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Mitochondrial ribosome assembly in health and disease

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The ribosome is a structurally and functionally conserved macromolecular machine universally responsible for catalyzing protein synthesis. Within eukaryotic cells, mitochondria contain their own ribosomes (mitoribosomes), which synthesize a handful of proteins, all essential for the biogenesis of the oxidative phosphorylation system. High-resolution cryo-EM structures of the yeast, porcine and human mitoribosomal subunits and of the entire human mitoribosome have uncovered a wealth of new information to illustrate their evolutionary divergence from their bacterial ancestors and their adaptation to synthesis of highly hydrophobic membrane proteins. With such structural data becoming available, one of the most important remaining questions is that of the mitoribosome assembly pathway and factors involved. The regulation of mitoribosome biogenesis is paramount to mitochondrial respiration, and thus to cell viability, growth and differentiation. Moreover, mutations affecting the rRNA and protein components produce severe human mitochondrial disorders. Despite its biological and biomedical significance, knowledge on mitoribosome biogenesis and its deviations from the much-studied bacterial ribosome assembly processes is scarce, especially the order of rRNA processing and assembly events and the regulatory factors required to achieve fully functional particles. This article focuses on summarizing the current available information on mitoribosome assembly pathway, factors that form the mitoribosome assembly machinery, and the effect of defective mitoribosome assembly on human health.

Introduction

Synthesis of all proteins is catalyzed by the ribosome. Functional ribosomes are macromolecular ribonucleoprotein nanomachines that are assembled from rRNA (rRNA) and proteins. Ribosomes are present in the cytoplasm of bacterial and eukaryotic cells. They are also present within semiautonomous eukaryotic organelles of bacterial ancestry, namely mitochondria and chloroplasts. In this review we will focus on discussing the current knowledge on the biogenesis of mitochondrial ribosomes (mitoribosomes), from yeast to mammals.

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Mammalian and yeast mitoribosomes were first isolated in the late 1960s/early 1970s.[1-3] Mitoribosomes reside in the matrix of the organelle and associate with the inner membrane to facilitate co-translational insertion of highly hydrophobic nascent polypeptides. Mitoribosomes are specialized in the synthesis of only a handful of polypeptides (8 in yeast, 13 in human cells), which are essential for oxidative phosphorylation (OXPHOS) that facilitates ATP production aerobically. As remnants of their eubacterial origin, mitoribosomes are sensitive to similar antibiotics as bacteria and have many conserved proteins and RNA moieties. However, owing to evolution, mitoribosomes differ significantly in structure and composition, not only in comparison to their bacterial ancestors but also among different species[4-7]

In all organisms, mature ribosomes are composed of 2 subunits, the large subunit (LSU) and the small subunit (SSU). The LSU is involved in catalyzing the peptidyl-transferase reaction, while the SSU provides the platform for mRNA binding and decoding. The LSU includes 3 rRNAs (rRNAs) in eukaryotes (28S, 5.8S, 5S in *Saccharomyces cerevisiae*) and 2 rRNAs in prokaryotes (23S and 5S in *Escherichia coli*). The SSU has instead only one rRNA in all kingdoms (18S in *S. cerevisiae* and 16S in *E. coli*). *S. cerevisiae* mitochondria contain 74S ribosomes composed of a 37S small subunit (mtSSU) formed by a 15S rRNA and 38 mitoribosomal proteins (MRPs), and a 54S large subunit (mtLSU) formed by a 21S rRNA and 46 proteins.[6,8] Mammalian mitoribosomes sediment as 55S particles, constituted of a 28S mtSSU, formed by a 12S rRNA and 29 MRPs, and 39S mtLSU, formed by a 16S rRNA and 50 MRPs.[9,10] These two mitochondrial rRNAs are universally transcribed from mtDNA genes. The rRNA content of the mtLSU, however, varies among species. For instance, yeast mtLSU lacks a 5S rRNA and the region where the cytoplasmic and bacterial 5S rRNA is occupied by mito-specific RNA and protein extensions. In that position, the mammalian mtLSU contains a structural mtDNA-encoded tRNA,[11] which is $tRNA^{Val}$ in the case of the human mitoribosome.[12]

Regarding the mitoribosomal proteins, progress in genome sequencing and proteomics technology led to the identification of the full complement of yeast and mammalian mitoribosomal proteins, which has been further confirmed by high-resolution cryo-EM structures (**Table 1**). It is important to note that the nomenclature of mitoribosomal proteins has been unified,

Table 1. Mitoribosomal proteins. The old nomenclature for mitochondrial ribosomal proteins (MRPs) has been substituted by an unifying nomenclature where proteins with a prefix "u" (for universal) are observed in all kingdoms of life, proteins with a prefix "b" are bacterial in origin and do not have a eukaryotic (or archaeal) homolog, and proteins with a prefix "m" are mitochondrion-specific. Modified from[13]

New Name	Old Nomenclature		Bacteria	New Name	Old Nomenclature		Bacteria
	Yeast	Human			Yeast	Human	
Mitoribosome Large subunit (mtLSU)							
uL1	Mrpl1	MRPL1	L1	bL36	RTC6	MRPL36	L36
uL2	Rml2	MRPL2	L2	mL37	—	MRPL37	—
uL3	Mrpl9	MRPL3	L3	mL38	Mrpl35	MRPL38	—
uL4	YML6	MRPL4	L4	mL39	—	MRPL39	—
uL5	Mrpl7	—	L5	mL40	Mrpl28	MRPL40	—
uL6	Mrpl6	—	L6	mL41	Mrpl27	MRPL41	—
bL12	Mnp1	MRPL7/L12	L7/L12	mL42	—	MRPL42	—
bL9	Mrpl50	MRPL9	L9	mL43	Mrpl51	MRPL43	
uL10	Mrpl11	MRPL10	L10	mL44	Mrpl3	MRPL44	
uL11	Mrpl19	MRPL11	L11	mL45	—	MRPL45	
uL13	Mrpl23	MRPL13	L13	mL46	Mrpl17	MRPL46	
uL14	Mrpl38	MRPL14	L14	mL48	—	MRPL48	
uL15	Mrpl10	MRPL15	L15	mL49	Img2	MRPL49	
uL16	Mrpl16	MRPL16	L16	mL50	Mrpl13	MRPL50	
uL17	Mrpl8	MRPL17	L17	mL53	Mrpl44	MRPL53	
bL19	Img1	MRPL19	L19	mL54	Mrpl37	MRPL54	
bL21	Mrpl49	MRPL21	L21	mL55	—	MRPL55	
uL22	Mrpl22	MRPL22	L22	mL56	—	MRPL56	
uL23	Mrp20	MRPL23	L23	mL57	Mrpl15	—	
uL24	Mrpl40	MRPL24	L24	ICT1	—	MRPL58	
bL27	Mrp7	MRPL27	L27	mL58	Mrpl20	—	
bL28	Mrpl24	MRPL28	L28	mL59	Mrpl25	—	
uL29	Mrpl4	MRPL47	L29	mL60	Mrpl31	—	
uL30	Mrpl33	MRPL30	L30	mL61	Mrp49	—	
bL31	Mrpl36	—	L31	mL63	—	MRP63	
bL32	Mrpl32	MRPL32	L32	MHR1	Mhr1	—	
bL33	Mrpl39	MRPL33	L33	bS18a		MRPS18-3	
bL34	Mprl34	MRPL34	L34	mS30		MRPS30	
bL35	YNL122C	MRPL35	L35	CRIF1		CRIF1	
New Name	Old Nomenclature		Bacteria	New Name	Old Nomenclature		Bacteria
	Yeast	Human			Yeast	Human	
Mitoribosome Small subunit (mtSSU)							
bS1	—	MRPS28	S1	mS25	Mrp49	MRPS25	—
uS2	Mrp4	MRPS2	S2	mS26	—	MRPS26	—
uS3	Var1?	MRPS24	S3/S24	mS27	—	MRPS27	—
bS4	Nam9	—	S4	mS29	Rsm23	MRPS29	—
uS5	Mrps5	MRPS5	S5	mS31	—	MRPS31	—
bS6	Mrp17	MRPS6	S6	mS33	Rsm27	MRPS33	—
uS7	Rsm7	MRPS7	S7	mS34	—	MRPS34	—
bS8	Mrps8	—	S8	mS35	Rsm24	MRPS35	—
bS9	Mrps9	MRPS9	S9	mS36	Ymr31	MRPS36	—
uS10	Rsm10	MRPS10	S10	mS37	—	MRPS37	—
uS11	Mrps18	MRPS11	S11	mS38	—	MRPS38	—
uS12	Mrps12	MRPS12	S12	mS39	—	MRPS39	—
bS13	Sws2	—	S13	mS40	Rsm22	—	—
uS14	Mrp2	MRPS14	S14	mS41	Mrps35	—	—
uS15	Mrps28	MRPS15	S15	mS42	Mrp51	—	—
bS16	Mrps16	MRPS16	S16	mS43	Mrp13	—	—
uS17	Mrps17	MRPS17	S17	mS44	Mrp1	—	—
mS18b	—	MRPS18-2	S18	mS45	Pet123	—	—
bS18c	Rms18	MRPS18-1	S18	mS46	Rsm26	—	—
bS21	Mrp21	MRPS21	S21	mS47	Mrp10	—	—
uS22	—	MRPS22	S22	mS48	Rsm28	—	—
uS23	Rsm25	MRPS23	S23	mS49	Ppe1	—	—

Alias names: mS37 CHCHD1; mS38 AURKAIP1; mS39 PTCD3

following a proposal by the ribosome community, as reported[13] and stated in **Table 1**. With the single exception of yeast Var1, which is encoded in the mtDNA, all mitoribosomal proteins are encoded in nuclear genes. In *S. cerevisiae* mitochondria, 26 of the 38 mtSSU proteins and 30 of the 46 mtLSU proteins are homologous to *E. coli* proteins, while the rest are specific to mitoribosomes.[6,14] In mammals, 16 of the 30 mtSSU proteins and 28 of the 50 mtLSU proteins are homologs of proteins present in *E. coli* while the rest are mito-specific proteins.[5,11,12,15,16] As a consequence, yeast 74S and particularly human 55S mitoribosomes differ from bacterial (70S) and cytoplasmic ribosomes (80S) in their lower RNA:protein ratio, where significant amounts of RNA have been replaced by mitochondrion-specific proteins. Over the course of evolution mitoribosomes in both yeasts and mammalian ribosomes have acquired multiple changes in both their RNA and protein moieties compared to bacterial ribosomes. Yeast mitoribosomes expanded into both mito-specific RNA and protein segments, compared to the bacterial ribosome, giving rise to a RNA:protein ratio of 1:1; as opposed to an RNA: protein ratio of 2:1 observed in bacteria. In comparison to the bacterial ribosome, the mammalian mitoribosome has lost a significant amount of RNA, which has been replaced by mito-specific proteins giving rise to a 1:2 RNA:protein ratio.

Recent developments in electron microscopy have allowed resolving the structure of yeast mtLSU[13] and both mammalian mtLSU[11,12,15] and mtSSU[16,17] at near-atomic resolution, which will be summarized in depth in this review. These structures have provided a wealth of novel information, raising the next obvious question as to how these ribonucleoprotein macromachines are assembled. The assembly of ribosomes involves coordinated processing and modification of rRNAs with the temporal association of ribosomal proteins. The process is better characterized in cytoplasmic[18] and bacterial ribosomes,[19,20] with the latter having been fully reconstituted *in vitro*.[21] In the reconstitution experiments, protein binding kinetic data and time-resolved fingerprinting have demonstrated that bacterial ribosomal assembly takes place co-transcriptionally where MRPs bind to the 23S rRNA in a 5′ to 3′ direction while the transcript is still being synthesized.[19,20] However, despite recent progress, knowledge of the molecular details of the mitoribosome assembly pathway, the factors involved and the compartmentalization of the process is still very limited.

In addition to their biological significance, mitoribosomes and mitochondrial translation are also relevant from a biomedical perspective, as mutations in most mtDNA-encoded tRNAs and 12S rRNA, as well as nuclear genes encoding mitoribosomal proteins, translation initiation, and elongation factors, lead to clinically and genetically heterogeneous infantile multisystemic diseases, such as Leigh's syndrome, sensorineural hearing loss, encephalomyopathy and hypertrophic cardiomyopathy.[22-24]

In this review we have summarized recent progress in the structural characterization of mitoribosomes, their biogenetic process and the factors involved during assembly. We also discuss human diseases related to disruption of mitoribosome assembly.

