Role of GTPase MTG1 in Mitochondrial Translation and heart Physiology

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UNIVERSITY OF MIAMI

ROLE OF GTPASE MTG1 IN MITOCHONDRIAL TRANSLATION
AND HEART PHYSIOLOGY

By
Hyun-Jung Kim

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of the University of Miami
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ROLE OF GTPASE MTG1 IN MITOCHONDRIAL TRANSLATION
AND HEART PHYSIOLOGY

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Mammalian mitochondrial ribosomes (mitoribosomes) synthesize thirteen proteins, essential components of the oxidative phosphorylation system. They are linked to mitochondrial disorders, often involving cardiomyopathy. Mitoribosome biogenesis is assisted by multiple cofactors whose specific functions remain largely uncharacterized. Here, we examined the role of human MTG1, a conserved ribosome assembly GTPase. *MTG1*-silencing in human cardiomyocytes and developing zebrafish revealed early cardiovascular lesions. A combination of gene-editing and biochemical approaches using HEK293T cells demonstrated that MTG1 binds to the large subunit (mtLSU) 16S rRNA to facilitate incorporation of late assembly proteins. Furthermore, MTG1 interacts with mtLSU uL19 protein and mtSSU mS27, a putative GTP-exchange factor (GEF), to enable MTG1 release and the formation of the mB6 intersubunit bridge. In this way, MTG1 establishes a quality control checkpoint in mitoribosome assembly. In conclusion, MTG1 controls mitochondrial translation by coupling mtLSU assembly with intersubunit bridge formation using the intrinsic GEF activity acquired by the mtSSU through mS27, a unique occurrence in translational systems.
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CHAPTER 1

INTRODUCTION

1.1 Overview of mitochondrial OXPHOS system biogenesis

Mitochondria are essential organelles of eukaryotic cells that produce ATP by oxidative phosphorylation (OXPHOS). In addition, mitochondria are important for many cellular processes, including apoptosis, biosynthesis of amino acids and nucleotides, biogenesis of iron-sulfur clusters and calcium homeostasis. Mitochondria contain multiple copies of their own genome (the mtDNA), which is a double stranded closed circular molecule of 16.6 kb. Mitochondria are believed to be organelles of endosymbiotic origin. The mtDNA is a vestige of the original bacterial genome. During evolution, most genetic information has been transferred to the nuclear genome \(^1\). Only a handful of hydrophobic proteins (8 in yeast and 13 in mammalian cells) remain encoded in the mtDNA. Mitochondria also contain their own translation machinery, which in mammals is specialized for the synthesis of the 13 mtDNA-encoded proteins. These mitochondrial translation products are co-translationally inserted into the mitochondrial inner membrane and form the core reaction centers of the OXPHOS system enzymes. The rest of OXPHOS components are encoded in the nucleus, synthesized on cytoplasmic ribosomes, and imported into mitochondria. In addition to 13 polypeptides, the mtDNA also encodes RNA components of the mitochondrial translation machinery: two ribosomal RNAs (rRNAs, 16S and 12S) and 22 tRNAs. All additional protein factors required to assemble the mitochondrial translation apparatus and make it
functional, including mitoribosomal proteins, mitoribosome assembly factors, translation factors, aminoacyl tRNA synthetases, RNA-modifying enzymes, and other auxiliary factors, are encoded by nuclear DNA (nDNA) and imported into mitochondria (Fig. 1.1).

Figure 1.1. Biogenesis of OXPHOS enzyme subunits. OXPHOS subunits are encoded by two genomes (mtDNA and nuclear DNA). Nuclear-encoded proteins are translated by cytosolic ribosomes as precursor proteins and imported into mitochondria through the translocase complexes of the outer membrane (TOM) and the inner membrane (TIM23). mtDNA-encoded proteins are synthesized by the mitoribosomes and co-translationally inserted into the mitochondrial inner membrane with the help of the insertase machinery. Proteins from these two origins are assembled together to form a functional OXPHOS complex. Figure modified from 2.

1.2 The mammalian mitoribosome: structure and composition

As mitochondria originated from endosymbiotic proteobacteria, the mammalian mitoribosome is more similar to the bacterial than to the cytoplasmic
ribosome, structurally and functionally. For example, the catalytic properties, decoding and peptide bond formation in human mitoribosomes are similar to those of bacterial ribosomes. However, along evolution, mitoribosomes have acquired specific components and specific functional properties.

In all living organisms, mature mitoribosomes are composed of two distinct subunits, the large subunit (LSU) and the small subunit (SSU). Whereas the LSU catalyzes the peptidyl-transferase reaction, the SSU provides the platform for mRNA binding and decoding. Mammalian mitoribosomes have a low sedimentation coefficient (~55S), including the 39S large subunit (mtLSU) and the 28S small subunit (mtSSU), large variation in the protein content, much more protein and less rRNA than bacterial ribosomes and yeast mitoribosomes. The mtLSU is formed by a 16S rRNA, a structural tRNA (tRNA^Val^ in human cells) and 53 mitoribosomal proteins (MRPs). The mtSSU is formed by a 12S rRNA and 29 MRPs. In the 55S mitoribosome, 36 out of its 82 proteins, 22 from the mtLSU and 14 from the mtSSU, are mitochondria-specific, whereas the rest are conserved in the bacterial ribosome. Most conserved proteins contain mitochondrion-specific N- or C-terminal extensions, which only in part serve to replace several of the major RNA stem structural elements, present in the bacterial ribosome that has been lost in the mammalian mitoribosome. In fact, the recent high-resolution cryo-EM reconstruction of the mammalian mitoribosome revealed that the MRP extensions predominantly interact with mitochondrion-specific proteins, whereas only a few participate in filling the space of deleted rRNA. Most mitochondrion-specific proteins are peripherally distributed over the solvent-accessible surface,
forming clusters at the central protuberance, the L7/L12 stalk, and contiguous to the polypeptide exit site. Similar to the protein extensions, these MRPs occupy novel positions rather than compensate for the missing rRNA\textsuperscript{10-12,15,16}. The protein-protein interaction network has dramatically increased from the bacterial to the mammalian mitoribosomes. The central protuberance (CP) of the mtLSU is enlarged due to the acquisition of several mitochondrial-specific proteins, and two crescent-shaped pentatricopeptide repeat (PPR)-fold proteins dominate the overall appearance of the mtSSU \textsuperscript{17}. An exceptional feature of the human mitoribosome is that tRNA\textsubscript{val} exists as a structural component of the mtLSU \textsuperscript{9}. Furthermore, the tRNA-exit site (E-site) is significantly altered and the central protuberance (CP) is markedly remodeled in the mitoribosome \textsuperscript{5}. Some mitochondrion-specific proteins serve to establish intersubunit bridges \textsuperscript{13}, a feature that is different from cytoplasmic ribosomes that typically contain RNA-RNA intersubunit connections. The number of intersubunit bridges is also lower in mammalian mitoribosomes than in the bacterial ribosome, mostly resulting from the reduction of rRNA segments, and possibly to allow the two subunits to have flexibility in the conformation enabling them to tilt freely (Fig. 1.2) \textsuperscript{15,16}. Another major remodeling from the bacterial ribosome occurs at the aminoacyl and peptidyl-tRNA binding sites, where some proteins present in bacterial ribosomes (e.g. uL5 or bL25) have been lost to accommodate human tRNAs, which contain highly variable loops at the elbow. A unique property of mitoribosomes is the acquisition of an intrinsic GTPase activity through mtSSU subunit mS29, a GTP-binding protein \textsuperscript{17}. The GTPase activity is probably relevant to subunit association,
given the localization of mS29 at the subunit interface, its involvement in coordinating two mitochondrion-specific intersubunit bridges, mB1a and mB1b, with elements of the remodeled central protuberance\textsuperscript{9,18}. The affinity for GTP is higher for the mtSSU than for the 55S monosome.

Mitoribosomes reside in the mitochondrial matrix and associate with the inner membrane to facilitate co-translational insertion of the nascent polypeptides into the inner membrane. In fact, the polypeptide exit tunnel is adapted to the transit of hydrophobic nascent peptides\textsuperscript{10,11}. The tunnel is formed by several conserved proteins that create a ring around the exit site, namely bL23m, bL29m, bL22m, bL24m and bL17m. This conserved core is surrounded by another protein layer consisting of bL33 and mL45, which promote anchoring of the mtLSU to the inner membrane\textsuperscript{10,11,18,19}. Another significant structural remodeling has been observed at the mRNA entrance of the mtSSU\textsuperscript{14-16}, to accommodate mammalian mitochondrial mRNAs, which lack or have very short 5’-untranslated regions\textsuperscript{20}. Since the structural data have disclosed the presence of mS39, a pentatricopeptide repeat (PPR) protein in the proximity of the channel entrance, it has been suggested that it could be involved in recruiting leaderless mRNAs during translation initiation\textsuperscript{14-16}. 
Figure 1.2. The structure of the yeast and human mitoribosome, and their intersubunit bridges. Intersubunit interfaces with residues that contribute to bridges highlighted. (A) Bridges also present in the bacterial ribosome are in blue; mitoribosome-specific bridges conserved in the human mitoribosome are in red; and yeast-specific bridges are in yellow. Residues that form additional bridges in class B are shown in teal. 21 (B) Contact surfaces (distance <4 Å) and surfaces in close proximity (<6 Å) are colored in blue and pale blue (C) or red and pale red (D), respectively, and labeled with the bridge number 18.
In yeast, proteins that facilitate insertion of newly-synthesized polypeptides into the inner membrane, also serve to anchor the mitoribosome to the membrane. Genetic and biochemical analyses identified three factors that compose the yeast mitochondrial inner membrane insertase machinery; Oxa1, Mba1 and Mdm38. Oxa1, the homologue of the bacterial YidC, serves as an integrase that inserts its client proteins into the inner membrane. Mba1, the yeast homologue of mammalian mtLSU protein mL45, has homology to the C-terminal domain of inner membrane translocon component TIM44 and locates next to the tunnel exit of the mitoribosome. Mdm38, the homologue of mammalian Letm1, is a conserved membrane receptor for mitochondrial ribosomes that is required for efficient cotranslational insertion of newly synthesized polypeptides into the mitochondrial inner membrane. Biochemical studies have shown that the yeast mitoribosome remains associated to the inner membrane even in mutants that lack the three identified anchoring proteins, indicating the presence of additional, so far unidentified membrane tethers. However, tomogram analyses have shown a second anchoring point for the yeast mitoribosome formed by the 21S rRNA helix 9S-ES1. Differently, the subtomogram average of the membrane-associated human mitoribosome has revealed a single major contact site with the inner membrane, mediated by the mitochondria-specific protein mL45. The second rRNA-mediated contact site present in yeast is absent in the human mitoribosome, which results in a more variable association of the human mitoribosome with the inner membrane. Despite extensive structural differences between the mammalian
and fungal mitoribosome structure, the main organization of the peptide exit tunnel and the mL45 homolog remains invariant, presumably to align the mitoribosome with the membrane-embedded insertion machinery.24

Figure 1.3. The in situ structure of the human and yeast mitoribosome. Human mitoribosome has only one mL45-mediated contact site, whereas the yeast mitoribosome has two contacts to be associated with the IMM, mediated by the mL45 homolog Mba1 and the long rRNA expansion segment 96-ES1. On both the human and yeast mitoribosome, mL45/Mba1 is positioned directly beneath the PET (peptide exit tunnel), bridging the ~30-Å gap between the ribosomal surface and the IMM.24

1.3 Mitochondrial ribosomal RNA processing

Mammalian mtDNA is a double-stranded circular molecule. The two strands of mtDNA have been designated as the light inner strand (L-strand) and the heavy outer strand (H-strand) (Fig. 1.4). The heavy strand encodes most of the information: 14 tRNAs and 2 rRNAs, and 12 polypeptides. The light strand
Figure 1.4. Structure of mammalian mitochondrial DNA. The outer and inner circles represent heavy and light strands, respectively. Protein-encoding genes (blue arrows) and ribosomal RNA genes (yellow arrows) are interspersed with 22 tRNA genes (orange arrows; single-letter amino acid codes). The D-loop regulatory region contains the L- and H-strand promoters (LSP, HSP1, and HSP2), with arrows showing the direction of transcription. $O_H$ and $O_L$ indicate the replication origins of the H- and L-strands, respectively. Term is a terminator for the short transcript from HSP1\textsuperscript{25.}

encodes 8 tRNAs and one polypeptide, the *ND* (NADH dehydrogenase) 6 subunit\textsuperscript{26.} Two genes, *ATP8*/*6* and *ND4*/*4L*, have overlapping of 46bp and 7bp, respectively. The long polycistronic RNAs of the H-strand and L-strand are transcribed from the H- and L-strand promoters by the mitochondrial transcription
machinery, including a monomeric RNA polymerase (POLRMT, polymerase (RNA) mitochondrial), TFAM (mitochondrial transcription factor A), TFB2M (mitochondrial transcription factor B2), TEFM (mitochondrial transcription elongation factor), mTERFs (mitochondrial transcription termination factors), and MRPL12 \(^{27}\) (Fig. 1.5). The entire mitochondrial genome give rise to three (HSP1, HSP2 and LSP) polycistronic primary transcripts, two of these transcripts correspond to each whole strand \(^{29}\) (Fig. 1.5). The HSP2 transcription from the H-strand results in an RNA unit that is processed into two rRNAs, 14 tRNAs and mRNAs that encoded 12 proteins. The LSP transcription of the L-strand

![Figure 1.5. Overview of human mitochondrial transcription, RNA processing and translation \(^{28}\). xARS and xARS2, aminoacyl-tRNA synthetases.](image)

\(^{27}\) HSP1, HSP2 and LSP are mitochondrial protein-coding genes.

\(^{28}\) xARS and xARS2 are mitochondrial RNA polymerases.
RNA that is cleaved to produce 8 tRNAs and only one mRNA that encodes a single protein. Unlike other two long transcripts, the third transcript is smaller and contains, in consecutive order, tRNA\textsuperscript{Phe}, 12S rRNA, tRNA\textsuperscript{Val}, and 16S rRNA, the last three being components of the mitoribosome. In exponential dividing HeLa cells, initiation at HSP1 is favored fifty to hundred times over initiation at HSP2. Once the entire mtDNA is transcribed, the long polycistronic transcripts require multiple processing and modification steps to become functional, individual RNA species. Most ORFs (open reading frames) and rRNAs in these transcripts are punctuated by tRNAs, and processing of primary transcripts occurs at tRNAs. These primary transcripts are processed by the action of mitochondrial ribonuclease P (mtRNase P; MRPP1, MRPP2 and MRPP3) and the RNase Z-type enzyme (ELAC2), which cleave the tRNAs at their 5' and 3' ends, respectively, and individual RNA species are released and subsequently matured. The mammalian mt-rRNAs are unknown to have subsequently microprocessing, as it occurs in bacteria. RNase P and ELAC2 are also involved in tRNA\textsuperscript{Val} excision. It remains unknown whether and how tRNA\textsuperscript{Val} excision is coordinated with assembly of 39S mtLSU. The SUV3 helicase and PNPase (polynucleotide phosphorylase) form a transient RNA-degrading complex, the degradosome, which efficiently degrades defective or excess mtRNAs.

1.4 Mitochondrial ribosomal RNA modifications

Mammalian mitoribosomes have smaller rRNAs than do bacterial ribosomes and they do not contain a 5S rRNA. The 12S rRNA in the human mtSSU is 954
nucleotides long (323 kDa), which is about 40% shorter than bacterial 16S rRNA. The 16S rRNA in the human mtLSU is 1558 nucleotides long (528 kDa), which is approximately half of bacterial 23S rRNA. Mammalian mitochondrial rRNAs contain fewer modified nucleotides. The few methylated nucleotides are concentrated at the subunit interfacial regions. Only a single pseudouridine locates at a conserved site within the peptidyl transferase domain.

rRNA modifications at conserved regions, frequently in the catalytic domains, is an important step in ribosome assembly. These modifications consist of base methylation, 2'-O-ribose methylation, and pseudouridylation, which can occur co-transcriptionally or immediately after transcription during ribosome assembly. These processes are conserved between prokaryotes and eukaryotes. Base methylation is catalyzed by specific methyltransferases (MTases), which recognize their targets directly. However, 2'-O-ribose methylation and pseudouridylation involve different mechanisms between prokaryotes and eukaryotes. Pseudouridine synthases catalyze rRNA pseudouridylation. Whereas in the eukaryotic system, small nucleolar RNAs (snoRNAs) are required to recognize the target nucleotides, the prokaryotic rRNA modifications are catalyzed by site-specific enzymes without the guide RNA. In a similar way, the mitochondrial MTases catalyze rRNA modifications without small RNA guidance.

In S. cerevisiae, the mtSSU 15S rRNA lacks methylated nucleotides and might have only one pseudouridylation site, although the position on the rRNA and the enzyme responsible remain unknown. However, the mtLSU 21S rRNA has three modified nucleotides; one pseudouridine (ψ2819) and two 2'-O-ribose
methylations (Gm2270 and Um2791) located in the peptidyl transferase center (PTC) implicated in the interaction of the ribosome with an aminoacyl (A)-site tRNA. In *E. coli*, three ribose methylations are found on universally conserved nucleotides within domain V located at PTC, G2251, C2498 and U2552. The corresponding methylation site of *E. coli* G2251 in yeast 21S rRNA is G2270. Gm2270 is methylated by a nuclear-encoded site-specific rRNA ribose MTase called Pet56 or Mrm1. Although Mrm1 does not co-sediment with the mtLSU, ribosome assembly is affected when Mrm1 expression is impaired. Methylation by Mrm1 can proceed in naked RNA, indicating that methylation could occur early in the mitoribosome assembly pathway. The methylation site corresponding of *E. coli* U2552 in yeast 21S rRNA is Um2791. The MTase responsible for Um2791 is Mrm2, which methylates U2791 only when the 21S rRNA is assembled into the LSU, indicating that this modification probably occurs at a later stage of mitoribosome maturation. Mrm2 co-sediments with the mtLSU on a sucrose gradient. However, accumulation of the ribosome assembly intermediats in the absence of Mrm2 is not reported. The 21S rRNA has only one pseudouridylation at residue ψ2819, which is synthesized by the pseudouridine synthase Pus5. This residue is conserved in the mtLSU rRNA from species, including gram-negative bacteria, mouse and human, but not in gram-positive bacteria or eukaryotic cytoplasmic ribosomes. ψ2819 is located in the most highly conserved region of the PTC domain, implicating that the ψ2819 residue plays a functionally important role during peptide bond formation.
In the mammalian mitoribosome, the rRNA modifications, mapped using hamster cells showed that only nine rRNA nucleotides are modified in mitochondrial rRNAs, which are comparable with more than 30 and 200 modifications in prokaryotic and eukaryotic cytosolic rRNAs, respectively. The mammalian mtSSU 12S rRNA has five methylated bases; m\textsuperscript{5}U429, m\textsuperscript{4}C839, m\textsuperscript{5}C841, m\textsuperscript{6}\textsubscript{2}A936, and m\textsuperscript{6}\textsubscript{2}A937. The two adenines (A936 and A937) at the stem loop structure of the 3’ end of the 12S rRNA are conserved in all domains of life, archaebacterial, eubacteria and eukaryotes. In bacterial SSU rRNA, these modifications are present in a region that contains the mRNA decoding center and the binding site for the LSU, which indicates the importance of this modification for translation regulation. In the mammalian mtSSU rRNA, the two adjacent adenines are methylated by mitochondrial transcription factor B (TFB1M), which is a functional homologue of bacterial MTase KsgA. The extract from KsgA deficient E. coli has showed a reduced affinity between the SSU and LSU. In addition, KsgA sterically blocks incompletely assembled small ribosomal subunits from entering the translational cycle prematurely. These data indicate that KsgA may have a role in the final stages of monosome assembly. The mtLSU 16S rRNA contains three 2’-O-ribose methylated nucleotides (Gm\textsubscript{1145}, Um\textsubscript{1369}, and Gm\textsubscript{1370}) as well as one pseudouridylated base, \(\psi\textsubscript{1397}\). The Um\textsubscript{1369} and Gm\textsubscript{1370} are predicted to locate in the LSU A-loop, which is an essential component of the PTC. The PTC structure is well conserved through evolution among bacterial, cytosolic, and mitochondrial ribosomes, in which they stabilize the RNA structure critical for the peptidyl-transferase reaction and tRNA
recognition. Significantly, highly conserved nucleotides in the A-loop often contain 2'-O-methylation of ribose, namely, 2'-O-methyluracil (E. coli, Um2552; yeast cytosol, Um2921) and 2'-O-methylguanosine (yeast cytosol, Gm2922) \(^{47}\). Three methyltransferases, MRM1, MRM2, and MRM3 (also called RNMTL1), modify the mammalian 16S rRNA. MRM1 catalyzes the methylation of G1370, MRM2 of Um1369, and MRM3 of Gm1370 of human 16S rRNA. MRM1 does not co-sediment with mitoribosomes in glycerol gradients. Instead, a small fraction of this proteins was found at the bottom of the gradient where nucleoids and probably other large complexes, such as the RNA granules, sediment \(^{46}\). On the contrary, MRM2 and MRM3 are associated with mitoribosomes, and their depletion affects both mitochondrial protein synthesis and respiration. These observations indicated that the modifications catalyzed by MRM2 and MRM3 are important for the proper maturation of the mitochondrial translation machinery \(^{47}\). Both Um1369 and Gm1370 locate in the A-loop of the mtLSU, which is an essential RNA component of the ribosomal PTC that directly interacts with aminoacyl (A)-site tRNA. Interestingly, human MRM2 interacts with the intact mitochondrial monosome, which was not detected from its orthologue of bacterial RrmJ nor yeast Mrm2p, implicating an extra role of MRM2 that could contribute to the very final stages of mitochondrial ribosome subunit joining into a functional monosome.

