object which best represents the data (Punjani et al., 2017). The first 3D structure, however, is an average of a not yet homogeneous population of particles, thus it is subjected to multiple processing steps designated to resolve heterogeneity. Eventually, high resolution refinement functions are applied to obtain high-resolution maps that can be used for accurate model annotation of atomic structures.

4 Results and Discussion

4.1 PAPER I: HUMAN GTPBP5 IS INVOLVED IN THE LATE STAGE OF MITORIBOSOME LARGE SUBUNIT ASSEMBLY

In **paper I** we investigated the involvement of GTPBP5 in mtLSU assembly. We used Flp-In T-Rex 293 cell line overexpressing GTPBP5 to examine its interactome network and HEK293T GTPBP5 knock-out cell line to assess its effects on mitochondrial functionality. Earlier studies on GTPBP5 *E. Coli* homologue ObgE and other bacterial homologues suggested its involvement in large subunit assembly; information that was further corroborated by the publication of the structure of ObgE bound to the interface of a 50S late-stage assembly intermediate (Lin et al., 2004; Jiang et al., 2006; Matsuo et al., 2006; Feng et al., 2014; Gkekas et al., 2017). Kotani and co-workers started working on human GTPBP5 showing that it binds to the mtLSU *in vitro*. However, siRNA experiments did not reveal any mitochondrial alterations (Kotani et al., 2013). We, therefore, decided to study this protein in more detail.

After generating GTPBP5 overexpressing cells, we performed Flag-immunoprecipitation experiments followed by mass spectrometry to detect GTPBP5 binding partners. We observed a clear enrichment in MRPs from the mtLSU, whereas mtSSU MRPs were lacking, excluding the possibility of GTPBP5 binding to the 55S monosome or participating in mtSSU assembly. Intriguingly, already reported mtLSU assembly factors were also among the highly enriched hits detected. The 16S mt-rRNA methyltransferase MRM2 was found (Lee and Bogenhagen, 2014; Rorbach et al., 2014) together with GTPBP7, MTERF4-NSUN4 complex and MALSU1-L0R8F8, all factors implicated in the late maturation stages of assembly (Cámara et al., 2011; Rorbach et al., 2012; Wanschers et al., 2012; Metodiev et al., 2014; Lee and Bogenhagen, 2014; Rorbach et al., 2014; Brown et al., 2017; Kim and Barrientos, 2018; Chandrasekaran et al., 2021; Cipullo et al., 2021; Hillen et al., 2021; Lenarčič et al., 2021; Cheng et al., 2021; Rebelo-Guiomar et al., 2022).
To better understand the mechanism of GTPBP5-mtLSU interaction and the role that GTP hydrolysis places in this, we repeated pull-down experiments in the presence of 5'-guanylyl imidodiphosphate (GDPNP), a non-hydrolysable GTP analog. Most assembly and translational GTPBPs reported in literature bind the ribosome in a GTP-bound state, as observed for ObgE (Feng et al., 2014; Zhang and Haldenwang, 2004). GDPNP presence induced the loss of GTPBP5 interaction to the mtLSU, initially suggesting that GTPBP5 binding is dependent on GTP hydrolysis. However, in **paper II**, we showed that GTPBP5, recruited to the ribosome by mtEF-Tu, binds the mtLSU

in the GTP-bound form. In light of these results, we now believe that the concentrations of GDPNP used in our experiments might have interfered with the mtEF-Tu function causing a consequent lack of GTPBP5 binding.

Additional work was carried out in GTPBP5 knocked-out cell line to examine its phenotypical impacts on mtLSU assembly. Two gRNAs targeting the first coding exon of the GTPBP5 gene were transfected in HEK293T cells, and the obtained knocked-out clone was validated via Sanger sequencing and Western blotting. The absence of GTPBP5 caused a strong impairment in mitochondrial functionality as shown by decreased OxPhos proteins' steady-state levels and decreased growth capability both in glucose and galactose. This was further supported by respiration analysis and OxPhos in-gel activity assays. Moreover, the sucrose gradient centrifugation experiment evidenced a reduction in monosome formation in GTPBP5 knock-out cells, which manifested in a 50% reduction of mitochondrial translational rates. The latter results endorsed the hypothesis of GTPBP5's role in mtLSU biogenesis and pushed us to clarify the exact composition of the maturation intermediates accumulated in its absence. A combination of sucrose gradient centrifugation experiments with SILAC revealed the presence of late-stage mtLSU assembly factors associated with GTPBP5 knock-out mtLSU intermediates, most of which were also found in our interactome analysis.