Lessons from High-Resolution cryo-EM Structures of Yeast and Mammalian Mitoribosomal Subunits

In 2009 the Nobel Prize in Chemistry was jointly awarded to Venkatraman Ramakrishnan (MRC Laboratory of Molecular Biology, Cambridge, UK), Thomas A. Steitz (Yale University, CT, USA) and Ada E. Yonath (Weizmann Institute of Science, Rehovot, Israel) "*for studies of the structure and function of the bacterial ribosome*." However, determination of the mitoribosome structure was halted for many years as the low yield of mitoribosomes from most tissues made the crystallization process nearly impossible. Amidst these challenges, in 2003 Rajendra K. Agrawal was able to resolve the first 3-dimensional cryo-electron microscopic (cryo-EM) map of the bovine mitochondrial 55S ribosome carrying a tRNA at its P site[5] at 13.5 Å resolution, and subsequently in 2009 the cryo-EM reconstruction of the mitochondrial ribosome with minimal RNA in the protist *Leishmania tarentolae* at 14.1 Å resolution.[4]

Nearly a decade after the first structure, 2 groups - namely those of V. Ramakrishnan (Cambridge, UK) and Nenad Ban (ETH Zurich, Switzerland) - made a rapid advancement in the mitochondrial ribosome structure field by resolving the mtLSU of both yeast and mammals at a much higher resolution. Utilizing the advancements in EM and algorithms for computer-based analysis for "noisy" EM images of single particles, the Ramakrishnan group resolved the yeast mtLSU at a resolution of 3.2Å,[14] the human mtLSU with nascent polypeptide being folded in the exit tunnel at 3.4Å resolution.[12] The Ban group focused mainly on the mammalian mtLSU, where the porcine mtLSU (*Sus scrofa*) was resolved at both 4.9Å and 3.4Å resolution.[11,15] Contributing to the era of mitochondrial structural revolution, Agrawal's group resolved the cryo-EM structure of the mammalian mtSSU at 7Å resolution.[16] To finalize the impressive race between the Ramakrishnan and Ban groups, during the preparation of this manuscript, they have resolved at 3.5Å or 3.8Å resolution the complete structures of the 55S human[25] or porcine[26] mitoribosomes, respectively.

The high-resolution structures have confirmed that the mammalian 55S mitoribosomes differ from bacterial (70S) and cytoplasmic ribosomes (80S) in their lower RNA:protein ratio, where mitochondrion-specific proteins have been recruited to prevent destabilization caused by rRNA reduction. In the yeast 74S mitoribosome, both protein and RNA moieties are substantially larger than their bacterial counterparts.[13] Almost all the mitochondrial ribosomal proteins with bacterial homologues have N- and/or C- terminal extensions, functions of some of which are unclear[6] and ~25% of mito-specific proteins serve to directly compensate for the lost RNA secondary structures in mammalian mitoribosomes.[5,17,27-29] However, the early cryo-EM map of the bovine mitoribosome has already shown that many of additional or larger mitoribosomal proteins occupy new positions within the ribosome and that missing RNA segments are not always replaced by any protein mass.[5] In addition, high-resolution structure of human mitoribosome that was trapped in 3 different conformations, revealed a wider spectrum of mtSSU orientations than was observed for bacterial or cytoplasmic ribosomes.[17]

Overall, the high-resolution reconstructions have provided a wealth of novel information, specifically noting the similarities and differences between yeast and mammalian mitoribosomes (**Fig. 1**).[4,5,13,15,16,25, 26]

Yeast mtLSU has a core conserved with the bacterial LSU, while the mitochondrion-specific proteins and extensions of known bacterial homologues and rRNA expansion segments lie on the surface of the conserved core.[13] The rRNA expansion segments act as scaffolding for the additional new proteins. With regard to the rRNA, there is an expansion of 392 additional nucleotides spreading over 11 expansion segments (ES) compared to the bacterial LSU. However, there was no evidence of a

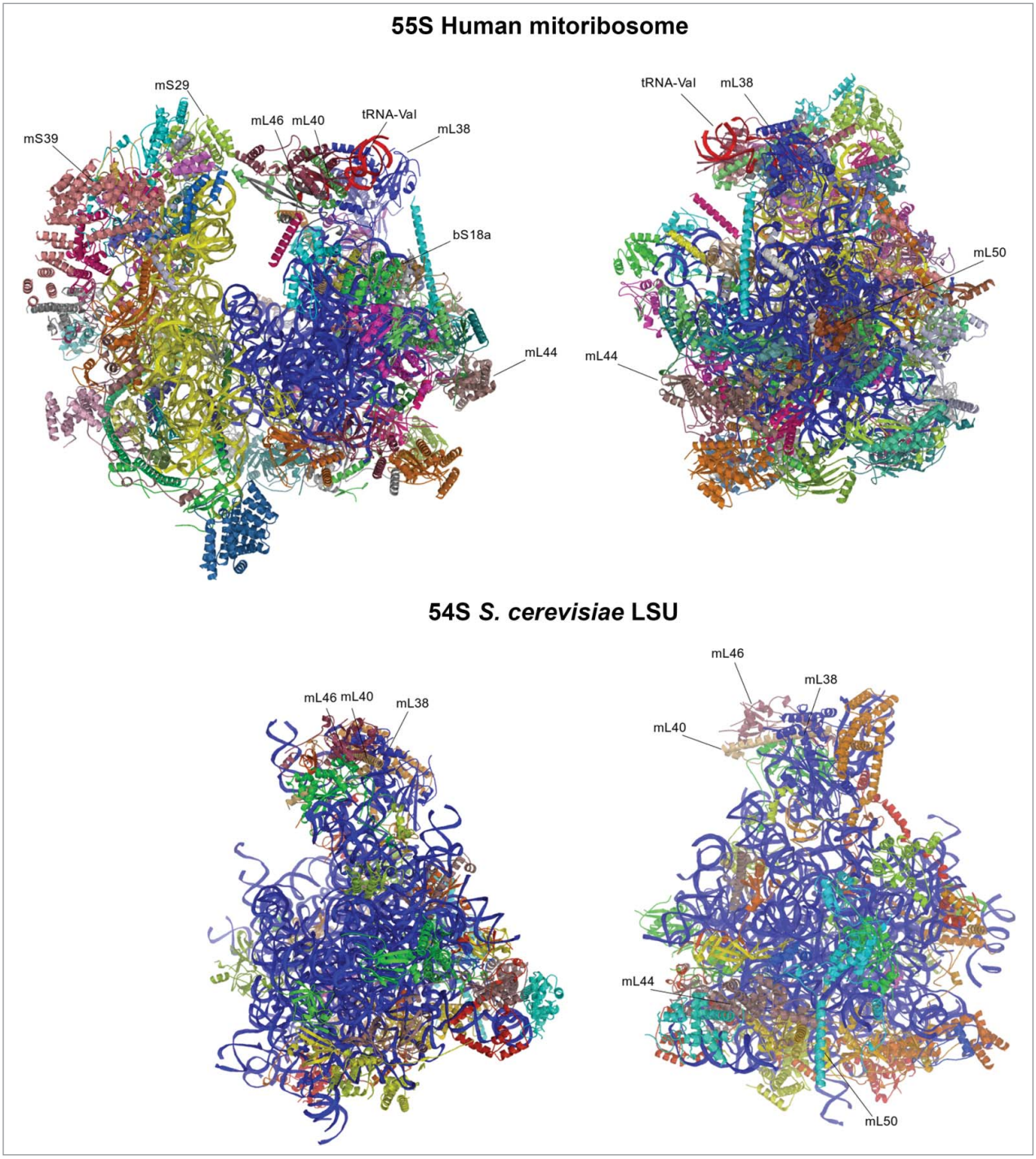

Figure 1. Structure of mitochondrial ribosomes. Top panel, human 55S; bottom panel, yeast LSU. LSU rRNA is shown in blue, SSU rRNA is yellow, tRNA-Val is red. Individual proteins are depicted in different colors, and those mentioned in the text are labeled.

5S rRNA or putative 5S rRNA binding proteins L18 and L25, as had been proposed for the mammalian LSU. In its place, this missing area is occupied by mitochondrion-specific proteins (mL38, mL40 and mL46), 16S rRNA expansion clusters (82-ES and 84-ES1) and extensions from proteins homologous to bacteria. All these additions do not structurally mimic the 5S rRNA but they made the yeast mtLSU central protuberance (CP) into triple the volume of the bacterial CP. A new protuberance is observed adjacent to the mouth of the tunnel exit site consisting of both mitochondrion-specific proteins and rRNA expansion segments. Owing to its location, this bulged structure is proposed to be involved in tethering the mitoribosome to the membrane, which is independent of nascent peptide insertion. Furthermore, the yeast mtLSU has an additional contact with the E site tRNA that is not seen in bacteria. This contact is mediated through the C-terminal extension of bL33 with the tRNA acceptor stem, thus creating additional stability for the incoming tRNA. The polypeptide tunnel channel and its exit sites are highly conserved among previously studied ribosomes. However, when the structure of the yeast LSU is superimposed onto the bacterial ribosome, the region where the path is located appears blocked by a mitochondrion-specific extension of uL23. Notably, a new path that deviates from the bacterial path was observed, presenting a new exit site, surrounded by mitochondrion-specific proteins mL44 and mL50. Interestingly, these proteins are also involved in forming the mitochondrial inner membrane-facing protuberance, an orientation expected to promote mitoribosome interaction with the membrane to facilitate the co-translational membrane insertion of newly synthesized, highly hydrophobic OXPHOS subunits. Several inner membrane proteins are known to mediate membrane binding of ribosomes such as the Oxa1 machinery, which enables the insertion of nascent polypeptides into the inner membrane.[30-36] The binding of mitoribosomes to the inner membrane is discussed more extensively in section 5.2.

The high resolution cryo-EM reconstruction of the mammalian mitoribosome revealed extensive differences not only with bacterial cytoplasmic ribosomes but also with yeast mitoribosome.[11,12,15,25,26] The mammalian mitoribosome is highly protein-rich compared to both yeast and bacterial ribosomes. While the total rRNA content has increased in yeast with only minor deletions at the tunnel exit site, the mammalian rRNA content has nearly halved owing to numerous deletions evenly distributed when compared with the bacterial rRNA. The ribosomal proteins that are homologous to bacteria have extensions, which are shorter and not conserved with yeast. These extensions mainly interact with mitochondrion-specific proteins while only a few engage in filling the space of deleted rRNA. The mitochondrion-specific proteins are mainly peripherally distributed over the solvent-accessible surface forming clusters at the central protuberance, the L7/L12 stalk, and adjacent to the polypeptide exit site. Similar to the protein extensions, these proteins accommodate novel positions rather than compensate for the missing rRNA.

In addition to the mitochondrion-specific protein cluster at the CP, a second L-shaped RNA molecule was discovered in the mammalian mitoribosome at the same position where the 5S rRNA is found in the bacterial ribosome. However, the structure of the human mtLSU[12] showed that it is not a 5S rRNA molecule as it had been long speculated[37] but rather $tRNA^{Val}$, which plays an integral structural role at the CP. In the mtDNA, the $tRNA^{Val}$ gene is located in the middle of the genes encoding for the 12S and 16S rRNAs. These three RNAs are transcribed together as a polycistronic transcript similar to that of the bacterial rRNA operon. Owing to the presence of $tRNA^{Val}$, the CP of the human mtLSU is substantially different from other ribosomes, including the yeast mitoribosome. Despite the major remodeling at the CP, the interactions it makes with the small subunit and the tRNAs bound to the ribosome are conserved. Nevertheless, in the yeast mitoribosome, this inter-subunit communication occurs via rRNA while in the mammalian mitoribosome it is coordinated entirely by mitochondrion-specific proteins,[5,11,12,14,16] a feature that is different from bacterial and cytoplasmic ribosomes that typically consist of only RNA-RNA intersubunit connections. Even though this suggests that, during evolution, mito-specific proteins have functionally replaced the RNA, it is more likely that much of the additional ribosomal proteins in mitochondria were recruited to stabilize the general structure of the ribosome.[7] Furthermore, the number of intersubunit bridges is lower than in the bacterial ribosome, probably to allow the 2 subunits to have flexibility in the conformation allowing them to tilt freely.[25,26] An additional major remodeling is observed at the A (aminoacyl) and P (peptidyl) tRNA binding sites, where some subunits present in bacterial ribosomes (e.g. uL5 at the P site, bL25 and the A site) have been lost in order to accommodate human mitochondrial tRNAs, which contain highly variable loops at the tRNA elbow. However the P-site finger, unique to the mammalian mitoribosome, compensates for these missing interactions.[25,26] A distinctive property of mitoribosomes is the acquisition of an intrinsic GTPase activity through the GTP–binding protein, mS29 of the 28S mtSSU subunit.[25,26] The GTPase activity is probably linked to subunit association, since mS29 locates at the subunit interface and is involved in coordinating 2 mitochondria-specific bridges.[25,26]

The polypeptide exit tunnel is adapted to the transit of hydrophobic nascent peptides.[11,12] The tunnel exit site consists of conserved proteins from bacteria, namely bL23, bL29, bL22, bL24 and bL17, which create a ring around the exit site. Moreover, this conserved core is surrounded by a second layer of protein density consisting of bL33 and mL45, which promote anchoring of the mtLSU to the inner mitochondrial membrane.[11,12] The structure of the human mitoribosome with nascent polypeptide shows its extensive interactions with specific hydrophobic residues of the tunnel wall.[17] Membrane anchoring aligns the polypeptide exit site with the OXA1L translocon to facilitate co-translational membrane insertion of newly synthesized proteins. The overall tunnel path is similar to that of bacterial and cytoplasmic ribosomes but different from yeast mitoribosomes. Thus, the upper tunnel path does not show the constriction and the alternative exit tunnel observed in yeast.

