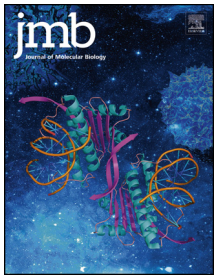
Review



Regulation of Mitochondrial Electron Transport Chain Assembly

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Abstract

Mitochondrial function depends on the correct synthesis, transport, and assembly of proteins and cofactors of the electron transport chain. The initial idea that the respiratory chain protein complexes (RCCs) were independent structures in the inner mitochondrial membrane evolved after the identification of higher quaternary structures called supercomplexes (SCs), whose formation is dynamically regulated in order to accommodate cellular metabolic demands. Due to the dual genetic origin of the mitochondrial proteome, electron transport chain and SCs formation must be tightly regulated to coordinate the expression and assembly of components encoded by both genomes. This regulation occurs at different levels from gene transcription to protein, complex or SCs assembly, and might involve the participation of factors that contribute to the formation and stability of the RCCs and SCs. Here we review the cellular pathways and assembly factors that regulate RCCs and SCs formation.

Introduction

Mitochondria, in addition to functioning as the powerhouse of the cell, host crucial biochemical processes including iron–sulfur (Fe–S) protein biogenesis [1,2], β-oxidation of fatty acids [3,4], and citric acid cycle [5,6]. Moreover, mitochondria are at the center of key cellular pathways implicated in calcium signaling and homeostasis [7,8], cell death [9–11], and *r*eactive *o*xygen *s*pecies (ROS) generation [12]. Indeed, they are at the core of metabolic adaptations and modulate anabolic and catabolic reactions according to the cytosolic conditions. All these functions are sustained by the activity of protein complexes that constitute the oxidative phosphorylation system (OXPHOS) [13]. The OXPHOS machinery is mainly located in the mitochondrial cristae membrane and comprises the complexes of the electron transport chain (ETC) and the ATP synthase (Complex V, CV). The ETC is formed by four complexes (respiratory chain complexes, or RCCs): complex I (NADH-ubiquinone oxidoreductase, CI), complex II (succinate-quinone oxidoreductase, CII), complex III (ubiquinol-cytochrome *c* oxidoreductase, cytochrome bc_1 complex, CIII), and complex IV (cytochrome *c* oxidase, CIV). These complexes couple electron transport with proton pumping using ubiquinone and cytochrome *c* as electron carriers. During this process, the generated proton gradient is used by the ATP synthase to phosphorylate ADP and produce ATP. The RCCs formation relies on the completion of the different processes that allow for the correct synthesis, transport, and assembly of the mitochondrial proteins encoded by both nuclear and mitochondrial genome. This is true for CI, CIII, and CIV, but not for CII, which is exclusively formed of nuclear (nDNA)-encoded proteins. During the formation of these complexes, nDNA-encoded accessory subunits, termed assembly factors, are necessary to give stability and/or protection against ROS produced by the accumulation of detrimental unassembled OXPHOS subunits [14]. Therefore, these factors are fundamental for the successful assembly and function of the ETC and include either proteins that participate in translation, heme and copper metabolism, or protein–protein interaction.

For many years, the organization of the ETC has been the subject of an intense debate. In 1947, Keilin and Hartree [15] proposed the *solid model,* where the complexes maintain a static and close association in order to allow for a rapid electron transport. This model was supported by later studies that defined the "units of mitochondrial electron transfer" corresponding to assemblies of two or more complexes [16,17]. Forty years later, after the isolation of the four complexes as active monomers, Hackenbrock and colleagues [18] discarded the *solid model* and proposed the *fluid model* where the complexes and cytochrome *c* are independent components freely shuttling into the mitochondrial membrane. In 2000, Schägger and Pfeiffer [19], using the native electrophoresis technique, were able to isolate superstructures of complexes together with unexpected associations between complexes I and III, and III and IV, in addition to the single complexes. Currently, the idea that independent complexes and supercomplexes (SCs) coexist and that the latter subsequent to the organization of the former is widely accepted and known as *plasticity model* [20,21]. This model has been recently confirmed by an elegant study of the CI assembly pathway [22]. This study using dynamic complexome profiling demonstrates that no intermediates of CI are assembled in SCs, and that SC formation starts only when the individual CI assembly is completed; similar results were obtained regarding CIII and IV formation [23]. The *plasticity model* infers that the ETC organization is not random but requires a high level of coordination. Therefore, key processes like transcription, translation, import, and assembly of protein products from nuclear and mitochondrial genomes are expected to be coordinated to build functional respiratory complexes.

The OXPHOS Machinery

The dual genetic origin of the mitochondrial proteome

The mitochondrial proteome is predicted to be composed of more than 1000 proteins [24,25], but only 13 of these mitochondrial proteins are encoded by mitochondrial DNA (mtDNA), a vestige of the endosymbiotic origin of the *organelle*; the remaining 99% of the mitochondrial protein content is nuclear encoded. Originally, the genome of the ancestral proteobacterium contained all the genes needed for its independent life including those dedicated to replication, transcription, and translation [26]. Early in evolution after the endosymbiotic event, many mtDNA genes were transferred to the nDNA or functionally replaced by already existing nuclear genes. Subsequently, the number of mtDNA genes decreased from several thousand to only three genes in some unicellular parasites [27–29]. The decrease in genes encoded by mtDNA throughout evolution has been postulated to be as a strategy to optimize cellular resources and energy saving. Interestingly, phylogenetic studies suggest that an alternative step in mitochondrial evolution has produced mitochondria-derived organelles lacking mtDNA in eukaryotes not requiring aerobic respiration because living in anoxic habitats [30,31]. Recently, the first secondarily amitochondriate eukaryote has also been described as a result of such evolutive adaptation [32]. Nevertheless, the presence of the mitochondrial genome in respiring eukaryotes supports that it is a requirement for mitochondrial existence in these organisms.

mtDNA-encoded proteins: nucleoid and RNA granules as centers of the transcription/translation control

The number of genes in mtDNA is highly variable in different organisms ranging from yeast to higher eukaryotes. However, certain mtDNA properties are conserved such as the presence of genes encoding for highly hydrophobic and crucial components of the respiratory chain, ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) that are necessary for protein translation. The human mitochondrial genome is a 16.5-kb circular double-stranded DNA molecule (H: heavy strand and L: light strand) coding for 13 polypeptides that are integral components of Complexes I, III, IV, and V (ATP synthase), 2 rRNAs and 22 tRNAs. The number of mtDNA molecules varies from hundred to thousand depending on the cell type, and this number reflects the potential energetic need of the cell: muscle mitochondria contain more DNA molecules [33] than leukocytes [34], and this is interestingly associated with a higher or lower number of mitochondrial cristae [35]. Whether and how mitochondria ultrastructure and mtDNA copy number and distribution are correlated is not known but might be worth further investigation. One possibility for why a mitochondrion would maintain such a high mtDNA copy number could be to ensure the rapid supply and homogeneous distribution of proteins. Moreover, a high mtDNA copy number could increase the probability to conserve wild-type mtDNA and thus the production of functional proteins despite the high rate of mtDNA mutations due to the local mutagenic environment and poor efficient mtDNA repair system [36–38].

The mitochondrial genome is organized into structures called nucleoids [39,40]. Nucleoids contain also proteins that contribute to compaction and replication of the DNA molecules and to the coordination of gene transcription [41–47]. Still, the protein composition of a nucleoid is, however, contentious [48]. A proposed model describes a core and a peripheral zone that contains proteins that tether the nucleoid to the inner mitochondrial membrane (IMM) and regulate the mtDNA segregation and transcription (Table 1)

Table 1. Nucleoid *bona fide* core components

Protein	Function	Mitochondrial defects/disorders	References
Twinkle	Helicase	Decrease of CI, CIII and CIV activities. PEO, recessive inherited infantile-onset spinocerebellar ataxia and mtDNA-depletion syndromes	[49,50]
mtSSB	Stabilize single strand during DNA replication	Regulates mtDNA copy number in cells and if absent is lethal in *Drosophila melanogaster*; preweaning lethality in mouse	[51,52]
PolG	Catalytic subunit of DNA pol gamma	PEO, ataxia neuropathy spectrum, Alpers–Huttenlocher syndrome, myoclonic epilepsy myopathy sensory ataxia, childhood myocerebrohepatopathy spectrum; MtDNA depletion and/or deletions, reduction of CI and CIV activities	[53,54]
TFAM	Transcription factor. MtDNA replication, packaging, and repair	mtDNA depletion syndrome (hepatocerebral type); lethal if absent in mice; low basal respiration and low mtDNA copy number	[55,56]
POLRMT	RNA polymerase	Mitochondrial dysfunction; defects in mitochondrial gene expression and hampered mitochondrial biogenesis	[57]
TFB2M	Transcription factor	Low mitochondrial-encoded gene expression, loss of mtDNA content and mitochondrial dysfunction; lethal if absent in *D. melanogaster*	[58,59]
TEFM	Transcription elongation factor	Regulate CI and IV stability and transcripts	[60,61]
LONP1	Lon protease	Cerebral, ocular, dental, auricular and skeletal anomalies syndrome (CODAS), mtDNA depletion syndrome. Low CI, CIII, and CIV protein synthesis, complex formation, and global activity	[62–65]

mtSSB, mitochondrial single-stranded DNA-binding protein; PolG, DNA polymerase subunit gamma; TFAM, transcription factor A mitochondrial; POLRMT, RNA polymerase mitochondrial; TFB2M: transcription factor B2 mitochondrial; TEFM: transcription elongation factor mitochondrial; LONP1: Lon protease-like protein 1.