Overall, our results confirmed the involvement of GTPBP5 in the mtLSU biogenesis pathway, proposing it works in cooperation with other assembly factors at the late stages of assembly.

4.2 PAPER II: STRUCTURAL BASIS FOR LATE MATURATION STEPS OF THE HUMAN MITORIBOSOMAL LARGE SUBUNIT

While the role of GTPBP5 was partially determined by us and by another group (Maiti et al., 2020), the exact function of GTPBP5 and its possible interplay with other assembly factors was still under investigation. Therefore, in **paper II** we decided to utilize the cryo-EM technology. For this purpose, we employed the GTPBP5 knock-out and overexpressing cell models generated in **paper I** and subjected them to sucrose gradient centrifugation and Flag-immunoprecipitation experiment, respectively. By capturing mtLSU sub-assembly intermediates accumulated in the absence and presence of GTPBP5, our aim was to visualize consecutive steps of mtLSU assembly. As a confirmation of our previous biochemical work, nine assembly factors detected in our proteomic analysis were also found in our structures.

The majority of particles obtained from the knock-out model represented the most immature intermediate found in our studies, which displayed highly disordered RNA and MALSU1 in complex with both L0R8F8 and mt-ACP, shown by previous work (Figure 6)(Brown et al., 2017). The MTERF4-NSUN4 complex was found to interact with the mtLSU interface in both our models, however with some differences. The dual-function methyltransferase NSUN4 is involved in the biogenesis of both subunits (Metodiev et al., 2014), but in both our states it was found with its active site facing the mtLSU core, hereupon suggesting that it does not engage in 12S mt-rRNA methylation at the captured state. Surprisingly, we showed that its partner, MTERF4, works as a chaperone protein as it leads to the folding of a premature rRNA helical turn (pre-H68-71), necessary for the next assembly steps. In fact, besides deciphering how these factors operate, these structures headed us towards a reconstruction of the sequential steps of mtLSU late assembly. The formation of pre-H68-71 caused, indeed, the exposure of the 16S mt-rRNA MRM2 methylation site (A-loop), leading to MRM2 recruitment in this position.
Flag-immunoprecipitation analyses as well as the proximity-dependent biotin identification (BioID) method evidenced a strong interaction between GTPBP5 and MRM2 (Cipullo et al., 2020; Maiti et al., 2020), substantiated by rescue studies performed in bacteria and yeast where ObgE/Mtg2p was able to recover FtsJ/Mrm2p deleterious effects (Datta, 2004; Tan et al., 2002). In our structures, we revealed that MRM2 sits at the PTC before and after GTPBP5 arrival (Figure 6). Prior to GTPBP5 recruitment, however, MRM2 active pocket surrounds the A-loop in a conformation favourable for methylation. GTPBP5 arrival dislocates the A-loop far from MRM2 active site, promoting MRM2 release.
In concomitance with MRM2 arrival, GTPBP7 is also recruited to the mtLSU (Figure 6). It binds near the pre-H68-71 and contacts the C-terminus of MALSU1, far from the PTC where it was found to bind in bacteria and *Trypanosoma brucei* (Seffouh et al., 2019; Jaskolowski et al., 2020; Tobiasson et al., 2021). Based on our work, it might function as a quality control checkpoint for the correct folding of pre-H68-71 or as an anti-association factor. Notably, another study showed it binds the PTC in the same position as MRM2 found by us (Chandrasekaran et al., 2021), opening the question of whether it changes position and conformation throughout the assembly pathway.