The recent cryo-EM reconstructions of the 55S human[25] and porcine[26] mitoribosome at high 3.5Å or 3.8Å resolution, have allowed to distinguish important features in the mammalian mtSSU. Similar to the mtLSU, several peripheral rRNA helices

present in bacteria are either truncated or missing in the 12S rRNA, but the tertiary structure of the 12S rRNA core and the overall positions of the MRPs with bacteria homologs are preserved in the structure. A significant structural remodeling is observed at the mRNA entrance of the mtSSU compared to the bacterial SSU.[16,25,26] This remodeling serves to accommodate mammalian mitochondrial mRNAs, which have either null or very short 5′-untranslated regions (UTR),[38] since the structural data shows that in the proximity of the channel entrance a pentatricopeptide repeat (PPR) protein (mS39) is bound, it could be involved in recruiting the leaderless mRNA during translation initiation.[16,25,26]

According to the mammalian mitoribosomal protein complement there are 3 isoforms for mammalian mtSSU protein bS18.[39,40] S18 isoforms are not uncommon. *E. coli* contains a single S18, but other prokaryotes such as *Streptomyces coelicolor* contain 2 S18 variants. A single bS18 form is present in yeast mitochondria, 2 in worms and 3 in flies and mammals.[39] Apparently two rounds of duplication of the gene encoding the mammalian bS18 occurred, giving rise to 3 very different variants termed bS18A, bS18B and bS18C. The primary sequences of these variants are very divergent and have conserved regions with prokaryotic S18s only in their central portion.[39] The existence of several bS18 variants led to the speculation that each mitoribosome would have a single variant leading to a heterogeneous population of mitochondrial ribosomes[9,39] for performance of specialized functions. However, the recent cryo-EM structures have identified bS18A to be in the mtLSU subunit (in porcine liver and human HEK293 cells),[11,12] but not in the human mtSSU. Instead, the human mtSSU contains bS18B at the position of S18 in bacterial ribosomes, and bS18C at a different position.[25,26] The precise role of these subunits remains to be disclosed.

Trans-acting factors Required for Mitoribosome Assembly from Yeast to Mammals

With the high-resolution mitoribosome structures resolved, the question still remains of how these macromolecular structures are assembled and what elements of the pathway are conserved across evolution. Bacterial ribosome assembly has been reconstituted *in vitro* and a large amount of information on the assembly pathway of bacterial and cytoplasmic ribosomes is already available. Ribosome assembly involves the coordinated processing and modification of rRNAs with the temporal association of ribosomal proteins. The entire process is regulated by several classes of ribosome biogenetic factors, including nucleases, rRNA modifying enzymes, DEAD-box helicases, GTPases and chaperones.[19,20,41] The current understanding of the mitoribosome assembly pathway and the factors involved (**Table 2**), which is still far from complete, will be summarized in this section.

Factors required for rRNA processing and modification

Nucleases

The rRNA genes are often organized in operons that are transcribed into a single polycistronic transcript that requires processing to liberate the individual large and small rRNAs. Subsequently, rRNAs are further processed at both 5′ and 3′ end to yield mature rRNAs. The processing activities are performed by exo and/or endonucleases. In the mitochondrial genomes, rRNA organization depends on the species, as will be discussed below. A common trait among all species is the polycistronic nature of the transcript and the large number of RNA-processing events to liberate not only rRNAs but also mRNAs and tRNAs for translation.

In yeast *S. cerevisiae* the mtDNA contains 35 genes. Eight genes encode proteins; 7 subunits of OXPHOS enzymes and one SSU ribosomal protein (Var1). Twenty-seven other genes encode RNAs required for translation, specifically 24 tRNAs and 2 rRNAs (*21S rRNA* and *15S rRNA*), and one gene for the RNA component of RNase P (*RPM1*). Only three genes contain either group I or group II introns,[42] namely one intron in *21S rRNA*, 5 in *COB*, and 7 in *COX1*. Ten of these introns contain ORFs coding for homing endonuclease genes (HEG)[43], which in some cases have gained maturase (intron splicing) activity. All yeast mitochondrial genes are transcribed as polycistronic precursors from 11 distinct promoters[44]. Regardless of the organization in the original transcript, all precursor tRNAs have 5′ and 3′ extensions that require further processing. The 5′-tRNA endonucleolytic processing is executed by an almost ubiquitous RNaseP, which contains the nucleus-encoded Rpm2 protein, and an RNA component encoded in the mtDNA *RPM1* gene in yeast.[45] The 3′-tRNA processing is an endonucleolytic cleavage catalyzed by tRNase Z,[46] which is encoded by the *TRZ1* gene in yeast.[47]

The main 3′-5′-exoribonuclease in yeast mitochondria is the mitochondrial degradosome (mtEXO). It is composed of 2 large subunits: an RNase and an RNA helicase encoded by nuclear genes *DSS1* and *SUV3*, respectively.[48] Lack of Suv3 or Dss1 attenuates RNA turnover, and also affects 5′- and 3′-processing of mitochondrial RNAs,[49] suggesting that turnover and processing of mitochondrial transcripts could be linked. All mRNAs of yeast mitochondria are processed at their 3′ ends within a conserved dodecamer sequence, 5′-AAUAAUAUUCUU-3′. It is relevant to mention that a dominant mutation in *SUV3* gene was found to suppress a dodecamer deletion at the 3′ end of the *VAR1* gene,[50] which is transcribed as part of a polycistronic transcript that contains $tRNA^{Ser}$ and *ATP9*.[44]

In *S. cerevisiae*, the mtLSU 21S rRNA precursor is a ~5.1–5.4 kb transcript containing a 1.2 kb intron (ρ1), $tRNA^{Thr2}$, $tRNA^{Cys}$ and $tRNA^{His}$.[44] The 3.1 Kb mature 21S rRNA transcript is formed by excision of the intron and removal of a ~900bp extension from the 3′- end.[51] Processing of 21S rRNA transcripts containing the ρ1 intron requires the Suv3 helicase.[52,53] The 15S rRNA is also a part of a primary transcript containing $tRNA^{Trp}$. However, the single 15S rRNA precursor transcript is a 15.5S RNA with approximately 80 additional nucleotides at the 5′- end than the mature15S rRNA.[54] While it is not clear how the processing occurs, since no evidence for an endonucleolytic cleavage mechanism has been reported, it has been shown that the precursor is not processed in rho^- mitochondria,[54] nor in isolated organelles,[48] suggesting that it may depend on functional mitochondrial translation and/or

Table 2. Role of mitoribosome assembly cofactors. Nucleases and RNA processing enzymes are not included

Protein			Effects of deletion/depletion		
Yeast	**Human**	**Function/Interaction**	**Subassembly**	**rRNA**	**Ref**
		Mitoribosomal LSU assembly cofactors			
Modifying enzymes					
Mrm1/Pet56	MRM1	Methylation of 21S rRNA during early assembly Methylation of 16S rRNA	Unstable LSU	Stable 21S rRNA-	70,164
Mrm2	MRM2	Methylation of 21S rRNA during late assembly Methylation of 16S rRNA	Unstable LSU	-Unstable 16S rRNA	71,82,164
	MRM3/RNMTL1	Methylation of 16S rRNA	Yes	Stable 16S rRNA	81,82
Pus5		Pseudouridylation of 21S rRNA	—	Stable 21S rRNA	72
GTPases					
Mtg1	MTG1	Δ*mtg1* defect suppressed by *21S ery*^{R} mutations GTPase important for mtLSU biogenesis	—	Stable 21S rRNA Stable 16S rRNA	95,97
Mtg2	MTG2/GTPBP5	Multicopy suppressor of *mrm2* thermo-sensitive mutant GTPase important for mtLSU biogenesis	Unstable LSU	Unstable 21S rRNA Stable 16S rRNA	99,97
	C7orf30	uL14 chaperone	Yes	Stable 16S rRNA	132
RNA helicases					
Mrh4	DDX28	Binds 21S rRNA Binds 16S rRNA	Yes	Stable 21S rRNA Unstable 16S rRNA	123,127,128
	DHX30		No		128
Other					
	mTERFD1/mTERF3	Stabilization of 16S rRNA	Unstable LSU	Unstable 16S rRNA	130
	mTERF4	Binds both 12S and 16S rRNA and forms a complex with NSUN4 and recruits it to mtLSU	Stable and increased level of both 28S and39S SU, 55S absent	Stable and increased levels of both 12S and16S rRNA	78
Yta10	AFG3L2	Processing of bL32	Yes	Stable 21S rRNA	134
Yta12	SPG7	Processing of bL32	Yes	Stable 21S rRNA	134
	FASTKD2	Binds 16S rRNA	No	Stable 16S rRNA	128
		Mitoribosomal SSU assembly cofactors			
Modifying enzymes					
	TFB1M	Dimethylates 2 highly conserved adenines at a stem loop structure close to the 3′ end of 12S rRNA	Reduced 28S levels; 55S absent	Unstable 12S rRNA	76
	NSUN4	Methylation of 12S rRNA, complex with mTERF4	Stable and increased level of both, 28S and 39S SU, 55S levels reduced	Stable and increased level of both12S and16S rRNA	77
GTPases					
Mtg3	C4orf14	GTPase important for processing of 15S rRNA, uL29 is a multicopy suppressor of Δ*mtg3* Interacts with the SSU via a GTP-binding mechanism	Unstable 30S Unstable 28S	Unstable and unprocessed 15S rRNA Unstable 12S rRNA	101,102
	ERAL1	Binds to 12S rRNA	Unstable 28S	Unstable 12S rRNA	104,105
Other					
	GRSF1	Found in mtRNA granules, important for post-transcriptional storage, handling and translation of mtRNA	28S assembly intermediate.	Unstable 12S and 16S rRNA	136
Mitoribosomal assembly cofactors affecting SSU and LSU					
	MPV17L2	Binds to 39S subunit	Unstable 28S and 39S. SSU proteins trapped in enlarged nucleoids.	—	163

metabolism. The RNA binding PPR-protein called Dmr1[55] is required to maintain 15S rRNA integrity. Even though the 15.5S precursor was detected in *dmr1*Δ mutants, it is not clear whether Dmr1 is directly involved in 15.5S processing. The only known mitochondrial 5′–3′ exonuclease activity in *S. cerevisiae* is performed by Pet127[56,57] that processes the 5′ end of the 15S rRNA precursor. Interestingly, this processing is not essential for ribosome assembly and function, as *pet127*Δ mutants are

respiratory-competent, even though they accumulate 15S rRNA with the unprocessed 5′ end.[56] Because *pet127* mutants were isolated as partial suppressors of mutations affecting the *COX3* translational activator Pet122,[58] it is possible that ribosomes with unprocessed 15S rRNA precursor exhibit slight changes in translation activation or specificity, yet these are not sufficient to significantly disrupt their function.

In humans, the mtDNA is a double-stranded molecule that encodes 13 proteins, 2 rRNAs (12S and 16S rRNAs) and 22 tRNAs. Mitochondrial transcription occurs on both DNA strands and gives rise to 3 polycistronic primary transcripts.[59] Two of these transcripts correspond to each whole strand. Most ORFs and rRNAs in these transcripts are punctuated by tRNAs.[60] Cleavage of tRNAs at their 5′ and 3′ ends by ribonuclease P (RNase P,[61] and the RNase Z-type enzyme ELAC2,[62,63] respectively, releases individual RNA species, which are subsequently matured.[38] The third transcript is smaller and contains, in consecutive order, $tRNA^{Phe}$, 12S rRNA, $tRNA^{Val}$ and 16S rRNA, the last 3 being components of the mitoribosome. RNase P and ELAC2 are also involved in $tRNA^{Val}$ excision. It remains unknown whether and how $tRNA^{Val}$ excision is coordinated with assembly of 39S mtLSU.

rRNA-modifying enzymes

An important step in ribosome assembly is the modification of rRNA at conserved regions, frequently in the catalytic domains. These modifications can take place co-transcriptionally or immediately after transcription, while others occur when the rRNA is assembled in the pre-ribosomal particle.[64] rRNA modifications are of 3 major kinds, namely pseudouridylation, base methylation and 2′-O-ribose methylation. Pseudouridylation of rRNA is synthesized by pseudouridine synthases. Methylations are introduced by methyltransferases (MTases). Base methylations are carried out by specific MTases that recognize their targets directly.[65] Unlike in the eukaryotic system, where a small nucleolar RNAs (snoRNAs) is required to locate the target nucleotide, the prokaryotic rRNA modifications are performed by site-specific enzymes without the help of a guide RNA.[66] Similarly in mitochondria, MTases catalyze rRNA modifications without a guided small RNA.

Compared with the prokaryotic and the eukaryotic cytoplasmic ribosomes, which contain more than 30 and more than 200 modifications, respectively, the mitochondrial rRNA is scarcely modified. In *S. cerevisiae*, the mtSSU 15S rRNA lack methylated nucleotides and might have only one pseudouridylation site,[67] in which the position on the rRNA and the enzyme responsible remain unknown. *S. cerevisiae* mtLSU 21S rRNA has 3 modified nucleotides, a pseudouridine (ψ 2819) and 2 2′-O ribose methylations (Gm2270 and Um2791) located at the Peptidyl Transferase Center (PTC) domain. Much of the understanding of the yeast mtLSU 21S rRNA modifications was facilitated by the previous studies in *E.coli* 23S rRNA, in which 3 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. Mrm1 does not co-sedement with the mtLSU but ribosome assembly is affected when Mrm1 expression is impaired.[68,69] Methylation by Mrm1 can proceed in naked RNA, indicating that methylation could occur early in the mitoribosome assembly pathway. The methylation site corresponding to *E.coli* U2552 in the yeast 21S rRNA is Um2791.[70] 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 sucrose gradients[71]; however, no data is currently available on the accumulation of ribosome assembly intermediates in the absence of Mrm2. The 21S rRNA has only one pseudouridylation at residue ψ2819 that is synthesized by the pseudouridine synthase Pus5. This residue, ψ2819, is conserved in the mtLSU rRNA from species such as humans and mouse and also in LSU rRNA from gram-negative bacteria (e.g., *E. coli*) but not in gram-positive bacteria (e.g. *Bacillus subitilis*) or in the 26S rRNA of eukaryotic cytoplasmic ribosomes. ψ2819 is located in the most highly conserved region of the PTC domain; hence, it is likely that the ψ2819 residue is playing a functionally important role during peptide bond formation.[72]

Modifications in the mammalian mitochondrial rRNA were first mapped using hamster cells,[73] which include a total of 9 modifications. The mammalian mtSSU 12S rRNA contains 5 base methylations but does not have any methylated ribose residues. These modifications include 2 adenine di-methylations (A936 and A937), one uracil methylation U429 and 2 cytosine methylations (C839 and C841/C842), numbered according to human 12S rRNA numbering.

