Evolution of Translation in Mitochondria

Aldo E. García-Guerrero, Angélica Zamudio-Ochoa, Yolanda Camacho-Villasana, Rodolfo García-Villegas, Adrián Reyes-Prieto and Xochitl Pérez-Martínez

1 Introduction

Around 1.5 billion years ago a bacterial cell related to modern α-proteobacteria established a symbiosis with a eukaryote that originated mitochondria [1]. It is well-established that mitochondrial origin is monophyletic (i.e., it happened only once in evolution) and that the organelle arose from an α-proteobacterium with identity yet to be established [2, 3]. The symbiotic event was followed by extensive reduction of the organelle's genetic material, either by gene loss or gene transfer to the nuclear genome. In addition, mitochondrial DNA (mtDNA) from different lineages diverged extensively in shape, size, content, mutation rate, and gene expression mechanisms. What mitochondria from different lineages have in common is that more than 1000 proteins are present in the organelle [4–7]. However, only a very limited number of proteins are encoded in mtDNA. For example, mtDNAs from the *Phylum* Apicomplexa have only three protein-coding genes [8], animal mitochondria code (in general) for 13 proteins [9], land plants code for more than 30–40 proteins [10], and members of the jakobid protists, which are considered to be relics of the endosymbiont bacterial ancestor, code around 65 proteins [11]. Thus, the majority of proteins necessary for function are

Aldo E. García-Guerrero and Angélica Zamudio-Ochoa have contributed equally to this work.

A.E. García-Guerrero · A. Zamudio-Ochoa · Y. Camacho-Villasana · R. García-Villegas · X. Pérez-Martínez (✉)
Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico
e-mail: xperez@ifc.unam.mx

A.E. García-Guerrero
e-mail: aldog.2602@gmail.com

A. Reyes-Prieto
Department of Biology, University of New Brunswick, Fredericton, New Brunswick, Canada

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imported into mitochondria from the cytosol (for a review, see [12]). The organization of mtDNA among lineages has also diverged. Some organisms have extended non-coding regions, including type I and type II introns, as is the case for land plants, while others, like metazoan, have very compact mitochondrial genomes with only a few hundreds non-coding regions [9]. While fungal and apicomplexan mtDNAs are lineal molecules, animals and some protists have circular mtDNA, trypanosome mtDNA is composed of minicircles and maxicircles, which are topologically intertwined [13], and some *Amoebidium* have several hundred linear DNA molecules with different gene contents in each molecule [14]. In land plants, mtDNA is arranged in circular molecules of DNA whose composition varies constantly as a high frequency of recombination events occurs in this clade [10].

Independent of the shape, coding capacity and size of mtDNA, these organelles contain a complete gene expression system that comprises DNA replication and maintenance, transcription, post-transcriptional processing, translation and post-translation functions, such as protein assembly and prosthetic group additions. Much of the mitochondrial expression machinery is nucleus-encoded, while only a limited set of mtDNA genes is coded in the organelle. The *Phylum* Apicomplexa has only two ribosomal RNAs coded by the mtDNA. Metazoans have around 22 tRNAs and the small and large subunits rRNAs, while land plant mtDNA in addition to tRNAs and rRNAs codes for a varied number of ribosomal proteins. Protists from the jakobid lineage code in addition a translation factor (TufA), 5S rRNA, RNA polymerase and a sigma factor, and three chaperones for protein processing [11]. In general, mitochondrial genomes code for subunits of respiratory complexes and ATP synthase. Apicomplexan mtDNAs code for only subunits 1 and 3 of cytochrome *c* oxidase and cytochrome *b* from complex *bc1* [8]. In contrast, the jakobid *Phylum* codes for 12 subunits from complex I (NADH dehydrogenase), 3 for complex II (succinate dehydrogenase), 1 for *bc*1 complex, 3 for cytochrome *c* oxidase and 6 for ATP synthase [11].

Since mitochondria evolved from an α-proteobacterial ancestor, one might expect that the mtDNA expression mechanisms have conserved bacterial features. Even when this is the case, many novel mechanisms to control mtDNA expression have emerged and diverged among the eukaryotic groups. Some are conserved among certain lineages, but others appeared later during eukaryote divergence. In the present chapter, we describe the most prominent features of the mitochondrial translation machinery across different eukaryotic lineages. This knowledge allows us to better understand the evolution of the translation process in mitochondria.

2 The Mitochondrial Genetic Code

Translation in jakobid and land plant mitochondria uses the universal genetic code in mitochondria [11, 15]. However, at least 27 genetic code alterations (i.e., codon reassignments) are detected in mitochondrial systems of diverse eukaryote lineages (reviewed in [16, 17]). One of the most common changes in mitochondrial genetic code is the reassignment of termination codons to sense codons, such as the use of

Table 1 Mitochondrial genetic code in different organisms

	Group/organism	Genetic code	Comments
Protists	Jakobida	Standard	
	Euglenozoa Alveolata Rhizaria Amoebozoa Malawimonads	*UGA* Stop → Trp	Alternative initiation codons *Trypanosoma spp*: *UUA*, *UUG*, *CUG* *Leishmania spp*: *AUU*, *AUA* *Tetrahymena spp*: *AUU*, *AUA*, *AUG* *Paramecium sp: AUA, AUU,AUC, GUG, GUA*
Fungi	All fungi	*UGA* Stop → Trp	
	Saccharomyces cerevisiae, Candida glabrata, *Hansenula saturnus*, *Kluyveromyces thermotolerans*	*AUA* Ile → Met *CUU* Leu → Thr *CUC* Leu → Thr *CUA* Leu → Thr *CUG* Leu → Thr *UGA* Stop → Trp *CGA* Arg → Absent *CGC* Arg → Absent	*AUA* is frequently used in *VAR1* gene
Metazoa	Invertebrates	*AGA* Arg → Ser *AGG* Arg → Ser *AUA* Ile → Met *UGA* Stop → Trp *UAA* Stop → Tyr	In some flat and round worms: *AAA* Lys → Asn In ascidians: *AGA*, *AGG* Arg → Gly
	Vertebrates	*AUA* Ile → Met *UGA* Stop → Trp	Alternative initiation codons: *Bos taurus*: *AUA* *Homo sapiens*: *AUA*, *AUU* *Mus musculus*: *AUA*, *AUU*, *AUC* *Gallus gallus*: *GUG*
Non-chloropycean algae	Rodophyta Haptophyta	*UGA* Stop → Trp	
Viridiplantae	Chlorophyta	*UAG* Stop → Leu	Also found in *Scenedesmus obliquus*: *UCA* Ser → Stp
	Embryophyta	Standard	Alternative initiation codons: Gymnosperms and angiosperms: *ACG* *Cycas taitungensis*: *GCG* Alternative termination codons: Gymnosperms and angiosperms: *CGA*

Based in http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi

the canonical *UGA* stop codon to decode tryptophan in numerous biological groups [18] (Table 1). Other cases include the use of the typical *UAG* triplet as a leucine codon in chlorophycean algae [19] and the codon *UAA,* which decodes tyrosine in the nematode *Radopholus similis* [20]. Non-standard stop codons are used in mitochondria from some lineages. For example, the chlorophycean algae *Scenedesmus obliquus* uses the *TCA* codon as a translation stop signal [21]. In bryophytes and vascular plants, the codons *CAA*, *CGA* and *GGU* are reassigned stop codons, while *AAA* and *AAU* are recognized as stop codons in *Oryza sativa* [22]. In vertebrate mitochondria, the *AGA* and *AGG* codons, which are universally assigned to arginine, were thought to become stop codons [23]. However, recent studies indicate that these codons are unassigned [24]. Other prevalent reassignment is the use of *AUA* in *Saccharomyces cerevisiae*, vertebrates and some invertebrates to decode methionine instead of the canonical isoleucine [25, 26]. The standard arginine codons *AGA/AGG* were reassigned to serine in certain invertebrate groups (Nematoda, Arthropoda) and decode glycine in Ascidians [27]. In *S. cerevisiae,* the typical arginine codons *CGA/CGC* are unassigned, and the triplets *CUU/CUC/CUA/CUG* are used for threonine instead of leucine [28]. In some invertebrates (flat and round worms), *AAA* was reassigned, from lysine to asparagine (for an example, see [29]). Atypical start codons are also present in mitochondrial systems. For example, humans use *AUA* and *AUU* as start alternatives [24]; other cases of alternative start codons occur in trypanosomatids, which use *UUA, UUG, CUG* and the ciliate *Tetrahymena* with *AUU, AUA* or *AUG* [30] and certain nematodes that use the *UUG* triplet to initiate protein translation [31].