GTPBP5 recruitment marks the passage from one consecutive step to the other. Besides inducing MRM2 release, it contributes to the final rearrangements of the 16S mt-rRNA, and it does it in cooperation with NSUN4. A density for NSUN4 N-terminus, not visible in our knock-out model, appears in the presence of GTPBP5, and by breaking the interaction between the P-loop and H89, it renders H89 accessible to

reach its mature conformation, which is facilitated by GTPBP5. Therefore, GTPBP5 and NSUN4 together contribute to the final folding steps of the 16S mt-rRNA.

An unexpected encounter during the resolution of these structures was to find a translational factor entailed in the assembly process. MtEF-Tu is mostly known to be essential for the delivery of aa-tRNAs to the translating mitoribosome. However, using GTPBP5 as a bait in immunoprecipitation and protein-proximity interactome analyses can specifically enrich mtEF-Tu (Antonicka et al., 2020; Cipullo et al., 2020). Moreover, GTPBP5 Obg-domain protrudes between H89 and H93, positioning it in a similar manner as an A-site tRNA. It was therefore tempting to propose that mtEF-Tu is responsible for the delivery of GTPBP5 during assembly as it is for the delivery of aa-tRNAs during translation. Of note, this is not the only evidence of a translation factor bound to an assembly intermediate, as it has also been shown for human mtIF3 and trypanosoma mtIF2 (Itoh et al., 2022; Lenarčič et al., 2022).

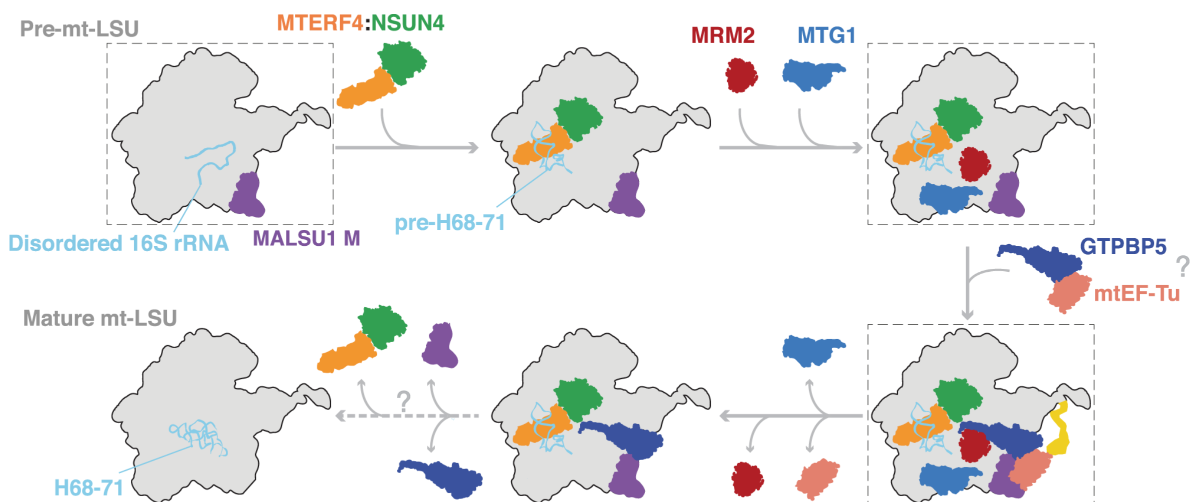

Figure 6: Schematics of mtLSU late-stage assembly. Reprinted from Cipullo & Valentín Gesé (Cipullo et al., 2021) under the terms of the Creative Commons Attribution Licence 4.0 (CC BY). Resolved structures are included in dashed boxes**.** The dashed arrow denotes an undetermined order of release of biogenesis factors. Question marks denote mechanisms that are still unknown.

In conclusion, in this paper we report the structural characterization of the final steps of mtLSU assembly, giving novel insights into the function of essential mtLSU assembly factors as well as delineating their interdependence.

4.3 PAPER III: MITORIBO-TAG MICE PROVIDE A TOOL FOR *IN VIVO* STUDIES OF MITORIBOSOME COMPOSITION

Most of the research carried out on mitoribosome assembly and translation is based on the use of cultured cell models, advantageous in that they are easy to handle and fast to grow, but limiting in that they might not reflect the physiological conditions of whole organisms. For these reasons, complementary studies on mice can help shed more light on how these systems operate *in vivo* in various tissues and under various physiological circumstances.

