Inventory control: cytochrome oxidase assembly regulates mitochondrial translation

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Abstract

Mitochondria maintain a genome and translation-machinery to synthesize a small subset of subunits of the oxidative phosphorylation system. These organellar gene products must assemble with imported subunits that are encoded in the nucleus to build up functional enzymes. New findings on the early steps in cytochrome oxidase assembly reveal how the mitochondrial translation of its core component Cox1 is directly coupled to the assembly of this respiratory complex.

Introduction

The four respiratory chain complexes in the mitochondrial inner membrane utilize electrons from NADH or $FADH_2$ as energy to generate a proton gradient across the membrane that drives ATP production by the F_1F_o-ATP synthase (complex V). Electrons are passed through the redox systems of these complexes and are finally transferred to molecular oxygen by cytochrome oxidase (complex IV). Thus, the energy of the oxyhydrogen reaction is utilized in an indirect way by the mitochondrial respiratory chain to produce ATP.

Three of the mitochondrial respiratory complexes (I, III, IV), and the F_1F_o-ATPase, comprise subunits of dual genetic origin. The majority of respiratory chain subunits are encoded by nuclear genes, translated on cytosolic ribosomes, and imported into mitochondria by the translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) import machineries[1,2] (FIG. 1). In contrast, the mitochondrial genome encodes only a small number of core complex subunits, most of which are exceedingly hydrophobic. Mitochondrial DNA (mtDNA) encodes seven subunits of complexes III, IV, and V in *Saccharomyces cerevisiae*, and thirteen subunits of complexes I, III, IV, and V in humans.

To express their small organellar genomes, mitochondria have maintained a transcription–translation apparatus during evolution, the components of which are mainly encoded by the nucleus and represent almost one third of the mitochondrial proteome[3]. Transcription and translation apparently occur at the matrix side of the mitochondrial inner membrane. Oxa1, a member of a widely conserved family of translocases, is a main constituent of the mitochondrial protein export machinery, which facilitates the insertion of hydrophobic domains into the bilayer and the export of hydrophilic protein domains to the mitochondrial intermembrane space[4]. (FIG. 1). Its C-terminal domain binds to mitochondrial ribosomes[5,6] near their exit-tunnel[7,8], consistent with a co-translational mechanism for the insertion of

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mitochondrially synthesized proteins into the inner membrane. Oxa1 also assists the post-translational insertion of some cytoplasmically synthesized proteins into the inner mitochondrial membrane after their import into the matrix[9,10]. This is essential for membrane protein domains that do not sort laterally via the TIM23 complex.

It is unclear whether the mitochondrial import of cytoplasmically synthesized respiratory chain subunits is spatially coordinated with the export and insertion into the membrane of those synthesized inside mitochondria. It is clear, however, that respiratory complex subunits do not simply self-assemble in the inner membrane. The assembly of cytochrome oxidase is the best-studied system, where many factors that assist at different steps of the assembly process have been genetically identified in yeast[11], and distinct assembly intermediates have been detected in yeast and human organelles (BOX 1).

**Box 1**

The cytochrome oxidase assembly line

The mRNAs coding for the mitochondria-encoded respiratory chain subunits Cox1, Cox2 and Cox3 have specific translational regulators that have been defined in yeast (orange spheres) and assembly factors (grey and red spheres) that positively or negatively regulate translation (see the figure, part 1). The remaining cytochrome oxidase (Cox) subunits are encoded by the nucleus.

Mss51 inactivation by the Cox14–Coa1–Coa3 complex (red spheres) shuts off Cox1 translation, preventing the assembly of cytochrome oxidase (see main text). In yeast, Cox2 is translated as a precursor (pCox2) with an amino-terminal 15 amino acid extension. Both termini are transported across the membrane[4] and Cox2 is kept in an assembly-competent state by Cox20[63].