The two adenines (A937 and A938) at the stem loop structure of the 3′ end of the 12S rRNA are conserved in all domains of life, archaebacteria, eubacteria and eukaryotes.[74] In bacterial SSU rRNA these modifications are present in a region that contains the mRNA decoding center and the binding site for the large ribosome subunit,[75] suggesting the importance of this modification for translation regulation. In the mammalian mtSSU rRNA, the 2 adjacent adenines are methylated by mitochondrial transcription factor B (TFB1M), which is a functional homolog of bacterial MTase KsgA.[76] Studies in mice indicated that knocking out (KO) *TFB1M* produces embryonic lethality. A conditional *TFB1M* KO in the heart led to mitochondrial cardiomyopathy owing to complete loss of adenine dimethylation of the SSU rRNA, which led to impaired mitoribosome assembly and abolished mitochondrial translation.[76]

The mammalian mitochondrial cytosine MTase NSUN4 is the homolog of *E. coli* YebU, known to methylate the cytosine at position 1407 of the SSU 16S rRNA. Remarkably, YebU cannot act on free rRNA, *in vitro*, but can exert its function only if the rRNA is assembled in the SSU. Studies in a conditional *Nsun4* KO mice showed that NSUN4 is required for mitochondrial translation.[77] Deep sequencing bisulfate-treated RNA showed that NSUN4 methylates C911 in 12S rRNA of the SSU, a modification that is lost in *Nsun4* KO mice. Further NSUN4 forms a

stoichiometric complex with MTERF4, a member of the mitochondrial transcription termination factor family.[78] The 3D crystal structure of the human MTERF4-NSUN4 complex showed a very stable interaction between both subunits that occurs at the carboxy-terminus of MTERF4.[79] MTERF4 is composed of structurally repeated MTERF-motifs that form a nucleic acid binding domain, while NSUN4 lacks an N- or C-terminal extension commonly used by other related RNA MTases for RNA recognition. Instead, NSUN4 binds to the C-terminus of MTERF4 and a positively charged surface forms an RNA binding path across both MTERF4 and NSUN4, suggesting that both proteins in the complex together contribute to RNA recognition. Interestingly, NSUN4 is still capable of methylating its target (C911 in mice SSU rRNA) in *Mterf4 KO* mice.[77] However, deletion of *Merf4* gene in the mice heart leads to inhibition of mitochondrial translation and prevents NSUN4 being targeted to the mtLSU. In the absence of MTERF4, the SSU and LSU levels are increased but mature mitoribosomes are not formed.[78] This implies that NSUN4 is a bi-functional protein, where NSUN4 alone methylates C911 in mtSSU 12S rRNA and also forms a complex with MTERF4 and targets mtLSU[77] to regulate mitoribosomal assembly.

Regarding uracil methylations in mtSSU rRNA, a single methylation at nucleotide 426 was identified in the 13S hamster mtSSU rRNA.[80] However, its presence has not been confirmed in other rodent or human mtSSU.

The human 16S rRNA has 3 2′-O-ribose methylations and one pseudouridylation. The three 2-O-ribose methylations are a Gm residue at G1145[81] and a UmGm at U1369G1370. These sites are conserved in both bacterial and cytoplasmic rRNA, in which they stabilize the RNA structure critical for the peptidyltransferase reaction and t-RNA recognition. The single pseudouridylation site is found at the U1397.

The three methyltransferases that modify the 16S rRNA are MRM1, MRM2 and RMTL1 (also called MRM3).[81] Specifically, MRM1 catalyzes Gm1145. However, MRM1 does not cosediment with mitoribosomes in glycerol gradients but a small fraction of this protein was found at the bottom of the gradient where nucleoids[81] and probably other large structures, such as the RNA granules, sediment. Both Um1369 and Gm1370 lie in the A-loop of the mtLSU rRNA. The A-loop is an essential RNA component of the ribosomal PTC that directly interacts with aminoacyl (A)-site tRNA. This structure is well conserved through evolution and the highly conserved nucleotides in the A-loop often contain 2′-O-ribose methylations. In human mitochondria, the enzyme responsible for the Um1369 is MRM2 protein, which is the ortholog of bacterial RrmJ and yeast Mrm2.[81,82] RNAi-mediated depletion of MRM2 decreases the stability of the mtLSU, impairs mitochondrial translation and consequently mitochondrial respiration.[82] Intriguingly, MRM2 was shown to interact not only with the mtLSU but also with the monosome,[81,82] suggesting a second function for this protein during mitoribosome maintenance or mitochondrial translation.

The Gm1370 is exclusive to the mammalian 16S rRNA and is not present in *E. coli* or yeast mitochondrial 21S rRNA. It is catalyzed by the MTase MRM3, which associates with the mtLSU, but not with the monosome.[81] RNAi-mediated depletion of MRM3 gave rise to mtLSU pre-ribosomal particles,[82] indicating that, if the pre-ribosomal particles were on pathway intermediates, yet to be demonstrated, MRM3 functions at a late stage of mitoribosome assembly.[82]

Even though no pseudouridylated nucleotides were identified in the hamster mitochondrial LSU rRNA, the human 16S rRNA could have one pseudouridylated base at U1397 (according to human 16 rRNA numbering).[83] However, the enzyme responsible and the precise role of this modification are yet to be identified.

Factors required for formation of the ribonucleoprotein particle

In addition to modification and processing of the rRNAs, formation of the ribonucleoprotein particle also requires non-ribosomal proteins, largely known as ribosome assembly factors. In the prokaryotic and the eukaryotic systems where ribosome assembly is studied in depth, many of these assembly factors include GTPases and ATP-dependent RNA helicases. These enzymes consume energy by hydrolyzing nucleotide triphosphates and provide directionality and accuracy at specific stages during assembly. Concerning mitoribosome biogenesis only a handful of assembly factors have been identified so far, raising the need for more intensive investigation in this area of study.

GTPases

Members of the G-protein super family are key regulators of many cellular processes found in all domains of life. GTPases can be considered as molecular switch proteins as they alternate an active GTP-bound conformation, an inactive GDP bound form and an inactive intermediate apo-state.[84] These proteins are composed of a G-domain that has a characteristic fold and 4–5 highly conserved motifs (G1-G5) with additional N- and C- terminal domains.[43] G-proteins play important roles in ribosome assembly.[20] So far, 3 conserved G-proteins have been identified in mitoribosome assembly in yeast and humans, namely Mtg1, Mtg2 and Mtg3 (mitochondrial GTPases 1,2 and 3). In addition, the mammalian-specific GTPase named ERAL1 (Era-like 1) is also involved in mitoribosome biogenesis.

MTG1 or GTP-binding protein 7 (GTPBP7) is a conserved YiqF/YawG family protein. Prokaryotes have one MTG1 homolog, the RbgA protein, also termed YIqF.[85] RbgA homologs are widely present in gram-positive bacteria such as *Bacillus subtilis*, archaea, and in a few gram-negative bacteria (but not *E. coli*). RbgA proteins have a highly conserved GTP-binding domain and belong to a family of circularly permuted GTPases (cpGTPases) in which the highly conserved 5 GTPase motifs are aligned in an unusual order (G5-G4-G1-G2-G3).[86-88] In *B. subtilis*, deletion of the highly conserved N-terminal region inhibits cell growth even in the presence of wild-type RbgA, thus acting as a dominant-negative variant.[89] RbgA in *B. subtilis* is essential for cellular growth and participates in the late steps of 50S LSU ribosome assembly and maturation. It interacts with the 50S subunit in a GTP-dependent manner and its GTPase activity

induces its dissociation from the ribosome.[90] According to *in vitro* studies the GTPase activity is stimulated more than 60-fold in the presence of the 50S subunit.[87,89-91] In RbgA depleted cells the mature 50S subunits are not formed; instead, a 45S complex (lacking ribosomal proteins –L16 and L36,[86,90-94] and reduced amounts of L27, L33, and L35[92]) is accumulated. Spontaneous *rbgA* suppressors have been mapped to the gene encoding L6, and *in vitro* maturation assays have suggested that RbgA is necessary for correct positioning of L6 on the ribosome, prior to the incorporation of L16 and other late assembly proteins.[92]

In eukaryotes, *MTG1* was first identified in mitochondria in *S. cerevisiae*.[95] In *yeast*, an *mtg1* null mutation induces a decrease in 21S rRNA and a severe defect in mitochondrial protein synthesis. This phenotype is partially suppressed by spontaneous mutations in the 21S rRNA conferring resistance to erythromycin,[95] thus linking the role of Mtg1 to mtLSU biogenesis. In *B. subtilis*, RbgA interacts with helix 38 (H38) of 23S rRNA and the mutual stabilization between the central protuberance and H38 ensures further maturation of the 23S rRNA.[89,96] H38 is conserved in the yeast 21S rRNA but studies exploring a possible interaction of MTG1 with H38 are still missing. In human, however, H38 has been lost and additional stabilizing interactions involve mitochondrion-specific MRPs and the $tRNA^{Val}$.[12] Human MTG1 shares some common characteristics with its yeast homolog, such as: i) Mtg1 localizes to the mitochondria inner membrane facing the matrix side, where it associates with the mtLSU; ii) mtLSU stimulates the GTPase activity of Mtg1, while the intrinsic GTPase activity is undetectable; iii) silencing of *MTG1* decreases mitochondrial translation activity[97]; iv) heterologous expression of human MTG1 partially rescues the mitochondrial protein synthesis defect in yeast *mtg1* null mutant cells,[95] suggesting a functional conservation of MTG1 from yeast to humans.

Mtg2 is an Obg family protein. The *Escherichia coli* Obg protein (ObgE) binds to the large and the small ribosomal subunit. Deletion of ObgE leads to a decrease in the 70S ribosome levels while increasing the 30S and 50S subunits, and accumulation of a 50S subunit assembly intermediates containing reduced amounts of L16, L33 and L34.[98] Yeast and human mitochondrial Mtg2 associate with the inner mitochondrial membrane facing the matrix, a location consistent with a role in mitoribosome assembly. Deletion of yeast *mtg2* protein leads to a loss of mitochondrial DNA, which is often related to a defect in mitochondrial protein synthesis[99]. Moreover, cells carrying temperature-sensitive mutant alleles are unable to carry out mitochondrial protein synthesis and contain lowered levels of mitochondrial ribosomal subunits. Sucrose gradient sedimentation patterns have shown co-sedimentation of Mtg2 with the mtLSU, indicating a role in the biogenesis of this subunit. Importantly, yeast Mtg2 was identified as a high copy suppressor of the thermosensitive respiratory defect of a 21S rRNA methyltransferase mutant, *mrm2*, suggesting that Mtg2 acts in the mtLSU assembly pathway at a step prior Mrm2. The accumulation of mtLSU assembly intermediates in yeast *mtg2* mutants remains to be investigated.

The mammalian MTG2/ObgH1, also known as GTP-binding protein 5 (GTPBP5), specifically associates with the LSU. However, unlike the mammalian MTG1, only an intrinsic GTPase activity was detected in ObgH1. Interestingly, knockdown studies have not shown any clear indication that ObgH1 is involved in ribosome assembly.[97]

Mtg3 belongs to the YqeH protein family. In *Bacillus subtilis*, the YqeH protein contains a circularly permutated GTP-binding domain (G4-G5-G1-G2-G3). YqeH binds to the 30S small ribosome subunit in a GTP/GDP-dependent fashion and is required for the efficient assembly of the 30S small subunit and 70S ribosomes. *B. subtilis* cells depleted of YqeH are greatly reduced in functional 70S ribosomes and 30S subunits.[55,100] In yeast, Mtg3 functions in the assembly of the mtSSU. A null mutation in *mtg3* prevents the maturation of 15S rRNA and the consequent accumulation of a 15S precursor transcript.[101] Notably, the respiratory deficiency and 15S rRNA precursor processing defect in the *mtg3* null mutant can be partially suppressed by overexpression of uL4, an mtLSU MRP located near the tunnel exit site. These data suggest that Mtg3, together with uL4, regulates assembly of the small subunit by modulating processing of the 15S rRNA precursor.[101]

The human homolog of Mtg3 is C4orf14, a protein that interacts with the 28S mtSSU.[102] Sucrose gradient sedimentation analysis showed the protein to co-sediment with the mtSSU. Gene silencing of C4orf14 specifically affected components of the small subunit, leading to decreased mitochondrial protein synthesis. The GTPase activity of C4orf14 is critical for its interaction with the 28S mtSSU and other factors involved in mitochondrial translation. It was therefore proposed that C4orf14, with bound GTP, binds to components of the 28S subunit facilitating its assembly and GTP hydrolysis acts as the release mechanism. C4orf14 was also found to be associated with human mitochondrial nucleoids. C4orf14 is capable of binding to DNA *in vitro* and silencing of the C4orf14 gene in human cells led to mitochondrial DNA depletion. The association of C4orf14 with both mitochondrial translation factors and the mitochondrial nucleoid has suggested that the 28S mtSSU could be assembled at the mitochondrial nucleoid, enabling the direct transfer of mRNA from the nucleoid to the mitoribosome,[102] as it will be discussed in section 5.