Regarding the human 16S rRNA base pseudouridylation at \(\psi_{1397}\), two recent studies have reported the identification of the enzyme involved. One group used a proximity-biotinylation assay to identify mitochondrial RNA granule components, which are platforms for post-transcriptional RNA modification and, at
least, for late stages of ribosome assembly. They identified a protein module containing three uncharacterized pseudouridine synthases, TRUB2, RPUSD3, and RPUSD4. Investigation of the molecular targets in mitochondrial RNA by pseudouridine-Seq showed that RPUSD4 plays a role in the pseudouridylation of a single residue in the 16S rRNA, a modification that is essential for its stability and assembly into the mitochondrial ribosome, while TRUB2/RPUSD3 were similarly involved in pseudouridylyating specific residues in mitochondrial mRNAs. The second group also found RPUSD4 to be a component of the RNA granules. They showed that RPUSD4 depletion leads to a severe reduction of the steady-state level of the 16S rRNA with defects in the biogenesis of the mtLSU. Interestingly, RPUSD4 not only pseudouridylates the 16S mt-rRNA, but also the mt-tRNA\textsuperscript{Phe}.  

### 1.5 Ribosome assembly

Ribosome assembly is a highly complicated pathway that requires coordination of rRNA synthesis, processing and modification with ribosomal protein synthesis and modification as well as their correct folding, maturation and assembly into functional particles. For mitoribosomes, the process is further complicated by the dual genetic origin of their RNA and protein components.

Although in pioneering experiments bacterial ribosomes were assembled \textit{in vitro} without the assistance of non-ribosomal components, the \textit{in vivo} process requires the assistance of dedicated assembly factors that increase the accuracy and the efficiency of the process. A large list of mitoribosome assembly factors are
involved in each stage of the biogenetic pathway to regulate proper folding and maturation of assembly intermediates (Table 1.1).

In bacteria and cytoplasmic eukaryotic ribosomes, where the process is known in more detail, ribosome assembly is tightly regulated by the action of several classes of assembly factors such as GTPases, RNA helicases, RNA modification enzymes and chaperone proteins\(^{51,52}\). To date, the proteins known to facilitate mitoribosome biogenesis are only a few\(^ {53-55} \), although the list is growing quickly. As for other ribosomes, the entire process of mitoribosome assembly is regulated by the same classes of ribosome biogenetic factors, including nucleases, rRNA modifying enzymes, helicases, GTPases and chaperones. Unexpectedly, two mitochondrial transcription factor family proteins (mTERF3 and mTERF4) play roles in mtLSU assembly, perhaps coordinating transcription and ribosome biogenesis. The precise role of mTERF3 (or mTERFD1) is not understood\(^ {55} \), but mTERF4 binds to the rRNA methyltransferase NSUN4 to promote its recruitment to the mtLSU\(^ {54} \), possibly to facilitate monosome assembly\(^ {53} \). A role in monosome formation has also been proposed for MPV17L2, a mtDNA maintenance factor\(^ {56} \), although its function remains intriguing. FASTKD2, a member of the Fas-activated serine-threonine kinase family of proteins also binds to the 16S rRNA and participates in mtLSU assembly\(^ {57} \). Furthermore, MALSU1, a member of the DUF143 family of ribosomal silencing factors, interacts with uL14 and promotes its incorporation into the mtLSU\(^ {58} \). Finally, GRSF1 (G-rich sequence factor 1) is an RNA-binding protein that interacts with several mtRNAs, including the 12S rRNA, and participates in mtSSU biogenesis\(^ {59} \). The list of known mitoribosome assembly
factors and their role are summarized in Table 1, some of which will be discussed in this section. For bacterial and eukaryotic ribosomes, assembly starts with the binding of ribosomal proteins to nascent rRNA while transcription proceeds, which remains to be identified in more detail in mitochondria.

1.5.1. Compartmentalization of the mitoribosome assembly pathway
Mitoribosome mtLSU assembly has been proposed to occur in contact with the inner membrane \(^6\), in the RNA granule compartment, which is located near the mtDNA nucleoid \(^5\). The RNA granules contain ribosomal proteins, ribosomal RNA modifying enzymes, all the mitoribosome assembly factors listed in Table 1.1 and Table 1.2 as well as a host of proteins involved in diverse aspects of mt-RNA metabolism \(^4\). The RNA granules condense around mtRNAs and therefore initially overlap with the mtDNA nucleoids. However, the granule structures progressively separate from the nucleoid forming an independent compartment \(^5\). Biogenesis of the mtLSU and the mtSSU may be differently compartmentalized. While both require rRNA and therefore will start at the nucleoid where transcription occurs, incorporation of mtSSU proteins could occur in this compartment as it has been reported by several groups \(^6\). Further discussion on mitoribosome assembly compartmentalization can be found in the Discussion section.
Table 1.1. Role of mitoribosome assembly factors in yeast and human\(^{40}\).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function/Interaction</th>
<th>Effects of deletion/depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yeast</strong></td>
<td><strong>Human</strong></td>
<td><strong>Subassembly</strong></td>
</tr>
<tr>
<td><strong>Mitoribosomal LSU assembly cofactors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modifying enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrm1/Pet56</td>
<td>Mitoribosomal LSU assembly cofactors</td>
<td></td>
</tr>
<tr>
<td>Mrm1/ Pet56</td>
<td>MRM1</td>
<td>Methylation of 21S rRNA during early assembly</td>
</tr>
<tr>
<td>Mrm2</td>
<td>MRM2</td>
<td>Methylation of 21S rRNA during late assembly</td>
</tr>
<tr>
<td>MRM3/RNMTL1</td>
<td>Methyltransferase</td>
<td>Yes</td>
</tr>
<tr>
<td>Pus5</td>
<td>Pseudouridylation of 21S rRNA</td>
<td>-</td>
</tr>
<tr>
<td><strong>GTPases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mtg1</td>
<td>MTG1</td>
<td>Δmtg1 defect suppressed by 21S ery(^{2}) mutations</td>
</tr>
<tr>
<td>Mtg2</td>
<td>MTG2/GTPBP5</td>
<td>Multicopy suppressor of mrm2 thermo-sensitive mutant</td>
</tr>
<tr>
<td>GTPBP10</td>
<td>GTPase important for mtLSU biogenesis</td>
<td>-</td>
</tr>
<tr>
<td><strong>RNA helicases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrh4</td>
<td>DDX28</td>
<td>Binds 21S rRNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binds 16S rRNA</td>
</tr>
<tr>
<td></td>
<td>DHX30</td>
<td>Binds 16S RNA and 21S rRNA</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTERFD1/mTERF3</td>
<td>Stabilization of 16S rRNA</td>
<td>Unstable LSU</td>
</tr>
<tr>
<td>mTERF4</td>
<td>Binds both 12S and 16S rRNA and forms a complex with NSUN4 and recruits it to mtLSU</td>
<td>Stable and increased level of both 28S and 39S SU, 55S absent</td>
</tr>
<tr>
<td>Yta10</td>
<td>AFG3L2</td>
<td>Processing of bL32</td>
</tr>
<tr>
<td>Yta12</td>
<td>SPG7</td>
<td>Processing of bL32</td>
</tr>
<tr>
<td>FASTKD2</td>
<td>Binds 16S rRNA</td>
<td>No</td>
</tr>
</tbody>
</table>
### TFB1M
- Dimethylation of two highly conserved adenines at a stem loop structure close to the 3' end of 12S rRNA
- Reduced 28S levels; 55S absent
- Unstable 12S rRNA

### NSUN4
- Methylation of 12S rRNA, complex with mTERF4
- Stable and increased level of both, 28S and 39S, 55S levels reduced
- Stable and increased level of both 12S and 16S rRNA

### GTPases

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>28S Assembly</th>
<th>39S Assembly</th>
<th>12S rRNA Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mtg3</td>
<td>GTPase important for processing of 15S rRNA, uL29 is a multicopy suppressor of ∆mtg3</td>
<td>Unstable 30S</td>
<td>Unstable and unprocessed 15S rRNA</td>
<td>Unstable 12S rRNA</td>
</tr>
<tr>
<td>C4orf14</td>
<td>Interacts with the SSU via a GTP-binding mechanism</td>
<td>Unstable 28S</td>
<td>Unstable 12S rRNA</td>
<td></td>
</tr>
<tr>
<td>ERAL1</td>
<td>Binds to 12S rRNA</td>
<td>Unstable 28S</td>
<td>Unstable 12S rRNA</td>
<td></td>
</tr>
</tbody>
</table>

### Other

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>28S Assembly</th>
<th>39S Assembly</th>
<th>12S rRNA Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRSF1</td>
<td>Found in mRNA granules, important for post-transcriptional storage, handling and translation of mRNA</td>
<td>28S assembly intermediate.</td>
<td>Unstable 12S and 16S rRNA</td>
<td></td>
</tr>
</tbody>
</table>

### Mitoribosomal assembly cofactors affecting SSU and LSU

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>28S Assembly</th>
<th>39S Assembly</th>
<th>12S rRNA Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPV17L2</td>
<td>Binds to 39S subunit</td>
<td>Unstable 28S and 39S. SSU proteins trapped in enlarged nucleoids.</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

#### 1.5.2 Mitoribosome assembly factors

##### 1.5.2.1 GTPases

GTPases are required for ribosome assembly as regulatory molecules. In bacteria, they present the largest class of essential assembly factors, suggesting that the requirement for regulation is evolutionally conserved. Classical GTPases depend on external proteins for its GTP hydrolysis activity (GAPs, GTPase activating proteins), and following the GTPase reaction to exchange GDP for GTP (GEFs, guanine nucleotide exchange factors), converting the GTPase into the active state again. However, there are many GTPases that do not follow this classical pattern.
Certain ribosome-associated GTPases are not reliant on other GEF proteins to exchange GDP for GTP \(^{66}\). Intriguingly, many of these G proteins are activated by RNAs and not by external GAPs. Numerous ribosome-associated GTPases, either required for ribosome biogenesis or directly participating in protein synthesis, have low intrinsic GTPase activities, but RNA plays a GAP-like role in GTPase activation \(^{66}\).

In bacteria, seven GTPases have been implicated in ribosome assembly \(^{52,67}\). In human mitochondria, three conserved GTPases are known to participate in mtLSU biogenesis; MTG1, a homolog of bacterial RbgA \(^{68}\), and two homologs of bacterial Obg, MTG2 (or GTPBP5 \(^{69}\)) and GTPBP10 \(^{70}\). Two other GTPases are required for mtLSU assembly; C4orf14 (a homolog of bacterial YqeH) \(^{71}\) and ERAL1 (bacterial Era1) \(^{72}\). Mammalian MTG1 belongs to the RbgA/YlqF GTPase subfamily, which is present in many bacteria except \(E.\ coli\). All members of the RbgA/YlqF GTPase subfamily, which contains a unique circular permutation of the GTP binding domain (cpGTPase), interact with RNA and are involved in ribosome assembly \(^{52}\). Whereas bacterial RbgA/YlqF is extensively characterized structurally and functionally, little is known regarding its mitochondrial MTG1 counterpart, particularly in human cells. The most studied \(Bacillus\ subtilis\) RbgA participates in the late steps of 50S ribosomal LSU assembly and maturation \(^{68}\). It interacts with the 50S (to specific positions in 23S rRNA) in a GTP-dependent manner, and its GTPase activity is stimulated more than 60-fold by association with the 50S subunit \textit{in vitro} \(^{73-75}\). After GTP hydrolysis, RbgA dissociates from the ribosome \(^{76}\). RbgA-depleted cells do not form mature 50S, but instead accumulate
a 45S intermediate complex that contains sub-stoichiometric amounts of late assembly LSU proteins: L16, L27, L28, L33, L35, and L36 \textsuperscript{51,67,74-78}. The \textit{rbgA} LSU assembly defect is attenuated by mutations in L6, which has suggested that RbgA could facilitate proper incorporation of L6 to the LSU assembly line, subsequently triggering the integration of late-assembly proteins \textsuperscript{51}. In mitochondria, MTG1 was first identified in the yeast \textit{Saccharomyces cerevisiae} as required for mtLSU 21S rRNA stability and mitochondrial protein synthesis \textsuperscript{79}. This phenotype is partially suppressed by spontaneous mutations in the 21S rRNA domain V, strongly suggesting a direct role of yeast MTG1 in mtLSU biogenesis. As in yeast, human MTG1 is known to localize to mitochondria, and \textit{MTG1} silencing in HeLa cells partially attenuates mitochondrial translation \textsuperscript{80}. The intrinsic recombinant MTG1 GTPase activity is undetectable, but it is stimulated \textit{in vitro} by the mtLSU \textsuperscript{80}. Importantly, heterologous expression of human \textit{MTG1} partially rescues the mitochondrial protein synthesis defect in yeast \textit{mtg1} null mutant cells \textsuperscript{79}. These observations suggest that human MTG1 could also participate in mtLSU assembly.

MTG2 (GTPBP10) belongs to the Obg subfamily, which is a class of highly conserved small monomeric P-loop GTPases principally involved in LSU assembly. In \textit{Bacillus subtilis}, Obg associates with the ribosome \textsuperscript{81}, possibly through an interaction with L13 \textsuperscript{82}. \textit{E. coli} ObgE or CgtA also associates with the LSU, interacts with the 23S rRNA and several ribosomal proteins \textsuperscript{69,83} and its GTPase activity is essential for LSU biogenesis \textsuperscript{84,85}. ObgE deletion in \textit{E. coli} leads to the accumulation of LSU assembly intermediates containing reduced levels of
L16, L33, and L34, further linking Obg proteins to roles in LSU biogenesis \(^8\)\(^6\). Furthermore, ObgE genetically interacts with the 23S rRNA methyltransferase RrmJ since ObgE overexpression suppresses the LSU 50S assembly defect observed in \(rrmJ\) mutant cells \(^8\)\(^7\). However, ObgE co-fractionates not only with 50S, but also with 30S ribosomal subunits, co-purifies with components of the two subunits and is required for efficient 16S rRNA processing \(^6\)\(^9\), suggesting a more global role in ribogenesis. In *S. cerevisiae*, the nucleolar Obg protein Nog1 is important for pre-60S particle assembly \(^8\)\(^8\),\(^8\)\(^9\). Within mitochondria, the Obg ortholog Mtg2 was initially identified in *S. cerevisiae* as a suppressor of mtLSU 21S rRNA methyltransferase mutant *mrm2*, suggesting a role during mtLSU biogenesis \(^9\)\(^0\). In mammals, two Obg proteins exist in mitochondria, MTG2 (alias OBGH1 or GTPBP5) and GTPBP10 (alias OBGH2), whose molecular roles are starting to emerge. MTG2 was shown to specifically associate with the mtLSU \(^9\)\(^1\), but partial *MTG2* silencing did not produce any apparent phenotype \(^8\)\(^0\),\(^9\)\(^1\). Regarding GTPBP10, it was localized to the nucleolus by immunofluorescence \(^9\)\(^1\). However, it is predicted to have a mitochondrial targeting sequence (MTS), and is listed in the MitoCarta inventory of mammalian mitochondrial proteins \(^9\)\(^2\). It has been recently reported that GTPBP10 is actually a mitochondrial protein that participates in late stages of mtLSU assembly in human cells \(^7\)\(^0\).

NOA1 (C4orf14) is a human homologue of Mtg3, a protein that interacts with the 28S mtSSU \(^7\)\(^1\). Sucrose gradient analysis showed that Mtg3 co-sediments with the mtSSU. Mtg3 depletion specifically affected the small subunit components, resulting in decreased mitochondrial protein synthesis. The GTPase
activity of Mtg3 is important for its interaction with the 28S SSU and other factors for mitochondrial translation. Therefore, it is proposed that Mtg3 binding with GTP facilitates mtSSU assembly, and GTP hydrolysis releases Mtg3 with GDP. Mtg3 was also found to be associated with human mitochondrial nucleoids. Mtg3 has capacity of binding to DNA in vitro and Mtg3 depletion in human cell caused mitochondrial DNA depletion. The association of Mtg3 both with mitochondrial translation factors and the mitochondrial nucleoids had suggested that the 28S SSU could be assembled at the mitochondrial nucleoids, which is capable of the direct transfer of mRNA from the nucleoid to the mitoribosome.  