In **paper III** MitoRibo-Tag knock-in mice expressing C-terminal Flag-tagged MRP mL62 were generated to study mitoribosome interactome and composition in different tissues. To ensure that the MitoRibo-Tag animals were appropriate for mitoribosome purification from various organs, mitochondria were extracted from kidney, liver, and heart and Flag-immunoprecipitation followed by mass spectrometry analyses were carried out. Although mitoribosomes were successfully purified, differences in interactome composition were found, with most of the mitoribosome-associated factors being enriched in heart. Several proteins involved in RNA metabolism and the assembly of respiratory chain complexes were detected, as well as assembly factors of the mtLSU and mtSSU. The latter included GTPBPs such as GTPBP5, GTPBP10, GTPBP7 and MTG3. Interestingly, the presence of proteins like Oxa1L, mtIF2 and mtIF3 suggested that mitoribosomes were also trapped in translationally active states.

As this method can be used under physiological conditions, it was opted to determine the makeup of the 39S assembly intermediates that build up in the absence of MTERF4 by using this approach on mice with MTERF4 conditional knockouts in the heart and skeletal muscle. Among the proteins upregulated in the proteome, two drew our attention: protein pseudouridine synthase-like 1 (PUSL1) and GTPBP10. PUSL1 belongs to the TruA family of pseudouridine synthases in bacteria and it is responsible for the pseudouridylation of uridines in bacterial tRNAs (Suzuki et al., 2001; Hur and Stroud, 2007). In mitochondria, however, the role of this protein is far from being understood. Flag-immunoprecipitation experiments of HE293T cells overexpressing Flag-tagged PUSL1 showed it co-purifies with MRPs from both subunits, hinting at a possible role of this protein in the regulation of mitochondrial translation. The stabilization of rRNAs to ensure proper translation has previously been demonstrated to be dependent on pseudouridine synthases; thus, PUSL1 may play a similar role (Antonicka et al., 2017; Perks et al., 2018). The development of PUSL1 knock-out HEK293T cells, which revealed a partial reduction in mitochondrial translation, provided more evidence in favour of this notion. However, whether it exploits its function to pseudouridylate mt-tRNAs or mt-rRNAs is still unknown.

The assembly intermediate derived from the conditional knockout of MTERF4 also co-purified assembly factor GTPBP10, confirming its involvement in the maturation of mtLSU, as established by previous work (Lavdovskaia et al., 2018b; Maiti et al., 2018). We also decided to study GTPBP10 interactome using HEK293T overexpressing a C-terminal Flag-tagged version of the protein, with the addition of GDPNP to examine its mechanism of action. GDPNP did stabilise the GTPBP10-mtLSU interaction, suggesting GTP-hydrolysis is required for its release.
The fact that GTPBP10 and GTPBP5 have the same bacterial homologue raised the possibility that these components serve identical roles. Nonetheless, GTPBP10 overexpression failed to reverse the effects of GTPBP5 knock-out (Maiti et al., 2020), and GTPBP5 was not enriched in its interactome analysis, and vice versa (Lavdovskaia et al., 2018; Busch et al., 2019; Cipullo et al., 2020; Maiti et al., 2018, 2020).
Additional research revealed that GTPBP10 knock-out cells had a severe growth defect in galactose media and modestly lowered steady-state levels of MRPs. Mitochondrial translation, on the other hand, was severely affected.
As of now, GTPBP10 was found to bind late states of mtLSU assembly (Cheng et al., 2021); its exact function, however, is not fully clarified.

In summary, MitoRibo-Tag mice offer an essential tool to study mitochondrial ribosome assembly and translation *in vivo* and can be potentially used to examine how the composition of mitoribosomes changes as a result of various physiological conditions or disease. This study was done in collaboration with Nils-Göran Larsson lab and most of the experimental work was conducted by Jakob Busch. Our contribution lies in the production of GTPBP10 data.