Another mitochondrial member of the conserved GTP-binding proteins with RNA-binding activity is ERAL1, a homolog of Ras-like protein Era protein in *E. coli*, present in high eukaryotes. The bacterial Era binds near the 3′ terminus of the 16S rRNA within the 30S small ribosomal subunit and plays a crucial role in ribosome assembly[103]. Human mitochondrial ERAL1 was identified to coimmunoprecipitate with either mitochondrial transcription factor A (TFAM), a main component of mitochondrial nucleoids, or with the mtRRF (mitochondrial ribosome recycling factor). ERAL1 localizes in the mitochondrial matrix, associates with mitoribosomal proteins, binds to the mtSSU 12S rRNA and plays an important role in the formation of the 28S mtSSU.[104,105] Bacterial Era associates with a 3′ unstructured nonanucleotide immediately downstream of the terminal stem-loop (helix 45) of 16S rRNA. This site contains a highly conserved AUCA sequence directly upstream of the anti-Shine-Dalgarno sequence, which is conserved in bacteria. This has

suggested that Era plays an essential role in ribosome maturation and quality control. In human mitochondria, the entire Era-binding region is absent from 12S rRNA and instead ERAL1 binds to a 33-nucleotide section on the 3′ terminal stem-loop region of 12S rRNA[105] that contains 2 dimethylated adenine residues, as mentioned in the previous section. Furthermore, in contrast with the bacterial ortholog, loss of ERAL1 leads to rapid decay of nascent 12S rRNA,[104,105] consistent with a role as a mitochondrial RNA chaperone. It is worth noting that some phenotypes observed in human cells depleted of ERAL1 are similar to those in cells from mice devoid of TFB1M. They include dramatically reduced levels of the 12S rRNA, whereas mitochondrial mRNA levels remain largely unaffected or even increase. This has suggested that binding of ERAL1 to the 12S rRNA may require or have a greater affinity for the adenine dimethylated stem loop.[105]

RNA helicases

RNA helicases bind and remodel RNA and RNP complexes in an ATP- (or NTP-) dependent manner. These proteins have a structurally conserved helicase core containing characteristic sequence elements and structural motifs. Additional domains confer specificity by recruitment to target RNPs, while the conserved helicase core interacts with RNA in a sequence-independent manner. One critical family of RNA helicases is the DEAD-box proteins, which received the name from the unique amino acid sequence Asp-Glu-Ala-Asp in one of their motifs (motif II). Members of the DExD/H-box family of ATP-dependent RNA helicases participate in virtually all aspects of cellular RNA metabolism, including pre-mRNA splicing, mRNA export, translation initiation, RNA turnover and ribosome biogenesis. One of the key differences of the DEAD box helicases from conventional helicases is their ability to unwind duplexes and/or remodel RNA-protein complexes locally within the strands in a non-processive way. The conventional helicases on the other hand act processively and translocate along single-stranded RNA in a defined polarity toward the duplex region.[106]

The highly conserved helicase core in DEAD-box proteins consists of 2 virtually identical domains that resemble the bacterial recombination protein recombinase A (RecA) connected by a flexible linker. The two domains bend in such a way that a cleft is formed that harbors the binding sites for both ATP and RNA at the 2 opposite ends.[107-109] The cleft between the 2 domains must be closed to productively bind and hydrolyze ATP.[107-109] Frequently the helicase core is flanked by variable N- and C-terminal extensions that might be critical for the specific function of the individual protein. DEAD-box proteins acquire substrate specificity by interaction with co-factors that target the enzymes to their place of action.[110]

The much-studied ribosomal assembly pathways of bacterial and eukaryotic systems have shown the involvement of many DExD/H-box proteins at various stages of the assembly process.[111] They often engage in unwinding of double-stranded RNA, assisting dissociation of RNA-binding proteins to favor recruitment of downstream factors or mediating local structural remodeling of pre-ribosomal complexes and providing accessibility for endo- or exonucleases and RNA modifying enzymes.[112]

E.coli has 5 DExD/H-ATPases, namely DeaD/CsdA, RhlB, RhlE, DbpA and SrmB. Some of these proteins are multifunctional as they participate in ribosome assembly, RNA degradation and/or translational regulation.[20,113] DeaD/CsdA and SrmB participate in ribosome biogenesis, by associating with the 50S subunit, and the absence of either protein causes ribosome assembly defects that result in accumulation of 40S late assembly intermediates.[114,115] DbpA interacts with 23S rRNA (rRNA), specifically with helix 92, an interaction that stimulates the ATPase and helicase activities of the enzyme.[116,117] Although *dbpa* mutants have normal ribosome assembly, a dominant negative mutant induces the accumulation of a ribosome intermediate.[118] RhlE plays a role in the interconversion of rRNA-folding intermediates that are further processed by DeaD/CsdA or SrmB during ribosome maturation.[21] No obvious orthologues of these proteins are known in mitochondria.

With regard to mitoribosome assembly, participating DEAD-box helicases have been identified only recently. When our group performed an *in silico* screen in search of mitochondrial putative RNA helicases required for mitoribosome biogenesis and/or translation in *S. cerevisiae*, our focus was directed to Mrh4 (4th putative mitochondrial DEAD box RNA helicase). Mrh4 is essential for mtLSU biogenesis. Mrh4 interacts with the *21S* rRNA, mitoribosome subassemblies and fully assembled mitoribosomes. In the absence of Mrh4, the *21S* rRNA is matured and forms part of a large on-pathway assembly of intermediate missing proteins bL9, uL16 and bL33. In *E. coli*, L16 and L33 play important structural and perhaps catalytic roles.[119] Whereas L33 does not seem to play a major role in ribosome assembly,[120] L16 accelerates the late steps of *in vitro* assembly[121] and induces a conformational change in the 50S, which may in turn affect the peptidyltransferase activity and subunit association of the ribosome.[122] The stable incorporation of these proteins is essential for ribosomal subunit association. Several bacterial ribosomal mutants accumulate 40S–45S particles, each lacking small sets of proteins that overlap with those missing in the Δ*mrh4* pre-54S particle. Particularly, the dominant-negative mutant of *dbpa*, when overexpressed in *E. coli*, induced a deficit in 50S subunits giving rise to a 45S particle containing reduced levels of L16, L25, L27, L28, L33, L34, and L35.[118] Several of the missing MRPs bind near the PTC, suggesting the requirement of a DbpA-mediated conformational change for the binding of these MRPs at this site.

For Mrh4 on the other hand, we have proposed that it plays an essential role during the late stages of mitoribosome assembly by promoting remodeling of the *21S* rRNA-protein interactions.[123] Because Mrh4 was found to interact with the monosome, the possibility exists that it could perform additional roles during translation.

Looking for putative mammalian homologues of Mrh4, we performed cluster analysis of all identified DEAD/H helicases across species, which grouped yeast Mrh4 with mouse/rat/human DDX28. Heterologous expression of DDX28 in a yeast *mrh4*

null mutant, however, did not rescue its mitoribosome assembly defect.[123] DDX28 displays RNA-sensitive Mg^{2+}-dependent ATPase activity *in vitro*.[124] It was shown that DDX28 has dual subcellular localization in monkey COS1 cells; whereas the majority of the protein permanently resides in mitochondria, a small portion was found in the nucleolus.[124,125] GFP-fused DDX28 was detected in punctate structures in COS1 cell mitochondria, about half of which co-localized with, or were adjacent to the mitochondrial nucleoids.[126] These results were confirmed in cultured human cells although biochemical studies localized most of the protein in mitochondria.[127] DDX28 interacts with the 16S rRNA and the mtLSU,[127] most probably with a helix in domain V.[128] RNAi-mediated *DDX28* silencing in HEK293T cells does not affect mitochondrial mRNA stability, 16S rRNA processing or modification. However, it leads to reduced levels of 16S rRNA and mtLSU proteins, impaired mtLSU assembly and deeply attenuated mitochondrial protein synthesis.[127] Our findings have identified DDX28 essential for 16S rRNA stability, perhaps during the early stages of mitoribosome mtLSU biogenesis.

The DHX30 helicase also participates in mtLSU assembly, although its precise role still remains to be investigated. This protein localizes to foci in the vicinity of nucleoids,[129] the RNA granules.[128] Silencing of DHX30 in human cells produces a very severe translation defect that correlates with a profound loss of monosomes. DHX30 seems to interact with the mtLSU, mtSSU and the monosome, suggesting that it remains closely associated with the ribosome at all stages of assembly.

Other mitoribosome assembly factors

In mammalian cells, 2 mitochondrial transcription factor family proteins play roles in mtLSU assembly, perhaps coordinating transcription and ribosome biogenesis. The precise role of mTERFD1 (or mTERF3) in promoting mtLSU biogenesis is not understood,[130] but mTERF4 forms a complex with the rRNA methyltransferase NSUN4 to promote its recruitment to the mtLSU[78] in order to facilitate monosome assembly,[77] as mentioned earlier.

Moreover, C7orf30, a member of the DUF143 family of ribosomal silencing factors, participates in mtLSU assembly[131-133] by interacting with uL14 and promoting its incorporation into the mtLSU.[132] Knockdown of C7orf30 by short hairpin RNA (shRNA) does not alter the sedimentation profile of the mtLSU, but results in mtLSU depletion and consequent decreased monosome formation that leads to a mitochondrial translation defect.

The matrix AAA-protease system is involved in the processing of the *S. cerevisiae* 54S mtLSU protein bL32, allowing its association with pre-ribosomal particles in the late stages of mtLSU assembly.[134] bL32 folding depends on its mitochondrial targeting sequence. The post-translocational processing by m-AAA protease is required as opposed to co-translocational cleavage by the general mitochondrial processing peptidase, as bL32 requires presequence-assisted folding for complete import.[134]

The pre-54S intermediate that accumulates in the absence of Mrh4 contains bL32, which supports the late-stage nature of the intermediate and suggests the requirement of Mrh4 subsequent to the incorporation of bL32. The role of the AAA-protease system in mitoribosome maturation is conserved in mammals, since maturation of bL32 and mitochondrial protein synthesis are also impaired in a hereditary spastic paraplegia (HSP) mouse model lacking the *m*-AAA protease subunit paraplegin.[134] Expression of the human homolog proteins AFG3L2/SPG7 in a *yta12/yta10* mutant yeast strain complements the lack of the yeast m-AAA protease,[135] further suggesting functional conservation.

GRSF1 (G-rich sequence binding factor 1) is the core component of mitochondrial RNA granules, where newly synthesized RNAs accumulate and where their processing, storage, sorting or translation is regulated.[136,137] Furthermore, a small portion of GRSF1 co-sediments with the mtSSU[127] and GRSF1 depletion induces a defect in the assembly of the mtSSU and leads to a marked attenuation of mitochondrial protein synthesis.[136,137] These observations indicate a role for GRSF1 in mtSSU biogenesis.

FASTKD2, a protein of the Fas-activated serine-threonine kinase (FASTK)- domain-containing family, also localizes to RNA granules, binds to the 16S rRNA and is required for mtLSU assembly.[128]

Defining the Assembly Pathway for Biogenesis of Mitoribosomes in Yeast and Mammals

Lessons from studies in vitro and on bacterial ribosomes

A wealth of information is already available on the assembly of the bacterial ribosome and these studies can provide valuable hints as to how this process could occur in mitochondria, as well as to what approaches can be used for that purpose. Readers interested in bacterial ribosome biogenesis are referred to several recent excellent reviews.[20,88,111] In general, the assembly of ribosomes involves the coordinated processing and modification of rRNAs with the temporal association of ribosomal proteins. Both studies *in vitro* and *in vivo* have identified precursors and assembly factors that lead to the formation of the bacterial ribosome.

Despite the complex composition of ribosomal subunits, earlier pioneering work in the 1960s led by Nomura[138,139] and Nierhaus[140,141] demonstrated that active bacterial subunits could be reconstituted *in vitro* from individual unmodified rRNAs and proteins. The process did not require any additional components (i.e., assembly factors), but has a marked dependence on non-physiological temperature and ionic conditions. Through numerous measurements of binding interdependences among different proteins, they generated general *in vitro* assembly maps for the 30S and 50S subunits.[138-142] Their data revealed that ribosomal proteins are incorporated in different orders, displaying both hierarchical and parallel manners. For example, in the early stages of 30S assembly, primary ribosomal proteins bind directly to the 5′-, central and 3′-domains of 16S rRNA, inducing changes in the rRNA structure, and facilitating subsequent binding of secondary and tertiary ribosomal proteins.[139,143]

More recent studies using time-resolved hydroxyl radical footprinting,[144] matrix-assisted laser description/ionization mass spectrometry (MS),[145] time-resolved electron microscopy[146] and

in vivo studies of *E. coli* cells treated with neomycin,[147,148] have revealed that even in the presence of ribosomal protein binding dependencies, assembly can proceed through multiple parallel pathways generating heterogeneous populations of assembly intermediates.