1.5.2.2 RNA Helicases

RNA helicases are enzymes that catalyze the unwinding of double-stranded nucleic acids utilizing energy, produced by hydrolyzing nucleotide triphosphates, preferentially ATP. The ribosomal RNAs (rRNA) are often transcribed as precursors, which require additional processing steps for maturation, prior to assembly into ribosomal subunits. A large number of RNA helicases, the majority of which belong to the DEAD/H-box family (a family of helicases that contain Asp-Glu-Ala-Asp (DEAD) or Asp-Glu-Ala-His (DEAH) motifs), are involved in this process. In bacterial and eukaryotic systems, helicases play a major role in ribosomal biogenesis, translation initiation and termination. A large number of RNA helicases are involved in bacterial and cytoplasmic ribosome biogenesis. In mitochondria, two RNA helicases are known to be involved in mtLSU biogenesis; DDX28, a homolog of yeast Mrh4, and DHX30.
S. cerevisiae Mrh4 (4th putative mitochondrial DEAD box RNA helicase) was first identified as a suppressor of mitochondrial splicing defects in COX1, one of the three S. cerevisiae mtDNA genes that contain introns. However, several other mitochondrial helicases have been found to suppress splicing defects when overexpressed. Therefore, this function is not necessarily specific for Mrh4. A GFP-fusion of Mrh4 was localized to mitochondria and its presence was important for the maintenance of the mtDNA even in an intron less background. However, it is important to note that mtDNA is highly unstable in strains defective in translation hence the genome instability could be secondary to the translation defect. In S. cerevisiae, Mrh4 is essential for mtLSU biogenesis, participating in the late stages of mitoribosome assembly. Mrh4 interacts with the 21S rRNA, mitoribosome assembly intermediates, and fully assembled mitoribosomes. In the absence of Mrh4, 21S rRNA was able to mature, but part of a large on-pathway assembly intermediate accumulated proteins Mrpl16 and Mrpl39, two late assembly proteins. Therefore, Mrh4 is essential for the stable incorporation of these proteins as well as ribosome assembly. In this way, Mrh4 could act as an RNA chaperone to facilitate RNA-protein interactions or to catalyze protein displacement from RNA, thereby participating in the structural reorganization of late 54S ribonucleoprotein assemblies, which allow efficient binding of late assembly proteins.

Human DDX28 is an RNA granule component essential for mtLSU biogenesis. RNA co-immunoprecipitation analysis showed that DDX28 interacts with the 16S rRNA, with specificity for the region corresponding to domain V of the 16S rRNA, which is present on the subunit interface side of the mtLSU.
Sucrose gradient analysis identified DDX28 interacting with mtLSU. Depletion of DDX28 does not affect mitochondrial mRNA stability or 16S rRNA processing or modification. However, 16S rRNA and mtLSU proteins were decreased and mtLSU assembly was impaired. As a consequence, mitochondrial translation and oxidative phosphorylation were deeply attenuated. DDX28 is proposed to be an essential factor in early stages of mtLSU biogenesis, which takes place within the compartment defined by the RNA granules.

DHX30 was initially identified at the central core region of mitochondrial nucleoids where it has been proposed that proteins related to mitochondrial replication and transcription accumulate. More recent studies have localized DHX30 to the mitochondrial RNA granules and shown to be mostly important for mitoribosome LSU assembly. However, the helicase interacts with both the 12s and 16S rRNAs, co-fractionates with the two mitoribosome subunits and the monosome and DHX30 depletion affects not only mtLSU biogenesis but also slightly the accumulation of the mtSSU.

The identification of DDX28, DHX30 and other proteins in both RNA granules and mtDNA nucleoids confirms that RNA granules and mtDNA nucleoids are located in close proximity and a portion of them dynamically overlap to attend the necessities of newly transcribed RNAs.

### 1.5.2.3 Other assembly factors

C7orf30 (MALSU1, mitochondrial assembly of ribosomal large subunit 1) is a member of the DUF143 family and participates in mtLSU assembly by interacting
with uL14 and promoting its incorporation into the mtLSU. Isokinetic sucrose gradient and 2D blue native (BN)-PAGE analyses showed that MALSU1 co-sediments with mtLSU \(^98\). MALSU1 depletion does not affect the sedimentation profile of the mtLSU, but results in the depletion of several mtLSU proteins and consequent decreased monosome formation. This leads to a mitochondrial translation defect, involving the majority of mitochondrial polypeptides, and a severe OXPHOS assembly defect \(^99,100\). Immunoprecipitation and mass spectrometry analyses revealed that uL14 is a specific interacting partner of MALSU1 in the mtLSU. The recent cryo-EM reconstruction redefined MALSU1 binding to the mtLSU in a higher resolution \(^101\). The face of the five-stranded beta-sheet of MALSU1 packs perpendicularly against the terminal helix of uL14. Upon MALSU1 binding, both uL14 and the neighboring bL19 display conformational changes relative to their positions in the mature mitoribosome. Besides MALSU1, two new assembly factors (L0R8F8 and mt-ACP) were identified in the human mtLSU structure.

GRSF (G-rich sequence binding factor1) is an RNA binding protein which has an important role as a mitochondrial RNA granule component, which as mentioned earlier, are involved in the processing, storage, sorting or translation of newly synthesized RNAs \(^59,63\). The mitochondrial isoform of GRSF accumulates in discrete foci located in the mitochondrial matrix, and nascent mitochondrial RNA and RNase P were present together in the same foci, implicating the role of GRSF1 in the mitochondrial RNA granules. GRSF1 preferentially binds RNAs transcribed from three contiguous genes on the light strand of mtDNA, the \textit{ND6} mRNA, and
the long noncoding RNAs for \textit{cytb} and \textit{ND5}, each of which contains multiple consensus binding sequences\textsuperscript{59}. GRSF1 depletion affects the cleavage of primary RNA transcripts and mtRNA stability, leading to impaired mitoribosome assembly and decreased expression of mitochondria-encoded proteins \textsuperscript{59,63}. In addition, GRSF was proposed to play a direct role in mtSSU assembly (the two original papers about rna granules) and it has been found to co-fractionate with the mtSSU in wild-type HEK293T cells\textsuperscript{61}.

1.5.3 Defining the mitoribosome assembly pathway

During the last decade, developments in high-resolution cryo-electron microscopy (cryo-EM) and X-ray crystallography have led to critical information regarding the 3D structure of yeast and mammalian mitoribosomes \textsuperscript{9,18}. Now that the yeast, porcine and human mitoribosome structures\textsuperscript{9} have been determined, interest has increased regarding how their assembly proceeds. The mechanisms by which mitoribosomal proteins and rRNAs are integrated into the assembly process, and the steps at which the various mitoribosomal assembly factors act are only starting to emerge. However, recent efforts have provided the first assembly maps for the yeast mtLSU \textsuperscript{22} and the human mitoribosome \textsuperscript{102}. As a reference, because the hierarchical ribosome assembly \textsuperscript{103} of bacterial ribosomes is well characterized \textit{in vitro} and \textit{in vivo} \textsuperscript{104-106}, the bacterial studies can provide information about how this assembly might occur in mitoribosomes and how to approach the study of mitoribosome assembly.
Efforts to define bacterial ribosome assembly pathway started in the 1960s when the groups of Nomura and Nierhaus reconstituted the assembly in vitro. They demonstrated that active bacterial ribosome subunits can be reconstituted in vitro with unmodified free rRNA and ribosomal proteins without any other components, but a marked dependence on non-physiological temperature and ionic conditions. Through numerous measurements of binding interdependence among different proteins, they categorized 30S reconstitution intermediates and 50S reconstitution intermediates into two and three hierarchical classes, respectively. With the data obtained from these experiments, they established the classic Nomura and Nierhaus assembly map representing the binding of ribosomal proteins in 30S and 50S subunits and found that ribosomal proteins are incorporated in different orders, displaying both hierarchical and parallel manners. Moreover, they found that a few primary binding proteins stably bind directly to the nascent rRNA and the remaining proteins (secondary and tertiary binding proteins) depend on the binding of the primary proteins.

More recently, different methods such as 1) time-resolved hydroxyl radical footprinting to map changes in the structure of the rRNA within 20 milliseconds (ms) after the addition of total 30S proteins in E. coli 30S ribosome subunit, 2) 2D gel electrophoresis and autoradiography to analyze ribosome compositions and assembly isolated from mitochondrial extracts from HEK293T cells, 3) combined pulse-labeling and quantitative mass spectrometry to analyze the dynamics of ribosome assembly and ribosomal assembly intermediates in vivo, have revealed that despite the dependency of ribosomal protein binding,
ribosome assembly can proceed through multiple parallel pathways generating heterogeneous populations of assembly intermediates. These studies suggested that a variety of methods can be applied to any system where ribosomal assembly intermediates accumulate, including strains with deletions or mutations of assembly factors, to study the dynamics of assembly and turnover of other macromolecular complexes that can be isolated from cells. Systematic chromosomal deletion of ribosomal protein genes in combination with cryo-EM applied to *E. coli* provided an idea about conformational maturation of the rRNA and of the growing ribonucleoprotein particles. The use of high-resolution high-throughput qMS in combination with SILAC (stable-isotope pulse labeling) approaches made it possible to characterize in vivo ribosome assembly intermediates and associated assembly factors in wild-type *E. coli* cells. They provided snapshots of the assembly intermediates of four subassemblies for the 30S subunit and 6 for 50S precursors, which correlated with *in vitro* reconstitution data.

As explained earlier, the mitoribosome is different from the bacterial ribosome and therefore, changes may be expected in its assembly kinetics and pathway. First, the mitoribosomes are anchored to the mitochondrial inner membrane, through the mtLSU, in order to facilitate protein synthesis and membrane insertion. Not surprisingly, the yeast mtLSU assembly process has been proposed to occur in contact with the inner membrane. In fact, a recent report has shown that several assembly intermediates are tethered to the inner mitochondrial membrane. Second, most mammalian mitoribosome assembly
mutants described so far apparently do not accumulate assembly intermediates that could be characterized. In yeast, several methodologies have been attempted to identify ribosome assembly; 1) thermo-sensitive mutant analysis to control the induction of mutant types, 2) expression of genetic suppressors to maintain mtDNA stability in the absence of mitoribosome assembly, and 3) expression of some truncated ribosome proteins resulting in ribosome assembly lesions without affecting mtDNA stability.

Recently, a hierarchical cluster analysis of mtLSU assembly subassemblies were published\(^\text{22}\). They analyzed by mass spectrometry analysis (LC MS/MS) the protein compositions of 54S subassemblies accumulated in 44 mutant strains each deleted for a different mtLSU subunit gene and established a mtLSU assembly map that was compared to that of bacterial ribosomes (Fig. 1.6). Their data revealed cooperative assembly of protein sets forming structural clusters and preassembled modules. In addition, orthologs or paralogs of the yeast mtLSU factors discussed here have been recognized as acting in similar stages of assembly of bacterial 50S LSU.

At the same time, the first human mitoribosome assembly model has been obtained by using SILAC pulse-labeling in a combination of structure-based kinetic analyses\(^\text{102}\). The authors analyzed the rate of mitochondrial import of MRPs and their assembly into intact mitoribosomes in HeLa cells by using SILAC pulse-labeling. This methodology has made it possible to track the appearance of newly synthesized MRPs in isolated mitochondria and in mature mitoribosomes. MRPs were classified and clustered by their binding stages to generate a timeline of
Figure 1.6. Comparison of the proposed bacterial LSU and yeast mtLSU assembly maps. On the left, the structure cartoons of the bacterial and yeast mitochondria LSU are presented. In the yeast mtLSU assembly pathway, in each cluster, proteins conserved in bacteria are boxed and marked in black, and mitochondria-specific proteins are boxed and labeled in blue. Names of non-essential proteins are underlined. The clusters proposed to assemble during intermediate stages are separated into 5+2 and 4 (corresponding to the central protuberance (CP)). At the bottom of the yeast 54S mt-LSU assembly pathway are listed in grey the 6 assembly factors so far described. Proposed bacterial orthologs and paralogs of these assembly factors are listed in grey at the bottom of the 50S E. coli LSU assembly pathway.

mitoribosome assembly. The authors found that proteins with similar assembly kinetics shared extensive binding surfaces, suggesting the coordinated assembly of adjacent proteins. For that purpose, the binding affinity of a protein-protein interaction in a crystal structure was calculated by the buried surface area (BSA) using PDBePISA site (www.ebi.ac.uk/pdbe/pisa). Based on previous analysis considering BSA values larger than 600-850 Å² as a biologically significant interface, BSA >1,000 Å² are estimated as strong interactions. The calculation of estimated interaction surface sub-grouped proteins following with their assembly kinetics and found that small sets of proteins with similar assembly kinetics can be grouped into modules that share extensive interactions. In summary, in the 39S mtLSU assembly map, as illustrated in Fig. 1.7A, the earliest 24 16S-binding
proteins in mtLSU reside in three relatively large groups, some of which have very extensive RNA contacts, including uL3-bL19, bL20, and uL4-uL5. Some early-binding groups include proteins that have no direct interaction with RNA, such as mL39 and mL50 (shown in bold brown characters). One group of early-assembly proteins with strong mutual interaction, formed by mL40, mL46 and mL48, binds

**Figure 1.7. Assembly Scheme for the mtLSU (A) and the mtSSU (B).** The assembly scheme showing protein-protein interactions between individual or grouped polypeptides, with their longer standard names truncated to numbers for simplicity. Heavy dashed lines indicate interaction surface areas greater than 1,000 Å², while lighter dashed lines indicate interactions between 1,000 Å² and 350 Å². (A) uL12m and uL1m are included in the early and intermediate groups based on the kinetic proteomic results but are outlined in red, since they were not identified in the cryo-EM structure. bL31m is shown as an atypical member of an early group that appears to join the structure at a later stage. mL39, mL45, and mL50 are shown in boldface brown type, since they are early-binding proteins but do not have extensive RNA contacts. (B) mS2, mS3, mS35, mS39, and bS1m are shown in boldface brown type, since they are early binding proteins lacking extensive RNA contacts. Since they depend on other proteins for assembly, they may be considered secondary binding proteins associated with the indicated early clusters. uS6m and mS38 are shown as independent proteins with variable association with the mS26 group but did not yield sufficient proteomic data for definitive kinetic assignment
one face of the tRNAVal, while a second group containing mL38, uL18, and bL27 binds the other. Intermediate-binding mtLSU proteins reside in a more scattered distribution, often sharing interactions with early-binding proteins. uL13-mL66, with a BSA of 2,312 Å², and uL11 tend to surround the uL10 stalk. A major set of intermediate-late proteins including uL23 joins the base of the mtLSU to contribute to the formation of the PTC. These proteins make few contacts with early proteins or with other intermediate-assembly proteins. At a late assembly stage, a group of proteins including uL2 joins the structure to add a prominent mass adjacent to uL23:uL29 approaching the interface of the mtLSU with the mtSSU. mL37 joins bL28 in extended contacts, wrapping along intermediate-binding protein uL29. mL37 and mL65 share a substantial protein-protein interface and serve to compensate for a large evolutionary RNA deletion in domain III of the 16S rRNA.

Similarly, in the 28S mtSSU assembly map (Fig. 1.7B), the early binding proteins in mtSSU were found in two relatively large groups at the head (a group including S7) and lower body/foot (a group including S16) of the elongated 12S RNA core. Subunits shown in bold brown characters do not directly contact with 12S rRNA but join at an early stage through its interactions with 12S-contacting proteins. A smaller set of early-assembly proteins, including S23, shares a binding surface of 1.797 Å² with uS2 and bS1, and strongly interacts with early-assembly proteins uS9 and uS5. Three early-assembly proteins, uS11, uS12 and uS17, do not contact other early binding proteins. Thus, their addition appears to depend upon their substantial interactions with the 12S rRNA. Late binding proteins reside into three major groups, one in the head region and the other in the body.
Interestingly, early-assembly proteins were shown to have a substantial presence in the nucleoid fraction and bind to the outer surface of the mtSSU, away from the interface with the mtLSU. In contrast, later-assembly proteins tend to exist closer to the interface with the mtLSU \(^{102}\). This coordinated modular assembly indicates that protein-protein interactions have a far greater relative importance in assembly of the mitoribosome than in the bacterial ribosome \(^{102}\). The composition of these modules will guide future experiments to define mitoribosome assembly in more detail.

The characterization of the late stages of human mtLSU assembly also has been approached by cryo-EM analysis of assembly intermediates isolated from a native pool at the intersubunit interface within a human cell line \(^{101}\). The data obtained revealed insights into the timing of rRNA folding and protein incorporation during the final steps of mtLSU maturation. Furthermore, the structures obtained refined the role of a member of the universally conserved ribosome silencing factor (RsfS) family, MALSU1, and identified two new assembly factors (L0R8F8 and mt-ACP). Upon binding of MALSU1, both uL14 and the neighboring bL19 display conformational changes, posing as in the mature mitoribosome. MALSU1 also forms electrostatic interactions with the sarcin-ricin-loop (SRL, helix H95) of the 16S rRNA, indicating that MALSU1 may position the H95 before the folding of the interfacial mt-rRNA \(^{101}\). Furthermore, a density-based fold-recognition pipeline identified two additional factors, a mitochondrial acyl carrier protein (mt-ACP) and a eukaryotic specific LYR-motif-containing protein, L0R8F8, as a binding protein located between MALSU1 and mt-ACP. In eukaryotic cells, mt-ACP and L0R8F8,
with a third catalytic subunit, form a cysteine desulfurase complex that mobilizes sulfur for incorporation into multiple biosynthetic pathways \(^{114,115}\). Also in mitochondria, two complex I accessory subunits LYRM6 and LYRM3 locate at the distal proton-pumping membrane arm facing the matrix space and at the matrix arm close to the ubiquinone reduction site, respectively. Those proteins are supposed to anchor mt-ACP independently to complex I similarly to the yeast homologues \(^{116}\). Therefore, both mt-ACP and L0R8F8 may be involved in fatty-acid and iron-sulfur biogenesis thus connecting these pathways to mitoribosome biogenesis \(^{115}\). Another potential role for the MALSU1-L0R8F8-mt-ACP module is that is located in a position expected to sterically obstruct the binding of the mtSSU, thus acting to prevent premature subunit joining, as will be further commented upon in the Discussion section.

The availability of these assembly pathway models is expected to fuel studies on the role of mitoribosome assembly factors and the identification of the assembly steps that they catalyze. In this thesis, I have focused on characterizing the role of the human mitochondrial GTPase MTG1 on mtLSU assembly, which is described in chapter 2.
2.1. MTG1 is a mitochondrial peripheral inner membrane protein that interacts with the mtLSU.

Human MTG1 was previously localized to mitochondria in HeLa cells\textsuperscript{80}. To confirm and expand these observations we used HEK293T cells to pursue several approaches. Cell fractionation experiments to isolate mitochondria followed by brief sonication to separate soluble and insoluble fractions, alkaline carbonate extraction to separate membrane intrinsic and extrinsic proteins, and proteinase protection assays to determine MTG1 submitochondrial localization and topology (Fig. 2.1A-B), in conjunction with fluorescence immunohistochemistry (Fig. 2.1C) allowed us to confirm that MTG1 is a mitochondrial protein. The data obtained (Fig. 2.1A-B) also lead us to conclude that MTG1 is a matrix protein loosely associated with the inner membrane, the location and behavior expected of a ribosome assembly protein.

To test whether the steady-state levels of MTG1 depend on the presence of mitochondrial ribosomes, we estimated MTG1 steady state levels in a derivative of an osteosarcoma 143B cell line devoid of mtDNA (rho\textsuperscript{0} cells) and therefore of all mtRNAs including rRNAs. Several ribosome proteins tested (uL16, uL19, bS18) did not accumulate in rho\textsuperscript{0} cells and other (mS27) were markedly decreased (Fig. 2.2A), whereas only traces of MTG1 were detected.
Figure 2.1. MTG1 is a mitochondrial inner membrane protein associating with the mitoribosomal large subunit.