Next, Cox1 assembles with the first nuclear-encoded subunits, Cox6 and Cox5[47–49](part 2). Whether this association occurs prior to or after the insertion of co-factors into Cox1 is unclear. Mutant analyses revealed that the Cox1–Cox5–Cox6 complex can subsequently form different assembly intermediates in the absence of either Cox2 or Cox3 (part 3). A proposed Cox1–5–6–2 intermediate was identified in patients lacking Cox3[64], whereas a Cox1–5–6–3 intermediate has been characterized in yeast *cox2* mutants[48]. Copper insertion into Cox2 is a prerequisite for the association with the Cox1–Cox5–Cox6 complex[65], whereas incomplete cytochrome oxidase lacking Cox2 was found to associate with complex III of the respiratory chain[18].

Little is known about the sequence of events that follow the association of Cox2 and Cox3 with the Cox1–Cox5–Cox6 complex, as no stable assembly intermediates have been found (part 4). However, Cox7, Cox8 and Cox9 form a complex prior to their incorporation, which is mediated by Pet100[66] (part 5), although the arrangement of this subcomplex (*) is difficult to reconcile with the structure of mature cytochrome oxidase.

The incorporation of Cox12 and Cox13 concludes the formation of the complex (part 6). However, neither is essential for the enzymatic activity of the complex, which remains stable in their absence[67,68]. (White boxes, structural Cox subunits; green boxes, reported assembly intermediate complexes; schematic drawings represent assembly intermediates in a structural context, color-code as in Supplementary Figure 1)
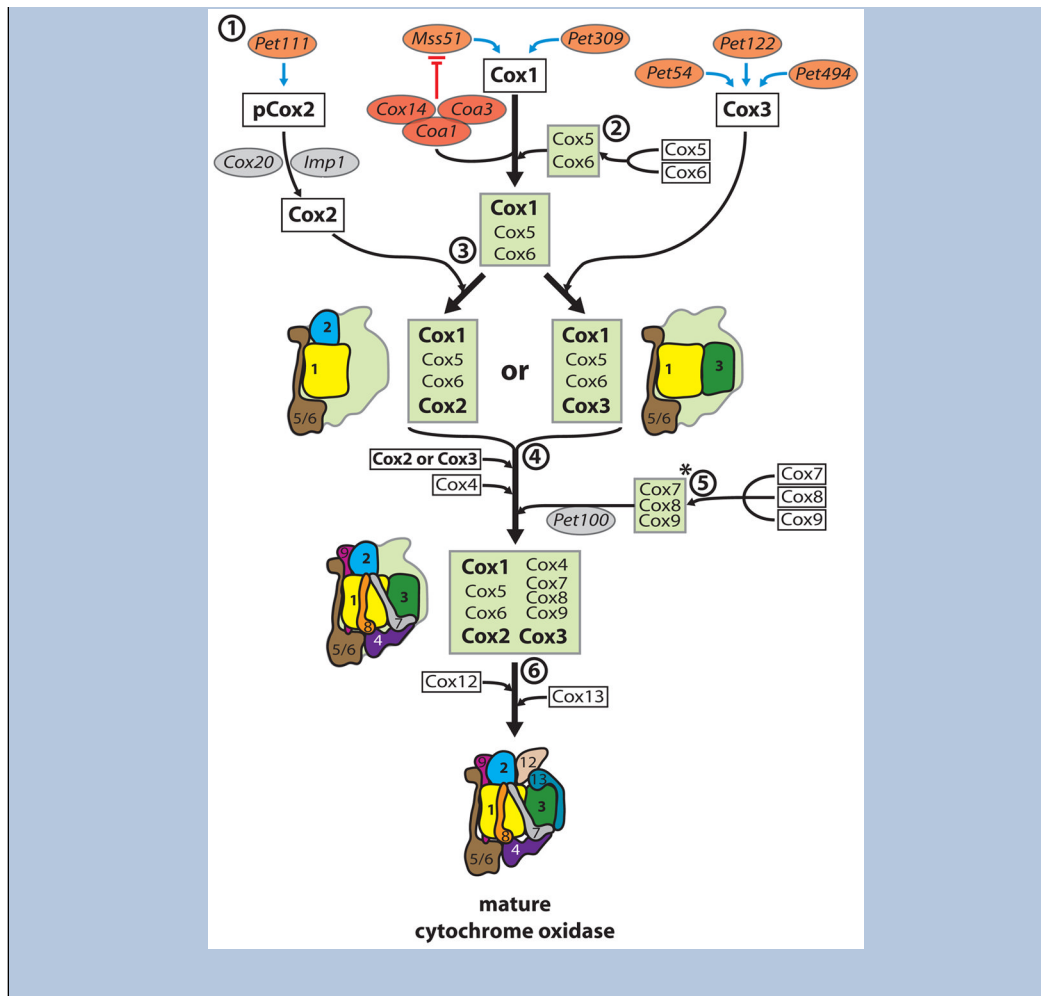
The goal of this Progress article is to discuss new insights into the molecular mechanism of cytochrome oxidase biogenesis and to highlight recent findings that link this assembly process to the regulation of the translation of its central subunit Cox1.