[39,49–65]. Because of their debatable composition and regulation, and based on different studies, it can be said that defect in nucleoid core components leads to a loss of mtDNA, low mtDNA-encoded gene expression, and reduced activity of the complexes. Accordingly, mutations in some of the core components have been described with a phenotype of mitochondrial disorder in humans, which in some cases is specifically denominated mitochondrial depletion syndrome (Table 1). In the case of proteins participating in the nucleoid anchoring to the membrane (peripheral zone), the proximity to the IMM would facilitate, similarly to bacteria, the co-translation insertion of new synthetized proteins (Fig. 1, step 6). Therefore, nucleoid and mtDNA instability can be a limiting step in OXPHOS synthesis by inducing the loss of wild-type mtDNA copy number under a not well-defined threshold.

The transcription of mtDNA is bidirectional and initiates at the non-coding region D-Loop from two different promoters on the H strand (HSP1 and HSP2, heavy strand promoter 1 and 2) and one at the L strand (LSP, light strand promoter) that generate two main long polycistronic pre-RNAs. The HSP2-transcript encodes 2 rRNAs, 14 tRNAs, and 12 polypeptides that are processed in single messenger RNAs (mRNAs), while the L-transcript encodes 8 tRNAs and only 1 mRNA (*ND6*). The rest of the L-strand contains non-coding regions complementary to the H-strand [66,67]. Interestingly, these regions generate antisense sequences for the genes located in the H-strand which function is not known but could regulate H-strand transcripts availability inside the mitochondria [68]. The HSP1 produces a third transcript encoding the two rRNAs positioned close to the promoter. Both H and L promoters are functionally independent and lead to a different rate of transcript synthesis, evidenced by the lower amount of *ND6* transcripts [69]. The diverse stoichiometry between transcripts has been attributed to protein-mediated termination events ~160–185 nucleotides downstream of the LSP, where the transcription of the L-strand is aborted [68,70,71]. The analysis of mitochondrial transcripts in different tissues in steady-state conditions revealed that mtDNA-encoded transcripts are more abundant than nuclear-encoded mitochondrial transcripts [72]; however, such increased abundance of mitochondrial transcripts does not correlate with higher protein synthesis. Interestingly, mtDNA copy number does not influence the transcription and synthesis of nuclear-encoded mitochondrial proteins, since mtDNA depleted cells are still able to transcribe and synthesize them [72]. Overall these results suggest that downstream processes like translation regulation and efficiency, and not mtDNA copy number or transcription rate, are crucial for the synthesis of the OXPHOS subunits [73].

The translation of the OXPHOS core components is mediated by mitochondrial ribosomes bound to the IMM. Therefore, it is not surprising that different studies propose the existence of a non-membranous subcompartment mediating RNA processing located in proximity of this membrane. In this compartment, known as mitochondrial RNA granules (MRGs) (Fig. 1, step 7), the mtRNA processing takes place concomitantly with transcription [74–76]. Indeed, the MRGs were identified as foci containing proteins involved in RNA-processing and maturation of nascent mtRNA, and regulators of mitochondrial

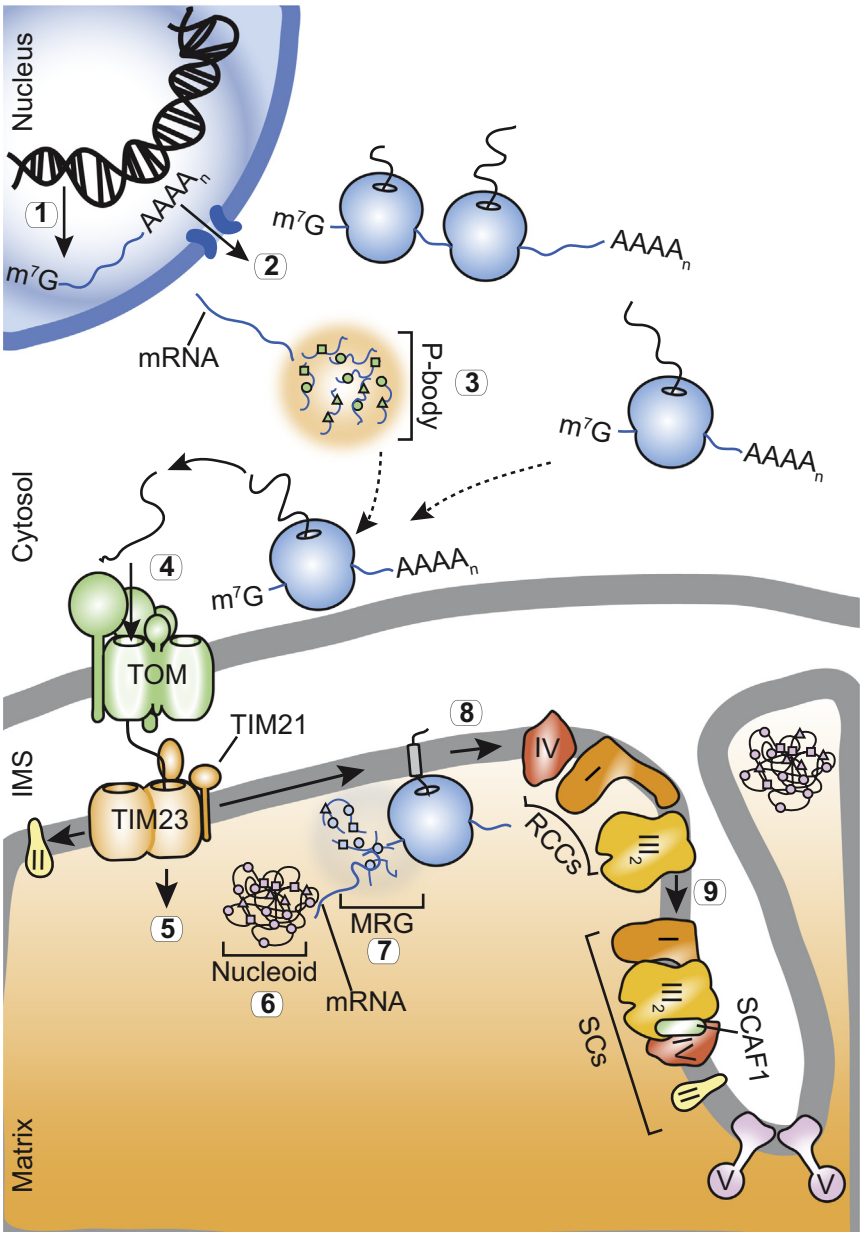


Fig. 1. Pathways of nuclear and mtDNA-encoded transcripts and proteins to mitochondria. Transcripts from the nuclear genome [1] are transported to the cytosol through the NPC [2] where they can either be translated by the cytosolic ribosome pool or aggregate and store in non-membranous compartments termed P-bodies [3]. Nuclear-encoded mitochondrial transcripts are translated at ribosomes in proximity of OMM to proceed with the protein import through the TOM complex [4] at the OMM and TIM23 complex (that includes the subunit TIM21) at the IMM. From here, proteins can be released to the matrix [5] or be inserted in the IMM where the proteins will be assembled in single complexes (CI-IV, and ATP synthase). In the case of CII, the only complex formed exclusively by nuclear-encoded proteins, the assembly takes place with the assistance of the assembly factors described in the Respiratory chain complexes section and Fig. 2. In the case of CI, CIII, CIV, and ATP synthase, their assembly requires the synthesis and export of mtDNA-encoded proteins to the IMM. For these proteins, transcription begins at nucleoid, structures containing the mtDNA [6]. The transcripts accumulate in non-membranous compartments overlapping with nucleoid called MRG [7]. The nucleoid and MRG are located in the mitochondrial matrix in proximity of the IMM where the co-translational insertion of mt-DNA-encoded proteins into the lipid bilayer takes place. Then, these newly synthesized proteins assemble with the nuclear-encoded imported subunits [8]. Here, with the assistance of the assembly factors described in the Respiratory chain complexes section and Fig. 2, the RCCs are completed independently. Afterward, SCs are formed with the aid of SCAF1, HIG2A, or other assembly factors not yet identified [9]. Within brackets are indicated the numbers corresponding to different regulatory steps.

Table 2. MRGs *bona fide* components

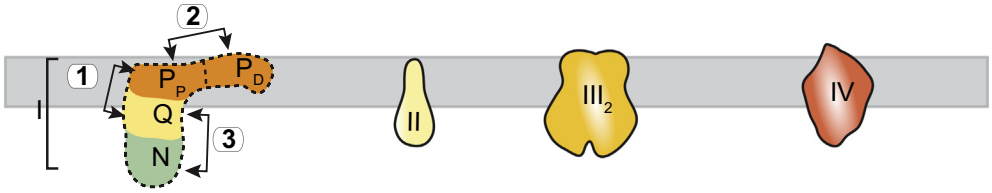
Protein	Function	Mitochondrial defects/disorders	References
GRSF1	RNA binding protein; RNA metabolism	Mitochondria translation defect, low expression of mtDNA-encoded transcripts, and aberrant mtRNA processing. Loss of OXPHOS complexes I, III, IV, and V	[74,77]
MRPP1,2,3	Endonuclease for the formation of 5′ ends and methylation of purine-9 of tRNAs	Epilepsy, developmental delay, static encephalopathy, optic atrophy and blindness, chronic; low CI and CIV activities	[74,78]
mtPAP	RNA polymerase Add poly(A) tail to mt-transcripts	Spastic ataxia with optic atrophy; severe loss of OXPHOS complexes I and IV, and perturbation of *de novo* mitochondrial protein synthesis	[79]
DHX30	RNA helicase	Neurodevelopmental disorder (ataxia, intellectual disability, motor developmental delay); mitochondrial translation defect with an accumulation of SSU subassemblies and low presence of the SSU	[80,81]
DDX28	RNA helicase	Low mitochondrial protein synthesis and activity of complexes I, III, IV, and V	[82]
ERAL1	RNA chaperone	Perrault syndrome; impaired respiration and low expression of mtDNA-encoded proteins	[83,84]
FASTK2&5	RNA binding protein, mRNA processing	Reduces COX1 synthesis (CIV deficiency), with a less evident decrease in other mtDNA-encoded protein synthesis	[76,85]
NLRX1	Binds FASTK5	Regulates processing of mtDNA-encoded transcripts	[86]
PUS1	Pseudouridylate synthase 1	Mitochondrial myopathy with lactic acidosis and sideroblastic anemia; low mitochondrial density	[87,88]
RPUSD4	Pseudouridine synthase	Defects in the biogenesis of LSU; mitochondrial translation defects; lower synthesis and activity of OXPHOS components	[89]