(A) Mitochondria isolated from HEK293T cells were first fractionated into soluble (S) and membrane-bound (P) proteins by brief sonication and centrifugation. The pellet was submitted to alkaline carbonate extraction to allow the separation of the extrinsic proteins present in the supernatant (CS) from the intrinsic proteins in the pellet (CP). Equivalent volumes of each fraction were analyzed by immunoblotting using antibodies against MTG1, the matrix-soluble protein Hsp70, the extrinsic membrane protein SDHA and the inner membrane intrinsic protein COX2.

(B) Proteinase K protection assay in mitochondria (MT) and mitoplasts (MTP) prepared by hypotonic swelling of mitochondria. The samples were analyzed by immunoblotting using antibodies against MTG1, the outer membrane protein TOM20, the inner membrane protein TIM50, the matrix protein Hsp60 and the inner membrane extrinsic protein CMC1.

(C) Sucrose gradient sedimentation analyses of MTG1 and mitoribosomal proteins in extracts from wild-type (WT) HEK293T mitochondria (WT-Mito), mitochondria from the MTG1-KO cells after silencing of residual MTG1 (1F5-siMTG1), or total cell lysates from WT cells (WT-WCL) s. For the whole protein lysate, cells were permeabilized with digitonin before extracting with 0.25% digitonin. Each different lysate was loaded on the top of 0.3-1M sucrose column and fractionated by sucrose gradient centrifugation.
These data could link MTG1 to mitoribosome biogenesis, similar to all its homologs, but in principle, also to mtDNA or mtRNA transactions.

To explore a possible association of MTG1 with the mitoribosome, we used sucrose gradient sedimentation analysis of mitochondrial extracts prepared in the presence of 5 mM EDTA to favor dissociation into ribosome subunits (Fig. 2.2B). In 143B-WT cells, a portion of MTG1 (~10% of total) co-sediments with mtLSU markers, whereas the rest accumulates in lighter fractions in which a calibration marker (LDH, 130 kDa) peaks. In 143B-rho<sup>0</sup> cells, the ribosome subunits are obviously not formed, and the residual MTG1 only forms slow sedimenting particles (Fig. 2.2B). Studies in HEK293T mitochondrial extracts consistently identified a portion of MTG1 molecules co-sedimenting with the mtLSU, either when they were prepared in the presence of EDTA (Fig. 2.2C) or in the presence of 10 mM Mg<sup>2+</sup> to favor the stability of the monosome (Fig. 2.2D). Further exploration of MTG1 sedimentation patterns in gradients prepared using whole HEK293T cell extracts allowed us to identify a small portion of MTG1 that additionally co-sediments with the 55S monosome (Fig. 2.2E). This interaction must be very transient or labile since it is not detectable following the mechanical stress the cells undergo during mitochondrial isolation. Therefore, MTG1 may play a role in mtLSU biogenesis that extends until the formation of the monosome.

2.2. MTG1 is required for HEK293T cell proliferation

To gain insight into the specific role of MTG1 in mitochondrial translation and
Figure 2.2. MTG1 is a mitochondrial protein that interacts with the mitoribosome large subunit (mtLSU).

(A) Immunoblot analyses of the steady-state levels of MTG1 and indicated mtLSU and mtSSU proteins in HEK293T, 143B, and 143B-206 rho0 cells. VDAC was used as loading control. (B) Sucrose gradient sedimentation analyses of mtSSU (mS27) and mtLSU proteins (bL12 and uL16) in mitochondrial extracts from 143B WT and 206 rho0 cells, prepared in the presence of 5 mM EDTA. (C-D) Sucrose gradient sedimentation analyses of MTG1 and indicated MRPs in mitochondrial extracts from HEK293T cells, prepared in the presence of either (C) 5 mM EDTA or (D) 10 mM MgCl2. (E) Sucrose gradient sedimentation analyses of MTG1 and indicated MRPs in whole cell extracts (WCE) from HEK293T cells, prepared in the presence of 10 mM MgCl2. Transparent red, purple and green colors mark the fractions where the 55S monosome, 39S mtLSU and 28S mtSSU sediment, respectively.
mitoribosome assembly, we attempted to engineer knock-out (KO) of \textit{MTG1} in HEK293T cells using a transcription activator-like effector nucleases (TALENs)-mediated approach. Although several transfection strategies and TALEN pairs were used and nearly 1,000 clones were analyzed, we did not identify any homozygous KO. Consistently, \textit{MTG1} could not be KO either in haploid HAP1 cells (Horizon Discovery, UK; \textbf{not shown}), which suggested possible essentiality for this gene. However, among the many heterozygous HEK293T clones obtained, as screened by immunoblot against MTG1, one clone obtained with the first-TALEN-pair (Fig. 2.3A) had detectable but particularly low residual MTG1 levels (Fig. 2.3B). By genotyping, the clone (\textit{MTG1}-KO 1F5) was found to harbor two null \textit{MTG1} alleles, and a third allele carrying an in-frame 24-nucleotide deletion within the TALEN spacer region (Fig. 2.3A). \textit{MTG1}-KO 1F5 has a proliferation rate in standard high glucose DMEM media considerably slower than wild-type cells (Fig. 2.3C).

To test whether the residual protein was still functional, the clone was subsequently treated with double strand siRNA for \textit{MTG1} gene-silencing. After five days of siRNA transfection, steady-state levels of MTG1 were undetectable (Fig. 2.3B), which further decreased the ability of the cells to proliferate (Fig. 2.3C), indicating that the truncated MTG1 protein is functional.

Attempts to use the \textit{MTG1}-KO 1F5 clone to generate homozygous KO clones by using additional TALEN constructs (second TALEN pairs in Fig. 2.3A) were not successful. However, the \textit{MTG1}-KO 1F5 clone is still a very useful
Figure 2.3. MTG1 is required for efficient mitochondrial translation and OXPHOS function in HEK293T cells.

(A) Schematics of the first exon of the MTG1 locus and the sequences recognition sites of two different TALEN pairs. The genotyping of a 3-allele compound heterozygous MTG1-KO clone, 1F5, is depicted. (B) Immunoblot analysis of the steady-state levels of MTG1 in HEK293T (WT), MTG1-KO clone 1F5 and the 1F5 clone treated for 5 days with siMTG1. VDAC was used as a loading control. Two exposures of the anti-MTG1 immunoblot (short and long) are presented. The lower panel shows the densitometry values normalized by the signal of VDAC and expressed relative to the WT; one-way ANOVA with a Tukey’s multiple comparisons test: **p < 0.01; ****p < 0.0001. (C) Cell proliferation assay of the WT, MTG1-KO clone 1F5 and the 1F5 clone treated for 5 days with siMTG1; one-way ANOVA with a Tukey’s multiple comparisons test: **p < 0.01; ***p < 0.001. (D) Metabolic labeling with 35S-methionine of newly synthesized mitochondrial translation products in whole cells from the indicated lines during increasing pulse times in the presence of emetine to inhibit cytoplasmic protein synthesis. Immunoblotting for VDAC was used as a loading control. Newly-synthesized polypeptides are...
identified on the right. The lower panel shows the densitometry values (average of ND2, COX2 and ATP8 signals) normalized by the signal of VDAC and expressed relative to the WT. (E) Immunoblot analysis of OXPHOS complex subunits in the indicated cell lines. NDUFB8 is a subunit of complex I, SDHB of CII, UQCRC2 or CIII, COX1 and COX2 of CIV, ATP5α of the F1F0-ATP synthase or CV. Immunoblotting for VDAC was used as a loading control. (F) Steady-state levels of OXPHOS complexes extracted with lauryl maltoside in the indicated cell lines, analyzed by BN-PAGE and detected by immunoblotting with the indicated antibodies. (G) Measurement of OXPHOS parameters in the indicated cell lines. The upper graph shows the endogenous cell respiration rate. The lower graph shows the enzymatic activity of CIV or cytochrome c oxidase (COX) normalized by the activity of citrate synthase (CS) and expressed as percentage of WT. In all graphs, data represent the mean ± SD of three independent repetitions; one-way ANOVA with a Tukey's multiple comparisons test: ***p <0.001; ****p <0.0001.

resource, because siRNA-mediated silencing of MTG1 is markedly more effective in this cell line than that in WT HEK293T cells (Fig. 2.4A).

The apparent essentiality of MTG1 was also manifested when performing siRNA-mediated silencing of its expression, a manipulation that induced severe cell death if extended for more than five days. Therefore, we used this timeframe in all our silencing experiments. In regular MTG1-KO 1F5 cultures, but not in WT cell cultures, abundant floating cells are commonly observed, indicating cell death, a number that markedly increases after MTG1 silencing. Analysis of floating and attached cells showed a significant increase in Caspase 3 cleavage upon MTG1 depletion (Fig. 2.4B-C). This phenotype was also observed in rho0 cells silenced for MTG1, indicating that the severe cell death induced by depletion of MTG1 is independent of the mtDNA genome and hence of mitoribosomes (Fig. 2.4C). Therefore, MTG1 may perform an extraribosomal role whose characterization is beyond the scope of this manuscript. Involvement of mitoribosome components and assembly factors in regulation of apoptosis has been previously documented. Recently, pro-apoptotic proteins DAP3 (death associated protein 3), BMRP (Bcl-
2-interacting mitochondrial ribosomal protein), and PDCD9 or p52 (programmed cell death protein 9), have been respectively identified as the mS29, mL41, and mL65 components of the mitoribosome. Contrary to MTG1, however, silencing of each of these proteins make cells resistant to apoptosis (reviewed in 117). Similarly, mouse embryonic fibroblasts KO for the GTPase NOA1 (C4orf14 or MTG3), a mt-SSU assembly factor, were found to be resistant to staurosporine-induced apoptosis 118. As for MTG1, however, depletion of ERAL1, another mitochondrial GTPase that plays an important role in the assembly of the 28S mt-SSU by acting as a chaperone for the 12S mt-rRNA, leads to apoptosis in HeLa 72 and HEK293T cells 119 although cell death occurred before a mitochondrial protein synthesis defect was manifested 119. Also, depletion of the human mitoribosome recycling factor (mtRRF) is lethal in HEK293T cells, where ROS overproduction preceded any measurable translational defect 120. The mechanisms connecting the mitoribosome to apoptosis regulation require therefore further investigations.

Here, we also observed that the MTG1-KO 1F5 cells undergo progressive adaptation and recovery of significant respiratory competence. This phenotypic suppression is not due to the acquisition of extragenic mutations in the 16S rRNA, as reported in yeast 79, nor to intragenic mutations in the allele expressing the truncated MTG1 protein, but to an increase in the steady-state levels of this protein (not shown). Therefore, we routinely assessed the MTG1 steady-state levels in the culture prior to performing any experiments.
Figure 2.4. Effect of MTG1 depletion in HEK293T cell physiology. (A) Immunoblot analyses of the steady-state levels of MTG1, OXPHOS complex VI subunits (COX1 and COX2) and mtLSU proteins after transfection during 6 days of non-targeting (NT) control or MTG1-targeting siRNA in wild-type HEK293T (WT) or partial MTG1-KO clone (1F5). Total cell lysates were separated by SDS-PAGE. (B) Immunoblot analyses of the steady-state levels of cleaved or total Caspase 3 in the indicated cell lines. A, attached cells; F, floating cells in the medium. VDAC was used as a loading control. The right-side panel shows the densitometry values normalized by the signal of VDAC. (C) Bright field microscope image of cells in culture vessels (X100). The wild-type HEK293T cells, 143B and 143B-206 Rho0 cells were treated with siRNA targeting MTG1 and incubated at 37°C for 3 days. The same number of cells from each group was seeded into new plates and maintained two more days with fresh transfection reagent before collecting the images.

2.3. MTG1 is required for efficient mitochondrial translation and OXPHOS function in HEK293T cells

Following the observation that MTG1 co-sediments with the mitoribosome, we examined the mitochondrial translation capacity of MTG1-depleted cells by following the incorporation of [35S]-methionine into newly synthesized
mitochondrial polypeptides in the presence of emetine to inhibit cytoplasmic protein synthesis. *De novo* mitochondrial protein synthesis rates were markedly decreased in *MTG1*-KO 1F5 and more deeply in *MTG1*-KO 1F5 siMTG1-treated cells (Fig. 2.3D) thus demonstrating a role for MTG1 in mitochondrial translation.

Consistent with their protein synthesis defect, MTG1-depleted HEK293T cells exhibited decreased steady-state levels of mtDNA-encoded OXPHOS components, especially NDUFB8 (a CI subunit), COX1 and COX2 (two CIV subunits), as analyzed by denaturing immunoblotting (Fig. 2.3E). Blue Native-PAGE analyses further disclosed a decrease in the accumulation of OXPHOS complexes I, III, IV and V, which contain mtDNA-encoded subunits, whereas the level of complex II, formed exclusively by nucleus-encoded subunits, was not affected (Fig. 2.3F). The OXPHOS complex deficiency resulted in decreased endogenous cell respiration in *MTG1*-KO 1F5 cells to a residual level of 30% of control that was further decreased to 10% after *MTG1* gene-silencing for five days (Fig. 2.3G). Under the same conditions, the residual activities of complex IV (cytochrome c oxidase, COX) were approximately 40% and 14% of control cell activities (Fig. 2.3G). We conclude that in HEK293T cells, MTG1 is necessary for mitochondrial protein synthesis and consequently, for the biogenesis and function of mitochondrial OXPHOS complexes.

### 2.4. MTG1 is necessary for proper human cardiomyocyte activity and zebrafish cardiac development

Mutations in both nDNA and mtDNA affecting mitochondrial protein expression...
Figure 2.5. MTG1 is necessary for proper human cardiomyocyte physiology and zebrafish cardiac development

(A) Immunoblot analysis of the steady-state levels of MTG1, OXPHOS complex subunits and mitoribosome proteins in iCell human cardiomyocytes left untreated (WT) or treated for 4 days with siNT or with siMTG1. VDAC was used as a loading control. The right panel shows the densitometry values normalized by the signal of VDAC and expressed relative to the WT. The bars represent average ± SD. n = 4; Student’s two-tailed t-test: *p < 0.05; **p < 0.01. (B) Metabolic labeling with $^{35}$S-methionine of newly synthesized mitochondrial translation products in whole cells from the indicated lines during 30 min pulse in the presence of emetine to inhibit cytoplasmic protein synthesis. Immunoblotting for VDAC was used as a loading control. Newly-synthesized polypeptides are identified on the right. The lower panel shows the densitometry values (average of all polypeptide signals) normalized by the signal of VDAC and expressed relative to the WT. The bars represent average ± SD. n = 3; t-test: *p < 0.05. (C) Cardiomyocyte beating rate. The bars represent average ± SD. n = 4; one-way ANOVA with Sidak’s multiple comparisons test: **p < 0.01. (D) Cardiomyocyte physiology estimated by measuring Ca$^{2+}$ decay. Data represent the mean ± SD of four independent repetitions. In each repetition, 5 cells from each group were analyzed; one-way ANOVA with a Sidak’s multiple comparisons test: ***p < 0.001. (E) Immunoblot analysis of the steady-state levels of COX1 as a mtDNA-encoded subunit marker in zebrafish embryos silenced for MTG1. The translation-blocking morpholino-targeting MTG1 exon 1 (zf-Mtg1, Gene Tools) or the standard scrambled morpholino (STD, Gene Tools) used as a control were injected at two different concentrations (25 or 50 nM) into 1–2 cell-stage embryos. At 48-hour post fertilization (hpf), ten morphants from each group were pooled, and used for immunoblotting. The graph on the right side shows
the densitometry values of COX1 signal normalized by the signal of VDAC and expressed relative to the WT. Data represent the mean ± SD of three independent repetitions; one-way ANOVA with a Tukey’s multiple comparisons test: *p < 0.05. (F) Anatomical and cardiac features of zebrafish embryos injected with STD or zf-Mtg1. The upper panel present representative images of zebrafish embryos at 96 hpf, depicting the several anatomical features observed. The lower panel graph presents the quantification of heart beating rates in the STD- or zf-Mtg1-treated groups. In each group, each symbol represents an individual embryo. On the right side of the graph, the zf-Mtg1 50 nM morphants were further divided into different groups based on their anatomical features. Data represent the mean ± SD of four independent repetitions. Two-tailed t-test with a Mann-Whitney post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. N, normal shape; E, edematous chest; K, kinked body; E+K, a combination of kinked body and edematous chest.

machinery and their constituents, including mitoribosome components, lead to devastating human disorders often manifesting as mitochondrial cardiomyopathies or encephalo-cardiomyopathies. For this reason, we decided to test the consequences of MTG1 depletion in cardiomyocyte physiology and heart development using two different models.

First, we used induced pluripotent stem cell-derived human cardiomyocytes (iCell cardiomyocytes) obtained from Cellular Dynamics (Madison, WI). After four days of full culture accommodation, transient MTG1 silencing for four days resulted in MTG1 steady-state levels attenuated to 40% compared to siNT transfection groups (Fig. 2.5A), and mitochondrial translation capacity decreased to 50% (Fig. 2.5B). As a result, steady-state levels of mtDNA-encoded proteins (COX1 and COX2) were reduced. Depletion of MTG1 also affected steady-state levels of mtLSU proteins (uL14, uL16, bL27 and bL36), suggesting a mitoribosome biogenesis defect (Fig. 2.5A). To examine the physiological effects of MTG1 depletion on cardiomyocyte functions, we measured intracellular Ca²⁺ transient ([Ca²⁺]i) and Ca²⁺ decay in a single cell base by using
an IonOptix iCCD camera as reported\textsuperscript{122}, to record spontaneous action potentials from individual cells using the whole-cell current clamp technique. After four days of *MTG1* silencing, the cardiomyocyte beating rate was significantly decreased (Fig. 2.5C), and the Ca\textsuperscript{2+} clearance was substantially delayed (Fig. 2.5D), indicating that MTG1 function is essential for maintaining optimal cardiomyocyte physiology.

Second, we tested the role of MTG1 in developing zebrafish. For this purpose, 1~2 cell stage zebrafish embryos were injected with zebrafish Mtg1 (zf-Mtg1)-targeting or standard control (STD) Morpholino to induce transient KO-Mtg1. Available antibodies against zebrafish mitochondrial proteins are scarce, and our anti-human MTG1 did not cross-react with the fish homolog. We only found the anti-human COX1 antibody to cross-react with the fish protein and used it as a surrogate to follow potential mitochondrial protein synthesis defects derived from MTG1 depletion. The silencing efficiency was tested in this way after 48 hpf (hours post fertilization) when the average steady-state levels of COX1 were below 40% compared to control (Fig. 2.5E). After 96 hpf when the embryos required a functional developed cardiovascular system for respiration, two different doses of zf-mtg1 morphants had significantly lower heartbeats than those in each same dose of STD morphants (Fig. 2.5F). Interestingly, whereas all STD morphants had a normal shape, most zf-Mtg1 morphants in the higher dose (78.75%) and a few at the lower dose (8.4%) displayed apparent anatomical abnormalities such as a kinked body, edematous chest, or a combination of both (Fig. 2.5F). To better understand the hierarchy of pathophysiological events, we dissected the data from
79 morphants treated with 50 nM zf-Mtg1 into four different groups based on their anatomical phenotypes. From the total, 36.25% of morphants displayed only edematous chest, 5% only kinked body and 37.5% exhibited both phenotypes (Fig. 2.5F). Notably, 21.5% of morphants had apparently normal shape, but their heartbeats were already significantly lower than those in the same dose of STD or lower dose of zf-Mtg1 morphants (Fig. 2.5F), indicating that the cardiovascular lesion induced by MTG1 depletion is an early event in developing zebrafish.