Why did mitochondria acquire modified codon assignments during evolution? One explanation is that codon reassignments might be a consequence of the organelle genome reduction, which encodes for a small set of proteins, and in most cases for a small number of tRNAs [8–10]. The diversity of mitochondrial genetic codes across eukaryotic groups might also reflect differential mutational rates in mtDNAs, a general increase in AT content and a diversification of genome expression mechanisms [17]. Interestingly, in silico studies suggest that genome size is not correlated to incident mutations that could lead to codon reassignments (i.e., the size of mitochondrial genomes does not correlate with mutation rates) [16]. The tRNAs' structure, the mitochondrial-targeted aminoacyl tRNA synthetases and in general the translation machinery are adjusted to the mitochondrial genetic code of each eukaryotic group. For example, reassignment of *UGA* for tryptophan (instead of the stop codon) is mediated by a tRNA where the wobble position carries a modified uridine. Modifications include 5-taurinomethyluridine (τm^5U), 5-carboxymethylaminomethyl-2-thio-uridine ($cmnm^5s^2U$) or 5-carboxymethylaminomethyluridine ($cmnm^5U$). These modifications expand the decoding capacity to R-ending codons, enabling the decoding of UGG and UGA as tryptophan [32]. Decoding of mammalian *AUG* and *AUA* as methionine is possible because the met-$tRNA^{Met(CAU)}$ has a 5-formylcytidine (f^5C) in the wobble position [33, 34]. Some theories try to explain how reassignments in the mitochondrial genetic code might have occurred during evolution. Two of the most established theories are the *Codon Capture* and *Ambiguous Intermediate* models.

The *Codon Capture,* also termed the *Codon Disappearance* theory, proposed by Osawa and Jukes in 1989, postulates that genetic code alterations are the result of neutral changes associated with the GC/AT content balance [35–38]. The theory posits that the disappearances of both the codon and the decoding tRNA are fundamental steps for further codon reassignment. Later, the "lost codon" can be reintroduced into the system by new mutations, but now is decoded at a relatively low efficiency by a different, noncognate tRNA, but with a similar anticodon sequence that allows the "capture" of the recently reestablished codon. Some reassignments are consistent with this *Codon Capture* model, such as the case of the frequent reuse of the *UGA* triplet to decode tryptophan [16]. A prediction derived from this model is that in mitochondrial genomes, which are high in AT content, GC-rich codons disappear at higher frequencies than AT-rich codons [36, 38, 39]. However, some codon reassignments in mtDNA do not follow the predictions of the GC/AT content balance. Thus, the *Codon Capture* theory does not explain satisfactorily the use of GC-rich codons in genomes with high AT content or the fact that some codons seem to be unassigned in some mtDNA genetic systems.

The *Ambiguous Intermediate* theory, proposed by Schultz and Yarus [40, 41], suggests that codon reassignment is the result of selective mechanisms that favor ambiguity in codon recognition during protein translation. The model postulates that codon recognition ambiguity, associated with structural changes in the tRNA molecules, is fundamental for the codon reassignment. The idea is that the codon in the spotlight is suddenly decoded by two different tRNAs, namely the "original" and the new "mutant," which is now able to form a cognate pair with the codon. Later the "mutant" tRNA takes over the codon in a selection-driven process. Thus, the triplet is reassigned to a new amino acid. During mitochondrial evolution, many repeated tRNAs for each amino acid were lost, and in general mitochondria contain only one tRNA for each amino acid [42]. In contrast to the *Codon Capture theory*, in this model the initial loss of the codon before the reassignment is not necessary [37]. Some examples consistent with the *Ambiguous Intermediate* theory are the reassignments of leucine to threonine in yeast mitochondria [17] and from serine to lysine in Arthropoda [43].

Overall, both models are not mutually exclusive, as reassignments might have arisen from combinatory events during evolution [17, 44]. Some changes in the mitochondrial genetic code are explained by the *Codon Capture* theory, while others by the *Ambiguous Intermediate* theory.

3 Mitochondrial tRNAs

Translation of mitochondrial mRNAs requires around 20 tRNAs, but the exact number varies depending on the wobble rules and the genetic code in each species. Mitochondrial tRNAs have nuclear and mitochondrial origins. Depending on the organism, the proportion of nuclear and mitochondrial tRNAs varies. While human and the jakobid *Andalucia godoyi* [9, 11] encode a complete set of mitochondrial

encoded tRNAs for reading all codons in mtDNA, protist-like *Trypanosoma brucei* and *Plasmodium falciparum* have no mtDNA-coded tRNAs [8, 45] and therefore have to import all tRNAs necessary for translation. Interestingly, the number of mtDNA-derived tRNAs among closely related organisms is variable. For example, in chlorophycean algae, *Chlamydomonas reinhardtii* codes for only 3 tRNAs in mitochondria, while *Nephroselmis olivacea* codes for a full set of 26 tRNAs [46]. It is expected that mitochondria would import only the necessary number of tRNAs to complete the ~22 tRNAs necessary for translation. However, in some cases, import of redundant tRNAs can take place. For example, in the yeast *S. cerevisiae* a $tRNA^{Lys}_{(CUU)}$ is imported from cytosol even when mtDNA codes for the full set of tRNAs necessary to decode all codons [28, 47]. This tRNA is particularly important to decode codons under stress conditions [48]. Mammalian mitochondria can also import redundant cytosolic tRNAs [49]. The unicellular algae *C. reinhardtii* imports 31 tRNAs instead of the expected 22 tRNAs necessary to decode all codons [50]. The mechanisms to import cytosolic tRNAs are particular to each eukaryotic group, indicating that import of tRNAs into mitochondria is a process that emerged independently several times during evolution (for a review, see [51]). Delivery of tRNAs to mitochondria is mediated by proteins, usually with a previously described function. *S. cerevisiae* Eno2 (involved in glycolysis) delivers the charged $tRNA^{Lys(CUU)}$ to the mitochondrial surface, where the mitochondrial lysyl-tRNA synthetase binds it and co-transports it via the general import machinery. In land plants, aminoacyl-tRNA synthetases might be involved in the delivery of tRNAs to mitochondria, and the Voltage Dependent Anion Channel (VDAC), together with the outer membrane receptors Tom20 and Tom40, functions in tRNA import. In *Trypanosoma*, the cytosolic EF1a, together with the import component Tim17 and Hsp70, Hsp60 and Hsp20 might participate in the delivery and import of cytosolic tRNAs.

The structure, sequence and post-transcriptional modifications of mitochondrial tRNAs have conserved features with cytosolic RNAs. However, many of these features have amazingly diverged in different eukaryotic groups and among specific tRNAs from the same organism. According to the structural characteristics, mitochondrial tRNAs are classified into five groups, named 1–5 [51]. Group 1 shares the most conserved features with cytosolic tRNAs. They carry canonical T and D arms, anticodon and acceptor arms, and L1/l2 connectors (involved in joining the acceptor and anticodon helices) [52]. This class of tRNAs is present in mitochondria from amoebozoans, alveolates, plants and fungi. Group 2 carries conserved anticodon and acceptor arms. However, T/D arms may be smaller in size and may have less conservation on bases involved in D/T-loop interactions (mainly bases G18, G19, U55 and C56). These tRNAs are present in amoebozoans, alveolates, plants, fungi and some metazoans (including mammals). Group 3 consists of tRNAs where the acceptor arm may be 1–3 nucleotides shorter; they are T-armless and carry a shorter D-loop. The L2 connector is also shorter (6–7 nt instead of 21–30 nt). This class of tRNAs is present in some nematodes, bryozoan and arachnid species. Group 4 is represented by some insect and bryozoan species and by mammals. They are D-armless and carry shorter T arms. The L1 connector is also shorter (5–12 nt instead of 19–20 nt). Group 5 carries both shorter anticodon and

acceptor arms; they are T- and D-armless and have shorter L1 and L2 connectors. These minimalist tRNAs are found in acaria and some nematodes. In this group, the only conserved features with cytosolic tRNAs are the presence of an acceptor arm with the 3′-single-stranded CCA terminus and an anticodon arm with the canonical anticodon loop of seven nucleotides. The shortest mitochondrial tRNA so far is 54-nt long ($tRNA^{Ser(UCU)}$) from the nematode *Ascaris suum* [53, 54].