5 Conclusion and future perspectives

The making of a mitoribosome is a tightly regulated process that requires the participation of many auxiliary factors. Over the past few years, numerous studies have employed different methods which span from biochemical and genetic tools to advanced proteomic technologies and, more lately, to cryo-EM, to identify and partly characterize the role of assembly factors involved in mitoribosome assembly. Nevertheless, we are only starting to understand how this intricate pathway works. Recent structural studies have elucidated late-stage assembly steps of both the mtLSU and mtSSU. However, very little is known about the early maturation of ribosomal subunits in mitochondria, and many unknown assembly factors may have remained undetected to date.

Our current approaches enable us to capture complexes and interactions in native conditions, but they lead to the accumulation of stable assembly intermediates while more dynamic and transient states are lacking. In bacteria, it is possible to reconstitute *in vitro* the assembly of the 50S and 30S subunits combining rRNA and proteins (Nierhaus and Dohme, 1974; Traub and Nomura, 1968). Setting up a similar system for the study of mitoribosome assembly could help visualize states where rRNA is almost naked or only partially surrounded by MRPs in a time-resolved fashion.
Time-resolved cryo-EM is another way to study more transient interactions such as the ones that are regulated by GTP or ATP-hydrolysis. If the kinetics are known, it is possible to precisely control the timing of a biochemical reaction prior to freezing the sample at the point where the short-lived state is most abundant (Amann et al., 2023). To add an extra layer of information, cryoelectron tomography (cryoET) can further give spatial resolution to the study of mitoribosome assembly, allowing visualization of larger molecules in situ in the cell context.

While studying mitoribosome assembly, it is also important to consider the possibility of the existence of multiple parallel assembly paths which might be regulated differently in various conditions, as well as the interrelation between this process and transcription or translation, which might unravel higher levels of regulation.

In conclusion, our studies provided further information regarding mtLSU assembly steps, both biochemically and structurally, and will hopefully be helpful for developing future diagnostic and therapeutic approaches to mitochondrial disorders.

6 Acknowledgements

Singlehanded scientific achievements are neither the norm nor the goal. I must therefore thank all the colleagues and collaborators that accompanied me during my PhD studies.

To my main supervisor Asia, thank you for giving me the opportunity to work in your lab, for teaching me and guiding me over the past years; your constant support has been invaluable. I couldn't have hoped for a more inspirational journey than this one.

I am grateful to my co-supervisors Nils and Chris, for always creating a stimulating academic environment. Your scientific insight has been priceless.

To Martin and Genís; uncovering with you the art of cryo-EM has been challenging and fun at the same time. I consider myself lucky to have worked with such caring and open-minded collaborators.

To Ilian, for your great support in the analysis of proteomic data, I have learned a lot thanks to your teachings.

My biggest thank you goes to the whole MolMet division, past and present members, you have been by far the highlight of my PhD. Being surrounded by brilliant scientists has made me a better one, just like being surrounded by a variety of cultures has made me a better person.

Camilla, Sussi and Leila, you are the beating heart of this amazing group of people; I hardly believe I will ever find a more organized and welcoming working environment than the one I have been gifted in the past years.

I strongly wish to acknowledge some of the people from whom I learned when I first stepped in the mitochondrial world: Sarah, Cami, Isabelle, Ola and Florian, you have helped me place the roots of my future academic career, always with fun and excitement.

Another acknowledgment goes to the whole Rorbach group: science and laughter are the best combination, and we had a lot of both.

Thank you, Ruth and Annika, I always found motivation and enthusiasm in our endless ribosome-centred conversations, and I am extremely grateful for having met two new friends *en route*.

Anas, Laura, Rodolfo and Roby, you have walked this road with me, and I couldn't have been happier. I was always inspired by your knowledge and your commitment, and I will treasure all the teachings you have given me along the way. I cannot thank you enough.

Last but definitely not least, thank you Shreekara and Jelena; sometimes a single person can make the difference between a good journey and an awesome journey. I've found twice as many.

Un grazie doveroso va alla mia amica Francy, per essermi stata a fianco tutto il tempo, fin dal primo giorno.

A Massi, perché sei da sempre il mio principale sostenitore e mi accompagni in ogni mia avventura. Sei la mia casa ovunque io vada.

Ed infine, alla mia famiglia, a mia sorella, e soprattutto ai miei due boomers preferiti, un grazie infinito, per tutto, sempre.

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