We believe that the phenotypes described in these models are not influenced by the secondary function of MTG1 on cell survival since even very low levels of the protein are sufficient to maintain viability in HEK293T cells. Our data support a role of MTG1 in the maintenance of cardiac physiology and cardiac development and presents the gene as a candidate when screening for the molecular bases of dilated and hypertrophic cardiomyopathies associated with mitochondrial translation and OXPHOS defects.

2.5. MTG1 is essential for the formation of functional mtLSU and monosomes

A function for MTG1 in preserving mitochondrial protein synthesis is conserved across the different models tested. Henceforth, for all mechanistic studies on the role of MTG1 at the molecular level, we have used the amenable HEK293T model cell lines. Data presented in Figure 2.2 supplies information about the interaction of MTG1 with mtLSU and a more transient interaction with the monosome.

To explore whether MTG1 depletion affects mitoribosome accumulation, we first tested the steady-state levels of mtLSU and mtSSU subunit markers (Fig.
Immunoblot analyses showed that several mtLSU proteins including uL11, uL14, and in particular the late assembly proteins uL16, bL27 and bL36 were significantly reduced (Fig. 2.6A). Incidentally, the B. subtilis counterparts of these subunits require the bacterial MTG1 homolog RbgA for their incorporation into late assembly intermediates \(^77,123\). Subsequently, we followed the formation of the mtLSU ribonucleoprotein particle in MTG1-depleted mitochondria by sucrose gradient sedimentation analysis of whole cell extracts prepared in the presence of magnesium. The most obvious effect of MTG1 depletion was the virtual absence of 55S monosomes (Fig. 2.6B). Otherwise, the sedimentation pattern of the mtLSU and mtSSU protein markers within the individual subunits was not changed in MTG1-KO 1F5-siNT- and MTG1-KO 1F5-siMTG1-treated cells, except for bL36, which accumulated in the top light fractions (Fig. 2.6B). These data suggest that while the 39S mtLSU particle can form near to completion when MTG1 is limiting, at least bL36 cannot be efficiently incorporated into the mtLSU assembly line. The residual MTG1 expressed in MTG1-KO 1F5-siNT- and MTG1-KO 1F5-siMTG1-treated mitochondria co- sedimented with the mtLSU, but monosome formation with the mtSSU appears to be impaired, as mentioned earlier (Fig. 2.6B).
Figure 2.6. MTG1 is essential for the formation of functional mtLSU and monosomes. 

(A) Immunoblot analysis of the steady-state levels of MRPs in HEK293T (WT), MTG1-KO clone 1F5 treated with siNT and the 1F5 clone treated for 5 days with siMTG1. VDAC was used as a loading control. Two exposures of the anti-MTG1 immunoblot (short and long) are presented. The lower panel shows the densitometry values normalized by the signal of VDAC and expressed relative to the WT. Data represent the mean ± SD of three independent repetitions; one-way ANOVA with a Tukey’s multiple comparisons test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. 

(B) Sucrose gradient sedimentation analyses of MTG1 and MRPs in whole cell extracts prepared from the same cell lines as in (A) in the presence of either 10 mM MgCl₂. Transparent red, purple and green colors mark the fractions where the 55S monosome, 39S mtLSU and 28S mtSSU sediment, respectively. 

(C) Immunoblot analysis of MTG1 and MRP levels in fractions from the sucrose gradients presented in panel (A) where the monosome (F4-5), mtLSU (F6-7), mtSSU (F8) and unassembled subunits (F12-13) peak.
Equal volumes of fractions corresponding to each cell line were loaded. (D) For each fraction in (A), total RNA was isolated and reverse transcribed, and 16S and 12S rRNA levels were quantified by qPCR. Values were normalized to the lowest fraction for each cell line, represented as percentage of total for each cell line, and plotted. (E) Northern blot analyses of the steady-state levels of mitochondrial rRNAs in WT or the MTG1-KO clone 1F5 carrying either an empty vector (EV) or a construct to overexpress MTG1. The right panel shows the densitometry values normalized by the signal of 18S rRNA and expressed relative to the WT. Data represent the mean ± SD of three independent repetitions. (F) Quantitative PCR (qPCR) analyses of the steady-state levels of mtDNA-encoded rRNAs (16S and 12S), mtDNA-encoded mRNAs (COX1, COX2 and ATP6), a mtDNA-encoded tRNA (tRNA-Val) and nuclear DNA (nDNA)-encoded MRP mRNAs (uL11, bL36 and uS22) in WT and the MTG1-KO clone 1F5 after five days of transfection with non-targeting control (1F5-siNT) or MTG1-targeting (1F5-siMTG1) siRNAs. Data represent the mean ± SD from three independent experiments.

To better compare the accumulation of MTG1 and mitoribosome protein markers in WT and MTG1-depleted extracts, we ran in parallel, in a single gel, samples from the relevant sucrose gradient fractions (Fig. 2.6C). We confirmed that the levels of mtLSU proteins were increased in the F6-7 fractions where the mtLSU peaks, except bL36. Furthermore, we can better appreciate how the increasing depletion of MTG1 progressively impairs 55S monosome formation (Fig. 2.6C). The same fractions from gradients presented in Figure 2.6B were used to analyze the rRNA distribution by RT-qPCR. Levels of 16S rRNA in the mtLSU fractions were similar in WT and MTG1-depleted cells, but the latter had enhanced relative levels of 12S rRNA in the mtSSU fractions (Fig. 2.6D), probably reflecting the lack of monosomes. In fact, quantification of total 12S and 16 rRNAs by northern-blot and RT-qPCR did not identify any significant changes in their steady-state levels, which was also true for tRNAVal and several mRNAs analyzed (Fig. 2.6E-F).

Finally, to obtain a more thorough picture of the differences in the composition and abundance of assembled mitoribosomal subunits, sucrose gradient fractions corresponding to the monosome, mtLSU and mtSSU were
methanol/chloroform precipitated and analyzed by mass spectrometry (Fig 2.7). Consistent with the data presented in Figure 2.6B-C, all mtLSU and mtSSU proteins from MTG1-depleted mitochondria showed a decrease in the monosome fraction. Interestingly, the abundance of some protein subunits tended to increase in the mtLSU fraction despite the reduced levels of some mtLSU proteins in total cell lysates (Fig 2.7A), suggesting that those proteins able to integrate into ribonucleoprotein particles could be more stable than when unassembled. bL36 was not detected, and bL35 was found particularly underrepresented.

These two proteins are among the late assembly mtLSU proteins that accumulate in substoichiometric amounts in the 45S particle found in RbgA-depleted B. subtilis cells 73,77,78 and Nicotiana benthamiana chloroplasts 124. Because mutations in B. subtilis L6 attenuate the growth defect of an rbgA mutant 51, it has been proposed that RbgA may facilitate proper interaction between L6 and the maturing 50S ribosome, subsequently triggering the incorporation of the late assembly proteins such as L16, L27, L28, L33a, L35, and L36. However, L6 is not present in the mitochondrial ribosome despite robust conservation of the rRNA to which it binds (see next section). This loss is structurally compensated by mitochondria-specific proteins mL66 (MRPS18a) and mL53 10, which are not particularly underrepresented in the mtLSU particles.
Figure 2.7. Identity and abundance of mitoribosome proteins and assembly factors that accumulate in mitoribosome particles in WT and MTG1-depleted HEK293T cells. Accumulation of mitoribosomal large and small subunit proteins in the fractions from sucrose gradient sedimentation studies presented in Fig 4. HEK293T cells (WT) and MTG1-KO 1F5 silenced for MTG1 for 6 days (1F5 siMTG1) were included. (A) The proteins in the fractions in which the monosome (F4-5), the mtLSU (F6-7) and the mtSSU (F8) peak, were precipitated using methanol-chloroform and identified by mass spectrometry. The bar graphs represent the mean ± S.E. of total unique spectral count difference between WT and 1F5-siMTG1 in three independent repetitions. Mitoribosome proteins are identified at the bottom. (B) Accumulation of mitoribosome LSU assembly GTPases (MTG1 and MTG2) and a helicase (DDX28) in the different fractions. Data represent total unique spectral counts in three independent repetitions (1, 2 and 3).

detected in the absence of human MTG1 (Fig. 2.6A and 2.7A). In yeast, the RbgA homolog Lsg1 has been proposed to play a role in the incorporation of the L16 homolog Rpl10 and other late-assembly proteins into the cytoplasmic LSU.125,126,
suggesting that RbgA and its homologs regulate a step during LSU biogenesis that must be at least broadly conserved during evolution. In human cells, total mitochondrial bL16 levels are attenuated (Fig. 2.6A) but they accumulated normally in the MTG1-less mtLSU particles (Fig. 2.6C and 2.7A).

Taken together, our data allow us to conclude that human MTG1 catalyzes a step that is required for the efficient incorporation of at least bL36 and bL35 during the late stages of mtLSU biogenesis.

2.6. MTG1 catalyzes an mtLSU late-assembly step during the hierarchical incorporation of MRPs

We aimed to further examine the role of MTG1 during the hierarchical incorporation of MRPs and the ordered action of assembly factors during mtLSU biogenesis. For this purpose, we introduced siRNA-induced silencing of selected mtLSU proteins (Fig. 2.8A) and assembly factors (AF)s in WT cells and tested genetic interactions among them by immunoblotting (Fig. 2.8B-C) and sucrose gradient sedimentation assays (Fig. 2.8D).

The selected mtLSU proteins included early-assembled proteins such as bL19 and mL45\textsuperscript{127}. As expected, silencing of bL19 or mL45 significantly lowered the steady-state levels of most mtLSU proteins, prevented mtLSU assembly and also attenuated MTG1 levels (Fig. 2.8B-C), further confirming that MTG1 steady-state levels depend on mtLSU components. mL45 is the homolog of yeast Mba1, a ribosome membrane anchoring protein\textsuperscript{6}, whose incorporation at an early stage may serve to tether the 39S mtLSU at the inner membrane during subsequent
steps in assembly. Hence, MTG1 acts beyond the incorporation of early assembly subunits.

We also included proteins that structurally compensate for the loss of L6, a proposed MTG1-functionally interacting protein, such as the mitochondria-specific protein mL66 (MRPS18a)\textsuperscript{10}, as well as for other structurally interconnected intermediate-binding proteins (uL14, uL16) and assembly factors, such as C7orf30 also known as MALSU1. MALSU1 acts as a chaperone of uL14\textsuperscript{58,100}, whose incorporation into the assembling 39S particle involves a conformational change of the sarcin-ricin loop (SRL, helix 95) in the 16S rRNA\textsuperscript{128}. The SRL is essential for assembly of the functional LSU core as well as for GTP-catalyzed steps of translation\textsuperscript{129}, specifically for anchoring elongation factor G on the ribosome during mRNA-tRNA translocation\textsuperscript{130}. The crystal structure of bacterial LSU revealed that SRL interacts with the body of the 50S LSU through the C-terminus domain of uL6 and loop-loop tertiary interactions with 23S rRNA h91. H91 then contacts h89, h90 and h92, which are connected directly to the A- and P-tRNA binding sites and the peptidyl transfer center (PTC). H91 also contacts h42, which connects to the GTPase-associated center (GAC; h43 and h44), indicating that SRL is linked with several critical regions of the 50S subunit by its tertiary interactions. In bacteria, uL6 is a two-domain protein. L6 N-terminus interacts with h97 of the 23S rRNA and its C-terminus interacts with the SRL. All mutations in L6 that suppress the mtLSU assembly defect in rbgA-deficient \textit{B. subtilis} cluster in a region where the L6 closely contacts with 23S rRNA H97\textsuperscript{51}. Also, the mutant L6 protein was stably incorporated into the 50S subunit, but
Figure 2.8. MTG1 catalyzes a mtLSU late-assembly step during the hierarchical incorporation of MRPs.

Knockdown (KD) of mitoribosome assembly factors and mitoribosome subunits in HEK293T cells using siRNAs for 8-9 days, verified by immunoblotting of whole cell lysates. (A) siRNA-
targeted mitoribosome proteins mapped to the human mitoribosome structure (PDB 3J9M) \(^{19}\). (B) Representative image of immunoblot analysis of the steady-state levels of mitoribosome proteins after silencing of target proteins. siRNA-NT is a non-targeting silencing control. Antibodies are listed on the right side, and VDAC was used as a loading control. (C) Following analysis in panel (B), the densitometric data obtained on the abundance of mitoribosome proteins and assembly factors accumulated after silencing of each target protein was used for cluster analysis (see STAR Methods). The heat map, generated with the R studio software, represents a log 2 scale of the normalized average levels of ratio to control (NT) in three independent repetitions of immunoblotting analyses. VDAC was used as a loading control. 2-way ANOVA followed by a Dunnett’s multiple comparisons test: \(^* p < 0.05; \,** p < 0.01; \,** * p < 0.001; \,** ** p < 0.0001\). (d) Sucrose gradient sedimentation analysis of MTG1 and MRPs from mitochondrial extracts from the indicated cell lines. For each experiment, siNT-treated wild type HEK293T cells were used as a control.

markedly disrupted the formation of intact 70S ribosomes as a result of the improper positioning of the intersubunit bridge involving h89, which makes direct contact with L6 and uL16 \(^{51}\). As explained earlier, uL6 is absent in mammalian mitochondria despite strong conservation of the uL6 binding rRNA helices, although the mitochondria-specific proteins mL66 and mL53 compensate for the loss of bacterial uL6 without functional replacement \(^{10}\).

Based on the observations made in bacteria, we hypothesized a potential structural interaction among uL14, uL16, mL66, MALSU1 and MTG1 around the mtLSU functional core. Silencing of uL14 or MALSU1 affected their reciprocal stability and decreased the steady-state levels of uL16 (the effects of siMALSU1 were milder), indicating that uL16 incorporates after uL14 (Fig. 2.8B-C). Also, the level of MTG1 was increased (Fig. 2.8B-C), but at least the absence of uL14 prevented MTG1 binding to the mtLSU (Fig. 2.8D), strongly indicating that MTG1 acts on the uL14-containing 39S pre-particle. We noticed an inverse correlation between the levels of MALSU1 and MTG1, which may reflect compensatory
mechanisms or some functional interaction between these two GTPases through uL14. The level of uL16 was not particularly affected by MTG1 silencing. However, silencing of mL66, which structurally balances the absence of uL6, dramatically decreased the steady-state level of uL16, and significantly increased MALSU1 and MTG1 (Fig. 2.8B-C). These data indicate that as for L6 in bacteria, mammalian mL66 is required for the proper incorporation of uL16 and other late assembly proteins. Yet, mL66 silencing did not prevent incorporation of at least a small fraction of MTG1 into the mtLSU (Fig. 2.8D). The decreased incorporation of MTG1 upon mL66 depletion indicates that MTG1 binding is enhanced or stabilized by the presence of mL66 and suggest that MTG1 might act to remodel the interaction of mL66 with the 16S rRNA after its incorporation into the mtLSU assembly line.

Our data exposed genetic interactions among the three S18 variants present in the mammalian mitoribosome (MRPS18a or mL66 in mtLSU, MRPS18b or mS40 and MRPS18c or bS18 in mtSSU), which were derived from gene duplication events from the same ancestral sequence. As expected, silencing of mL66 did not affect the levels of mS40 or bS18, and silencing of mS40 resulted in significantly decreased bS18 levels. mS40 silencing also enhanced mL66 levels, which could be a compensatory mechanism for the loss of mtSSU, since a similar (although not significant) tendency was observed in the absence of mS27 (Fig. 2.8B-C). Silencing of mtSSU proteins did not affect the overall levels of mtLSU proteins or the interaction of MTG1 with the mtLSU (Fig. 2.8D), thus supporting the notion that the mtLSU assembles independently of the mtSSU.
Finally, our study also incorporated silencing of mtLSU late-assembly protein bL36. In the mature mtLSU structure, bL36 was found only in a class of particles with folded 16S rRNA at the intersubunit interface. This observation suggested that the recruitment of bL36 and the folding of mt-rRNA may be interdependent in a way that bL36 stabilizes tertiary interactions among h89, h91 and h97. Because bacterial L6 has broad interactions with these helices, we asked how bL36 genetically interacted with other mtLSU proteins around the functional cores. Whereas depletion of bL36 did not significantly affect the levels of the mtLSU proteins analyzed, it leads to the marked accumulation of MTG1 as well as other mtLSU assembly factors, MALSU1 and the DEAD-box helicase DDX28 (Fig. 2.8B-C). MTG1 co-sediments with the bL36-depleted mtLSU pre-particle (Fig. 2.8D), which together with the observation that the steady-state levels of bL36 are remarkably decreased following depletion of MTG1 (Fig. 2.6A-C and 2.8B), allowed us to conclude that bL36 is incorporated following the action of MTG1, similar to what has been described for bacterial RbgA.

Mass spectrometry analysis of the 55S monosome and 39S mtLSU from MTG1-depleted cells allowed us to further evaluate the hierarchy of incorporation of assembly factors during mtLSU assembly (Fig. 2.8C and 2.7B). Levels of the DEAD-box helicase DDX28 were maintained constant, suggesting early incorporation and stable interaction with the mtLSU particle assembled in the absence of MTG1 (Fig. 2.8C and 2.7B). On the contrary, levels of the GTPase MTG2 on the mtLSU were reduced following MTG1 depletion, indicating a labile interaction. Considering that yeast Mtg2 acts in steps before Mtg1, it is
conceivable that human MTG2 could be released from the growing particle before MTG1 incorporation.

2.7. **MTG1 directly interacts with the 16S ribosomal RNA**

The bacterial homolog of MTG1, RbgA, interacts with the LSU rRNA to promote a conformational change that permits incorporation of late-assembly LSU proteins\(^{76}\). Thus, we set to examine whether MTG1 directly binds to the 16S rRNA that could also allow us to propose the conformation rearrangement hypothesis. For this purpose, mitochondrial extracts from WT cells stably expressing MTG1-FLAG were subjected or not to UV-mediated protein-nucleic acid crosslinking, anti-FLAG beads-driven IP as well as non-FLAG beads-driven IP as a control, and isolation of the co-immunoprecipitated (co-IPed) RNA. Following reverse transcription and quantitative PCR analysis, the 12S rRNA was poorly detected in all the different groups. In contrast, the 16S rRNA was significantly enriched in the FLAG Co-IP eluted samples compared to those in the control IP. We amplified the 16S rRNA using primers targeting three different regions of the gene (Fig. 2.9A), and all three regions of 16S rRNAs were highly enriched in MTG1 pulled-down eluates, thus demonstrating interaction of MTG1 with the 16S rRNA in vivo (Fig. 2.9B). Importantly, levels of detected 16S rRNA were significantly lower in the UV-irradiated samples compared to those non-UV-irradiated, except those corresponding to the 3’-end of 16S rRNA (Fig. 2.9B).
Figure 2.9. MTG1 directly binds to the mtLSU through the 16S ribosomal RNA and mitoribosome protein uL19.