GRSF1, G-rich sequence factor 1; MRPP, mitochondrial RNase P; mtPAP, poly(A) RNA polymerase mitochondrial; DHX30, DExH-Box helicase 30; SSU, small subunit of ribosome: DDX28, DEAD-Box helicase 28; ERAL1, era like 12S mitochondrial rRNA chaperone 1; FASTK, Fas activated serine/threonine kinase; NLRX1, NLR family member X1; PUS1, pseudouridylate synthase 1; RPUSD4, RNA pseudouridylate synthase domain containing 4.

ribosome assembly (Table 2) [74,76–89]. The nucleoid and MRG domains are in part spatially overlapping and functionally linked. Indeed, the absence of mtDNA results in loss of MRG [74]. Because of the few studies on MRG composition, the characterization of this compartment is still incomplete, as well as the effective spatial independence from the nucleoid. The MRGs represent a transitory mtRNA maturation step as demonstrated by pulse-chase experiments with bromouridine in which the efficiency of nascent RNA processing determines its release from the MRG. Afterward, the processed RNA is directed to the mitochondrial translation machinery assembled at the IMM for the protein synthesis and insertion into the membrane. Alterations in the *bona fide* MRG components provoke mitochondrial disorders with very similar phenotype to nucleoid associated disorders, with a general decrease in OXPHOS synthesis and activity (Table 2). The existence of functionally distinct subcompartments (nucleoids, MRG, and translation machinery) located at the IMM suggests the participation of the inner mitochondrial lipid bilayer in the regulation of the following processes: (i) mtDNA segregation, (ii) mt-mRNA transfer from the nucleoid to the MRG, (iii) mt-mRNA transfer from the MRG to the mitochondrial translation machinery, and (iv) synthesis and insertion of proteins into the IMM and assembly with other OXPHOS components to form the RCCs and SCs. Most of the mechanisms regulating these processes are poorly understood.

The pathway of nuclear-encoded mitochondrial proteins to mitochondria

The transcription of nuclear-encoded mitochondrial proteins follows the canonical pathway: pre-mRNA are processed in the nucleus and subsequently translocated through the nuclear pore complex (NPC) to the cytoplasm (Fig. 1, steps 1–2), a process assisted by associated proteins and factors [90,91]. Once in the cytoplasm, mRNA molecules can aggregate in non-membranous compartments known as P-bodies (Fig. 1, step 3), which also contain proteins devoted to mRNA remodelling (DDX6 and other helicases), translation repression (e.g., eukaryotic translation initiation factor 4E transporter, or 4ET), mRNA decay (e.g., 5′–3′ exoribonuclease 1, or XRN1) and microRNA-mediated silencing factors (e.g., argonaute 1–4) [92,93]. Remarkably, no nDNA-encoded rRNA has been found in P-bodies, suggesting that they are not directly involved in translation. The protein composition and the presence of not degraded mRNA suggest that P-bodies play a role in storing untranslated mRNA for later translation [94]. The signals that regulate P-bodies mediated mRNA storage and release for translation remain elusive. Noteworthy, recent data show that nuclear transcripts of mitochondrial proteins are not enriched in P-bodies [92], a result that could be interpreted as that these transcripts are directly recruited to the translation machinery, hypothesis that needs further validation. While cytoplasmic-targeted proteins are directly translated by cytosolic ribosomes



Stability and Assembly

P-module	Q-module
ACAD9	NDUFAF2
ATP5SL	NDUFAF3
DMAC1	NDUFAF4
ECSIT	NDUFAF5
FOXRED1	NDUFAF7
MITRAC15	TIMMDC1
NDUFAF1	
TIMMDC1	**N-module**
TMEM126B	NDUFAF2
TMEM186	
NDUFAF6	
NUBPL	

Stability and Assembly

SDHAF1
SDHAF2
SDHAF3
SDHAF4

Stability and Assembly

BCSL1
CCHL
LYRM7
UQCC1
UQCC2
UQCC3
TTC19

Stability and Assembly

CMC1	MITRAC12
COX16	MITRAC15
COX18	MR-1S
COX20	PET100
C12ORF62	PET117
MITRAC7	TMEM177

Copper and Heme Insertion

ADR	COX15
FDX2	COX17
COA6	COX19
COX10	SCO1-2
COX11	SURF1

Fig. 2. Assembly factors described playing a role in the assembly of RCCs. CI assembly factors are divided based on the module they participate to assemble: P, Q, or N modules. In the CI assembly pathway, the assembly initiates with the association of P_P and Q modules [1], followed by the addition of the P_D module [2], and it is completed with the incorporation of the N module [3]. In general, all the factors are considered to participate in the stability and assembly, except for NDUFAF6 and NUBPL (gray box), which function in the pathway is unclear. Assembly factors are described also for CII, $CIII_2$, and CIV and are divided based on their function in the assembly pathway. Within brackets is indicated the order in which the different modules are assembled. Translation activators and factors with unknown function are not included in the figure.

and do not require any site-specific protein translocation, mitochondrial-targeted proteins need to be imported into mitochondria to fulfill their function in the *organelle*. This process is facilitated by the *in loco* translation of mt-transcripts in close proximity to the outer mitochondrial membrane (OMM) [68,95]. In general, the delivery of mitochondrial precursors to the *organelle* is a post-translational process assisted by chaperones [96]. Interestingly, proximity-specific ribosome profiling performed in yeast has shown that most of the IMM proteins are co-translationally imported [97–100], most likely by a subset of specialized ribosomes localized at the cytosolic side of the OMM. The concept of specialized ribosomes implies differences in ribosome composition that could control the anchorage or interaction with the organelle membrane. Although recent studies have identified proteins that regulate the ribosome–OMM interaction [101–104], it is not clear whether such an association is stable, or dynamically regulated by recycling cytosolic ribosome components. Reciprocal regulation of mitochondrial protein translation both inside and outside of mitochondria might ensure that nDNA- and mtDNA-encoded mitochondrial proteins assemble in the proper stoichiometry to build the RCCs and SCs (Fig. 1, steps 8–9). Such regulatory control would also prevent the excessive and useless production of proteins, which otherwise are degraded and, as such, would represent a waste of energy and resources.

Interestingly, the nDNA- and mtDNA-derived transcripts that encode the OXPHOS machinery do not increase concordantly, indicating a delay in the communication between both compartments [105]. Recent studies propose a mechanism in which ribosomal activity remains in a standby state (stalled ribosomes) with only a portion of transcript already translated. Following a yet uncharacterized signal, translation is completed and the resulting peptide inserted and assembled into the IMM (Fig. 1, step 4). The import and sorting pathways into the different mitochondrial subcompartments are well established and have been extensively reviewed in [106]. Here, we review how the translation machinery interfaces with the ETC assembly, and whether and how the import machinery modulates the process.

Respiratory chain complexes

The assembly of the OXPHOS subunits into RCCs and SCs is a conserved event in eukaryotes, yet there are differences in the SCs composition and/or stoichiometry among organisms. The first step required for the proper oxidative phosphorylation activity is the formation of the individual RCCs:

CI, or NADH-ubiquinone oxidoreductase, is the largest enzyme of the OXPHOS system and is formed of 44 subunits, 7 of them encoded by the mtDNA. CI catalyzes the transfer of two electrons from NADH to ubiquinone coupled to the translocation of four protons across the IMM. Moreover, CI is a main site for ROS generation [107]. Its L-shaped structure can be divided into three structurally and functionally distinct modules: (i) the N-module responsible for NADH oxidation, formed by three core subunits (NDUFS1, NDUFV1, NDUFV2 in the human nomenclature) and a flavin mononucleotide moiety; (ii) the Q-module that transfers electrons to ubiquinone and contains four core subunits (NDUFS2, NDUFS3, NDUFS7, NDUFS8) with Fe–S cluster cofactors; and (iii) the P-module responsible of proton pumping and composed of seven highly hydrophobic mtDNA-encoded subunits (ND1-6 and ND4L). CI assembly proceeds in modules and requires a growing number of assembly factors (Fig. 2) that are essential for the completion of the complex [22,108]. The assembly begins with the P_P–Q-modules association (Fig. 2, step 1) that then allows the P_D incorporation (Fig. 2, step 2) and last the N-module (Fig. 2, step 3). Recently, a detailed study sheds light on the stepwise incorporation of all CI subunits into the different assembly intermediates, as well as the assembly factors involved [22], including three new putative factors. Mutations in CI subunits or assembly factors lead to severe mitochondrial diseases [109].