As a universal feature, mitochondrial tRNAs are also post-transcriptionally modified to define the structure and decoding capabilities. The best understood model is *Bos taurus*, where all mitochondrial tRNAs were isolated and analyzed. There are 15 types of modifications at 118 positions (representing 7.5 % abundance in mitochondrial tRNA bases) [55]. However, the occurrence of modified nucleotides can be as low as one residue in mitochondrial $tRNA^{Ser}$ of the rodent *Mesocricetus auratus* (representing 1.7 % abundance) [56]. To date, 15 out of 18 conserved modifications (present throughout kingdoms of life) are observed in mitochondrial tRNAs, with the exception of ac^4C, m^3U and $m^{66}A$, which are not yet detected (reviewed in [51]). Comparative analyses of tRNA sequences indicate that mitochondria have the highest number of modified positions that are not universally conserved. The acceptor stem is particularly rich in Ψ residues, and the number of modifications located in positions 46–50, 5′ to the T arm, is also relatively low in mitochondrial tRNAs [55, 56]. There are mitochondria-specific base modifications, like τm^5U and τm^5s^2U, discovered in ascidian mitochondria [32], f^5C, f^5Cm, present at the wobble position 34 in bovine and the nematode *A. suum* [33, 54], and k^2C in potato [57].

4 Mitochondrial mRNAs

Mitochondrial mRNAs have conserved some prokaryotic features, but some other characteristics have diverged. Mitochondrial mRNAs from some lineages, such as jakobid protists, have a putative Shine-Dalgarno-like sequence to locate the ribosome at the correct AUG start codon [11]. Other lineages lack a Shine-Dalgarno-like sequence and therefore must have different, unknown mechanisms to initiate translation. This is the case for flowering plants [58] and mammal mitochondria [59]. Similar to what is observed in prokaryotes, mitochondrial mRNAs do not have a 7-methylguanylate cap (5′-cap), as is found in cytosol mRNAs. Moreover, mitochondrial mRNAs undergo post-transcriptional modifications before they are ready for translation. The major post-transcriptional RNA processing events in mitochondria include 3′-end polyadenylation, intron/exon splicing and editing. Polyadenylation of RNA is present in all kingdoms of life and is a near-universal feature of RNA metabolism, although it can trigger different signals among cells and organelles. Today, the function of polyadenylation in mitochondrial gene expression is not fully understood. An additional interesting feature of mitochondrial mRNAs from some lineages is the requirement of RNA edition before translation. RNA editing might be important to correct transcript sequences that otherwise would affect the translation product's function [59, 60].

An example is editing in land plants, where the amino acid encoded by an edited mRNA is frequently more conserved than the one predicted from the gene sequence.

4.1 Polyadenylation of Mitochondrial mRNAs

Polyadenylation is the non-template addition of adenosine residues to the 3′ end of RNAs. In the eukaryotic cytoplasm, the majority of nuclear-encoded mRNAs require a poly(A) tail for stability, nuclear export and translatability (for reviews, see [61, 62]). In contrast, in prokaryotes, RNA polyadenylation functions to tag the mRNA for exonucleolytic degradation [63, 64]. Although mitochondria have a monophyletic origin, many features of polyadenylation have extensively diverged within eukaryotes.

In mammalian mitochondria, 12 out of 13 mRNAs have stable poly(A) tails of 45 nt on average. However, there are slight variations between cell types and between transcripts within the same cell type [65]. For example, only the *ND6* transcript lacks a poly(A) tail [66]. The precise function of polyadenylation is not entirely understood. However, one function of polyadenylation is to complete the UAA codon, since several mammalian RNAs contain incomplete translational stop codons. The same feature is observed in general in metazoans, where some coding regions lack a complete UAA stop codon, suggesting that polyadenylation also plays an important role in translation [67, 68]. Although polyadenylation produces stable transcripts [66, 69], truncated, adenylated transcripts may coexist, suggesting that human mitochondria use transient poly(A) tails to degrade RNA [70]. The mechanism of a possible differential polyadenylation on stabilizing and destabilizing RNAs remains to be elucidated. In plants, similarly to the bacterial system, addition of a poly(A) tail targets exonucleolytic degradation of RNA [71]. In trypanosomatid mitochondria, most protein-coding transcripts suffer a massive edition (insertion or deletion of uridines) necessary to render translatable mRNAs [59]. The addition of a poly(A) tail in these organisms seems to render both stable and unstable transcripts. Polyadenylation in these organisms has an intricate relation to edition and translation. Poly(A) tails are 20–200 nt long, and the length of the tail seems to correlate with the state of edition. Short tails ($\sim$20 nt) stabilize edited or non-edited mRNAs. Long (100–200 nt) poly(A/U) tails are added to fully edited RNAs, and this extension might render the transcript translationally competent [72, 73].

Yeast mitochondria are so far the only organelles that do not polyadenylate their mRNAs. This was found to be the case in *S. cerevisiae, Schizosaccharomyces pombe* and *Candida albicans* [74–77], suggesting that it might be a general phenomenon of fungal mitochondria. Instead, the 3′ ends of some, but not all fungal mitochondrial mRNAs possess a conserved dodecamer sequence that is encoded in the mitochondrial genome and seems to be vital for mRNA stability and translatability [75, 76, 78, 79].

4.2 Edition of Mitochondrial mRNAs

Some organisms require mitochondrial (and plastid) transcript edition before they can be translated. RNA editing consists of nucleotide substitutions, post-transcriptional or co-transcriptional insertion/deletions. These three processes occur in very different taxonomic groups, suggesting that they arose as several independent acquisitions [80]. This process is present in dinoflagellates (variable one-nucleotide substitutions), excavates (U insertions/deletions), unikonts (co-transcriptional insertion of 1 or 2 nucleotides), metazoa (U to C substitution) and archaeplastida (U to C and C to U substitutions) [80]. Editions throughout a transcript can be limited in number, as is the case for land plants [60, 81]. In other cases, extensive edition of a transcript is required to transform an unrecognizable sequence into a conserved protein sequence, as is the case of trypanosomatids [59, 82] and calcaronean sponges [83].

In land plants, C to U (and less frequently U to C) editing often results in changes of the amino acid sequence from what the genomic sequence predicts. This process evolved in land plants [84] and was most likely subsequently lost in some marchantiid liverworts [85]. The number of edited nucleotides among plant lineages is: *Physcomitrella patens* edits 11 sites [86], *Arabidopsis thaliana* edits 600 cytidines [87], while the lycophytes *Isoetes engelmanii* and *Selaginella moellendorfii* edit more than 1,700 and 2,100 nucleotides, respectively [88, 89]. The composition of the RNA editosome is not yet fully understood, although *cis*- and *trans*-factors are essential for the editing process. The *cis* elements that specify the editing of the C target are present in close proximity to the edition site. *Trans*-factors include members of the pentatricopeptide repeat (PPR) motif-containing family, which are site-specific recognition factors. While the cytidine deaminase catalyzing C-to-U conversion has not been identified, considerable evidence points to the C-terminal DYW domain found on some PPR proteins, which exhibits sequence similarity to known cytidine deaminase motifs (for reviews, see [60, 80, 81]).