Inside the cell, subunit assembly is highly efficient, as the process is facilitated by a variety of cofactors with diverse functions (nucleases, GTPases, RNA helicases, chaperones) that prevent the formation of kinetic traps during the assembly process. Disruption of cofactors by genetic deletion or mutation leads to growth defects and accumulation of subunit precursors, whose analyses by quantitative MS (qMS) have provided hints regarding the step in which they intervene and of rate-limiting steps in the assembly pathway (reviewed in[20]). In the same line, studies based on systematic chromosomal deletion of ribosomal protein genes in *E. coli* have shown that although most ribosomal proteins are essential, several proteins (L9, L15, L21, L24, L27, L29, L30, L34, S9, and S17) appear to be dispensable for ribosome assembly and translation. However, these proteins can be important for efficient and accurate assembly and protein synthesis capacity under some conditions.[41] These studies, combined in some cases with Cryo-EM structures of usually late-stage assembly intermediates, have provided an idea about conformational maturation of the rRNA and of the growing ribonucleoprotein particle.[93,96] Until recently the identification and characterization of *in vivo* assembly intermediates in wild-type bacterial cells have been challenging owing to the fewer number of ribosomes undergoing biogenesis (2–5%), the rapid kinetics and asynchronous transcription from rDNA loci.[149] Nevertheless, with the use of high-resolution high-throughput qMS methodology[19,150] the Williamson group was able to characterize *in vivo* ribosome assembly intermediates and associated assembly factors in wild-type *E. coli* cells. They analyzed the composition of sucrose gradient fractions prepared using an *in vivo* stable isotope pulse-labeling (SILAC) approach to distinguish degradation products and dead-end assembly intermediates from on-pathway intermediates.[19,150] This methodology provided snapshots of the assembly intermediates that confirmed 4 subassemblies for the 30S subunit and 6 50S precursors that correlated well with the *in vitro* reconstitution data, although they were not identical to the ones found *in vitro*.[19]

For the 30S subunit, analyses of sucrose gradient fractions revealed the existence of 5 subassemblies, whose composition is summarized in **Figure 2**.[19,146] In one of the reports, sucrose gradient fractions were analyzed in parallel using single-particle negative stain EM to elucidate the structures of assembly intermediates and mature ribosomes present in each sample, which revealed the heterogeneity of the 5 assembly intermediates.[146] Another profitable approach to study the 30S assembly pathway, applied by Culver's group, consists of aptamer-based RNA-tagging and affinity-purification systems to isolate SSU or LSU assembly intermediates from wild-type cells.[151,152] This strategy elucidated one predominant specie of pre-16S rRNA (17S rRNA) to be associated with all purified assembly intermediates in the SSU assembly pathway.[152] As in Williamson's experiments, Culver's group observed several independent pathways for maturation of 17S rRNA in SSU subassemblies, results that further suggest that flux through SSU biogenesis involves multiple pathways.[152]

For the 50S subunit, the qMS approach identified 6 distinct assembly groups of ribosomal proteins,[19] whose composition is also depicted in **Figure 2**. These studies have shown that the central protuberance assembles last.[19] This has suggested that key functional sites of the 50S subunit, such as the tRNA binding sites, remain unstructured until the late stages of maturation, preventing the incomplete subunit from prematurely engaging in translation.[93]

Current knowledge on mitoribosome assembly pathways

Unlike the bacterial ribosome, the mitochondrial ribosome is permanently bound to the inner mitochondrial membrane in order to facilitate protein synthesis with membrane insertion,[153] and the assembly process has been proposed to occur in contact with the inner membrane.[123,154] Even though during the last decade information on mitoribosome assembly factors and the complement of MRPs was greatly enhanced, a detailed map of the assembly pathway has yet to be determined (**Fig. 2**). The delay in information could be due to 2 major causes. First, yeast ribosome assembly mutants tend to lose their mitochondrial DNA, therefore complicating their analyses. Second, the mammalian ribosome assembly mutants described so far apparently do not accumulate assembly intermediates that could be characterized.

The challenge of yeast has been bypassed by the use of thermo-sensitive mutants that allow for controlled induction of the mutant phenotype.[95,101] A better approach has resulted from the identification of genetic suppressors that maintain mtDNA stability in the absence of mitoribosome assembly, thus allowing the study of assembly intermediates.[123] Also, expression of some truncated ribosome proteins have resulted in ribosome assembly lesions without affecting mtDNA stability.[154] Three studies have demonstrated the existence of late-assembly intermediates, which are tethered to the inner mitochondrial membrane. First, the LSU r-protein bL32 is imported into the mitochondrial matrix as a precursor that needs to be proteolytically processed by m-AAA proteases Yta10/Yta12 prior to bL32 insertion into the mitoribosome.[134] In the absence of the protease, LSU assembly is perturbed at a late step prior to bL32 incorporation.[134] As a result a membrane-bound large pre-54S assembly intermediate is accumulated in *yta10/yta12* mutants. Second, deletion of the C-terminal mitochondria-specific domain of uL23 (*mrp20*Δ*C*) results in accumulation of a membrane-bound pre-54S assembly intermediate of approximately 150kDa.[154] Interestingly, this intermediate is composed of uL23, uL24, uL4, and mL41,[154] all located at the LSU tunnel exit site[13,35] as well as mL59. Hence, it has been proposed that different modules of the 54S ribosome could assemble independently before completion of the full ribosomal subunit. And third, a recent report by our group on the characterization of Mrh4 described the accumulation of a membrane-bound on-pathway pre-54S late-stage assembly intermediate (containing bL32), missing only 3 late assembly MRPs, namely bL33, uL16 and bL9 in the absence of the DEAD-box

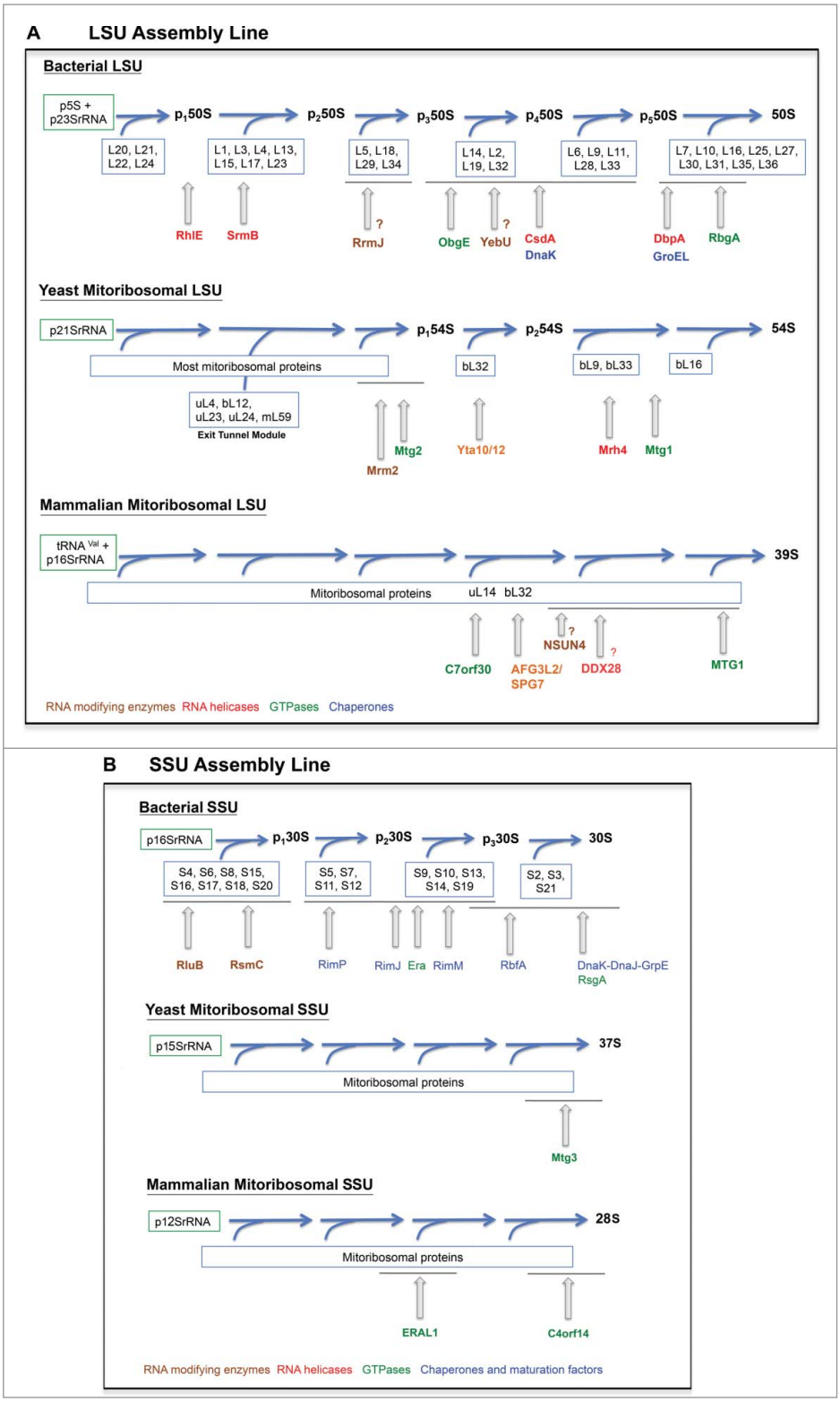

Figure 2. Mitoribosome assembly lines. Assembly pathway of the (**A**) mitochondrial large subunit (SSU) and (**B**) mitochondrial small subunit (SSU). The assembly pathways for the bacterial subunits are presented as a reference. Only some ribosome biogenetic factors are included. The assembly stage at which they act is depicted only approximately.

helicase.[123] Putting together this data, the assembly map that can be drawn for the 54S LSU particle is extremely incomplete (**Fig. 2**) and thus further in-depth investigation in this area of research is much needed. Currently, no assembly intermediates for the yeast 37S SSU have been identified.

Studies on human mitoribosome assembly have adapted the proteomics approach developed to study the *in vivo* assembly of bacterial ribosomes.[155] The only available SILAC pulse-chase experiments have discriminated the newly synthesized proteins enriched in the nucleoid fraction (and its vicinity) in comparison to the ribosome-enriched fraction. Higher resolution is required to identify mitoribosome assembly intermediates.

Compartmentalization of Mitoribosome Assembly

Nucleoids, RNA granules and mitoribosome assembly

A recent breakthrough in mitoribosome assembly relates to the mitochondrial compartmentalization of this process. At least 3 distinct types of foci relevant to mtDNA expression have been identified and visualized within the mitochondrial matrix of human cells. Those are the mitochondrial nucleoids, RNA granules and the RNA degradosome.[156] Here we will discuss the available data suggesting that the nucleoid and the RNA granule could be the factories where mitoribosomes are assembled.

The mammalian mitochondrial nucleoid is a nucleoprotein complex that contains mtDNA and proteins involved in mtDNA maintenance, replication and transcription.[129,157-160] Within the nucleoid, which can contain up to 5–6 mtDNAs, each genome replicates and transcribes independently of the others.[161] Early studies aiming to identify proteins associated with mt-nucleoids were based on affinity purification of known nucleoid components (mitochondrial transcription factor A (mtTFA or TFAM) and mitochondrial single strand binding protein (mtSSB))

followed by mass spectrometry. They identified proteins involved in mtDNA metabolism and additionally many mitoribosome proteins and proteins required for mitochondrial translation such as ATAD3,[162] suggesting an intimate association between nucleoids, components of the mitochondrial translation machinery and foci containing ribonucleoprotein complexes. For example, the human GTPase C4orf14 that functions in the assembly of the 28S mtSSU, associates with mitochondrial translation factors and the mitochondrial nucleoid.[102] Furthermore, the absence of MPV17L2, a protein proposed to be required for the assembly of the monosome and whose stability depends on mtDNA, induces the trapping of 28S mtSSU proteins in enlarged nucleoids.[163] This has suggested the hypothesis that the 28S subunit is assembled at the mitochondrial nucleoid, enabling the direct transfer of mRNA from the nucleoid to the mitoribosome.[102] Similar observations were recently reported in a proteomics study that found that mitochondrial RNA processing enzymes involved in tRNA excision - ribonuclease P (RNase P),[61] and the RNase Z-type enzyme ELAC2,[62,63] as well as a subset of mitochondrial ribosomal proteins (MRPs) - associate with nucleoids or foci located near the nucleoids to initiate RNA processing and ribosome assembly.[155] In an important experiment, formaldehyde cross-linking was performed to determine the nucleoid proteins that are in close contact with the mtDNA.[129] A set of core nucleoid proteins was found in both native and cross-linked nucleoids, including 13 proteins with known roles in mtDNA transactions. Several other metabolic proteins and chaperones identified in native nucleoids, including ATAD3 and ribosomal proteins, were not observed to cross-link to mtDNA.[129] These results suggested a model for a layered structure of mtDNA nucleoids in which replication and transcription occur in the central core, whereas RNA processing and translation may occur in the peripheral region.[129]

However, analysis of bromouridine (BrU) labeling of newly transcribed RNA in human mitochondria has identified distinct BrU-positive RNA foci in close proximity to the nucleoids.[161] These foci, recently termed RNA granules, contain the protein GRSF1 (G-rich sequence binding factor 1) and RNase P, and accumulate mtRNAs to regulate their processing, storage, sorting or translation.[136,137] These foci also contain ribosomal proteins[137] and rRNA modifying enzymes.[164] Furthermore, GRSF1 depletion induces a defect in the assembly of the mitoribosomal SSU and a marked attenuation in mitochondrial protein synthesis.[136,137] Consistently, our sucrose gradient fractionation data has shown co-sedimentation of GRSF1 with mtSSU markers.[127] This has raised the possibility that ribosome biogenesis could occur near or within the RNA granules. Affinity purification of tagged GRSF1 identified ribosomal proteins[137] and the 16S rRNA methyltransferases MRM1, MRM2 and MRM3/RMTL1.[164] Affinity purification of tagged MRM3 co-eluted ribosomal proteins and ribosome assembly factors that co-localize to the RNA granules, such as GRSF-1 and the DEAD-box helicase DDX28.[164,165] Recent comprehensive affinity purification studies of GRSF1 and DDX28 followed by mass spectrometry analysis by 2 independent groups have yielded similar results.[127,128] In the study performed by our group, DDX28 was found associated with 4 major groups of relevant proteins.[165] The first group included most mitoribosomal proteins (45 mtLSU and 29 mtSSU MRPs). The second group was comprised of several previously identified factors involved in the biogenesis of the mtLSU (MRM3, MTG1, C7orf30, FASTKD2 and MTERF-D1), the mtSSU (ERAL1) or both (DHX30), together with a set of potential mitoribosomal assembly/maintenance factors including proteases, GTPases and chaperones. The third group involved mitochondrial RNA metabolism proteins, including GRSF1, LRPPRC, and SLIRP involved in the stability, polyadenylation and coordination of translation of mitochondrial mRNAs[166,167]; RNase P; the 16S methyltransferase RMTL1/MRM3; the pentatricopeptide repeat domain protein 1 (PTCD1) involved in the processing of mitochondrial polycistronic transcripts that contain leucine tRNA[168]; and the translational regulator PTCD3.[150] And the fourth consisted of a set of translation factors and most aminoacyl tRNA synthetases. These studies also identified other proteins previously associated with mitochondrial nucleoids, e.g., the helicase DHX30 that acts as a transcriptional regulator, and ATAD3 mentioned earlier.