CII, or succinate-quinone oxidoreductase, functions in both the mitochondrial ETC and the Krebs cycle, where it couples the oxidation of succinate to fumarate. In eukaryotes, CII is composed of four subunits: SDHC and SDHD are embedded in the IMM, and SDHA and SDHB face the matrix [110]. Four assembly factors (Fig. 2) have been reported to play a role in the maturation of the inactive holoenzyme-SDH: SDH assembly factor 1 (SDHAF1), SDHAF2, and the chaperone-like proteins SDHAF3 and SDHAF4. SDHAF2 flavinates SDHA and covalently binds FAD, and SDHAF4 binds SDHA to reduce auto-oxidation [111]. SDHAF1 is necessary for the insertion of the [Fe–S] clusters into SDHB and later SDHAF3 in order to protect the system from the oxidative damage [112]. The subunits SDHC and SDHD are then incorporated into the holocomplex SDHA–SDHB by a still unclear process [113]. Mutations in all the CII subunits and in part of the assembly factors are associated with cancers that affect the paraganglia [114], the gastrointestinal tract [115,80], and kidneys [115]

CIII, cytochrome bc_1 complex or ubiquinol-cytochrome c oxidoreductase, catalyzes the transfer of electrons from ubiquinol (reduced coenzyme Q) to cytochrome *c* (cyt *c*) coupling this redox reaction to the translocation of protons across the IMM. CIII is a symmetrical homodimer composed of one mtDNA-encoded protein (MT-CYB) and 10 nDNA-encoded subunits. Its catalytic center is composed of three subunits: cytochrome *b* (MT-CYB), cytochrome c_1 (CYC1), and the Rieske protein (UQCRFS1 or RISP). Those, along with other eight extra subunits of unknown function, (UQCRC1, UQCRC2, UQCRH, UQCRB, UQCRQ, Subunit 9, UQCR10, and UQCR11), form the complete complex [116]. The current model of CIII assembly has largely been designed with data from *Saccharomyces cerevisiae* [117,118], and only a few of the established assembly steps have been experimentally proven in mammalian models with the identification of some assembly factors (Fig. 2). In mammals, the first step of CIII assembly begins with the release of the MT-CYB from the mitochondrial ribosome and its insertion into the inner membrane by two assembly factors: UQCC1 and UQCC2 [119,120]. An additional assembly factor, UQCC3, is also involved in the early stages of complex assembly downstream of UQCC1 and UQCC2 [121,122] and stabilizes MT-CYB. The subsequent step leads to the formation of an early-assembly intermediate that involves the recruitment of UQCRB and UQCRQ to the complex containing MT-CYB, UQCC1, UQCC2, and UQCC3. As a result, the two assembly factors UQCC1 and 2 are released from this complex and become available for another round of translation of MT-CYB [118,123]. The latter step is considered being a regulatory mechanism that couples assembly with translation. The assembly factor BCS1 regulates the last assembly step consisting in the incorporation of the Rieske protein to the CIII [123]. The assembly factor LYRM7, which is localized in the matrix, participates in the transfer of the Fe–S cluster to UQCRFS1 in the CIII dimeric intermediate within the IMM [119,124]. As a result, the mature CIII dimer ($CIII_2$) is formed. During the assembly process, the N-terminal tail of UQCRFS1 is cleaved and usually removed from the complex if the assembly factor TTC19 is present. The lack of TTC19 leads to the accumulation of these fragments provoking CIII dysfunction [125,126]. However, the exact mechanism of CIII dimerization is not known.

CIV, or cytochrome c oxidase (COX), is the most studied of the RCCs. The mammalian COX is formed of 14 subunits: three core subunits (COX1, COX2, and COX3) encoded by the mtDNA, and the remaining 11 encoded by the nDNA. This complex catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen, a redox reaction that requires co-factors such as copper ions and heme prosthetic

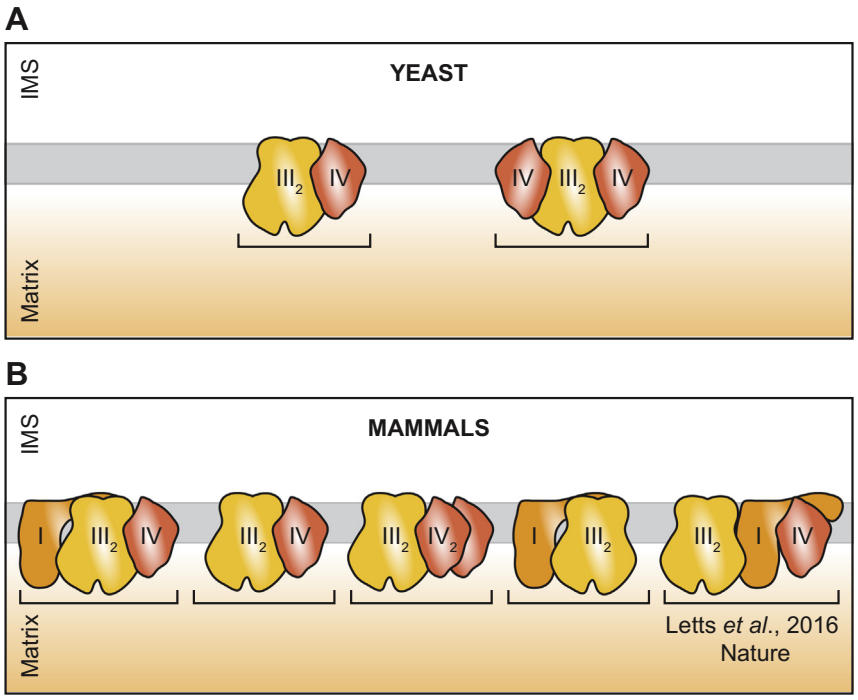


Fig. 3. Composition of the SCs identified in yeast and mammals. (A) Two main yeast SCs have been identified containing CIII and CIV (CI is absent in yeast) with different stoichiometry: $CIII_2$–CIV and $CIII_2$–CIV_2. (B) Mammalian SCs mainly contain CI, $CIII_2$, and CIV with different stoichiometry: CI–$CIII_2$–CIV (respirasome), $CIII_2$–CIV, $CIII_2$–CIV_2, and CI–$CIII_2$. An additional SC defined by Sazanov and co-workers (on the right) has the same stoichiometry of the respirasome, but here CI mediates $CIII_2$–CIV interaction ($CIII_2$–CI–CIV). The other described sub-SCs, not represented in the figure, are considered assembly intermediates.

groups inserted in the subunits COX1 and COX2. The formation of the complex engages numerous assembly factors that pre-assemble independent modules containing COX1, 2, or 3. These modules are platforms for the incorporation of additional subunits until the completion of the complex [127]. At present, 30 different proteins have been described to participate in copper and heme insertion, stability, and assembly of the complex (Fig. 2) [128–130]. Still, the function of some of these factors needs to be better characterized.

COX has an intricate but fascinating regulation. Indeed, nDNA-encoded tissue-specific subunits and others factors, differentially expressed under certain stress conditions, define different assembly complexes [131–133]. Some of these subunits determine the CIV monomer *versus* dimer conformation (Fig. 4A). In particular, the subunit COX7A1, mainly expressed in heart and muscle tissue, favors CIV dimerization. This conformation is further stabilized in the presence of COX6A2, also heart and muscle specific. Tissues lacking these two subunits, like the liver, contain mainly the monomeric CIV. In this conformation, the heart-specific subunits COX7A1 and COX6A2 are replaced by the liver-specific COX7A2 and COX6A1, which do not favor dimerization. The tissue specificity affecting the SCs stoichiometry presumably reflects on the respiratory capacity. These results open the question about how many unknown tissue-specific subunits might define SC formation and stoichiometry, differences that might explain metabolic specificities. It might be also interesting to investigate differences in assembly factors and SCs association in stem *versus* differentiated cells, or in tumorigenic *versus* non-tumorigenic cells.

Respiratory chain SCs

The supramolecular assembly of RCCs to SCs varies greatly among organisms raising questions about the functional implications of the different SCs. In plants, CI is mainly associated with CIII, while CIV is mainly in the monomeric form and partly associated with CIII [134,135]. In yeast, *S. cerevisiae* lacks CI, and the CIV either as monomer or dimer is associated with $CIII_2$ [19] (Fig. 3A). The filamentous fungi *Neurospora crassa* [136] and *Podospora anserina* [137] exhibit dimers of CI and CI + III + IV SCs in different stoichiometries. In mammalian cells, CI is mainly associated in SCs, and this association is important for its stabilization [138–140]. $CIII_2$ and CIV are either present as individual complexes or assembled in $CIII_2$ + IV and CI + III_2 + IV whose stoichiometries