In trypanosomatids, edition is a post-transcriptional process, where uridines are inserted or deleted from mRNA precursors [90]. Edition introduces start and stop codons, restores frame shifts and often completes the coding sequence of mRNAs. Mitochondrial editing can occur at different extensions: transcripts that are never edited, transcripts where edition is restricted to a small region, with minimal edition, and transcripts that are extensively edited or pan-edited, where a single mRNA is altered by 553 insertions and 89 deletions [80]. The process in trypanosomatids includes mRNA cleavage, U deletion or insertion, and mRNA ligation [91]. The maxicircle molecules of mtDNA code for guide RNAs (gRNAs), which are derived from scattered intergenic regions. A partial hybrid is formed between the 5′ portion of the gRNA and the complementary sequence on the pre-edited mRNA. Cleavage of the mRNA at the 3′ end of the first base that is not paired with the gRNA leaves a free 3′OH. The uridine addition or deletion is followed by immediate relegation of the two molecules. Many proteins have been implicated in the edition process (reviewed in [59, 82]). However, these proteins are not related to the proteins involved on plant edition.

5 The Mitoribosome

Mitochondrial ribosomes (mitoribosomes) are located in the matrix, and are closely associated with the inner membrane [92, 93]. This location facilitates the insertion of newly synthesized products, which are mainly hydrophobic proteins. All mitochondrial genomes currently sequenced encode ribosomal RNAs (rRNAs). In contrast, almost all mitochondrial ribosomal proteins (MRPs) are nuclear encoded. Thus, assembly of functional ribosomes requires a coordinated expression of both genomes and a proper import of the necessary components into the organelle [94, 95]. The mechanism of this process is almost unknown, but evidence supports that several MRPs assemble with rRNAs in a co-transcriptional fashion [96, 97].

In contrast to the cytosolic ribosomes, mitoribosome composition is highly variable between different eukaryotic lineages. Their sedimentation coefficient ranges from 80S in ciliates, to 70–74S in fungi, to 77–78S in vascular plants and 55S in animals. These variable sedimentation values are the result of the difference in the protein:RNA ratio, while bacterial ribosomes contain a protein:RNA proportion of 1:2, in mitoribosomes this proportion varies from 1:1 in yeast to 2:1 in bovine [98].

The α-proteobacterial ribosome is composed of 54 proteins [99], which were also likely to be present in the ancestor of mitochondria. It is proposed that, in the earliest stage of eukaryotic evolution, several novel proteins were recruited for ribosomal function, and only one, Rps20, was lost, resulting in an ancestral mitoribosome of 72 proteins (Fig. 1) [100]. An interesting feature of several mitoribosomal proteins of bacterial origin is that they increased in length sequence. Accordingly, this stage in mitoribosome evolution is known as the "constructive phase", as the total size of the ribosome was increased considerably [101].

The cause of the constructive phase of the mitoribosome is proposed to be the accumulation of slightly deleterious mutations on the mitochondrial genome, as this genome, with the exception of land plants, presents a higher mutation rate than the nuclear one [103, 104]. Slightly deleterious mutations could trigger the recruitment of new proteins because a mutation in an original component of the complex is compensated by the interaction with a new component [105]. This process is called Constructive Neutral Evolution (CNE), a universal evolutionary ratchet that leads to complexity [106]. Accordingly, genes coding for MRPs show higher levels of amino acid replacements than cytoplasmic ribosomal proteins, which suggests a compensatory modification [107, 108]. Gain of complexity throughout the evolution of mitochondria is not exclusive to the mitoribosome. The respiratory chain complexes have also acquired new proteins that are usually important for regulation, assembly and stability [101]. These eukaryotic subunits are in general localized in the peripheral regions of the enzymes. This feature is also observed for the mitoribosomes [109, 110]. The extensive gain of protein mass observed for mitoribosomes does not reflect the fate of all endosymbiotic organelles, as the plastid ribosomes only gained approximately 170 kDa [111, 112]. Several evolutionary mechanisms have led to the increase of protein mass in the mitoribosome.

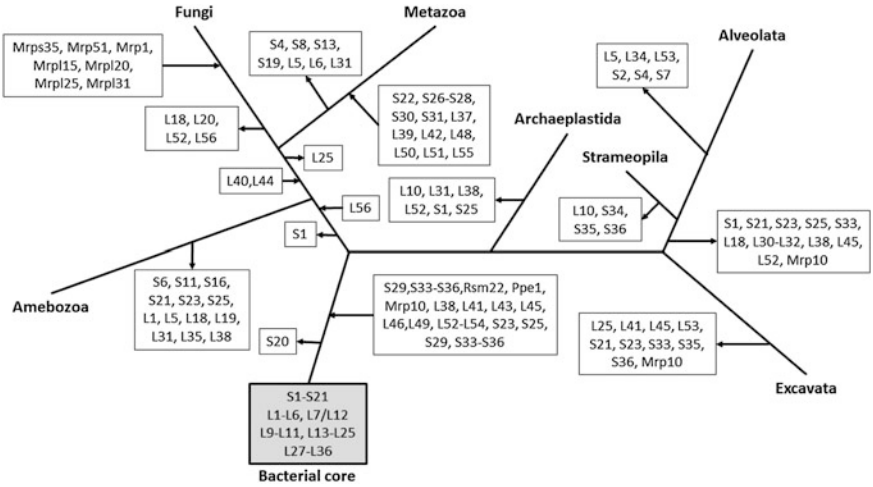

Fig. 1 Reconstruction of the evolutionary history of the mitochondrial ribosome proteome. Incoming and outgoing arrows indicate the gains and losses of the ribosomal proteins that are showed in the box. This figure is based on the data given by [102] and [100]. The models considered for the construction of this figure were: for fungi *Neurospora crassa, Aspergillus fumigatus, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Cryptococcus neoformans, Ustilago maydis* and *Encephalitozoon cuniculi*; for metazoa *Mus musculus, Homo sapiens, Danio rerio, Drosophila melanogaster, Caenorhabditis elegans* and *Monosiga brevicolis*; for amoebozoa *Dictyostelium discoideum* and *Entamoeba histolytica*; for Archaeplastida *Arabidopsis thaliana, Oryza sativa, Chlamydomonas reinhardtii, Ostreococcus tauri* and *Cyanidioschyzon merolae*; for Strameopila *Thalassiosira pseudonana and Phytophthora ramorum*; for Alveolata *Tetrahymena thermophila, Paramecium tetraurelia, Theileria annulata, Plasmodium falciparum, Plasmodium yoelii* and *Cryptosporidium parvum* and for Excavata *Leishmania brasilensis, Leishmania infantum, Leishmania major, Trypanosoma cruzi, Trypanosoma brucei, Naegleria gruberi, Trichomonas vaginalis, Giardia lamblia* and *Reclinomonas americana*

One of them was the recruitment of existent proteins, such as the case of Mrpl45, a homolog of Tim44 (a subunit of the mitochondrial protein translocase machinery), which is present in several bacteria but is not part of the prokaryote ribosome [102]. Mrpl39, a metazoan protein, was recruited later in evolution and is homologous to threonyl-tRNA synthetases [113]. It is proposed that addition of Mrpl39 to the mitoribosome compensated for the loss of bacterial proteins involved in tRNA binding [102]. Numerous new ribosomal proteins emerged through gene duplication. For instance, Mrps10 gave rise to Mrpl48 through this process in metazoans. Interestingly, the duplicated gene product became part of the other ribosomal subunit [102]. Another case is Mrps18, which in *Caenorhabditis elegans* has three variants originated by gene duplication. It is believed that each ribosome contains only one copy of the protein, suggesting that mitoribosomes exist in heterogeneous populations [102].

The increment in protein mass in mitoribosomes is not only due to the addition of new subunits, but also to the gain of new domains in the prokaryotic proteins.

MRPs are sometimes almost twice the size of their bacterial counterparts [102]. In *P. falciparum*, Mrpl4 has an AAA domain, which is not present in the bacterial counterpart. This domain is known to participate in chaperone-like functions [114]. Another case is the presence of an RRM (RNA recognition motif) domain in Mrps19 of *A. thaliana*, which could be involved in the association of the protein with rRNAs [115]. In the yeast *S. cerevisiae*, the carboxyl-terminal end of Mrp20, which is mitochondria-specific, plays a role in ribosome assembly [116].