(A) Scheme depicting the human 16S rRNA gene and the position of three different oligonucleotide pairs used for gene amplification of domains II, V or VI. (B) qPCR analyses of reverse-transcribed control (Ct) or MTG1-FLAG (FLAG) co-immunopurified RNAs after 4-thiouridine (4SU) treatment and non-crosslinked (NC) or UV-mediated protein-RNA crosslinking (CL). (C) Two-dimensional structure of 16S rRNA domains V and VI presenting helices, and VI-interacting LSU proteins indicated. (D) Identification of mtRNA targets of MTG1 by single-read-RNA-seq analysis of RNA isolated from IP experiments presented in panel (B). The graph represents a heatmap of the normalized average density of the reads across the
mitochondrial genome. Control sample represents non-specific binding of mitochondrial RNA to the naked beads (no antibody used for immunoprecipitation). Three independent repetitions are presented. Reads were preprocessed to remove sequencing adapters using Trimmomatic and then mapped with Tophat version 2.1.0 to the reference human genome (assembly GRCh37). Mitochondrial coverage was computed using Bedtools. Raw data can be accessed through the Gene Expression Omnibus (GEO) database repository (accession number GSE116688). (E) Co-IP analysis of MTG1-FLAG and native interacting mitoribosome proteins with anti-FLAG agarose beads (F) or plain beads used as control (Ct). (F) Localization of the highly ranked MTG1 interacting mitoribosomal proteins (uL19, mS27, mL37, mL39 and mL65) and 16S rRNA domain VI (red) in the human mitoribosome structure (PDB 3J9M) 19. (G) IP analyses of endogenous bL19, mL39 or mS27 using Abs targeting each subunit from HEK293T cells stably expressing MTG1-FLAG. The interaction was fixed by incubation with the cleavable crosslinker DSP. IP with IgG was used as a negative control (IgG), and IP with anti-FLAG beads was used for pull-down of MTG1 as a positive control (MTG1). See also Supplementary Table S1. The statistical tests of MTG1-rRNAs interaction were analyzed with 2-way ANOVA followed by a Tukey post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Since UV irradiation is known to induce RNA cleavage and degradation 132-134, we interpret these data to indicate that MTG1 binds near the 3’ end of the 16S rRNA and protects that domain from UV-induced RNA cleavage. The 16S rRNA 3’-end corresponds to domain VI, which includes helices 95-99 (Fig. 2.9C).

As an alternative approach to identify and narrow the MTG1 target mitochondrial RNA transcripts, we isolated the co-IPed RNA, and performed pair-end RNA sequencing (RNA-seq) experiments (Fig. 2.9D). This analysis clearly showed that MTG1 bound the 16S rRNA, with major specificity for the region encompassing domain VI of the 16S rRNA helices H95 (the SRL), H96 and H97. A schematic of the secondary structure of this domain indicating the helices with which mtLSU proteins interact is presented in Figure 2.9C.

Hence, our data show that MTG1 binds to a 16S rRNA region conserved in bacteria that is remodeled following interaction with RbgA to facilitate incorporation of L35, L36, and other late-assembly proteins.
2.8. MTG1 interacts with uL19 and mS27

In addition to interacting with rRNA, MTG1 could directly guide insertion of ribosomal proteins into the growing mtLSU particle. To identify potential mitoriboprotein interacting partners of MTG1, we used an array of approaches. Although we have detected a portion of total MTG1 co-sedimenting with the mtLSU, the interaction could be labile depending on GTP hydrolysis. To stabilize potential interactions, we performed immunoprecipitation (IP) assays with mitochondria incubated in the presence or absence of a non-hydrolysable GTP analogue (GMPPCP or β,γ-methyleneguanosine 5′-triphosphate sodium salt) or chemical crosslinkers.

In one assay, mitochondria purified from HEK293T cells stably expressing FLAG-tagged MTG1 were pre-incubated with 2.5 mM GMPPCP for 40 min at 4°C to trap the transient MTG1-mitoribosome complex by blocking MTG1 dissociation. After lysis with mild 0.25% digitonin to maintain native complexes intact, the MTG1-FLAG-interacting proteins were isolated by FLAG-affinity IP and analyzed by immunoblotting. Whereas mtSSU proteins were not detected in the bound fractions, all mtLSU proteins analyzed were detected in the FLAG Co-IP eluate, but not in the control-IP, demonstrating the native interaction of MTG1 with mtLSU proteins (Fig. 2.9E). It is mechanistically relevant, however, that following pre-incubation with GMPPCP, we observed only a mild increase in the portion of MTG1 that cosediments with the mtLSU (Fig. 2.10), which suggests that the number of GTP-bound MTG1 molecules interacting with the mtLSU in our preparations is near saturation.
In another set of assays, we explored the interaction of MTG1 with mitoribosomal proteins in the presence of cross-linkers followed by extraction under denaturing conditions. First, we explored the direct interacting partners of MTG1 by using the cleavable crosslinker DSP (dithiobis[succinimidylpropionate]). DSP has NHS-ester ends that react with amino groups, with a 12.0 Å spacer arm.

![Sucrose gradient sedimentation analyses](image)

**Figure 2.10.** The Fraction of MTG1 that co-sediments with the mtLSU is not markedly enhanced by pre-incubation with non-hydrolyzable GTP analog (GMPPCP). Sucrose gradient sedimentation analyses of MTG1 and mitoribosomal proteins in extracts from wild-type (WT) HEK293T mitochondria preincubated or not with non-hydrolyzable GTP analog (GMPPCP). The proportion of total MTG1 that co-sediments with the mtLSU is indicated.
that contains a cleavable disulfide bond. Mitochondria were incubated in the presence DSP or the vehicle DMSO as a negative control before extraction with 1% SDS, dilution to final 0.1% SDS and FLAG affinity IP. In these conditions, MTG1 was IPed with several mtLSU markers tested, and also with some mtSSU proteins such as mS22, mS27 and mS40 (data not shown).

Subsequently, we used the non-cleavable crosslinker AMAS (N-α-maleimidoacet-Oxysuccinimide ester). AMAS has NHS-ester and maleimide groups, which crosslink amino to sulfhydryl groups with a 4.4 Å spacer arm. Mitochondria purified from cells stably expressing MTG1-FLAG were incubated in the presence of AMAS or DMSO as a negative control, pulled-down with anti-FLAG agarose beads and analyzed by SDS-PAGE. After Coomassie staining, a band of ~75 kDa was observed only in the crosslinked-IPed sample (not shown). The band was cut, and its composition was analyzed by mass spectrometry. In two independent trials, we found that 13 MRPs were detected from each experiment, nine of which were overlapping (Table 2.1). Given the MW of these proteins, MTG1 could be expected to interact with one of them, although it is conceivable that more than one MTG1 complex of similar MW could conform to the ~75 kDa adduct. Also, some pairs of MRPs could be crosslinked and co-migrate with the MTG1-MRP adduct/s. While mapping these MRPs to the structure of the mitoribosome, we noticed that the five common MRPs with the higher rank, mS27, uL19, mL37, mL39 and mL65 locate adjacent to each other around the area where the mitochondria-specific bridge mB6 is formed by uL19 and mS27 (Fig. 2.9F). Furthermore, these
MTG1-interacting MRPs map to the location where the 16S rRNA 3’ end resides (Fig. 2.9F).

Finally, to confirm the interaction of those MRPs with MTG1, we performed multiple reciprocal pull-down analyses using DSP-crosslinked mitochondrial extracts and anti-MRP-specific antibodies with protein A-sepharose beads. In these assays, we detected a co-immunopurified fraction of MTG1 from elutes that was incubated with a uL19 or mS27 antibody (Fig. 2.9G). Beads conjugated to IgG or to mL39 were used as negative controls. Reanalyzing the mass spectrometry data of the AMAS-crosslinked adducts, we found that MTG1

Table 2.1. Mitoribosome proteins directly interacting with MTG1.
We explored the directly interacting partners of MTG1 by using a non-cleavable crosslinker, AMAS (N-α-maleimidoacet-oxysuccinimide ester). Mitochondria purified from cells stably expressing MTG1-FLAG were incubated in the presence of AMAS or DMSO as a negative control, pulled-down with anti-FLAG-sepharose beads and analyzed by SDS-PAGE. After Coomassie staining, a band of ~ 75 kDa was observed only in the crosslinked-IPed sample. The band was cut, and its composition was analyzed by mass spectrometry. The table presents the proteins identified in two independent experiments, sorted by their Mascot score.

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co-immunopurified with uL19 and mS27 with over 95% probability (Table 2.2), confirming that MTG1 directly interacts with uL19 and mS27. These results are in agreement with the observations made in sucrose gradient sedimentation analysis of whole cell extracts that detected a larger portion of MTG1 co-sedimented with the mtLSU, but also a minor fraction co-sedimenting with the monosome (Fig. 2.2).

During translation, multiple molecular contacts between the mature ribosomal subunits termed intersubunit bridges, keep the monosome together and serve to control dynamics that enable the various steps of translation. Whereas in bacteria and certain eukaryotes, these bridges mainly involved conserved RNA-RNA interactions, the interface of the human mitoribosome is

Table 2.2. Proteins directly interacting with bL19, mL39, mS27 or MTG1.

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Data was evaluated by total unique spectral count with the Scaffold4 software. The approximate molecular weight (MW) of each band is shown under the name of IPed protein.
richer in protein-mediated connections \(^{18,19}\). Several bridges present in bacterial ribosomes are absent in mitoribosomes, which in turn contain six mammalian mitochondrion-specific bridges, among them, bridge mB6 (also called B9) that is formed between uL19 and mS27 \(^{18,19}\). Whereas the formation and function of mitoribosome intersubunit bridges remain to be understood, our observations allow us to propose a model in which MTG1 could directly or indirectly participate in either connecting or preventing the premature connection of uL19 and mS27.

In this model, MTG1 would bind to the 16S rRNA to catalyze a late-stage mtLSU assembly step involving remodeling of the uL19-containing mtLSU domain. MTG1 would subsequently remain bound to the 39S mature particle and eventually be released, hence facilitating the formation of the mB6 intersubunit bridge.

### 2.9. MTG1 release from the 39S mtLSU is stimulated by mS27

GTPases work by cycling between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound states. The interconversion of these states is facilitated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs stimulate the release of GDP and then catalyze the GDP−GTP exchange, and GAPs accelerate GTP hydrolysis \(^{137}\). Unlike prototypical GTPases, the characterized bacterial GTPases acting in ribosome assembly bind nucleotides weakly and are thus not expected to be dependent on GEFs to exchange GDP for GTP \(^{138}\). Therefore, it has been proposed that most ribosome assembly GTPases could be directly regulated by the GTP/GDP ratio, which could allow these proteins to directly couple ribosome
assembly to energy status in the cell $^{52,138}$. There is, however, at least one exception, *E. coli* RsgA, which catalyzes fast GTP hydrolysis in the presence of 50S LSU, but then turnover is limited by GDP release, which is stimulated over 100-fold by the 30S ribosome acting as a GEF $^{139}$.

For the bacterial MTG1 homolog RbgA, GTP and GMPPNP were sufficient to promote RbgA association with a 45S intermediate, whereas only GMPPNP was able to support binding to the 50S subunit, likely due to the stimulation of GTP hydrolysis. Hence, RbgA has been proposed to promote a late step in ribosome biogenesis (discussed earlier) and one role of GTP hydrolysis is to stimulate its dissociation from the ribosome, which apparently does not need a GEF.

The case of MTG1 may be different. As for RbgA, a previous study reported that the intrinsic recombinant MTG1 GTPase activity is undetectable, but it is stimulated *in vitro* by the mtLSU and particularly by the 55S monosome $^{80}$. In human mitochondria, however, mS27 has been suggested to play a role as a GEF, as it shares sequence homology with the DH (dibble-homology) domain of the Dbl GEF family proteins $^{140}$. The DH domain interacts with the G protein and mediates the GEF activity. The alignment presented in Figure 2.11A shows weak but relevant primary sequence homology involving the C-terminus of mS27. The DH domain is constituted of three structurally conserved regions (CR1-3) separated by more variable regions $^{141}$. The three conserved regions, which are present in mS27 (Fig. 2.11A), are known to contain some of the critical
Figure 2.11. The mtSSU protein mS27 shares homology with Dbl GEF family proteins and promotes the release of MTG1 from the mitoribosome. (A) Schematics showing the structural domains present in several members of the Dbl GEF (GTP-exchange factor) family proteins (S. cerevisiae Cdc24 and human DBS, DBL and TIAM) in comparison with mS27. The catalytic region of most Rho-GTPase GEFs consists of a conserved DH/PH domain tandem. The DH (dibble-homology) domain, which interacts with the G protein and mediates the GEF activity, is conserved in mS27. The PH domain, commonly considered to be a phosphatidylinositol phosphate-binding protein domain structurally and functionally divergent in the individual GEFs, is absent in mS27. The lower panel shows a Clustal X alignment of human mS27 (NP_055899) with the DH domain present in the Dbl GEF family proteins Cdc24 (DAA06945), DBL (P10911), DBS (O15068) and TIAM1 (NP_001340617). Conserved regions 1, 2 and 3 (CR1-3) are labeled in blue. A single asterisk indicates conserved residues and
double asterisks identify identical residues. **(B)** Co-IP analysis of uL19 with MTG1-FLAG using anti-FLAG agarose beads, in the presence or absence of non-hydrolysable GTP (GMPPCP), hydrolysable GTP, recombinant mS27 (mS27p) or combination of GMPPCP with mS27. Ct, plain beads used as control; F, anti-FLAG agarose beads. The lower panel shows the densitometry values of co-immunopurified uL19 normalized by the signal of purified MTG1 and expressed relative to the untreated control. Data represent the mean ± SD of five independent repetitions; one-way ANOVA with a Dunnett’s multiple comparisons test, ****p < 0.0001.

determinants of GEF function 141. Conserved residues include E185 and L197 in CR1, F235 in CR2 and LVKE (373-376) in CR3 (**Fig. 2.11A**).

mS27 was initially thought to be a GEF for mS29 142, a GTPase embedded in the SSU head, where it contributes to the formation of intersubunit bridges 18,19. However, the cryo-EM structures of the mammalian mitoribosome have located mS27 to the mtSSU foot, making impossible the mS29-mS27 functional association 18,19. Now, the physical interaction between MTG1 and mS27 described here (**Fig. 2.11B**) opened the possibility for a functional relationship. We proposed that mS27 is a GEF of MTG1 that stimulates the release of GDP-bound MTG1 from uL19 in the mature mtLSU. To test this hypothesis, we used crude mitochondrial extracts from cells expressing FLAG-tagged MTG1 and performed IP assays to assess the amount of co-IPed uL19 when the extract was supplemented or not with recombinant mS27 (**Fig. 2.11B**) obtained from Proteintech (Cat # Ag11223). The presence of excess GTP or GMPPCP did not affect the levels of co-IPed bL19, probably because the MTG1 molecules interacting with the mtLSU are already GTP bound. Importantly, however, incubation with excess recombinant mS27 significantly decreased the levels of co-IPed bL19 (**Fig. 2.11B**). These data would be compatible with a scenario in which mS27 stimulates GDP-GTP interconversion in MTG1, promoting in this way the
dissociation of GDP-bound MTG1 from the mature 39S mtLSU. Future studies on the \textit{in vivo} role of mS27 as a GEF of MTG1 are warranted.
3.1 Role of MTG1 in 39S mtLSU assembly

Until the late 1990s, bacteria were known to harbor several GTPases, which were essential for bacterial growth, but had no function assigned. The bacterial GTPase RbgA (homolog of MTG1) was one of the first to be characterized. Now, RbgA is widely known as a ribosome associated GTPase, which has homologs that participate in the biogenesis of bacterial and cytoplasmic ribosomes as well as mitoribosomes. The mechanism of action and precise role of MTG1 in human mitoribosome assembly was poorly understood, which motivated the development of the thesis presented here.

Our data shows that depletion of MTG1 in HEK293T cells significantly reduces mitochondrial protein synthesis and steady-state levels of OXPHOS complexes, and as a consequence, abolishing OXPHOS enzyme activity, aerobic respiration, and cell viability. The MTG1 mutation, however, does not affect the steady-state levels of mitochondrial RNAs. MTG1 interacts with mitoribosomal proteins, specifically uL19 and mS27. MTG1 binds to the 3’ end of the 16S rRNA and protects the 16S rRNA from UV-induced degradation. Further characterization of MTG1 in ex vivo cardiomyocytes and in vivo zebrafish embryos revealed that MTG1 is required for efficient function and development of the heart.

Collectively, the data presented in this manuscript leads to a model for mitoribosome biogenesis in which MTG1 connects late-stage mtLSU assembly
with mtSSU joining (Fig. 3.1). In our model, MTG1 acts on mtLSU maturation in a manner broadly similar to its bacterial counterpart RbgA, with which it shares mechanistic principles \(^{73,77,78}\), and also significant differences to attend to the specific mitoribosome composition and assembly pathway. MTG1 acts after MALSU1 whose binding to uL14 also induces conformational changes in the neighboring bL19 relative to their positions in the mature mitoribosome \(^{128}\). Subsequently, GTP-bound MTG1 interacts with domain VI helices in the 16S rRNA and with protein bL19, presumably to induce a conformational change that facilitates incorporation of late-stage subunits, at least bL36 and bL35, to complete the mature 39S mtLSU particle. Then, MTG1 is not readily released from the mtLSU, but remains bound to it, a behavior probably sustained by a slow MTG1 GDP-GTP exchange. The interaction of mtLSU/bL19-MTG1(GDP) with mS27 in the mtSSU promotes the previously unrecognized mS27 GEF activity on MTG1 that will facilitate GDP-GTP interchange and MTG1 release from the 55S monosome.

### 3.2. MTG1 as a quality control checkpoint molecule

The proposed model has implications for translational regulation. We propose that MTG1 acts as an mtLSU quality control checkpoint molecule as it serves to avoid premature subunit joining. Redundant mechanisms exist to prevent premature subunit joining and translation initiation. An anti-subunit-association activity has been also proposed for MALSU1, which belongs to the universally conserved ribosome silencing factor (RsfS) family. RsfS protein sterically inhibits the
association of the small and large ribosomal subunits. The binding of uL14 to RsfS has parallels in other kingdoms of life. For example, the structurally and evolutionarily unrelated eIF6 also binds uL14 and sterically hinders the formation of cytosolic ribosomes in eukaryotes and archaea. In mammalian mitochondria, the structural change in the mtSSU indicates that the globular domain of MALSU1 also cannot obstruct subunit joining. Therefore, MALSU1 forms a complex with two proteins, the mitochondrial acyl carrier protein (mt-ACP) and the LYR-motif-containing protein L0R8F8, that sterically obstruct the binding of the mtSSU.

Figure 3.1. MTG1 is a GTPase that couples mtLSU assembly with inter-subunit bridge formation. Model of 39S mtLSU biogenesis depicting the role of MTG1 in late steps of assembly and how these are connected to subsequent mtSSU subunit joining. The translation initiation complex is not represented for simplification. See explanation in the text.
Similar to bacteria, binding of RsfS to uL14 prevents the formation of bridge B8 with helix 14 of 16S rRNA. The subunit anti-association role of MTG1 is conserved for other members of its family, such as the S. cerevisiae protein Lsg1. In the cytoplasm, Lsg1 GTPase activity is required to release two 60S assembly factors, Nmd3, positioned across the peptidyl transferase center (PTC), and Tif6, from the 60S LSU and, that allows 60S LSU interaction with 40S-initiation complexes.