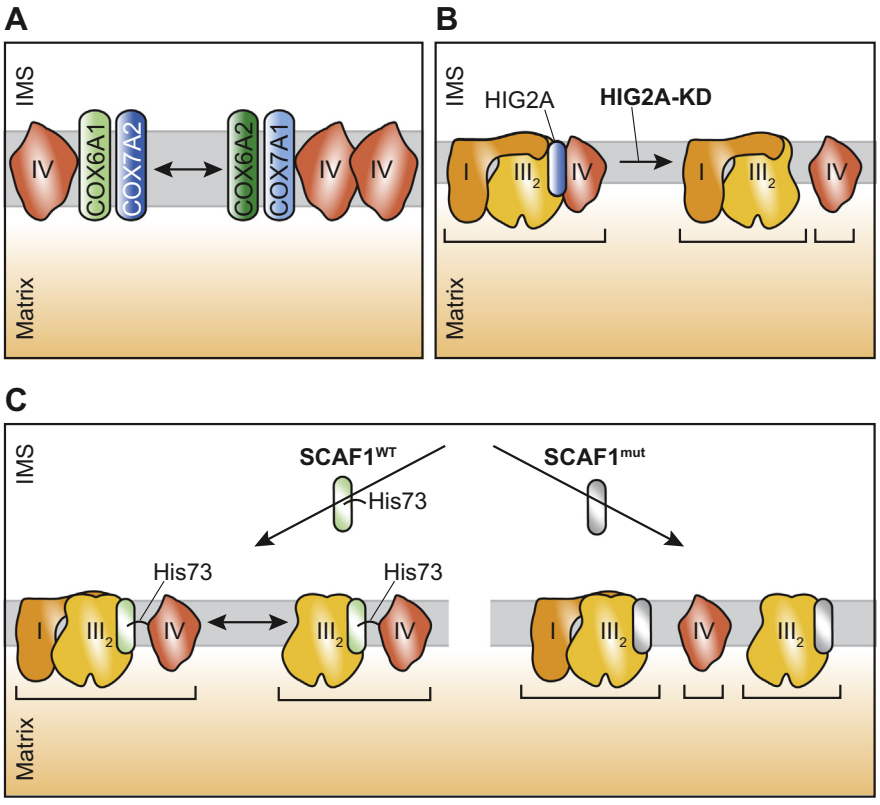


Fig. 4. SC assembly regulation and factors implicated. (A) Dimerization of CIV is affected by the presence of tissue-specific subunits. COX7A1 favors the CIV_2 form and this is stabilized by the association of COX6A2. These two subunits are specific of the heart and muscle tissue. On the contrary, the monomeric CIV is defined by COX7A2 and COX6A1, which are highly expressed in liver. (B) The mammalian protein HIG2A is a CIV subunit that regulates the $CIII_2$–CIV interaction. The HIG2A knockdown results in the loss of SCs containing CIII and CIV, with a general decrease in the activity of CIV. (C) SCAF1 is an SC assembly factor for $CIII_2$–CIV-containing SCs. It is stably bound to $CIII_2$ and binds CIV through the amino acid His73. SCAF1 consists of 113 amino acids ($SCAF1^{WT}$), but a mutated unfunctional protein of 111 amino acids lacking the His73 ($SCAF1^{mut}$) has been identified in the mouse strain C57BL/6J and Balb/cJ. $SCAF1^{mut}$ is not able to bind CIV and therefore to form $CIII_2$–CIV-containing SCs.

have not been established yet [19,21] (Fig. 3B). It is worth to mention that CII is described mainly as an independent complex, and that in mammals, different tissues and cells have different patterns of SCs, defined by tissue-specific subunits [141,142]. A prerequisite for SC assembly is that the individual complexes must be previously fully assembled [21,22]. In the case of mammalian CI, it is incorporated into SCs immediately after its formation supporting the evidence that its assembly into SCs stabilizes CI [138–140]. Indeed, when CI is not able to assemble, it is unstable and degraded. How the assembly of complexes into SCs is coordinated and what are the chaperone/s involved are still an open question. In the last decade, a few studies have identified proteins whose ablation compromises SC formation. However, it is not clear whether some of these factors promote SC assembly by stabilizing the independent complexes or if they are physical bridges between two single complexes.

In 2012, three independent groups identified Rcf1 and Rcf2 as SC assembly factors in yeast [143–145]. These proteins interact with CIV and indirectly with $CIII_2$, and they are necessary for the maturation of CIV. Their deletion causes a defect in CIV activity that could be cause or consequence of the observed loss of CIV-containing SCs [145]. In mammals, there are five homologous of Rcf1 grouped in two classes, among which HIG1A and HIG2A are the broadest expressed. Depletion of HIG2A in C2C12 myoblast mirrors what is described in yeast. HIG2A destabilizes the SCs containing CIV increasing the single and an incomplete form of the complex. These data suggest that HIG2A can participate in SC assembly but cannot exclude that its role in assembly is also mediated by the stabilization effect on the independent CIV (Fig. 4B). The depletion of the homolog of Rcf1 in the fungal aging model *P. anserina* destabilizes CIV and reduces the amount of CIV-containing SCs, impairing mitochondrial activity and reducing

life-span. Recently, it has been proposed that Rcf1 regulates the latest steps of CIV assembly by incorporation of lipids [146]. However, further studies are needed to clarify the function of these proteins.

In 2013, Cox7a2l has been identified in mice as the first *bona fide* SC assembly factor taking the name of SCAF1 (supercomplex assembly factor 1). In mice, this protein of 113 amino acids is required for the interaction between $CIII_2$ and CIV that are found in SCs with stoichiometry $CIII_2$ + IV and CI + III_2 + IV. SCAF1 binds $CIII_2$ through its N-terminus domain and CIV through the conserved His73 [141]. Interestingly, the SCAF1 protein sequence differs in the commonly used CD1 and SV129 mice with respect to C57BL/6J and Balb/cJ strains. The former have the wild-type protein ($SCAF1^{wt}$), while C57BL/6J and Balb/cJ have a microdeletion of two amino acids that encode for a 111-amino-acid protein ($SCAF1^{111}$). As a result of this modification, C57BL/6J and Balb/cJ mice lack $CIII_2$ + IV and CI + III_2 + IV SCAF1-containing SCs. Therefore, SCAF1 is able to segment the ETC in separated routes used either by NADH (CI + III_2 + IV and CI + III_2) or $FADH_2$ (CII) preventing the saturation of the ETC by only one substrate [147]. The presence of CI + III_2 + IV-containing SCs in the heart and muscle of C57BL/6J persuaded some authors to conclude that the presence of $SCAF1^{wt}$ is not required for the assembly of the respirasome but only for the $CIII_2$ + IV [148,149] and that it does not have any effect on the respiratory performance by different substrates [149]. Enriquez and co-workers demonstrated that C57BL/6J mice having $SCAF1^{111}$, lose completely the $CIII_2$ + IV SC, while they display a significant reduction in the CI + III_2 + IV SC, which only remains in a relevant amount in skeletal muscle and heart. Interestingly, the remaining SC CI + III_2 + IV does not have SCAF1 but COX7A2. These results demonstrate that there are different pools of CI + III_2 + IV-containing SCs that differ in the presence or absence of SCAF1 (Fig. 4C). The data obtained by Letts and collaborators [150] support this idea proposing two different conformations of CI + III_2 + IV depending on $CIII_2$ and CIV proximity. These results might be explained by the presence of SCAF1 that connects $CIII_2$ + IV resulting in a closer conformation. It cannot be excluded that other factors like HIG2A somehow influence the assembly. Whether the diverse CI + III_2 + IV-containing SCs described might function differently has still to be proven.

From the functional point of view, the *plasticity model* considers that the structure and assembly of complexes and SCs are dynamic and they adapt according to the metabolic demand and cytosolic cues. The demonstration that different SC organizations result in different respiratory performance upon different substrates supports the hypothesis that different SC organizations can modulate the metabolism not only in term of disease but also in the range of healthy phenotypes. This is observed in the liver after starvation, where a decrease of $CIII_2$ + IV interaction is observed [147]. Moreover, SCs increase both in human [151] and in rat upon physical activities and show an antioxidant effect [152]. In mitochondrial rat cortex, SCs containing CI decrease with age [153]. Heart failure in the canine model of intracoronary microembolization induces a dramatic decrease of SCs but retains complexes [154]. Interestingly, SCs decrease in muscle from diabetic individuals. Overall, these data support the correlation between SC organization and metabolism.

Nowadays, it is widely accepted that the OXPHOS is a dynamic system that modulates its organization to adjust the metabolic conditions, but a lot has still to be discovered about the pathways that modulate the formation and dynamic organization of SCs, especially during metabolic fluctuations.

Critical Steps in OXPHOS Translation, Protein Insertion, and Early Assembly

The formation of the OXPHOS pre-assembly intermediates and SCs requires specialized protein translocases to import nDNA-encoded proteins from the cytosol to the different mitochondrial compartments and to export and insert mtDNA-encoded proteins from the matrix into the IMM.

Mitochondrial protein export: the role of Oxa1

Most of our current knowledge about the export of OXPHOS subunits comes from genetic and biochemical studies performed in the yeast *S. cerevisiae*. Also in human, the core subunits of the yeast OXPHOS complexes (Cyt*b*, subunit of CIII; Cox1, Cox2 and Cox3, subunits of CIV; Atp6, Atp8, and Atp9, subunits of CV) are highly hydrophobic conserved transmembrane proteins translated on ribosomes bound to the matrix side of the IMM, and concomitantly inserted from the ribosomal exit tunnel [155], which guides newly translated proteins into the IMM [156,157]. Structural studies of the yeast mitochondrial ribosome have shown that its large subunit is tethered to the membrane by two distinct sites: the 21S rRNA expansion segment 96-ES1 (missing in the mammalian ribosomes), and the mitoribosomal binding protein Mba1 (Multi-copy Bypass of *AFG3* mutant 1, MRPL45 in mammalian), which specifically binds to the ribosome exit tunnel [158,159]. Moreover, the structure of the ribosome in yeast has evolved, in comparison to bacteria, to sustain mitochondria-specific functions [160,161], conceivably to assist co-translational insertion of the newly synthesized core subunits of the OXPHOS complexes.

The insertion of these mtDNA-encoded proteins from the ribosomal exit tunnel into the lipid bilayer is guided by the mitochondrial membrane protein insertion system, the highly conserved translocase Oxa1 (oxidase assembly mutant 1) [155,162]. Oxa1

belongs to the YidC/Alb3/Oxa1 family, which comprises proteins of bacterial, chloroplast, and IMM, respectively. The bacterial YidC complex inserts poorly charged small proteins into the membrane on its own, while for the assembly of larger substrates, it acts in conjunction with a second protein complex, the SecYEG translocon [163–169]. To date, among the YidC/Alb3/Oxa1 family, only the structure of YidC has been solved from prokaryotic organisms [170–172] and, similarly to other members from this group, it contains a catalytic domain consisting of five transmembrane spans crucial for its function [173].