The evolution of mitochondria involved numerous independent losses of ribosomal proteins in different lineages (Fig. 1). Bacterial-exclusive S20 protein seems to have been lost early during mitoribosome evolution. This protein is not essential for bacterial growth. However, its absence causes a decrease in the association of the ribosomal subunits [117, 118]. In contrast, S1 protein, which was lost early in the evolution of unikonts, is an essential protein in bacteria [119]. Moreover, there is no apparent pattern favoring protein loss from either bacterial or eukaryotic origin, suggesting that there is no tendency in protein dispensability [100].

Whereas protein gain in mitoribosomes is a general phenomenon in all lineages, the rRNA content varies greatly. While bacteria have an rRNA content of 1.4 MDa, in mitochondria this number varies from 0.5 MDa in *C. elegans* to 1.6 MDa in *Neurospora crassa*. Since animals show an important reduction of rRNA, it was previously thought that the proteins acquired during mitoribosome evolution replaced the lost helices of rRNA [120]. However, now it is clear that the high content of proteins in mitoribosomes is not a consequence of the lower concentration of rRNA, as the increase in MRPs occurred previously to the reductive phase of rRNA [101]. This is consistent with the structural data in which the extra proteins of the ribosome do not substitute the lost portions of rRNA [121–123]. Furthermore, it is proposed that rRNA reduction might be driven by the reduction of the mitochondrial genome size and not necessarily by adaptive changes of the translational machinery [124].

Reduction of rRNA had triggered an important mitoribosome remodeling. For example, the bacterial ribosomal protein L24 contacts the helices H7 and H19 of the 23S rRNA, stabilizing its binding to the 39S subunit. The mammalian mitochondrial counterpart, Mrpl24, lacks both helices. However, mitochondria-specific protein elements maintain Mrpl24 in the same place and orientation as the prokaryotic counterpart [125].

An almost general phenomenon in mitochondria is the loss of 5S rRNA, which is present only in plants and some algae. An extension of the 23S rRNA replaces the resulting gap in *N. crassa*. On the contrary, in the mammalian mitoribosome this space is occupied by protein [121–123].

An interesting aspect of the evolution of mitoribosomes is their assembly mechanisms. This process has probably evolved differently in each lineage, as the components of the ribosome are partially different among eukaryote groups. In some lineages mitochondrial-encoded rRNAs are fragmented, need edition or lack 5S rRNA [8, 9, 126]. As stated above, the composition of proteins also diverged among eukaryote lineages. The understanding of ribosome assembly, the order of

rRNA processing and protein addition, the chaperones involved in such events and the role of mitochondrial RNA granules in ribosome biogenesis are just starting to emerge, especially in mammals and yeast models [97, 127].

6 Mitochondrial Translation Initiation

Translation initiation in bacteria is carried out by three conserved factors: IF1, IF2 and IF3 [128]. There are important differences between prokaryote and mitochondrial initiation factors. While mitochondrial $IF2_{mt}$ is universally present, $IF3_{mt}$ is semi-universal and $IF1_{mt}$ was completely lost from the mitochondrial machinery [129]. In addition, there are important structural variations in the mitochondrial initiation factors. In agreement with the prokaryotic origin, mitochondria seem to initiate translation with formylated methionine, at least for the studied cases. Initially, by in vitro experiments, it was demonstrated that the initiation machinery in mammals does not need a formylated Met-tRNA. However, recent experiments demonstrate that a failure in formylation is a cause of disease in humans [130, 131]. In the yeast *S. cerevisiae* it was previously shown that a mutant *Δfmt1* (coding for a methionyl-tRNA formyltransferase) does not affect translation initiation [132]. However, an accessory factor, Aep3, was compensating the lack of *Δfmt1*. Double mutant *Δfmt1* and *Δaep3* affect respiratory growth [133]. The mechanisms of translation initiation regulation have extensively diverged from the bacterial counterpart. Despite the differences between bacterial and mitochondrial translation initiation factors, the general steps for initiation are conserved.

6.1 Structural and Functional Conservation of $IF2_{mt}$

In bacteria, IF2 interacts with initiator fMet-tRNA and promotes binding with the small ribosomal subunit and with mRNA. It also contains a GTPase activity to release all initiation factors from the completely assembled ribosome into the mRNA. IF2 triggers the binding of tRNA to the incomplete P site on the 30S subunit. After binding of the 50S subunit to the initiation complex, IF2 GTP hydrolysis assists the release of all initiation factors from the completely assembled ribosome [128]. Bacterial IF2 contains six domains (I–VI). To date, the function of domain I is not completely understood. Domain II stabilizes the interaction of IF2 with the ribosomal 30S subunit; this region is not conserved among bacterial species. Domain III is a linker between domains II and IV. Domain IV contains the GTPase activity. Domain V interacts with the ribosomal 30S subunit, and domain VI recognizes the fMet-tRNA [134]. In mitochondria, $IF2_{mt}$ consists only of domains III–VI (Fig. 2). This short version of $IF2_{mt}$ is not particular for mitochondrial systems, as shorter versions of IF2 factors are also present in some bacterial groups, like extremophiles [135]. Instead, in mammals $IF2_{mt}$ interaction

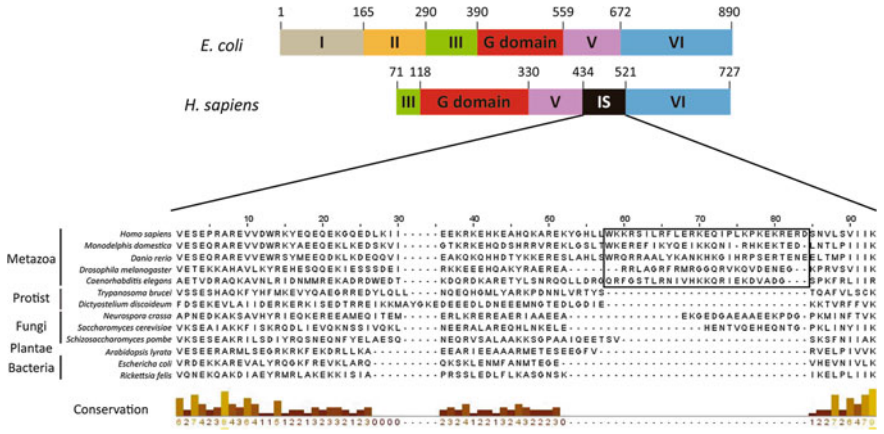

Fig. 2 Alignment of the insertion sequence on mIF2 among species from different phyla. Variations in domain composition between *E. coli* IF2 and *Homo sapiens* mIF2 are presented in the upper panel. Numbers indicate amino acid positions. The insertion sequence (IS) is amplified, and an alignment among different species is shown. Alignments of the insertion sequences were made with the MAFFT software, with the Blosum70 matrix as in [129]

with the 28S is performed by domain III, and this interaction is even stronger when GTP is bound to domain V [136]. Domain III of $IF2_{mt}$ is not conserved in all eukaryote lineages, but its function might be compensated by the differences in the small ribosomal subunit protein and RNA content among eukaryotes [137]. Domain IV is the most conserved region of $IF2_{mt}$ in structure and sequence similarity, sharing 99 % (metazoans) to 50 % (fungi) identity with bacterial counterparts [138–140]. Domain V in $IF2_{mt}$ is modestly conserved with the bacterial IF2, sharing identity of 35–50 %. However, the function of this region is not completely understood [140]. It might be important for interaction with the small ribosomal subunit because structural modeling of $IF2_{mt}$ suggests that this region is similar to domain II of EF-Tu and EF-G. This region is important for contact with small ribosomal subunits [141]. Interestingly, domain V in metazoa $IF2_{mt}$ contains an insertion of variable length and sequence (Fig. 2). This region might perform the same function as IF1 [142] (discussed below). Domain VI in bacteria and mitochondria is divided into subdomains C1 and C2. Subdomain C2 is important for binding of IF2 to the fMet-tRNA [143]. Mutagenic analysis in *Bacillus stearothermophilus* shows that there are two critical cysteines at the 668 and 714 positions necessary for this interaction [144, 145]. These amino acids are usually present in $IF2_{mt}$, suggesting that $IF2_{mt}$ subdomain C2 conserved the same function as in bacteria. $IF2_{mt}$ contains the C1 subdomain. However, as in bacteria, the function of this domain is still unknown. By NMR studies it was suggested that this subdomain from *Bacillus stearothermophilus* has a similar structure as domain III from eukaryotic eIF5B. This region is implicated in transmitting and amplifying structural changes to the G-domain after GTP binding [144, 146].