3.3. Mechanism of GTPase activity: a GEF for MTG1?

Yeast and mammalian mitoribosomes have acquired an intrinsic GTPase in the small subunit, mS29, unique feature among ribosomes, which participates in subunit association. The identification of another mitochondrion-acquired mtSSU protein, mS27, absent in yeast, that acts as a GEF of MTG1 to catalyze the return of MTG1 from the inactive GDP-binding conformation to the active GTP-binding conformation, is also unprecedented in translational systems. Close examples include the protein elongation factor EF-Tu, which requires elongation factor EF-Ts for rapid exchange of GDP for GTP. In prokaryotes, free peptide release factor 3 (RF3) is in vivo stably bound to GDP, and ribosomes in complex with RF1 or RF2 act as GEFs. The ribosome itself also acts as the GEF for elongation factor G (EF-G) that catalyzes the translocation of peptidyl-tRNA from the A site to the P site of the ribosome. The uniqueness of mS27 lies on the conservation of GEF motifs, its integration into the mitoribosome and its specificity for MTG1.
Rho family GTPases serve as molecular switches, which are activated by DbI family GEFs with tissue- and subcellular-specific localization. A Rac-1 specific GEF, named STEF, is a mammalian homologue of Drosophila SIF. STEF contains a catalytic DbI homologous (DH) domain flanked by a pleckstrin homologous (PHc) domain, which are a hallmark of DbI family GEFs, indicating its essential and conserved function. As mentioned earlier, a mtSSU protein, mS27, shares sequence homology with the catalytic DH domain found in proteins that stimulate the dissociation of GDP from GTP binding proteins. Since the mtSSU contains an intrinsic GTPase, mS29, it was initially thought that mS27 could be a functional partner of mS29. However, cryo-EM structural reconstruction of the mtSSU revealed a localization of both proteins (mS27 at the lower/foot and mS29 at the head) incompatible with such a possibility. Moreover, since we identified a physical interaction between MTG1 and mS27 in this study, we have proposed an alternative hypothesis that mS27 may have a functional interaction with MTG1. We found that incubation with excess recombinant mS27 significantly stimulated the dissociation of GDP-bound MTG1 from the mature 39S mtLSU, suggesting the physical as well as functional partnership between MTG1 and mS27. The role of mS27 as a GEF for MTG1 needs, however, to be better studied to answer whether MTG1 is a specific interacting partner of mS27, and how mS27 recognizes GDP-bound MTG1. A scenario is conceivable in which mS27 works as a sensor rather than a regulator and recognizes a particular state of the ribosome, such as a GDP-MTG1-mtLSU complex, and this recognition may catalyze the GEF activity.
Further proof would involve the purification and *in vitro* characterization of the GEF activity of mS27 in the presence of endogenous or recombinant MTG1. Three lines of experimentation could be implemented. First, we would quantitatively measure the release of GDP during *in vitro* incubation of MTG1 with mS27 using a commercially available bioluminescent assay kit \(^{154}\). Second, we would clarify at which state of mitoribosome assembly mS27 is activated. For this purpose, purified mitoribosome subunits or monosomes will be incubated with MTG1 in the presence of GTP or GDP. Third, we would explore the reaction specificity between MTG1 and mS27. Recombinant DbI family GEFs or Ras GTPases will be cross-paired with MTG1 or mS27, and the release of GDP will be measured.

### 3.4. Compartmentalization of the mitoribosome assembly process

As commented upon earlier, yeast mitoribosome mtLSU assembly has been proposed to occur in contact with the inner membrane \(^{60}\), a possibility that should also be explored in human cells. Regarding the question of whether mitoribosome assembly occurs in specific compartments in the mitochondrial matrix, data obtained over the last few years has pointed toward the mtDNA nucleoids and the mitochondrial RNA granules as such compartments.

The functional connections and physical dynamics of these two compartments are highlighted by the observation that the RNA granules corresponding to BrU-labeled foci are found in close proximity to nucleoids \(^{62}\), but only fewer than 10% of RNA granules overlap with the mtDNA-containing
structures, probably reflecting actively transcribing nucleoids. The intensity of Br-RNA labelling per focus increases progressively and declines exponentially ($t_{1/2} \sim 45$ min) during a chase. A quantitative assessment of the dynamics showed that after a short pulse of 20 min, most BrU-RNA foci co-localize within 200 nm of mtDNA, but they become randomly distributed after longer periods of a pulse-chase. The kinetics are consistent with Br-RNA having a residence half-life in mtDNA foci of $\sim 45$ min, which is much longer than the $\sim 5.5$ min for complete transcription. These data indicate that mtRNAs remain at transcription sites for extended periods after they have been synthesized. Thus, mitoribosome assembly could be initiated near the nucleoids, possibly in a co-transcriptional manner as shown for their ancestors, the bacterial ribosomes.

However, the granule structures progressively separate from the nucleoid forming an independent compartment. Biogenesis of the mtLSU and the mtSSU may be differentially compartmentalized. While both require rRNA and therefore will start at the nucleoid where transcription occurs, incorporation of early-assembly mtSSU proteins could occur in this compartment as has been reported by several groups. At the same time, several groups have proposed that later stages of mtSSU assembly and the bulk of mtLSU assembly occur at the RNA granule compartment.

Although the assembly pathways of the mtLSU and mtSSU are considered to be largely independent, there is at least one case reported in which connections between the two pathways have been established. Gene silencing of the mtLSU assembly factor MPV17L2, which co-sediments on sucrose gradients with the
mtLSU and the monosome, results in marked decreases in these structures and also in the mtSSU \(^5^6\). Furthermore, although MPV17L2 depletion does not affect mtDNA levels, it also results in aggregation of the mitochondrial nucleoids where mtSSU proteins were shown to accumulate \(^5^6\). It was proposed that if mtSSU assembly starts at the mtDNA nucleoids, MPV17L2 may coordinate mtLSU biogenesis with the release from the nucleoids of mtSSU assembly intermediates or fully assembled particles \(^5^6,^6^3\).

3.5. Mitoribosomal proteins and assembly factors in apoptosis

In this study, we found that depletion of MTG1 causes severe cell death, and the apoptotic pathway is one of cell death mechanisms induced by the absence of MTG1. This phenotype is mtDNA and mitoribosome-independent, indicating that MTG1 has an alternative role in apoptosis, independent as its role as a mitoribosome assembly factor. Like MTG1, three more mitoribosome assembly factors (ERAL1, MPV17L and NOA1) and three mitoribosomal proteins (mS29, mL41 and mL65) also have been shown to be involved in apoptosis regulation.

Era (E. coli Ras-like protein) is an essential GTPase in \textit{E. coli}, and its eukaryotic orthologue ERAL1 (Era G-protein-like 1) has been identified in various species. Depletion of the chicken homologue ERA (GdERA) \(^1^5^5,^1^5^6\) arrested cells at G1 phase and accumulated apoptotic cells. Also, depletion of the mammalian orthologue ERAL1 significantly reduced cell growth in normal rho\(^+\) cells, but not in rho\(^0\) cells, and rapidly increased apoptosis without affecting mitochondrial protein synthesis \(^1^1^9\). These data indicate that ERAL1 regulates cell viability depending on
the presence of mtDNA, and ERAL1 is involved in the apoptotic pathway independent of its role in mtSSU assembly and mitoribosome biogenesis. Another example is MPV17L. MPV17 is a mitochondrial protein of unknown function, and its mutations induce mtDNA maintenance disorders. Its close paralogue, MPV17L2 (sequence identity/similarity to MPV17 is 27/44), is associated with mitoribosome assembly. MPV17L2 co-sediments with the mtLSU and the monosome on sucrose gradients, and depletion of MPV17L2 significantly decreased the formation of monosome and both subunits, and consequently abolished mitochondrial translation. Another paralogue, MPV17L (sequence identity/similarity to MPV17 is 25/39), interacts with the HtrA2 protease and mediates antioxidant and anti-apoptotic functions in mitochondria. The other example, NOA1, has a different mode of action than the two factors discussed previously. NOA1 (nitric oxide associated-1, a bacterial homologue, YqeH) is essential for mitochondrial translation and mitoribosome biogenesis. Endogenous NOA1 locates in mitochondria colocalizing with cytochrome c. Immunoprecipitation followed by immunoblotting analysis identified that NOA1 directly interacts with DAP3 (or mS29), a positive regulator for \( \gamma \)-interferon-mediated apoptosis. Interestingly, NOA1-depleted HeLa cells were less responsive to staurosporine-induced and \( \gamma \)-interferon-induced apoptosis than controls or DAP3-depleted cells. In addition, primary embryonic fibroblasts isolated from NOA1 knockout mouse embryos exhibit resistance to staurosporine-induced apoptosis. These data indicate that NOA1 is necessary for caspase-dependent apoptosis activation.
Regarding mitoribosome proteins involved in apoptosis regulation, mS29 was first discovered as a member of the death-associated protein (DAP) family and termed DAP3\textsuperscript{160,161}. mS29/DAP3 is known as one of the major positive mediators of apoptosis\textsuperscript{162}. The intrinsic pro-apoptotic role of DAP3 is further highlighted by the fact that overexpression of human, mouse or yeast DAP3 leads to cell death resulting in mitochondrial fragmentation, perhaps affecting mitochondrial fission\textsuperscript{163,164} in a manner dependent on the GTP-binding ability of DAP3 and its mitochondrial localization\textsuperscript{164}. The mtLSU protein mL41 has also been named BMRP (BCL-2 interacting mitochondrial ribosomal protein)\textsuperscript{165}, based on the observation that overexpressed in human cells, mL41 induces cell death which is counteracted by BCL-2. mL41-induced cell death is also repressed by p35, a caspase inhibitor, further suggesting its involvement in the regulation of apoptosis via its interaction with anti-apoptotic members of the BCL-2 family of proteins. In fact, the BCL-2 binding sites have been found near the N-terminus of mL41\textsuperscript{166,167}, which is required to be buried within the 16S rRNA\textsuperscript{10,11}. Therefore, the interaction of mL41 with BCL-2 and its apoptotic activity are likely to occur in the cytosol before its import into mitochondria and incorporation into mitoribosomes\textsuperscript{167,168}. Finally, mL65 (previously known as mS30), which in the ribosome forms a dimer with its homolog protein mL37\textsuperscript{11}, has homology to the chicken pro-apoptotic protein p52. When overexpressed in mouse fibroblasts, mL65 induced apoptosis, upregulation of transcription factor c-Jun and activation of c-Jun N-terminal kinase 1 (JNK1), through a pathway distinct from the extrinsic death receptor-induced apoptosis\textsuperscript{169}. As the human counterpart of p52, mL65 was
initially termed PDCD9 (programmed cell death 9), and the corresponding gene mapped to chromosome 5q11\textsuperscript{170}. However, the molecular mechanisms underlying the pro-apoptotic roles of mL65 are still unclear.

So far, a total of seven mitoribosome-related proteins including MTG1, have been reported to have apoptotic activities, although the mechanisms involved remain ill-defined. Further studies are needed to unravel the precise mechanism/s by which mitoribosomal proteins induce apoptosis, and whether their role in the regulation of mitochondrial translation is coupled to their role in the regulation of apoptosis.

3.6. Clinical prospect of MTG1

Finally, from a biomedical perspective, our genetic and physiological studies on HEK293T cells, human cardiomyocytes and zebrafish developing heart demonstrate the requirement of MTG1 for mitochondrial translation and OXPHOS function in multiple biological systems. Given the frequent clinical outcome of mitoribosome assembly and mitochondrial translation defects as pure or syndromic mitochondrial cardiomyopathies, our data highlights the deleterious potential of \textit{MTG1} as a mitochondrial cardiomyopathy gene.
CHAPTER 4

MATERIALS AND METHODS

Human cell lines and culture conditions
HEK293T embryonic kidney cells (CRL-3216), and 143B osteosarcoma cells (CRL-8303) were obtained from ATCC. The 143B.TK^- rho^0 derivative (143B206) was obtained from Dr. M. King. The three cell lines were cultured at 37°C under 5% CO_2 in high-glucose DMEM medium supplemented with 10% FBS, 1 mM pyruvate and 50 µg/ml uridine (complete DMEM medium). Analysis for mycoplasma contamination was routinely performed.

iCell cardiomyocytes derived from induced pluripotent stem (iPS) cells were obtained from Cellular Dynamics (Madison, WI). These human heart cells consist of a 95% pure population of ventricular, atrial, and nodal cells. They are known to recapitulate healthy human cardiac biology. Plating and maintenance media were obtained from Cellular Dynamics (https://cellulardynamics.com/products-services/icell-products/icell-cardiomyocytes/).

siRNA oligoribonucleotides and transfection
The sources of the Non-targeting siRNA and siRNAs targeting MTG1, DDX28, DHX32, MALSU1, uL14, bL19, bL36, mL45, mL66, mS27, or mS40 are listed in the Supplementary Table.
HEK293T cells or the partial MTG1-KO cell line (1F5) grown on a 6-well plate at 30% confluency were transfected on days 0, 3 and 6 with the indicated mRNA-targeting siRNA or Non-targeting (NT) control siRNA at a final concentration of 20 nM using 5 μl of Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's specifications.

**Plasmid transfection**

To create a stable human MTG1 knockout (KO) line in HEK293T cells, we used TALEN constructs obtained from Cellectis (Paris, France). Two pairs of TALENs (#1 and #2) were designed. The left and right TALENs for each pair were designed to bind the sequences shown in **Fig 2A**. HEK293T cells grown on a 6-well plate at 30% confluency were transfected with 4 μg of each right and left TALEN plasmid as a pair using 5 μl of Lipofectamine 2000 pre-incubated in 300 μl of Opti-MEM (ThermoFisher). After 4 hours of incubation, the media were changed to complete DMEM medium. After 3-6 times of repetitive transfections every three days, cells were collected, diluted in complete DMEM medium and seeded as single cells in multiple 96 well plates. Upon growth, each clone was tested by immunoblotting to assess the steady-state levels of MTG1 as well as the mtDNA encoded COX2 protein as a surrogate of mitochondrial protein synthesis capacity. Clones that had attenuated MTG1 and COX2 levels were further analyzed by genotyping.

To establish stable lines expressing tagged or untagged versions of MTG1 or other genes of interest, 4 μg of pIRESpuro empty vector or pIRESpuro2 containing FLAG-tagged MTG1 were transfected to HEK293T cells using a
standard Lipofectamine 2000 protocol. Two days after transfection, the medium was supplemented with 2.5 µg/ml puromycin for three weeks.

**Cell Proliferation Assay**

Wild-type HEK293T, MTG1-KO cells and cells treated with siMTG1 or siNT were seeded at 5x10^3 cells/well in a 96-well plate in complete high-glucose DMEM medium and incubated in standard culture conditions. From the day of cell seeding (day 0), cell numbers were counted every 24h using a hemocytometer (Hausser Sci. Co., PA).

**Cell Death Assay**

MTG1 siRNA or non-targeting siRNA as an experimental control were transiently transfected into the 1F5 cell line. After three days, the medium was replaced with fresh transfection mixture. WT HEK293T cells were cultured in parallel keeping the same passage. On day five post-silencing, cells both attached on the bottom and floated in culture medium were harvested separately and analyzed by immunoblotting with antibodies targeting apoptotic markers: cleaved caspase-3 and caspase-3.

**Pulse Labeling of Mitochondrial Translation Products**

Mitochondrial protein synthesis was determined by pulse-labeling 80% confluent human HEK293T and cardiomyocyte cultures in the presence of 100 µl/ml emetine to inhibit cytoplasmic protein synthesis as described\textsuperscript{172,173}. Cells were labeled for
15 mins or at indicated time points at 37°C with 150 μCi/ml [35S] methionine (PerkinElmer Life Sciences, Boston, MA). After incubation, the cells were washed once with 1X PBS (Phosphate-buffered saline), collected by trypsinization, and whole-cell extracts were prepared by solubilization in RIPA buffer (1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 150 mM NaCl, 2mM EDTA (ethylenediaminetetraacetic acid), and 50 mM Tris-HCl, pH 8.0) supplemented with 1 mm PMSF (phenylmethylsulfonyl fluoride) and 1× EDTA-free mammalian protease inhibitor cocktail (Roche). One hundred micrograms of each sample were separated by SDS (sodium dodecyl sulfate)-PAGE on a 17.5% polyacrylamide gel, transferred to a nitrocellulose membrane and exposed to Kodak X-OMAT X-ray film. The membranes were then probed with a primary antibody against the mitochondrial protein VDAC as a loading control.

**Isolation of Mitochondria**

Mitochondria from wild-type (WT) HEK293T cells or HEK293T cells stably expressing MTG1-FLAG were isolated as described previously. Briefly, cells were harvested by centrifugation at 1,000 rpm for 7 min. The pellet was washed with pre-cooled 1x PBS. Following weighing of the pellet, it was resuspended in pre-cooled buffer (0.15 g/ml; 10 mM Hepes-KOH, pH 7.4, 10 mM KCl, 0.5 mM MgCl2), and incubated for 5 min on ice. The cells were lysed with 10 strokes with a glass homogenizer (Kimble/Kontes, Vineland, NJ, USA), and buffer (1 M sucrose, 10 mM Hepes-KOH, pH 7.4) was added to become the final concentration of 0.25 M sucrose. To separate the cell debris and nuclei from mitochondria the
solution was centrifuged twice at 1,500 x g for 3 min, and the supernatant was centrifuged at 8,000 x g for 10 min. The crude mitochondria pellet was gently resuspended in buffer A (20 mM Hepes, pH 7.4, 0.25 M sucrose, 40 mM KCl, 10 mM MgCl₂) using a glass homogenizer, then centrifuged at 1,500 x g for 3 min, followed by further centrifugation at 8,000 x g for 10 min. Mitochondria were resuspended in buffer A, snap-frozen in liquid nitrogen, and stored at -80°C.

**SDS-PAGE, BN-PAGE and Immunoblotting analyses**

To estimate steady-state levels of individual proteins, whole cell protein extracts (WCE) or isolate mitochondria were analyzed by denaturing SDS-PAGE and immunoblotting. Total cellular proteins were extracted in RIPA buffer (1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 150 mM NaCl, 2mM EDTA, and 50 mM Tris-HCl, pH 8.0) with freshly added 1 mm PMSF and 1x EDTA-free protease inhibitor cocktail (Roche). Mitochondria were isolated as explained earlier. Protein concentration in WCE and mitochondrial preparations was measured with the Folin phenol reagent 175. In general, 20–60 μg of mitochondrial proteins or 40–60 μg of WCE was separated by SDS–PAGE in the Laemmli buffer system 176. After transfer to a nitrocellulose membrane, the membranes with immobilized proteins were blocked with 5% skim milk and then incubated with antibodies against the indicated proteins followed by a second reaction with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase. Signals were detected by chemiluminescence incubation and X-ray film exposure.
To estimate the steady-state levels of assembled OXPHOS complexes we implemented Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) using a linear 3-12% gradient native gel. 2.5 × 10⁶ cells were incubated at 4°C for 10 min in 200 µl PBS supplemented with 70 µl of 8 mg/ml digitonin. Following dilution of the detergent with 1 ml PBS, cells were spun at 10,000 x g for 5 min at 4°C and washed twice in PBS. Cell pellets were resuspended in 100 µl of 1.5 M aminocaproic acid, 50 mM Bis-Tris pH 7.0 and 0.6% lauryl maltoside. After spinning at 22,000 x g for 30 min at 4°C, the extracts were supplemented with 10 µl of 750 mM aminocaproic acid, 50 mM Bis-Tris pH 7.0, 0.5 mM EDTA and 5% Serva Blue G. For each sample, extracts from ~2.5 to 5 × 10⁵ cells were loaded on a NativePAGE Bis-Tris linear 3–12% gradient gel-precast system (Invitrogen) and run at 4°C using NativePAGE anode and cathode buffers (Invitrogen). Following electrophoretic separation, the samples were transferred to a PDVF (polyvinylidene difluoride) membrane, and processed for immunoblotting as described.