Over the course of evolution, the insertion system was reduced to Oxa1 only (YidC homolog in *S. cerevisiae*). Oxa1 forms a pore across the IMM that according to electrophysiology studies done in yeast is regulated by the membrane potential [174,175]. The C-terminal tail of the Oxa1 insertase interacts with the large subunit of the mitochondrial ribosome [156,157,176,177], consistent with its proposed role in the co-translational membrane insertion of mtDNA-encoded proteins [178,179]. Mba1, in turn, cooperates with Oxa1 to mediate an efficient insertion process, and experimental evidence suggests that Mba1 is responsible for aligning the ribosome exit-tunnel with Oxa1 [180–184]. However, the mechanisms by which Oxa1 coordinates mitochondrial-protein synthesis and membrane insertion of newly synthesized polypeptides are not known.

Several studies support a fundamental role for the ribosome–Oxa1 interactions in the assembly of all the yeast OXPHOS complexes [185–187]. On the other hand, human OXA1L, which also interacts with the large subunit of the ribosome, partially complements the respiratory-deficient phenotype of yeast Oxa1. Indeed, it has been shown that OXA1L depletion affects the assembly of CI and CV [187]. Recently, Thompson and colleagues [188] have described a patient with an OXA1L mutation showing an additional CIV defect, raising the question about a wider defect on OXPHOS assembly. Future work should be undertaken to solve the structure of Oxa1 and to identify additional players of the human mitochondrial protein insertion machinery.

Early steps of OXPHOS assembly coordinate mitochondrial translation

Once in the IMM, single unassembled subunits need to be incorporated into assembly intermediates to proceed with the full assembly of complexes. Assembly of these complexes requires sophisticated regulation mechanisms described here below.

The translational feedback loop in yeast mitochondria

Following the insertion of newly synthesized mitochondrial proteins by Oxa1, mtDNA-encoded subunits assemble with nDNA-encoded subunits into sequential maturing assembly intermediates. During this process, redox-centers (heme or copper) essential for electron transfer are incorporated into the mitochondrial encoded core subunits tightly regulated by assembly factors. These ancillary factors, some conserved in human, have different functions, including the insertion of redox-centers or translation regulation [23,127,189–195].

In contrast to mammals, yeast mitochondrial transcripts contain 5′ untranslated regions (5′ UTRs) which support the binding of nDNA-encoded translational activators. Most of the latter associate with mitochondrial ribosomes and are bound to the IMM to spatially delimitate mitochondrial translation at the membrane surface. Feedback regulation mechanisms, implying translational activators, have been defined to coordinate the synthesis of mtDNA-encoded proteins to the state of assembly of the OXPHOS complexes. So far, three feedback translation mechanisms have been reported to regulate the synthesis of *ATP6/ATP8*, *COX1,* and *CytB* in yeast mitochondria. The translation of Atp6/Atp8 is exclusively linked to the state of assembly of the ATP synthase [196,197], while Cox1 and Cyt*b* synthesis are regulated by the insertion of co-factors such as heme moieties.

One of the best-studied examples of mitochondrial protein synthesis coupled to OXPHOS assembly is the core subunit of the cytochrome *c* oxidase, Cox1. Cox1 houses the heme as redox-center and in *S. cerevisiae* (Fig. 5A), and its translation is activated by the binding of the translational activators Mss51, Pet309, and Pet54 to the 5′ UTR of *COX1* mRNA [198–204]. Then, Mss51 binds the newly synthesized Cox1 (Fig. 5A, step 1) and the early assembly factors Coa3 (Cox25), Cox14, and Coa1 (Fig. 5A, step 2) [205–210]. The sequestration of Mss51 into this assembly intermediates preventing it from activating further cycles of Cox1 translation. The association of Shy1 and other nuclear subunits releases Mss51 from the sub-complex comprising Coa1, Coa3, Cox14, and Cox1 (Fig. 5A, step 3). The interaction of Mss51 with Pet54 may drive Mss51 initiation to further rounds of protein synthesis (Fig. 5A, step 4) [211,212]. Soto and collaborators [195] have shown that Mss51 binds heme-*b* and that its interaction with the heme moiety is necessary for efficient Cox1 synthesis. Through this mechanism, Mss51 enables the cell to adjust the rate of Cox1 synthesis to the state of complex assembly, as well as to heme or oxygen availability, which is needed for heme biosynthesis. The recently identified translational activator Mam33, conserved in eukaryotic organisms, is another example of the regulation of Cox1 synthesis. Indeed, Mam33 is required for efficient Cox1 synthesis when cells shift from fermentative to respiratory metabolism [213,214].

The regulation of yeast CIII assembly follows a similar route to the Cox1 pathway [117]. The mitochondrially encoded cytochrome *b* (Cyt*b*) is the central

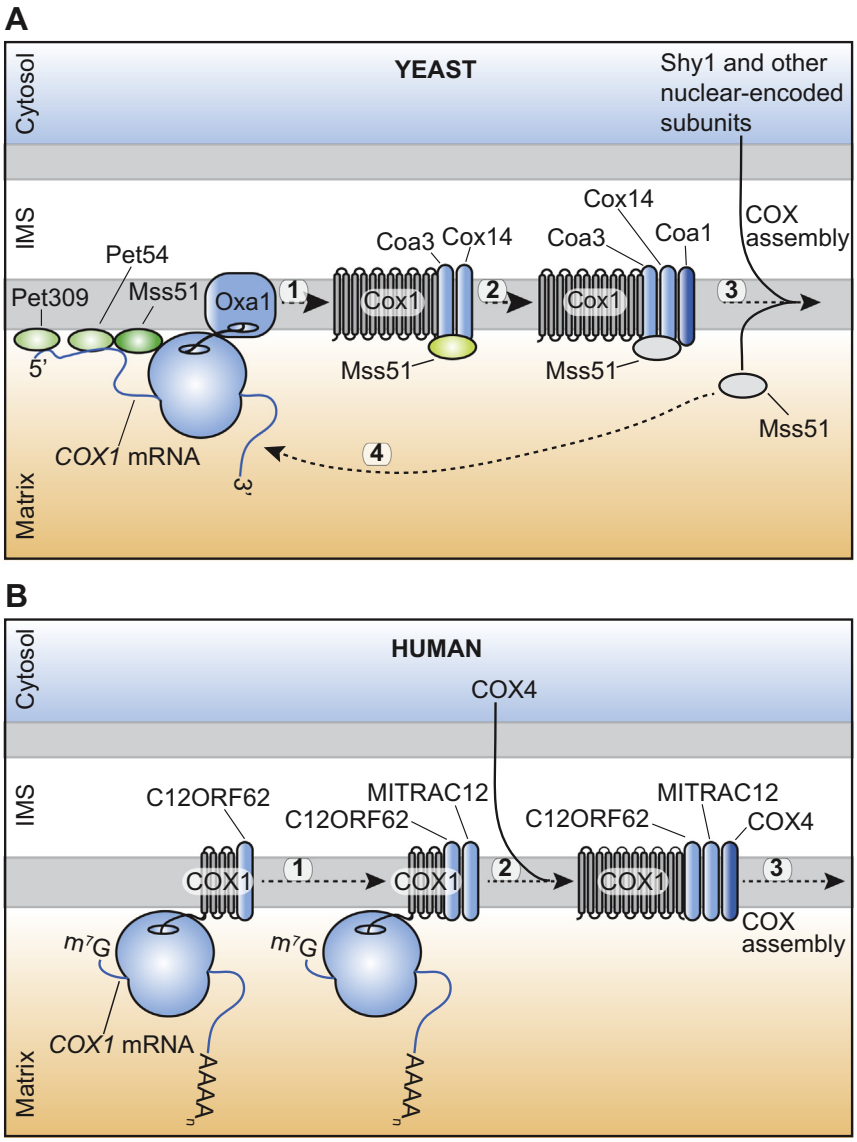


Fig. 5. Model of translation regulation of Cox1. (A) Yeast model. The Cox1 polypeptide is synthesized on mitochondrial ribosomes, which are bound to the matrix side of the IMM. In yeast, activation of translation requires the action of the specific translational activators Pet54, Pet309, and Mss51 (green), which associate with the 5′UTR of *COX1* mRNA. During the translation process, Cox1 is concomitantly inserted into the lipid bilayer by the translocase Oxa1 [1]. Newly translated Cox1 interacts with Coa3 and Cox14, which promote association of Mss51 with Cox1. At this stage of the Cox1 assembly process, Mss51 (yellow) is still capable of initiating further cycles of Cox1 synthesis [2]. Subsequent association of Coa1 with the complex sequesters Mss51 (gray) in an inactive state and is therefore unable to initiate translation. The interaction of Shy1 and other nuclear-encoded subunits with the assembly intermediate leads to the release of Mss51 [3], allowing it to promote Cox1 synthesis [4]. Within brackets are indicated the numbers corresponding to different regulatory step. (B) Human model. C12ORF62 associates with COX1 and it is recruited to mitochondrial ribosomes that are actively synthesizing COX1, specifically upon translation of 4 to 6 of the 12 transmembrane spans of COX1. Afterward, the specific C12ORF62–ribosome–nascent chain complex associates with MITRAC12 [1]. Consequent to the import of the nuclear-encoded COX4, COX1 is fully translated [2] and the cytochrome *c* oxidase assembly process can proceed [3]. Lack of MITRAC12 or COX4 stalls partially synthesized and membrane-inserted COX1 in association with C12ORF62. Within brackets are indicated the numbers corresponding to different regulatory steps.

subunit of the complex and harbors heme *b*. Cyt*b* synthesis is coupled to the incorporation of heme *b* into CIII by the assembly intermediate comprising newly synthesized Cyt*b* and the Cbp3 and Cbp6 assembly factors. The hemylation event induces a conformational change and binding of Cbp4, which leads to the release of the Cbp3–Cbp6 complex and consequent activation of further rounds of Cyt*b* translation. Failure of heme *b*

incorporation into Cyt*b* leads to the downregulation of Cyt*b* synthesis rate [215,216]

In conclusion, it becomes increasingly evident that mitochondrial translation must be tightly coordinated to link the state of assembly of the OXPHOS complexes to cellular metabolic demands. In further support of this coordination, a recent work in yeast demonstrates that the synthesis of nDNA-encoded genes coordinates mitochondrial translation of the OXPHOS system following a nutrient shift from fermentable to non-fermentable carbon sources [105]. The synchronization of both translational machineries could be explained by modulating synthesis of translational activators or influx of OXPHOS components regulating the assembly of mtDNA-encoded subunits. Further studies determining the factors that regulate the biogenesis of the OXPHOS complexes consequent to metabolic shifts will be relevant to dissect mitochondrial-nuclear coordination of the OXPHOS assembly machinery, as well as to extend our knowledge on the mechanisms that link cofactors insertion and mitochondrial gene expression.