6.2 The Mystery of the Lost IF1 in Mitochondria

In bacteria, IF1 plays an important role in the recognition of the correct *AUG* initiation codon. IF1 interacts with the A site of the 30S ribosomal subunit and prevents binding of the initiator aminoacyl-tRNA to the A site. In addition, bacterial IF1 increases the affinity of IF2 for 30S, has a role in small subunit dissociation and assists the release of IF2 form the 70S complex [128, 147]. So far, biochemical and bioinformatic approaches have failed to identify mitochondrial IF1. This suggests that IF1 was lost at the earliest stage of eukaryotic evolution [129, 148]. Mitochondria may be able to bypass the need for IF1: Experimental evidence indicates that, in the presence of mammalian $IF2_{mt}$ and $IF3_{mt}$, the bacterial ribosome does not need IF1 for the formation of the 70S particle or translation in general [149]. As discussed above, metazoan $IF2_{mt}$ contains an insertion between domains V and VI [129] (Fig. 2). Even though there is no conservation of the insertion sequence among eukaryotic $IF2_{mt}$s, it is possible that this insertion substitutes the function of IF1, at least in metazoa. Through cryo-electron microscopy and nuclease digestion experiments, it was observed that bacterial IF2 associates with the interphase of the 30S subunit [134, 148]. Modeling of mammalian $IF2_{mt}$ suggests that the extension is close to the small subunit A site, similar to bacterial IF1 [140]. How the need for IF1 is bypassed in other eukaryotic lineages remains an open question.

6.3 Mitochondrial $IF3_{mt}$

Bacterial IF3 plays a critical role in translation initiation. It binds the 30S ribosomal subunit in order to prevent association with the 50S subunit. This interaction is necessary for the initiation complex to recognize the Shine-Dalgarno sequence in the mRNA and enhances the interaction and activity of IF2 [140, 150]. Simple BLAST-P analysis failed to detect orthologs of IF3 in mitochondria. However, the existence of $IF3_{mt}$ was hypothesized because orthologs of the bacterial ribosomal proteins S7, S11 and S18, which are in proximity to IF3, are present in mitochondria [129]. More sensitive searching algorithms, like PSI-BLAST, identified $IF3_{mt}$ in fungi, animal, plant and excavates mitochondria [129, 140]. Structural data show that the IF3 C-terminal end is necessary for interaction with the 30S subunit through residues in two helical segments, designated H3 and H4. In most $IF3_{mt}$ orthologs the C-terminal domain is the least conserved region of the protein. However, some residues from the H3 segments are conserved in $IF3_{mt}$ [140, 151, 152]. Biochemical and structural approaches will clarify the mechanism of action of $IF3_{mt}$.

6.4 How Is the AUG Start Codon Recognized in Mitochondria?

As discussed above, most mitochondrial mRNAs seem to lack Shine-Dalgarno-like sequences to direct the ribosome to the AUG start codon. This is the case for metazoans, flowering plants and fungi [57, 58, 153]. In the case of metazoans it is even more puzzling because the start codon locates at or very near the 5′ end of the mRNA. This implicates that cells developed different mechanisms to localize the ribosome to the correct start codon.

Mammalian mitochondria have developed an initiation codon selection that relies on leaderless mRNAs. Addition of three nucleotides prior to the *COX2* 5′ AUG decreased translation by 40 %, and addition of 12 nucleotides reduced translation by 80 % [154]. It is proposed that the movement of the ribosome is

Table 2 Orthologs of known translational activators in mitochondria. Taken from [134, 157, 165, 168, 169, 225]

Mitochondrial target gene	Translational activator(s)	Reported species	Orthologs	Conserved function?
COB	Cbs1	*S. cerevisiae*	No	
	Cbs2	*S. cerevisiae*	No	
	Cbp3	*S. cerevisiae*	No	
	Cbp6	*S. cerevisiae, S. pombe*	Yes (only in fungi)	No, in *S. pombe* is only a chaperone
	Cbp1	*S. cerevisiae*	Yes (only in fungi)	Not known
COX1	Pet309	*S. cerevisiae, S. pombe*	Yes (only in fungi)	No, in *S. pombe* Ppr5 is activator and Ppr4 is repressor
	TACO1	*S. cerevisiae, H. sapiens*	Yes	Not known in *S. cerevisiae*
	C12orf62	*H. sapiens, S. cerevisiae*	Yes	No, in *S. cerevisiae* is a chaperone (Cox14)
	Mss51	*S. cerevisiae, S. pombe, M. musculus*	Yes	No, in *S. pombe* is only a chaperone and in mammals is a metabolic regulator
COX2	Pet111	*S. cerevisiae, H. sapiens*	Yes	No, in *H. sapiens* is a nuclear protein
COX3	Pet54	*S. cerevisiae*	No	
	Pet122	*S. cerevisiae*	No	
	Pet494	*S. cerevisiae*	No	
ATP6/8	Atp22	*S. cerevisiae*	No	
ATP9	Aep1	*S. cerevisiae*	No	
	Aep2	*S. cerevisiae*	No	
Accessory factors in translation initiation				
Aep3		*S. cerevisiae*	No	
Rsm28		*S. cerevisiae*	No	
Rmd9		*S. cerevisiae*	No	

paused after the first 17 nucleotides of the mRNA enter the ribosome. The small subunit then inspects the mRNA 5′ end. If there is a start codon at the P site, then a stable initiation complex is formed [58, 154].

Study of the mechanisms for initiation codon selection in the yeast *S. cerevisiae* has made important progress. A group of proteins, named translational activators, plays a role in the localization of the mitoribosome in the correct AUG start codon. Each one of these proteins interacts with specific mitochondrial mRNAs and with the ribosome to pose it on the start codon [155–157]. In addition, translational activators interact with each other and with the mitochondrial inner membrane, probably to tether translation initiation to the site where nascent peptides will be inserted [158–160] (Table 2). Many of these proteins are members of the pentatricopeptide repeat (PPR) family or RNA recognition motif (RRM). Other translational activators have no detectable RNA-binding motifs whatsoever. Many efforts have been made to find orthologs of these proteins in other organisms. Some translational activators may be present in other fungi [161–163] and probably also in humans [164]: However, in mammalian mitochondria, the mechanisms of action of the putative activators remains to be elucidated, as human mRNAs have either very short or no 5′-UTRs. Translational activation is also observed in plastids [165–167], suggesting that this mechanism arose several times during eukaryotic evolution.

7 Translation Elongation

Translation elongation in mitochondria is highly conserved with bacteria. During this process three elongation factors (EF) assist the mitoribosome for addition of new residues to the nascent polypeptide chain. EF-Tu_{mt} forms a ternary complex with the aminoacylated tRNA and GTP and enters the mitoribosome A site. Cognate codon-anticodon pairing triggers GTP hydrolysis by EF-Tu_{mt} and release of EF-Tu_{mt}-GDP. The mitoribosome catalyzes the peptide bond formation at the PTC. Thus, deacetylated tRNA is left in the P site and the elongated peptidyl-tRNA in the A site of the ribosome. This process is assisted by EF-$G1_{mt}$, which catalyzes the translocation of peptidyl-tRNA from the A to the P site, and removing the deacetylated tRNA from the ribosome. EF-Ts_{mt} exchanges GDP to GTP from EF-Tu_{mt} to allow a new round of elongation [58, 140]. Mitochondrial elongation, at least for mammalian and yeast models, seems to be a more conserved process than initiation and ribosome recycling. However, many components of the translation machinery have extensively diverged in different phyla, leading to adaptations of the elongation machinery. For example, as previously discussed, the structure of tRNAs and of mitoribosomes has diverged from the bacterial ancestor. In the next section, we discuss the main changes observed in the elongation factors.