The overlapping pool of proteins associated with either mitochondrial nucleoids or RNA granules reflects their proximity and the lack of membranous boundaries sealing these compartments. Their functional and physical dynamics is highlighted by the observation by fluorescence microscopy that approximately 10–15% of nucleoids and RNA granules do actually co-localize in most studies, probably reflecting sites of active transcription.[136,137,156,161] A quantitative assessment of these dynamics showed that after an initial short pulse of 20 min, most BrU-positive RNA foci colocalize within 200 nm of mtDNA, but after longer periods (or a pulse-chase) the BrU-positive RNA foci become distributed randomly.[137] Thus, mitoribosome assembly could be initiated within or near the nucleoids,[155,163] possibly in a co-transcriptional manner as it occurs in bacteria.[20] SILAC pulse-chase experiments showed that most newly synthesized mitoribosome proteins from the 2 subunits were initially recruited to the nucleoid-containing fraction and the authors concluded that the first steps in ribosome biosynthesis occurred at the nucleoids.[155] However, it is more likely that RNA granules co-sedimented with the nucleoids in sucrose gradients and therefore the SILAC results cannot exactly discriminate where mitoribosome assembly occurs. Studies of the composition of human DDX28[165] and GRSF interactomes[128] mentioned earlier point toward the conclusion that most steps of mitoribosome biogenesis could actually occur in the RNA granules. It is necessary, however, to stress the concept of RNA granules as dynamic structures. As portrayed in **Figure 3**, newly transcribed rRNAs and/or early mitoribosome assembly intermediates are probably transferred from nucleoids to the RNA granules, where mitoribosome assembly is completed. These mitochondrial matrix subcompartments are therefore reminiscent of the nucleolus. Within the nucleus, the membrane-less nucleolus is organized around the chromosomal regions that contain the genes for the rRNAs, and is the site of rRNA transcription and processing, and of ribosome assembly. Equivalent features pertain to the mitochondrial RNA granule, which we have proposed to term "the mitochondriolus" (**Fig. 3**).[127]

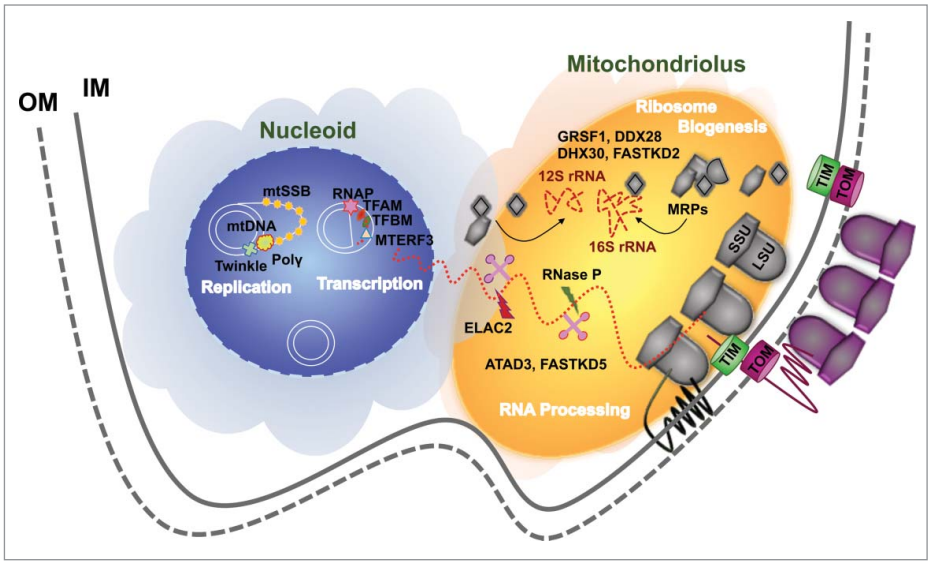

Figure 3. Compartmentalization of mitoribosome biogenesis. The processes of mtDNA replication and expression occur in submitochondrial matrix compartments. The mtDNA and proteins involved in mtDNA metabolism form a nucleoprotein complex called the mitochondrial nucleoid. Upon transcription, RNAs are sorted at the mitochondriolus (RNA granule), where ribosome assembly largely occurs.

Furthermore, the nucleoids are tethered directly or indirectly through both mitochondrial membranes to kinesin, and to microtubules in the surrounding cytoplasm.[161,169] On the other hand, the RNA granules colocalize with components of the cytoplasmic machinery that imports nuclear-encoded proteins such as the translocase in the outer membrane (TOM22). Therefore, it has been hypothesized that the mitochondrial nucleoids and RNA granules could organize the mitochondrial and cytoplasmic translation machineries on both sides of mitochondrial membranes to facilitate efficient assembly of proteins synthesized by the 2 distinct translation machineries into mitochondrial complexes.[161]

In yeast, the mitochondrial gene expression system is organized in distinct ribosome-containing clusters.[170] Similar to what has been described in mammalian cells, a subset of these clusters is engaged in a large complex with or near the nucleoid,[170] reminiscent of the mammalian mitochondrial RNA granules. Studies of BrU-RNA staining in yeast mitochondria, currently missing, will help clarify whether the "mitochondriolus" appeared early in eukaryotic evolution and whether this is the universal mitoribosome factory.

Membrane localization of mitoribosomes and mitoribosome assembly

Yeast and mammalian mitochondrial ribosomes are bound to the inner membrane, presumably as a result of their specialization in the synthesis of hydrophobic membrane proteins, which are co-translationally inserted into the inner membrane (*2*). Even synthesis of the yeast ribosomal subunit Var1 is membrane-localized.[171]

In yeast, several inner membrane proteins mediate membrane binding of ribosomes such as those in the Oxa1 machinery, which facilitates the insertion of nascent polypeptides into the inner membrane.[30-36] Oxa1 interacts with the mitoribosomal LSU *via* its C-terminal α-helix ribosome-binding domain.[30,32] Also Mba1 and Mdm38 tether the mitoribosome to the membrane in both translationally active and inactive states[34,36] and additionally exhibit overlapping regulatory functions in translation of selected mitochondrial mRNAs.[34] Studies of the proteins located at the polypeptide tunnel exit of the yeast mitochondrial ribosome showed that Mba1 is positioned in proximity to the ribosomal proteins uL29 and uL22.[35] This sub-ribosomal localization of Mba1 differs from that of Oxa1, which is located near uL24 and uL23.[30,31] Furthermore, yeast mitoribosomes remain docked to the membrane even in the absence of Oxa1 machinery associated with the tunnel exit, indicating that additional and as yet uncharacterized anchors must exist.[172] The yeast LSU structure has shown that the LSU central protuberance has a membrane-facing location, which makes it a candidate to be involved in the tethering of mitoribosomes to the membrane.[13]

On the other hand, the 4.9 Å Cryo-EM reconstruction of the mitoribosomal 39S LSU subunit from porcine (*Sus scrofa*) liver tissue has indicated that the mammalian mitochondrion-specific mL45 (MRPL45) subunit, localized at the polypeptide tunnel exit, anchors the mitoribosome to the inner mitochondrial membrane.[15] mL45 displays homology to the C-terminal domain, membrane-binding segment of TIM44, a subunit of the inner membrane translocase essential for protein import.[173] Notably, mL45 also presents homology to the yeast mitochondrial protein Mba1,[6] which tethers the mitoribosome to the inner membrane as discussed earlier. Thus, mL45 may bind the mitoribosome to the inner membrane in a manner that would expose the translated nascent polypeptide to the solvent matrix milieu,[15] making it accessible to specific maturation and assembly factors involved in the biogenesis of OXPHOS complexes. As in yeast, mitoribosomal membrane-anchoring would also facilitate alignment of the nascent polypeptide tunnel exit with mammalian OXA1L (homolog of yeast Oxa1) to facilitate co-translational membrane insertion of newly synthesized proteins[174,175]

Recent data has suggested that the inner mitochondrial membrane could act as a platform for the ribosome assembly process.[123,154] The expression of a truncated form of uL23 (Mrp20) in yeast Δ*mrp20* cells resulted in the accumulation of a ribosomal assembly intermediate composed of tunnel-exit-site proteins, which was found associated with the inner membrane.[154] Likewise, the late pre-54S particle that accumulates in the absence of the DEAD-box helicase Mrh4 is associated with the inner membrane. Strikingly, similar membrane-binding behavior occurs for

non-assembled ribosomal proteins in cells devoid of mtDNA (rho zero cells) and thus of mtrRNA, which suggests the possibility of specialized membrane-associated compartments to which ribosomal proteins become targeted after their import into mitochondria,[123,154] prior to being recruited for binding to mtrRNA or perhaps where ribosome assembly actually occurs.

Defective Ribosome Assembly and Human Disease

Defects in mitochondrial translation are responsible for devastating human disorders (**Table 3**). They constitute a specific class of mitochondrial diseases, biochemically characterized by alterations in the 4 OXPHOS enzymes that contain mtDNA-encoded subunits. In this group of disorders, mutations in most mtDNA-encoded tRNAs, as well as in nuclear genes encoding mitoribosomal proteins, translation initiation and elongation factors, are responsible for clinically and genetically heterogeneous infantile multisystemic diseases, such as Leigh's syndrome, sensorineural hearing loss, encephalomyopathy and hypertrophic cardiomyopathy.[22-24,176] The existence of many patients afflicted by mitochondrial disorders with multienzymatic OXPHOS defects of unknown genetic cause predicts that numerous genes involved in the biogenesis and function of the mitochondrial translation machinery remain to be identified.

Table 3. Mitoribosome assembly and human diseases. CPEO, chronic progressive external ophthalmoplegia; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes; MNGIE, Myo-, neuro-, gastrointestinal encephalopathy. For most mutations, only one or a few references were included. A complete set of references can be found in MitoCarta[221]

Gene	Mutation	Clinical Manifestations	Ref.
Mutations in mitoribosome structural components			
Mutations in mitorRNAs			
12S rRNA	T669C, A735G, A745G, C792T, A801G, A827G, A839G, A856G, T961G, T961C, T961delT+C(n) T990C, T1005C, A1027G, T1095C, A1116G, C1192T, C1192A, T1291C, C1310T, A1331G, A1374G, A1452C, C1494T, A1517C, C1537T, A1555G	Non-syndromic antibiotic induced hearing loss	177,178,222,223
16S rRNA	G3090A	Mitochondrial myopathy	194
	T2336C	Hypertrophic cardiomyopathy	195
MT-TV	C1628T; G1644A	Encephalocardiomyopathy	199
	G1644A	Adult-onset Leigh Syndrome	203
	G1642A	MELAS	204
	A1630G	MELAS	201, 202
	C1624T	MNGIE	200
	G1606A	Leigh Syndrome	198
	T1658C	Complex neurologic syndrome	196,197
		CPEO	205
Mutations in mitoribosomal proteins			
MRPS7	Homozygous c.A550G p.Met184Val	Congenital sensorineural deafness, progressive hepatic and renal failure, and lactic acidemia	206
MRPS16	Homozygous c.C331T p.Arg111Ter	Fatal neonatal lactic acidosis	207
MRPS22	Homozygous c.G509A p.Arg170His	Cardiomyopathy, tubulopathy, hypotonia, skin edema	208
	Homozygous c.T644C p.Leu215Pro	Cornelia de Lange-like dysmorphic features, encephalocardiomyopathy,	209
MRPL3	Compound heterozygous c.C950G p.Pro317Arg / large deletion	Hypertrophic cardiomyopathy, Psychomotor retardation	210
MRPL12	Homozygous c.C542T p.Ala181Val	Growth retardation, neurological deterioration	211
MRPL44	Homozygous c.T467G p.Leu156Arg	Infantile cardiomyopathy	212,224
Mutations in mitoribosomal assembly factors			
FASTKD2	Homozygous c.C1246T p.Arg416Ter	Mitochondrial encephalomyopathy	214
AFG3L2	Heterozygous missense mutations	Spinocerebellar ataxia type 28	216
	Homozygous c.1847G>A; p.Y616C	Spastic ataxia-neuropathy syndrome	217
SPG7	Mostly homozygous but also some heterozygous missense mutations	Optic neuropathy	225
		Hereditary spastic paraplegia	218
		CPEO	219,220

Focusing on changes in ribosomal components associated with human diseases, only a few mutations have been so far described in rRNAs and mitoribosomal proteins. On the contrary, several mutations have been identified in the *MT-TV* gene encoding for $tRNA^{Val}$ (see **Table 3**).