Coupling assembly and translation in mammals

Unlike in yeast, the absence of significant 5′UTR in mitochondrial transcripts in mammals necessitates alternative mechanisms to coordinate the assembly of the OXPHOS complexes to the translation of mitochondrial encoded proteins [217–219]. Indeed, most of the yeast translational activators are not conserved in humans. For example, TACO1, the first specific COX1 translation regulator identified in mammals has no clear yeast homolog [220]. Although in mammals, mtDNA-derived mRNA lacks the 5′ UTR, TACO1 seems to bind mainly to the 5′ end of the *COX1* mRNA and is needed for efficient COX1 synthesis through its interaction with the ribosome [221]. Mutations in TACO1 lead to reduced CIV activity [220].

Recently, Mick and co-workers [222] identified the early assembly intermediates of CIV, the MITRAC12 (mitochondrial translation regulation assembly intermediate of cytochrome *c* oxidase 12, Coa3 in yeast) complexes, providing insights into the mechanism linking COX1 synthesis to its assembly. The MITRAC12 protein [223,224], previously identified as CCDC56 [225], defines the MITRAC complexes as MITRAC12 containing complexes. Multiple assembly factors constitute it, comprising MITRAC15 (Coa1 in yeast), surfeit locus protein 1 (SURF1, Shy1 in yeast) [226,227], C12ORF62 (Cox14 in yeast), COX16 (Cox16 in yeast) [128,129], and COX assembly mitochondrial protein 1 (CMC1, Cmc1 in yeast) (Fig. 2) [228,229]. A recent study shows that MITRAC12 and C12ORF62 associate with ribosomes that specifically synthesize COX1 (Fig. 5B, step 1) [194]. In the absence of the early assembled nuclear-encoded subunit COX4, COX1 translation and membrane insertion is stalled, and ribosome–COX1 nascent chain complexes accumulate in association with C12ORF62 (Fig. 5B, step 2). This pool of COX1 can be released to proceed in translation when COX4 is not limiting (Fig. 5B, step 3). These findings demonstrate that ribosomes modulate the synthesis and insertion of nascent chain polypeptides in response to the assembly steps of the pathway [194]. The biological relevance of the MITRAC complex is underlined by the fact that mutations in some of its components, such as SURF1, MITRAC12, and C12ORF62, are associated with CIV deficiency and lead to severe human disorders [223,230–236]. Recently, an alternative translation-independent mechanism regulating COX1 biogenesis has been described. Bourens and Barrientos [229] have shown that the non-essential intermembrane space twin CXC9 protein CMC1 regulates the stability of newly synthesized COX1 prior and during its assembly with other CIV subunits without affecting its rate of synthesis. The authors conclude that CMC1 teams up with MITRAC12 and C12ORF62 to stabilize COX1 and is subsequently released from the complex when COX1 acquires prosthetic groups (heme/copper) or associates with COX4I or COX5A. These findings imply that non-assembled COX1 is turned over; hence, proteolysis restores the established stoichiometry of the complexes.

Taking in consideration that MITRAC12 is present in more than one complex, it could be speculated that different MITRAC12- and C12ORF62-containing complexes coexist and act differently at distinct steps of the CIV assembly pathway from the mitochondrial ribosome to later stages of assembly. It is plausible to hypothesize that COX4I assembles either on early complexes comprising the mitochondrial ribosome, MITRAC12 and C12ORF62 or/and on later complexes containing MITRAC12, C12ORF62, and CMC1. In this way, synthesis and stability of COX1 would be regulated at different stages. To date, it is not known if similar MITRAC-dependent mechanisms are restricted to COX1 translation or extend to other subunits of the OXPHOS complexes.

Human CIII assembly may be subjected to a regulation comparable to the one described for CIV by Richter-Dennerlein and colleagues [194]. The ubiquinol-cytochrome *c* oxidoreductase complex assembly factor 2, UQCC2 (Cbp6 in yeast), cooperates with UQCC1 (Cbp3 in yeast) in the synthesis of the mitochondrial-encoded MT-CYB (cytochrome *b*) [119]. It is appealing to hypothesize that other OXPHOS complexes such as complexes I and V, which also harbor mtDNA-encoded subunits, are subjected to feedback regulatory mechanisms linking translational machinery and assembly intermediates [219]. Future studies in defining how the assembly of single OXPHOS subunits and the mitochondrial translation machinery are coordinated will be essential to extend our understanding of the biogenesis of the OXPHOS system.

Coupling mitochondrial protein import and folding machinery to respiratory chain assembly

The nDNA-encoded mitochondrial subunits must enter mitochondria, fold correctly, and reach the right subcompartment to proceed with their assembly in OXPHOS complexes. This function is appointed to the mitochondrial import machinery and firstly requires the TOM complex (translocase of the outer membrane). Once inside mitochondria, according to the precursor protein destination, specific protein machineries are required for the folding and transport into the specific mitochondrial subcompartments: IMM, intermembrane space (IMS) or matrix. In general, proteins targeted to the IMM or matrix containing an N-terminal presequence, consisting of a positively charged amphipathic α-helix, are imported by the presequence translocase of the inner membrane (TIM23). From here, proteins are sorted based on sequence features: (i) precursor proteins with downstream hydrophobic sorting signal are laterally sorted into the IMM; (ii) matrix targeted precursor proteins are translocated by the presequence translocase-associated import motor (PAM), process that requires ATP. Afterwards, the presequence is cleaved by a mitochondrial processing peptidase (MPP), which results in protein maturation; (iii) hydrophobic inner membrane proteins and mitochondrial carrier proteins transported across the TOM complex into the IMS will be delivered by small TIM chaperones (Tim9, Tim10, and Tim12) to the TIM22 complex that drives their insertion into the IMM in a membrane potential-dependent process; and (iv) soluble cysteine-rich proteins of the IMS require the MIA (mitochondrial intermembrane space import and assembly) pathway for their oxidation and trapping within this mitochondrial compartment (the details on the mitochondrial import system are recently reviewed in Ref. [237]).

In general, the TIM-mediated import of OXPHOS components needs the membrane potential and ATP to be carried out that, in turn, is generated by the OXPHOS system itself, a line of evidence for the functional interdependence of both systems. Supporting this hypothesis, many studies in yeast demonstrate that some components of the OXPHOS, import, and MIA pathway are interacting. In this model, Tim21, a subunit of TIM23 translocase, interacts with the $CIII_2$ + IV-SC through Qcr6, a subunit of $CIII_2$ [238]. Moreover, the binding of Tim21 to the TIM23 translocase is mediated by one of its components, Mgr2 (mitochondrial genome required 2), which couples the RCCs to the translocase [239]. Independently of Tim21, the two regulatory PAM subunits Pam16–Pam18 copurify with CIII + IV [240].

In human, using an extensive panel of biochemical and proteomic techniques, Mick and co-workers [222] described that TIM21 shuttles imported respiratory chain subunits from the TIM23 translocase to the MITRAC complex, fundamental for CIV formation. Indeed, TIM21 is identified among the constituents of the MITRAC12-containing complexes and has been co-purified with CI assembly intermediates, data further confirmed by the dynamic complexome profiling of CI assembly performed by Guerrero-Castillo and co-workers [22]. These data support the import–OXPHOS machinery functional regulation and interdependence. Since TIM23 translocase is the main entry pathway of OXPHOS proteins, it is presumptive that TIM21 link TIM23 function with the regulation of the respiratory chain assembly. Moreover, since previous works demonstrated that CIII is structurally required for the stabilization of CI [138], it is very likely that the import of proteins for CI and CIII has to be coordinated. A similar regulatory mechanism can be hypothesized for the assembly of $CIII_2$ + IV. A further indication that supports the import–OXPHOS machinery interdependence comes from data demonstrating the CII subunit Sdh3 to be also a component of the TIM22 carrier translocase, and its presence is required for the assembly of the subunit TIM18 into the carrier [241]. Overall, the data support a functional interdependence of both OXPHOS and import systems.

Perspectives

The mitochondria ultrastructure consents a wide variety of mitochondrial functions, thanks to their compartmentalization. The presence of a double membrane and their own genome increases the complexity of processes such as protein import and targeting, and the assembly of nDNA- and mtDNA-encoded proteins to form functional RCCs and SCs. The assembly pathway requires the so-called assembly factors that intervene at the level of (i) mRNA by regulating translation or (ii) by coordinating and leading the assembly of different subunits. Therefore, RCC and SC formation can be regulated at different levels from gene expression to SC assembly.