7.1 Mitochondrial EF-Tu$_{mt}$

EF-Tu$_{mt}$ must be able to bind tRNA with canonical conformations (e.g., fungi, plants and some protist lineages) and shorter tRNAs versions (metazoans) [51]. In some cases the tRNAs reduction is so extensive that EF-Tu$_{mt}$ should use alternative binding modes. The divergence of EF-Tu is evident inside the nematode group: nematodes have 2 EF-Tu$_{mt}$ homologs [53]. While EF-Tu1$_{mt}$ is unable to bind cloverleaf type tRNAs, it is the only factor that binds T-armless tRNAs [170, 171]. *C. elegans* EF-Tu1$_{mt}$ has a C-terminal extension of around 60 amino acids that likely interacts with the D arm of T-armless tRNAs [172]. In the *Trichinella* lineage EF-Tu1$_{mt}$ binds T-armless tRNAs, D-armless tRNA and cloverleaf type tRNAs [170, 171]. Nematode EF-Tu2$_{mt}$ has a short C-terminal extension of 7–15 amino acids that is necessary for interaction with the D-armless tRNA^{Ser} [170]. *C. elegans* mt EF-Tu2$_{mt}$ is unique because it interacts with phosphates on the T arm on the opposite side from where canonical EF-Tu binds [173].

In trypanosomatids, EF-Tu$_{mt}$ has a highly charged insertion of approximately 30 amino acids near the C terminus. This trypanosomatid-specific motif is dispensable for the union of EF-Ts$_{mt}$, but critical for EF-Tu$_{mt}$ function [174]. This extension might be necessary for interaction with tRNAs or with the mitoribosome, which has less RNA content than mammalian ribosomes [175]. Since trypanosomatid mitochondrial tRNAs are imported, EF-Tu$_{mt}$ has evolved to interact with eukaryotic-type tRNAs, suggesting that the appearance of this motif is an adaptation of the mitochondrial machinery to recognize imported tRNAs [174]. Interestingly, complete loss of tRNA genes from mtDNA is also observed in apicomplexans [176, 177], and therefore their EF-Tu$_{mt}$s have to bind imported tRNAs. However, in this case EF-Tu$_{mt}$ is closer to the bacterial factor, indicating that each group has their own mechanisms for imported tRNA-EF-Tu$_{mt}$ binding [174]. Another distinctive feature of EF-Tu$_{mt}$ is found in hemi-ascomycete yeasts, where EF-Ts$_{mt}$ seems to be lost [178]. *S. cerevisiae* EF-Tu$_{mt}$ displays greater affinity for GTP, like the self-recycling GTPases EF-G or IF2 [179]. It is functionally equivalent to the *S. pombe* EF-Tu$_{mt}$/EF-Ts$_{mt}$ [180].

7.2 Mitochondrial EF-G1$_{mt}$ and EF-G2$_{mt}$

Bacterial EF-G participates in translation elongation and ribosome recycling. However, in some bacterial groups these functions are separated in two specialized paralogs. This is the case of the majority of Spirochaetes, Planctomycetes, Lentisphaera and some species of δ-proteobacteria. Mitochondria of most organisms have specialized EF-G$_{mt}$s paralogs as well, and these are phylogenetically related to the specialized bacterial EF-Gs. EF-G$_{mt}$s probably were acquired before the eukaryotic last common ancestor, since at least one EF-G$_{mt}$ paralog is present in all mitochondriate eukaryotes [181].

So far, aerobic eukaryotes that possess a unique EF-G_{mt} (which is an EF-$G1_{mt}$ paralog) are the plastid/apicoplast-carrying eukaryotes: Archaeplastida, stramenopile algae and Apicomplexa [181]. Interestingly, instead of a second EF-G_{mt}, these species have a plastid/apicoplast-targeted EF-G (termed EF-G_{cp} or EF-G_{api}). Outside of these groups, some *Cryptococcus* species have only one EF-$G1_{mt}$. Curiously, they do not have plastids. Until now, it is estimated that all mitochondriate eukaryotes have either two specialized EF-Gs or one EF-$G1_{mt}$ and an EF-G_{cp}/EF-G_{api} [181, 182]. Until now, it is estimated that all mitochondriate eukaryotes have either two specialized EF-G_{mt}s or one EF-G_{mt} and an EF-G_{cp}/EF-G_{api} [181, 182].

There are limited studies about the function of each mitochondrial paralog. Mammalian EF-$G1_{mt}$ is specialized in translation elongation, while EF-$G2_{mt}$ participates in ribosome recycling [183]. In contrast, *A. thaliana* EF-$G1_{mt}$ carries both functions, translocation and ribosome recycling [182]. *P. falciparum* EF-$G1_{mt}$ participates in ribosome recycling, although its translocation activity was not investigated [184, 185].

8 Termination and Ribosomal Recycling

Translation ends when the ribosome reaches one of three stop codons, *UGA*, *UAA*, or *UAG*. These codons are recognized by releases factors (RF) that enter the ribosomal A site and induce release of the nascent peptide (class-I RFs). Bacterial RF1 recognizes *UAA* and *UAG*, while RF2 recognizes *UAA* and *UGA* [186]. These factors assist the hydrolysis of ester bonds on the peptidyl-tRNA, which is located in the ribosomal P site, releasing the newly synthesized protein. Bacterial class-II RFs are GTPases that trigger dissociation of the class-I RF from the ribosome after peptide release. RFs have a conserved GGQ motif that is involved in ester bond hydrolysis (peptidyl-hydrolase domain, PTH), whereas RF1 has a PAT or PVT motif and RF2 SPF motif important for stop codon recognition (codon-recognition domain, CR) (reviewed in [187]). As mentioned in a previous section, mitochondria recognize non-conventional codons as stop codons (Table 1). Thus, understanding the mechanisms of termination and stop codon recognition is a fertile ground for research. Despite recent advances (mostly in mammalian mitochondria), it is still unclear how translation terminates in mitochondria. The most challenging subject is to understand how non-standard stop codons are decodified in mitochondria.

Mitochondrial release factors divide in five distinct subfamilies: mtRF1a, mtRF2a and ICT1, derived from bacterial ancestors, C12orf65 and mtRF1, so far found only in vertebrates. While mtRF1a, mtRF1 and mtRF2a conserved both the PTH and CR domains, ICT1 and C12orf65 have lost the CR domain [188]. Because the release factor family seems particularly prone to genetic expansion and functional divergence [188], there are high probabilities that the mechanism of translation termination varies among different phyla.

- mtRF1a is present in every eukaryotic organism and evolved from an α-protobacterial ancestor [188]. This protein recognizes *UAA/UAG* stop codons, both in vitro and in vivo [189, 190].
- mtRF1 is a vertebrate-specific mitochondrial protein [190], and it may originate from duplication of the mtRF1a gene at the root of this clade [191]. The function of mtRF1 is controversial. It may recognize the non-standard stop codons AGA and AGG [191]. However, posterior structural predictions and experimental data could not find evidence that mtRF1 recognizes any of the stop codons used in mitochondria [190, 192, 193]. In human mitochondria, the AGG and AGA codons may not function as stop codons. Instead, they promote a −1 frameshift that creates a standard TGA stop codon that can be decodified by mtRF1a [24]. Nonetheless, an analysis from all the vertebrate genomes showed that a −1 frameshift (or even a −2 frameshift) could not originate a canonical TGA stop in every ORF ending in AGG or AGA [188].
- ICT1 (immature colon carcinoma transcript-1) is widely distributed in mitochondria from all eukaryotic phyla [188]. ICT1 is a codon-independent release factor that lost the CR domain. In addition, it is an integral component of the mitoribosome and a crucial component for its assembly [194]. ICT1 is the eukaryotic ortholog of bacterial ArfB. This protein is a rescue factor of stalled ribosomes in prematurely truncated mRNAs and is also part of the bacterial ribosome [195, 196]. ICT1's role in mitochondrial translation is still not completely understood. The position of ICT1 in the mitoribosome is incompatible with the mechanism used by ArfB [193, 195]. In fact, the ICT1 integrated to the mitoribosome has no release factor activity [192]. This protein can rescue ribosomal complexes not only at the ends of mRNAs, but also in the middle of mRNAs, and even can rescue ribosomes depleted of mRNAs [192, 194]. ICT1 might terminate translation of ORFs ending in AGG and AGA since these codons are unassigned in mammalian mitochondria, and mitoribosomes stalled at AGG/AGA codons might be recognized by ICT1 [192, 193].
- C12orf65 is a release factor that probably derived from ICT1. It has a wide phylogenetic distribution and is only absent in viridiplantae [188]. Contrary to ICT1, C12orf65 is a mitochondrial soluble matrix protein that does not exhibit ribosomal-dependent peptidyl hydrolase activity. However, ICT1 overexpression partially complements C12orf65's absence, indicating that both proteins must have some overlapping functions [197].
- mtRF2s lack experimental data about their function or mitochondrial localization. mtRF2 has a narrow phylogenetic distribution, consistently found in land plants, red algae, dictyosteliida and some stramenopiles (brown algae, oomycetes and Blastocystis). It has been lost at least five times during eukaryotic evolution, in concordance with the reassignment of the UGA codon to Trp [188].