Mutations in the 12S rRNA are an important cause of hearing loss. Specifically, the homoplasmic A1555G and C1494T mutations at the highly conserved decoding site of the 12S rRNA gene are well documented as being associated with either aminoglycoside-induced or nonsyndromic hearing loss in many families of various ethnic backgrounds.[177-180] Aminoglycosides (such as gentamicin, kanamycin, and streptomycin) are used as antibacterial therapeutic agents that inhibit protein synthesis by interacting with the A-site of the 16S rRNA in the 30S ribosomal subunit.[181,182] They exert pleiotropic actions during the 3 phases of protein synthesis. They affect initiation, perturb peptide elongation at the 30S ribosomal subunit and impair translational proofreading, leading to misreading of the RNA message, premature termination, or both.[183,184] In the human mitochondrial 12S rRNA A-site, C1494 forms a non-canonical RNA base pair with A1555.[185] The bacterial sensitivity to aminoglycosides involves their direct binding to a 16S rRNA C–G base pair at positions 1409–1491.[186] The A1555G transition results in a decrease in flexibility of the decoding center and makes the secondary structure of rRNA more similar to the corresponding region of *E. coli* 16S rRNA therefore contributing to create a binding site for aminoglycoside drugs.[25,26,187] The effect of creating an A-T pair by the C1494T transition is apparently similar.[25,26] As a consequence, exposure to aminoglycosides can induce or aggravate hearing loss in individuals carrying one of these mutations. Phenotypic expression of the deafness-associated homoplasmic A1555G mutation varies from profound congenital hearing loss to normal hearing.[177,188] This variability in clinical expression in most patients is due to the complex inheritance of multiple nuclear-encoded modifier genes. Such modifier genes include the mitochondrial transcription factor B1 (TFB1M),[189] which methylates adenine residues in the adjacent loop of the A1555G mutation in the 12S rRNA gene.[74] Other modifiers are the genes *MTO1*[190,191] and *GTPBP3*[192] - human homologs of yeast MTO1 and MSS1 genes, respectively. Yeast Mto1 and Mss1 form a heterodimer complex, which interacts with the small mitochondrial ribosomal subunit at a site in the small rRNA close to or inclusive of paromomycin-resistance mutations, and may play a role in optimizing mitochondrial protein synthesis by a proof-reading mechanism.[193] Each human gene complements the respective yeast mutant, what indicates functional conservation during evolution[190,192] and explains their roles as disease modifier genes.

While numerous patients carrying mutations in the 12S rRNA have been described, only 2 mutations in the 16S rRNA associated with a primary mitochondrial disorder have been reported. The first case, a young woman with severe myopathy, presented ragged red fibers with enlarged abnormal mitochondria, profound and combined deficiency of OXPHOS complexes, with the exception of complex II, not accompanied by mtDNA depletion.[194] An almost homoplasmic G>A substitution at the 3090 conserved position in the 16S rRNA was detected by sequencing in muscle, the more affected tissue, but was absent in fibroblasts, follicles and blood cells. The mutation was not detected in the blood and urine cells of the patient's mother, suggesting a *de novo* origin during embryonic development.[194] More recently, the mutation T2336C has been associated with hypertrophic cardiomyopathy in the 4 affected members of a Chinese family.[195] The mutation is homoplasmic in blood, urine, hair follicles and oral epithelium and it is predicted to disturb the T2336-A2438 base pairing in the first position of a stem loop in the 16S rRNA domain III. In lymphoblastoid cell lines derived from these patients, mitochondrial morphology is altered and oxygen consumption is reduced by approximately 37%.[195] However, data on the status of mitoribosome assembly or mitochondrial translation efficiency are not available for either one of the 2 pathogenic mutations reported until now in the 16S rRNA.

Mitochondrial $tRNA^{Val}$ is a recurrent target for mutations causing mitochondrial disorders. Reported nucleotide variants in the *MT-TV* gene include: (i) Heteroplasmic G1606A, affecting the acceptor stem of $tRNA^{Val}$, which is associated with adult onset complex neurologic syndrome that included hearing loss, cataracts, ataxia, myoclonus, and dementia[196,197]; (ii) Homoplasmic C1624T affecting a base pair in the dihydrouridine loop produced multiple neonatal deaths and one surviving child with Leigh syndrome.[198] In one of the deceased patients, a selective and severe reduction of the steady-state $mttRNA^{Val}$ level was found in cardiac and skeletal muscle; (iii) Homoplasmic C1628T in a child presenting mitochondrial encephalocardiomyopathy.[199] Intriguingly, the patient had isolated complex I deficiency in skeletal muscle,[199] although complex V was not measured and the biochemical defect could be deeper in affected tissues. In all other patients reported and mentioned below, $tRNA^{Val}$ mutations caused a combined respiratory chain deficiency; (iv) Heteroplasmic A1630G in a patient with MNGIE-like gastrointestinal dysmotility and cachexia[200]; (v) Heteroplasmic G1642A associated with MELAS[201,202]; (vi) G1644A has been found in several patients. It results in progressive encephalocardiomyopathy with adult onset when present in homoplasmy[199] or when present in heteroplasmy, with adult onset Leigh syndrome[203] or with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes[204]). In the homoplasmic case, it was reported that the levels of mutant $tRNA^{Val}$ were ~20% of controls, suggesting that it was probably degraded. And (vii) heteroplasmic T1658C mutation was identified in a patient with chronic progressive external ophthalmoplegia (CPEO) in which the T loop structure of mitochondrial $tRNA^{Val}$[205] was changed. From all these studies, only 2 reported the levels of mutant $tRNA^{Val}$, which were found markedly decreased. At present, there are no reports analyzing the effect of these mutations on mitoribosomal assembly.

Reported mutations in genes coding for mitochondrial ribosomal proteins affect 3 SSU subunits: uS7 (MRPS7), bS16 (MRPS16) and uS22 (MRPS22), and 3 LSU subunits, uL3 (MRPL3), bL12 (MRPL12) and mL44 (MRPL44). A

homozygous mutation in *MRPS7* was described in 2 affected siblings from one family suffering from congenital sensorineural deafness and progressive hepatic and renal failure.[206] A homozygous mutation in *MRPS16* was described in one family with agenesis of corpus callosum, dysmorphism and fatal neonatal lactic acidosis.[207] *MRPS22* mutations lead to cardiomyopathy, hypotonia and tubulopathy in a first family[208] and Cornelia de Lange-like dysmorphic features, brain abnormalities and hypertrophic cardiomyopathy in a second family.[209] *MRPL3* mutations were identified in 4 siblings of the same family presenting cardiomyopathy and psychomotor retardation.[210] A homozygous *MRPL12* mutation was associated with growth retardation and neurological deterioration in a single patient born to consanguineous parents.[211] Finally, a homozygous *MRPL44* mutation was identified in 2 siblings suffering from recessive hypertrophic cardiomyopathy.[212] Since the mammalian mitoribosome (55S) contains more than 80 protein components that make up the 28S SSU and 39S LSU, it is likely that more pathogenic mutations in the constituent polypeptides will be uncovered in the near future. Notably, the A181V amino acid substitution in bL12 affects a highly conserved residue in the C-terminal domain of the protein, whose bacterial homolog has been involved in the interaction of the ribosome with translation elongation factors,[213] suggesting a potential detrimental effect of the mutation on mitochondrial translation efficiency and/or accuracy.[211] The information gained through the study of prokaryotic ribosomes can be highly valuable in the analysis of conserved features. However, uS22 and mL44 are mitochondrion-specific subunits, and the uL3 reported missense mutation is located in a mitochondrial C-terminal extension of the protein absent in the *E. coli* homolog subunit, hampering homology modeling.[210]

Remarkably, mutations in SSU and LSU proteins lead to decreased steady state levels of 12S rRNA and 16S rRNA, respectively[206,207,209-212] and apparent failure to accumulate assembly intermediates. Indeed, *MRPL12* and *MRPL44* mutant fibroblasts present with a reduction in fully assembled LSU without accumulation of subassembly particles at least containing the few subunits tested by immunoblotting (uL3, bL12, mL44 and uL13).[211,212] Unfortunately, no information on the mitochondrial ribosome assembly status is available for SSU pathogenic mutants. In cells and/or tissues from patients carrying mutations in mitochondrial ribosomal subunits, 12S and 16S rRNA amounts tightly correlate with the levels of residual mitochondrial translation and OXPHOS complexes, as well as with the severity of the clinical phenotype. In the case of the homozygous *MRPS16* mutation, the patient died at 3 days of age of untreatable acidosis. The decrease of 12S rRNA steady-state level to 12% of control was accompanied by a dramatic defect in mitochondrial protein synthesis and a combined deficiency of OXPHOS complexes, with the exception of complex II, in muscle and liver homogenate.[207] The known pathogenic mutations in LSU subunits are instead associated with a partial reduction in 16S rRNA level, which results only in a moderate decrease of mitochondrial translation.[210-212] It is intriguing that this mildly reduced efficiency in mitochondrial gene expression does not affect equally the biogenesis of all OXPHOS complexes. In most cases, patients' muscle and fibroblasts present with a combined complex I – complex IV or isolated complex IV deficiency, while activities and steady state levels of complexes II and III are unaffected.[211,212] Complex V is additionally reduced in *MRPL3* mutant cells.[210] Taken together, these observations suggest that the expression of mitochondrial-encoded complex IV subunits is more susceptible to partial deficiencies of the mitochondrial translational apparatus. Moreover, 2 siblings have been reported carrying the same homozygous mutation in *MRPL44*.[212] While the first patient died at 6 months of age of cardiac failure, the second one was an asymptomatic teenager at the time of publication. Fibroblasts from the latter showed a partial reduction of the fully assembled LSU and a dramatic complex IV defect despite an overall unaffected rate of mitochondrial translation, suggesting the existence of additional genetic and environmental factors responsible for the phenotypic variability.[212]

Until present, no mutations have been found in rRNA modification enzymes but there are patients carrying mutations in mitoribosome assembly factors. A mutation in *FASTKD2* was found in a patient with mitochondrial encephalopathy associated with an isolated complex IV deficiency,[214] although the molecular mechanism involved was not investigated. Mutations have been also identified in the 2 components of the heterodimeric AFG3L2/SPG7 mitochondrial m-AAA protease, also called paraplegin. The yeast Yta12/Yta10 m-AAA homolog protease is involved in protein quality control and additionally regulates ribosome assembly by controlling proteolytic maturation of the LSU protein bL32,[134] as explained earlier. Loss of function mutations in AFG3L2 result in either spinocerebellar ataxia type 28[215,216] or spastic ataxia.[217] SPG7 mutations are instead a common cause of hereditary spastic paraplegia,[218] although they have been also identified in cases of optic neuropathy and progressive external ophthalmoplegia.[219,220]

Concluding Remarks and Perspectives

The high-resolution cryo-EM data has revealed unexpected differences between bacterial and mitochondrial ribosomes. The evolution of the mitoribosome is reflected by the differences observed between the yeast and mammalian counterparts. The structural knowledge is expected to speed up our understanding of how mitoribosomes are assembled by facilitating the generation of hypotheses of how specific stages of the process may occur. Mitoribosome biogenesis is complicated by the dual genetic origin of their components. The synthesis of rRNAs within the mitochondrial matrix should ensure co-transcriptional ribosome assembly, perhaps initiating near the nucleoid and finishing at the mitochondriolus. The precise compartmentalization of the different ribosome biogenesis stages needs further investigation. The list of mitoribosome assembly factors, although incomplete, keeps growing. We are still facing the necessity to expand the list, but importantly also the challenge to determine their precise molecular functions, which is most cases remain to be elucidated. New approaches, imported from the bacterial field and adapted to mitochondrial experimental needs, seem necessary. For example,

SILAC-based approaches should allow characterizing the mitoribosome assembly landscape by quantitative mass spectrometry, and high-resolution cryo-EM will facilitate determining the structure of pre-ribosomal intermediates. Classical genetic and biochemical approaches will be always instrumental to analyze the proteins and/or RNA domains that interact with specific assembly factors. New developments in gene-editing in mammalian cells will allow the creation of cell line KOs for ribosome proteins or assembly factors, thus opening a new world of possible investigations. Overall, the next few years are anticipated to witness a further expansion of the mitoribosome assembly field, expected to crystallize on the discovery of intricate molecular details and new biogenetic factors occurring along the maturation pathway of yeast and mammalian mitoribosomes. Furthermore, this knowledge is expected to be instrumental for a better understanding of the pathogenic mechanisms underlying mitochondrial diseases associated with mitochondrial translation defects

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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