At the genome level, the signaling and molecular mechanism coordinating both genomes are of great interest but largely unknown. The mtDNA *per se* is not regulating transcription and synthesis of nDNA-encoded mitochondrial proteins since cells lacking mtDNA are still able to synthesize these proteins. However, coordination between both nuclear and mitochondrial genome has been proposed to be led by the nucleus and regulated by cytosolic cues. The retrieval of some unexpected nuclear transcription factors inside the mitochondria leads to hypothesize their involvement in the regulation of the mtDNA gene expression. In mammals, the estrogen receptor [242], CREB (*c*yclic-AMP *r*esponse *e*lement *b*inding protein) [243], the T3 receptor p43 [244], and STAT3 (*s*ignal *t*ransducer and *a*ctivator of *t*ranscription 3) [245] have been described. Except for the estrogen receptor, other transcription factors can bind the D-Loop and hypothetically modulate mtDNA transcription

when available. Interestingly, the expression of these proteins is associated to a diverse range of stimuli and signaling pathways [246], and in some cases, the expression is tissue-specific [247]. All these observations fit in a theoretical pathway of nuclear-mitochondrial genome communication pathway.

Also, the role of compartments such as P-bodies and MRG in the translation regulation that precedes OXPHOS protein synthesis is very intriguing. In the cytoplasm, P-bodies store transcripts that can re-enter translation after unknown specific signals. Up to date, nDNA-encoded mitochondrial transcripts have not been found in the P-bodies, a result that excludes their storage and a mechanistic regulation from this compartment. These data lead to suggest that these transcripts go in continuous translation. However, further studies are required to optimize P-bodies isolation and clarify their impact on OXPHOS synthesis. Inside the mitochondria, the MRG appear as a spatial compartment dedicated to RNA maturation, but it is unclear whether they can be considered as a nucleoid-independent subunit. Currently, there is no evidence to attribute additional roles to this compartment like RNA storage, but future studies will shed light on this question.

A critical step for RCC formation is the export of mDNA-encoded proteins into IMM and the import into the mitochondria of nDNA-encoded OXPHOS subunits. Many studies in yeast have evidenced the role of translation activators in the protein insertion and assembly of RCCs. In mammals, this is less known and the only translation activator identified is TACO1 interacting with *COX1* mRNA in a 5′-UTR-independent manner. Differences between yeast and mammalian translation regulation are expected due to structural RNA features. Thus, the finding of new translation regulators, like TACO1, that participate in RCC formation is foreseeable.

Moreover, mammals present a higher variety of SCs species that implies a higher request of assembly factors. Up to date, SCAF1 and HIG2A mediate the $CIII_2$–CIV association, but the presence of some remaining SCs with the same stoichiometry and composition in the absence of SCAF1 indicates the involvement of additional assembly factors, inducible or constitutive, that favor the SCs maintenance to respond to specific requirements. The existence and identification of these factors needs further investigation.

Last, whether and how the import machinery participates in the assembly of RCCs at the IMM appears to be somehow linked to an organization and distribution issue. TIM21, a TIM23 subunit, has been showed shuttle nDNA-encoded subunits to CIV creating a functional link between both the import and OXPHOS system. A model in which TIM21 drives specifically the CIV subunits from TIM23 to the complex leads to hypothesize that different TIM23 complex extra subunits might contribute to the assembly of a specific complex, acting as "complex-specific import-assembly factors.". This idea is interesting but speculative, with many open questions to be clarified and addressed in the near future.

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Abbreviations used:
IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; IMS, intermembrane space; ETC, electron transport chain; RCCs, respiratory chain protein complexes; SCs, Supercomplexes; ROS, reactive oxygen species; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NADH, Nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; OXPHOS, oxidative phosphorylation system; CI, complex I (NADH-ubiquinone oxidoreductase); CII, Complex II (succinate-quinone oxidoreductase); CIII, complex III (ubiquinol-cytochrome *c* oxidoreductase, cytochrome bc_1 complex); CIV, complex IV (cytochrome *c* oxidase); CV, complex V (ATP synthase); nDNA, nuclear DNA; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; tRNA, transfer RNA; mRNA, messenger RNA; H strand, heavy strand; L strand, light strand; HSP1, heavy strand promoter 1; HSP2, heavy strand promoter 2; LSP, light strand promoter; ND6, NADH-ubiquinone oxidoreductase chain 6; MRGs, mitochondrial RNA granules; NPC, nuclear pore complex; DDX6, DEAD box protein 6 or Probable ATP-dependent RNA helicase; 4ET, Eukaryotic translation initiation factor 4E transporter; XRN1, 5′–3′ exoribonuclease 1; P-bodies, processing bodies; NDUFS1, NADH-ubiquinone oxidoreductase 75 kDa subunit; NDUFV1, NADH dehydrogenase-ubiquinone flavoprotein 1; NDUFV2, NADH dehydrogenase-ubiquinone flavoprotein 2; NDUFS2, NADH dehydrogenase-ubiquinone iron–sulfur

protein 2; NDUFS3, NADH dehydrogenase-ubiquinone iron–sulfur protein 3; NDUFS7, NADH dehydrogenase-ubiquinone iron–sulfur protein 7; NDUFS8, NADH dehydrogenase-ubiquinone iron–sulfur protein 8; ND1-6, NADH-ubiquinone oxidoreductase chain 1-6; ND4L, NADH-ubiquinone oxidoreductase chain 4L; SDHA, succinate dehydrogenase ubiquinone flavoprotein subunit; SDHB, succinate dehydrogenase ubiquinone iron–sulfur subunit; SDHC, succinate dehydrogenase cytochrome b560 subunit; SDHD, succinate dehydrogenase [ubiquinone] cytochrome *b* small subunit; SDHAF1, SDH assembly factor 1; SDHAF2, SDH assembly factor 2; SDHAF3, SDH assembly factor 3; SDHAF4, SDH assembly factor 4; cyt *c*, cytochrome *c*1 heme protein; MT-CYB (Cyt*b*), mitochondrial cytochrome *b*; CYC1, cytochrome *c*1; UQCRFS1/RISP, cytochrome bc_1 complex subunit Rieske; UQCRC1, cytochrome bc_1 complex subunit 1; UQCRC2, cytochrome bc_1 complex subunit 2; UQCRH, cytochrome bc_1 complex subunit 6; UQCRB, cytochrome bc_1 complex subunit 7; UQCRQ, cytochrome bc_1 complex subunit 8; UQCR10, cytochrome bc_1 complex subunit 9; UQCR11, cytochrome bc_1 complex subunit 10; UQCC1 (cbp3), ubiquinol-cytochrome-*c* reductase complex assembly factor 1; UQCC2, ubiquinol-cytochrome-*c* reductase complex assembly factor 2; UQCC3, ubiquinol-cytochrome-*c* reductase complex assembly factor 3; BCS1, (cytocrome BC1 synthesi, mitochondrial chaperone); LYRM7, LYR motif-containing protein 7; TTC19, tetratricopeptide repeat protein 19; COX1 (cox1), cytochrome *c* oxidase subunit 1; COX2 (cox2), cytochrome *c* oxidase subunit 2; COX3 (cox3), cytochrome *c* oxidase subunit 3; COX4, cytochrome *c* oxidase subunit 4; COX7A1, cytochrome *c* oxidase subunit 7A1; COX6A2, cytochrome *c* oxidase subunit 6A2; COX7A2, cytochrome *c* oxidase subunit 7A2; COX6A1, cytochrome *c* oxidase subunit 6A1; Rcf1, respiratory supercomplex factor 1; Cox7a2l, cytochrome *c* oxidase subunit 7A-related protein; Rcf2, respiratory supercomplex factor 2; HIG1A, hypoxia-inducible gene domain family member 1A; HIG2A, hypoxia-inducible gene domain family member 2A; SCAF1, supercomplex assembly factor 1; Atp6, ATP synthase subunit a; Atp8, ATP synthase protein 8; Atp9, ATP synthase subunit 9; Mba1, multi-copy bypass of AFG3 protein; MRPL45, 39S ribosomal protein L45; OXA1L (Oxa1), oxidase assembly mutant 1; 5′ UTRs, 5′ untranslated regions; Mss51, mitochondrial splicing suppressor protein 51; Pet309, pentatricopeptide repeat-containing protein PET309; Pet54, petite colonies protein 54; MITRAC12 (Coa3), cytochrome *c* oxidase assembly factor 3; C12ORF62 (Cox14), cytochrome *c* oxidase assembly protein COX14; MITRAC15 (Coa1), cytochrome *c* oxidase assembly factor 1; COX16 (cox16), cytochrome *c* oxidase assembly protein COX16; SURF1 (Shy1), cytochrome oxidase assembly protein SHY1; CMC1 (cmc1), COX assembly mitochondrial protein 1; Mam33, mitochondrial acidic protein MAM33; Cbp4, cytochrome *b* mRNA-processing protein 4; Cbp6, cytochrome *B* pre-mRNA-processing protein 6; TACO1, translational activator of cytochrome *c* oxidase 1; TOM, translocase of the outer membrane; TIM23, mitochondrial import inner membrane translocase subunit Tim23; PAM, presequence translocase-associated import motor; MPP, mitochondrial processing peptidase; Tim9, mitochondrial import inner membrane translocase subunit Tim9; Tim10, mitochondrial import inner membrane translocase subunit Tim10; Tim 12, mitochondrial import inner membrane translocase subunit TIM12; TIM22, mitochondrial import inner membrane translocase subunit Tim22; MIA, mitochondrial intermembrane space import and assembly; Tim21, mitochondrial import inner membrane translocase subunit Tim21; Qcr6, cytochrome *b*–*c*1 complex subunit 6; Mgr2, mitochondrial genome required 2; Pam16, presequence translocated-associated motor subunit PAM16; Pam18, presequence translocated-associated motor subunit PAM18; sdh3, succinate dehydrogenase [ubiquinone] cytochrome *b* subunit; TIM18, mitochondrial import inner membrane translocase subunit TIM18; CREB, cyclic-AMP response element binding protein; STAT3, signal transducer and activator of transcription 3.

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