Sucrose gradient analysis

The sedimentation properties of MTG1 and mitoribosomal proteins were analyzed by sucrose gradient sedimentation. Mitochondria from WT or the partial MTG1-KO (1F5) cells after treatment with control (siNT) or MTG1-targeting siRNA (siMTG1) for 5 days were solubilized in 400 µl of a buffer containing 20 mM HEPES-KOH, pH 7.4, 100 mM KCl, either 10 mM MgCl₂ or 5 mM EDTA, 0.25% Digitonin, 0.5 mM PMSF and 1× EDTA-free protease inhibitor cocktail. The lysate was spun at
22,000 x g for 30 min at 4°C, and the supernatant was loaded onto a 5 ml linear 0.3–1 M sucrose gradient containing 20 mM Hepes-KOH pH 7.4, 100 mM KCl, 10 mM MgCl₂ or 5 mM EDTA, 0.1% Digitonin, 0.5 mM PMSF and 1× protease inhibitor cocktail, and centrifuged at 40,000 rpm for 3 h 10 min at 4°C in SW 55Ti (Beckman Coulter). The gradients were collected from the bottom into 14 or 15 fractions, TCA (trichloroacetic acid)-precipitated and the pellets resuspended in 1x Laemmli buffer and analyzed by immunoblotting. For RNA profiling experiments, total RNA was isolated from the sucrose fraction by using miRNeasy mini kit (Qiagen, Boston, MA). RNA extracts were reverse-transcribed to cDNA and analyzed by quantitative qPCR analysis using SYBR Green (Applied Biosystems) and specific primer pairs for each gene following standard procedures. Primers used in this study are listed in the key resource table. For mass spectrometry analysis, monosome, mtLSU and mtSSU fractions determined by immunoblotting were pooled and precipitated with methanol/chloroform and analyzed at the Keck Biotechnology Resource Laboratory (Yale University School of Medicine, New Haven, CT). For sucrose gradient sedimentation analysis with whole cell lysates, cells were permeabilized with 70 μl of 8 mg/ml digitonin in 200 μl of PBS at 4°C 10 min and washed with 1x PBS twice before solubilizing proteins with 0.25% digitonin.

RNA analysis
Total RNA was prepared from whole cells by using Trizol (Invitrogen) following the manufacturer instructions for Northern-blot and quantitative qPCR analyses. For
Northern-blot analyses, the RNA extracts were separated on a denaturing 1.8% agarose gel containing 2 M formaldehyde. The quality of the RNA was assessed by staining the gel with ethidium bromide prior to transferring the RNA onto a nylon membrane (Nytran®, SuPerCharge, Schleicher and Schuell, Keene, NH). The RNA bound to the nylon membrane was cross-linked with UV light and pre-hybridized at 65°C for 30 minutes. Subsequently, the specific probes, in a solution containing 7% SDS, 1 µM EDTA, 0.5 M Na₂HPO₄/NaH₂PO₄, were added to the membrane and was allowed to hybridize overnight at the respective melting temperatures of the probes. The probes were created by PCR on genomic DNA, column-purified (Promega, Madison, WI) and labeled with [α-³²P] dATP by random priming. For qPCR analysis of the steady state levels of mitoribosomal RNAs, total RNAs extracted from whole cells by using Trizol (Invitrogen) were reverse-transcribed to cDNA and analyzed with specific primer pairs for each gene following standard procedures. Primers used in this study are listed in the key resource table.

MTG1 interactome analysis
For native interactome analysis, mitochondria isolated from HEK293T cells after stably expressing MTG1-FLAG were solubilized in 500 µl of extraction buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 20 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF, 1x protease inhibitor cocktail) supplemented with 2.5 mM β,γ-methyleneguanosine 5’-triphosphate sodium salt (GMPPCP) at 4°C rotation for 40 min, then incubated with 0.25% digitonin supplementation at 4°C for 10 min. The lysates were cleared
by centrifugation at 30,000 rpm for 20 min at 4˚C in a TLA 100 rotor (Beckman Coulter). The lysate was incubated with 50 µl of α-agarose beads (control) or α-FLAG M2 beads at 4˚C for 4 hours with gentle rotation. The supernatant containing unbound material was subsequently collected, and the beads were washed twice with high-salt buffer (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 1% NP-40, 0.1% SDS, EDTA-free protease inhibitor cocktail), three times with wash buffer (PBS, 0.02% Tween-20, EDTA-free protease inhibitor cocktail). The bound proteins were eluted using SDS-PAGE loading buffer and incubated at 95˚C for 5 min.

For specific interacting partners analysis, mitochondria isolated from HEK293T cells after stably expressing MTG1-FLAG were incubated in the presence of a cleavable crosslinker (DSP or dithiobis[succinimidylpropionate], Thermo) or the vehicle DMSO as a negative control, and then the reactions were quenched by adding 20 mM Tris (pH7.4). DSP has NHS-ester reactive ends that react toward amino groups, with a 12.0 Å spacer arm that contains a cleavable disulfide bond. Cell pellets were incubated with 1% SDS extraction buffer at 4˚C for 10 min. After dilution to 0.1% SDS, the lysates were separated by centrifugation at 30,000 rpm for 20 min at 4˚C in a TLA 100 rotor (Beckman Coulter). MTG1-FLAG and bound proteins were pulled down with anti-FLAG-agarose beads, separated by SDS-PAGE, and analyzed with immunoblotting. By means of another strategy to investigate specific interacting proteins, HEK293T cells stably expressing FLAG-tagged MTG1 were incubated in the presence of the non-cleavable crosslinker AMAS (N-α-maleimidoacet-oxyssuccinimide ester) (Thermo Scientific) or the vehicle DMSO as a negative control, and then the reactions were
quenched by adding 20 mM Tris (pH 7.4). AMAS has NHS-ester and maleimide groups, which crosslink amino to sulphhydryl groups with a 4.4 Å spacer arm. Cell pellets were incubated with 1% NP40 extraction buffer at 4°C for 10 min, and the lysates were separated by centrifugation at 30,000 rpm for 20 min at 4°C. MTG1-FLAG co-purified proteins were separated by SDS-PAGE. After Coomassie staining, a single faint band of ~75 kDa (which is above the ~37 kDa band corresponding to the MTG1-FLAG protein) was cut from the gel and analyzed by mass spectrometry. For reciprocal immunoprecipitation analysis, instead of FLAG beads incubation, cross-linked lysates were incubated with protein A-conjugated Dyna-beads and protein-specific antibodies for overnight. Data was evaluated by total unique spectrum count, which represents number of unique spectra attributed to a single protein/protein group including those shared with other proteins. The approximate MW of each band is shown under the name of the immunoprecipitated protein.

**MTG1-RNA interaction analysis**

The protocol was modified from PAR-CLIP assay \(^{179}\). Cells stably expressing Flag-tagged MTG1 proteins were grown in medium supplemented with 100 uM 4-thiouridine (SU) for 14 hr, and subjected to UV-mediated protein-RNA crosslinking once with 150 mj/cm\(^2\) at 365 nm using a Stratalinker UV crosslinker (Stratagene) before disrupting them with 1% NP40 lysis buffer (PBS, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with EDTA-free protease inhibitor cocktail (Roche), and ribonucleoside vanadyl complexes (NEB) for 10 min at 4°C.
4SU-labeled non-irradiated cells were used as control. The lysate was spun at 30,000 rpm for 20 min at 4°C in a TLA 100 rotor, and the eluted protein-RNA complexes were applied for immunoprecipitation with anti-FLAG M2 beads. Bound protein-RNA complexes were treated with DNase for 30 min at 37°C, and RNA was extracted from the beads by proteinase K treatment and QIAzol extraction (Qiagen). In one set of experiments, RNA extracts were converted to cDNA and analyzed by quantitative qPCR analysis using SYBR Green (Applied Biosystems) and specific primer pairs for each gene following standard procedures. Primers used in this study are in the key resource table.

In another set of experiments, DNA-free RNA was used to prepare a library using the KAPA Stranded RNA-Seq kit for Illumina platforms (Kapa Biosystems # KR0934), size distribution of the libraries was verified using a bioanalyzer (see PDF bioanalyzer file), and then analyzed by single-read-RNA sequencing (RNA-seq) on a NextSeq 500 system (Illumina) at the Genomic Platform of the Institute for Research in Immunology and Cancer (IRIC, Montreal, Canada). Reads were processed to remove sequencing adapters using Trimmomatic 0.35 and then mapped with Bowtie version 2.1.0 to the reference human genome (assembly GRCh37). Mitochondrial coverage was computed and normalized using Bedtools version 2.26.0. Raw data can be accessed through the Gene Expression Omnibus (GEO) database repository (accession number GSE116688).
**Cellular respiration and mitochondrial enzyme activities**

Potassium cyanide (KCN)-sensitive endogenous cell respiration was measured polarographically in cells from a 70% confluent flask. The culture medium was changed 3 h prior to harvesting the cells by trypsinization. Cell pellets were resuspended in DMEM with galactose + 1 mM pyruvate, and oxygen consumption was measured using a Clark oxygen electrode in a micro water-jacketed, magnetically stirred cell set at 37°C (Hansatech Instruments Limited, Norfolk, UK). After measuring intact cell-coupled endogenous respiration, the reaction was inhibited with KCN (700 μM) to obtain the baseline rate, as described \(^{183}\). All values were normalized to the total cell number used in the assay. The activities of the OXPHOS complexes CIV and the TCA enzyme citrate synthase were determined spectrophotometrically in 3X-frozen-thawed cells as described previously \(^{183}\). The rate of complex CIV activity was normalized by the activity of the citrate synthase.

**MTG1 Localization Experiments**

Mitochondria from HEK293T were briefly sonicated, and then soluble and insoluble fractions were obtained by centrifugation at 35,000 rpm for 15 min at 4 °C. The membrane pellet was then resuspended in 0.1 M Na\(_2\)CO\(_3\), pH 11.5. After 30 min on ice, the sample was centrifuged at 35,000 rpm for 15 min at 4 °C to separate the soluble extrinsic from the insoluble intrinsic membrane proteins. Purified mitochondria were also resuspended in buffer (10 mM Tris pH 7.4, 10 mM KCl, 0.5 mM MgCl\(_2\)) with or without 0.25 M sucrose to allow mitochondria swelling and conversion to mitoplasts. Where indicated, samples were treated with 0.6 mg/ml
proteinase K for 20 min on ice. Mitochondria and mitoplasts were recovered by centrifugation at 10,000 rpm for 10 min at 4 °C and analyzed by immunoblotting.

**Immunofluorescence assays**

HEK293T cells were plated on coverslips and grown overnight at 37°C. Cells were stained for 30 min with 50 nM Mitotracker red (Molecular Probes, Invitrogen), fixed with 2% paraformaldehyde and treated with methanol before incubation with an anti-MTG1 primary antibody as described in 2% BSA. A secondary antibody labeled with Alexa Fluor 488 (Molecular Probes, Invitrogen) was used for immunofluorescence detection. Images were obtained in an Olympus IX81 confocal microscope and processed using FluoView 10-ASW (Olympus) and Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

**Assays in Cardiomyocytes**

iCell Cardiomyocytes purchased from Cellular Dynamics (6.9 x 10^6 cells/vial) were thawed and plated into fibronectin-coated slide grass plates for single cell analysis or 12-well cell culture plates for immunoblot analysis and metabolic labeling. The medium was refreshed every two days, and on day 4 post-plating, MTG1 siRNA or control siRNA as an experimental control were transiently transfected at a final concentration of 50 nM/well using TransIT-TKO Transfection Reagent (Mirus Bio) according to the manufacturer's specifications. After 48 hrs, the medium was replaced with fresh transfection mixture. On day 4 post-silencing, iCells were
analyzed by immunoblotting assays, mitochondrial translation product labeling, or single cell-based beating rate, Ca\(^{2+}\) transient and decay rate analyses.

For Ca\(^{2+}\) transient and decay rate analyses, iCell human cardiomyocytes (N = 4–5) were loaded with the membrane-permeable Ca\(^{2+}\)-sensitive dye, Fura-2-AM (Molecular Probes, Grand Island, NY), for 15 min at room temperature as described previously\(^{122}\). The sarcomere length (SL) was recorded with an IonOptix iCCD camera. Ca\(^{2+}\) was measured by dual excitation (340/380 nm) and Fura-2 fluorescence acquired at 510 nm on an IonOptix spectrofluorometer (IonOptix LLC, Milton, MA). The in vivo calibration was performed using solutions containing 10 \(\mu\)M ionomycin (Sigma-Aldrich)\(^{122}\). Changes in myofilament responsiveness to Ca\(^{2+}\) were assessed by acquiring pairwise measurements of SL and \([\text{Ca}^{2+}]_i\) in intact single cardiomyocytes tetanized by high-frequency (10 Hz) stimulation after disabling the reuptake of Ca\(^{2+}\) to the sarcoplasmic reticulum (SR) by using thapsigargin (0.2 \(\mu\)M, Sigma-Aldrich)\(^{122}\).

**Zebrafish husbandry**

Zebrafish were freshly supplied from University of Miami Zebrafish Core Facility and maintained in the facility during analysis. Experiments were carried out using *Danio rerio* wild type strains AB. Embryos were obtained from natural crosses after removing a divider at first light and microinjections were performed into one-cell stage embryos. Embryos were reared in petri dishes of system water in a 28°C incubator with a 14-hour light/10-hour dark cycle. All experiments were
conducted in accordance with the University of Miami Institutional Animal Care and Use Committee guidelines.

**Assays in Zebrafish**

Zebrafish were freshly supplied from University of Miami Zebrafish Core Facility and maintained in the facility during analysis. The translation blocking Mtg1 morpholino (zf-Mtg1, Gene Tools) was targeted to the MTG1 exon1, and the standard scrambled morpholino (STD, Gene Tools) was used for the control. Stock solutions (10 mM) of the morpholinos were diluted in 1% Fast-Green dye to a final concentration of 2.5 mM and 5 mM. One ml of each morpholino was loaded into a micropipette and injected into approximately 100 WT zebrafish embryos at 1~2 cell stage. The injection was visualized with co-injected Fast Green, and eggs that had improper injection following uneven cell division were removed. At 48-hour post fertilization (hpf), ten morphants from each group were pooled, and applied for SDS-PAGE and immunoblotting using antibodies. At 96 hpf, embryos were analyzed for morphology and heart beat rate under the microscope using a Zeiss LSM710 microscope using 23x air lens.

**Statistical Analysis**

All of the experiments were done at least in triplicate, or otherwise indicated. Data in X-ray films was digitalized and analyzed using the Image J software. Statistical analyses were performed using the Prism-6 software. The data are presented as the means ± S.D. of absolute values or percentages of control. The values
obtained for WT, a MTG1 partial KO (IF5) and the clone after silencing of MTG1 (1F5-siMTG1) strains for the different parameters studied were compared using a Student’s two-tailed t-test for comparison of two groups. For comparison of multiple groups, we performed one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for all the groups of the experiment. The statistical significance of cardiomyocyte and zebrafish studies were tested with one-way ANOVA followed by a Sidak’s or a Tukey’s multiple comparison test, respectively. The statistical tests of MTG1-rRNAs interaction were analyzed with 2-way ANOVA followed by a Tukey post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).
5.1 List of antibodies used in the study

The list of antibodies against *Homo sapiens* mitoribosomal proteins includes in parenthesis the old nomenclature.

<table>
<thead>
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<th>Source</th>
<th>Reference</th>
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<td>DHX32</td>
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### 5.2 List of siRNA oligonucleotides

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### 5.3 List of recombinant DNAs

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### 5.4 List of oligonucleotides.

<table>
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<tbody>
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</table>
| *EcoRI* MTG1-FLAG Reverse| **GCGCGAATTCTTTACTTGTTCGTCATCGTCTTTTG**
|                           | **TAGTCACCCGGGGGTGGCCCCCCGC**                                                                   |
| 16S-1 rRNA Forward        | **AGAGAGTAAAAAATTTAACACCAT**                                                                   |
| 16S-1 rRNA Reverse        | **TTCTATAGGGTGATAGATTGGTC**                                                                   |
| 16S-4 rRNA Forward        | **TTCACCAGTCAAAGCGAACT**                                                                     |
| 16S-4 rRNA Reverse        | **TTAATAGCGGCTGCACCAC**                                                                      |
| 16S-5 rRNA Reverse        | **CCCTGTTCTTGGGTTGGTGTTTGAT**                                                                 |
| 12S rRNA Forward          | **TAGAGGAGCCTGTCTTGTAATCGA**                                                                   |
| 12S rRNA Reverse          | **TGCAGCTTTATTTGTAGCCCTCC**                                                                    |
| 18S rRNA Forward          | **CCAGTAAGTCCGCCCACATAAA**                                                                    |
| 18S rRNA Reverse          | **CCTCACTAAAAACATCCAA TCGG**                                                                  |
| COX1 mRNA Forward         | **CTTCCTCGTCTTGATCCCT**                                                                       |
| COX1 mRNA Reverse         | **ATTCGAAGCCTGTCTGTAATCGA**                                                                    |
| COX2 mRNA Forward         | **ACGAGTACACCAGCTACGC**                                                                       |
| COX2 mRNA Reverse         | **CGGGATTTGCATCTGTTTTT**                                                                      |
| ATP6 mRNA Forward         | **TTTCCCCCTCATTGATCC**                                                                        |
| ATP6 mRNA Reverse         | **TTTCCCCTCTATTGATCC**                                                                        |
| uL11 mRNA Forward         | **ATTTCAGAGGAGGTAGTTGCAAAAA**                                                                  |
| uL11 mRNA Reverse         | **TCATTCAAGGTGATGAAACATC**                                                                    |
| bL36 mRNA Forward         | **AGTTACCACATCAGGTTTTGAT**                                                                    |
| bL36 mRNA Reverse         | **TTTTAGGGAACGTTTTGGAAT**                                                                      |
| uS22 mRNA Forward         | **AGGACTATGTATAGCCAGGACAGG**                                                                   |
| uS22 mRNA Reverse         | **TATACTCTGTGGAATCTGGCTCAA**                                                                   |
| *mt-VAL* tRNA Forward     | **CAGAGTGTAGCTTAACACAAAA**                                                                    |
| *mt-VAL* tRNA Reverse     | **CAGAGCGGCTCAAGTAAAGTTG**                                                                     |
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Camara, Y. et al. MTERF4 regulates translation by targeting the methyltransferase NSUN4 to the mammalian mitochondrial ribosome. *Cell Metab.* 13, 527-539 (2011).


88 Kallstrom, G., Hedges, J. & Johnson, A. The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. *Mol. Cell Biol.* **23**, 4344-4355 (2003).