The final step of mitochondrial translation consists of recycling of the mitoribosomes. Once the nascent chain is released, the ribosome recycling factor 1 ($RRF1_{mt}$) and the specialized $EF\text{-}G2_{mt}$ (see above) separate ribosome subunits to

allow new cycles of translation [181, 198]. In addition, $IF3_{mt}$ may attach to the ribosomal SSU to prevent futile association of the mitoribosome until an initiation complex is formed [199].

9 Mitochondrial Translation Is Coupled to Protein Assembly

The majority of proteins encoded by mitochondrial DNA are subunits of the respiratory chain complexes and the ATP synthase. These proteins are usually hydrophobic, with two or more transmembrane stretches. Thus, it is expected that mitochondrial translation machinery is physically coupled to the mitochondrial inner membrane. Mitochondrial and cytoplasmic subunits have to assemble and acquire the necessary prosthetic groups in coordination to make active enzymes. Indeed, major progress in the field has come from beaker's yeast *S. cerevisiae*. Defects in the coordination of mitochondrial-encoded subunit synthesis and assembly are proposed to affect the cell physiology. When cytochrome *c* oxidase is not assembled, then Cox1 synthesis in mitochondria is downregulated [200, 201]. Cox1 is part of the central core of the enzyme and has 12 transmembrane stretches. Downregulation of Cox1 synthesis may prevent generation of pro-oxidant species, because the poorly assembled heme *a* present in Cox1 has peroxidase activity [202]. When the ATPase F1 sector is not assembled, then translation of the *ATP8/ATP6* transcript is downregulated [203, 204]. This prevents accumulation on the membrane of Atp6–Atp9 rings that could interfere with the membrane potential [205, 206]. Translation of the mitochondrial *COB* mRNA, coding for the cytochrome *b* subunit from the *bc*1 complex is also tightly linked to enzyme assembly [207]. Coordination of mitochondrial translation and assembly is an intricate process that requires many factors, many of them specific for each mitochondrial-coded protein [159, 203, 208, 209] (Fig. 3). In general, initiation of mitochondrial mRNA translation is achieved by translational activators (discussed above), which assist in the positioning of the ribosome on the initiator *AUG*. Translational activators are themselves associated with the inner membrane and with other translational activators to tether translation initiation to the localization of assembly of nascent peptides [155, 156]. Some of these mRNA-specific activators act exclusively on the 5′-UTR of the target mRNA and are no longer required in downstream events after translation initiation. Some other translational activators have dual functions. Together with specific chaperones, they physically interact with the newly synthesized peptide and are key players in the coordination of translation and assembly. In general, it is proposed that once the respiratory complex proceeds to assembly, these chaperones/translational activators are released from the assembly intermediary and recycled for new rounds of translation [159, 208]. Equivalent processes were described for *C. reinhardtii* photosynthetic complexes in chloroplasts [165].

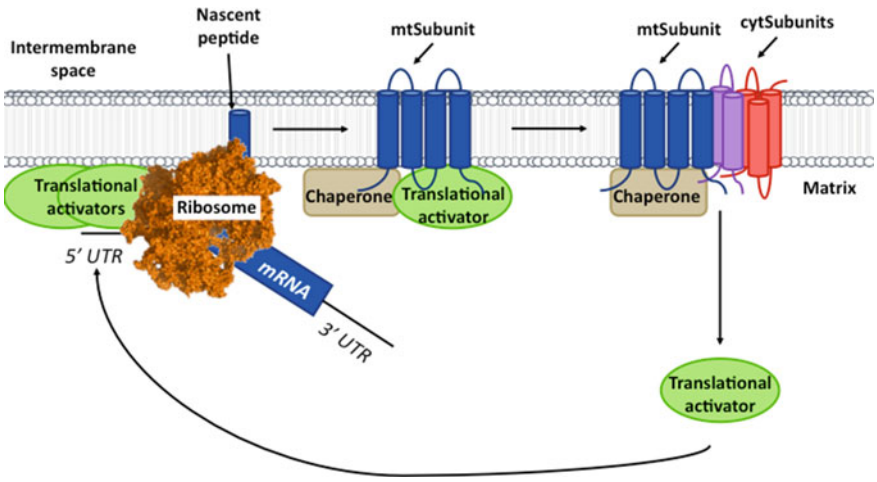

Fig. 3 General model for the coupling of protein synthesis and membrane assembly in yeast mitochondria. Translational activators assist the ribosome in localization of the AUG start codon through recognition of specific sequences within the 5′-UTR of each mRNA Cbs1/Cbs2/Cbp3/Cbp6 (cytochrome *b* synthesis) [207, 210]; Pet309/Mss51 (Cox1 synthesis) [157, 200, 201, 211]; Pet111 (Cox2 synthesis) [212]; Pet494/Pet122/Pet54 (Cox3 synthesis) [213–215], Atp22 (Atp6 and Atp8 synthesis) [204]; Aep1, Aep2 (Atp9 synthesis) [216, 217]. Some of these activators play a second role in coordination of translation/assembly. They physically interact with newly made peptides (mtSubunits) and with additional chaperones to form assembly intermediaries. This is the case for Cbp3/Cbp6 [207] and Mss51 [201]. Once the respiratory complexes assemble with cytosolic, imported subunits (CytSubunits), then the chaperones/translational activators are released and recycled. Translational activators are now ready for new rounds of translation

In human mitochondria, the scenario is not as clear as with yeast. However, some orthologs of the yeast translational activators and chaperones are present in humans, and some of them may have similar roles [164, 209, 218, 219].

10 Concluding Remarks

Mitochondrial translation evolution is an exciting field in biology. Many efforts have been made to understand mitochondrial translation by studying yeast, mammals, plant and trypanosomatid species, and to a lesser degree apicomplexans, nematodes and *Drosophila*. Since the mitochondrial translation machinery has extensively diverged among eukaryotes, it is necessary to study it in many disparate groups of eukaryotes in order to understand its evolution. To date, many questions remain open in this field. For instance: (1) *W*hat are the role and mechanism of action in the translation of mitochondrial-encoded small and large non-coding RNAs discovered a few years ago [220–222]? (2) How do mRNA polyadenylation and edition regulate stability, editing and translation in different species? (3) Are

there specialized ribosomes devoted to translating a specific mRNA in mitochondria? If this is the case, how are populations of each type of ribosome regulated in different conditions or cell types? (4) What are the mechanism and regulation of the recently discovered "programmed translational bypass"? This process was first discovered in the yeast *Magnusiomyces capitatus* [223, 224]. In this organism, almost all protein-coding genes in mtDNA have insertions of 27–55 nucleotides, called byps. Translation of these insertions would lead to frameshifts and premature termination of translation, so a precise mechanism to bypass these elements is necessary. Is translation bypass present in different eukaryotic groups? (5) What is the role of the recently discovered mitochondrial RNA granules in translation [127]? Are they present exclusively on mammalian mitochondria, or do they have a broader prevalence in eukaryotes? (6) Since Shine-Dalgarno sequences are absent in many eukaryotic lineages, how is the start codon *AUG* recognized by the initiation complex among different phyla? (7) Are the translation and assembly of nascent peptides prevalent processes in eukaryotes? The field of mitochondrial evolution awaits answers to these exiting questions.

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