The cytochrome oxidase complex

Cytochrome oxidase shuttles electrons from cytochrome c to molecular oxygen to capture energy in the membrane potential by asymmetric proton uptake and proton pumping. The complex is assembled from 13 subunits in humans and 11 subunits in $S.$ $cerevisiae$. These subunits are termed Cox for cytochrome oxidase, with a non-unified nomenclature (Supplementary Figure 1). The three core subunits, Cox1, Cox2 and Cox3, which are encoded inside mitochondria, are highly conserved among all respiring organisms. The remaining subunits are encoded in the nucleus and imported into mitochondria from the cytosol. The overall dimeric structure of the bovine enzyme reveals that the redox co-factors heme and copper are inserted into the core proteins Cox1 and Cox2[12] (Supplementary Figure 1). Cox1 carries two heme molecules and a copper ion in the CuB site, while the CuA site is formed by two copper ions in Cox2. The electrons from cytochrome c enter the enzyme from the CuA site in Cox2 and end at the CuB site that together with a heme molecule forms the active center that is deeply embedded in the membrane. Little is known about the enzymatic function of Cox3. As the cytochrome oxidase subunits encoded by the nucleus are less conserved than those encoded by mitochondria, the nomenclature differs

depending on the organism (Supplementary Figure 1). We will use the yeast nomenclature in this article.

Given the complexity of this multi-protein, multi-cofactor enzyme, and the fact that its subunits enter the assembly process from two sides of the membrane, it is not surprising that the assembly of cytochrome oxidase requires a large number of assembly factors. While the functions of many assembly factors remain largely enigmatic, some have been assigned to distinct processes such as translational regulation, heme *a* synthesis and copper or heme insertion (see Supplementary Table 1).

One of the best-characterized and highly conserved assembly factors is Shy1 (SURF1 homolog in yeast). In yeast, loss of Shy1 leads to severe reduction of cytochrome oxidase and a growth defect on non-fermentable carbon sources[13]. Recent analyses support the idea that Shy1/SURF1 plays a role in the insertion step for heme a_3. However, it remains unclear whether it acts as a protein chaperone or has a more direct role in heme insertion, as suggested by heme-binding activity of a bacterial homolog[14,15].

Genetic and biochemical studies in yeast, taking Shy1 as a starting point, have led to the identification of several new factors involved in cytochrome oxidase assembly (COA)[16–20], including Coa1–Coa4. While Coa2 and Coa4 appear to cooperate with Shy1 for heme insertion[16,19,21], Coa1 and Coa3 link Shy1 functionally to early assembly intermediates of Cox1.

In the following sections we will focus on the factors and processes that link the translation of the central subunit Cox1 to these early assembly intermediates.

From mitochondrial mRNA to protein

Translation of mitochondria-encoded mRNAs occurs on mitochondrial ribosomes bound to the inner membrane, where they interact with several membrane proteins[5,6,22–25]. The intimate link between mitochondrial translation and the inner membrane is underlined by the protein synthesis defects observed in mitochondria deficient in anionic phospholipids[26].

In *S. cerevisiae*, translation of mitochondria-encoded mRNAs specifying subunits of respiratory complexes requires dedicated translational activator proteins, which recognize the 5′-UTRs of their cognate mRNAs[27] (BOX 1). In the case of cytochrome oxidase subunits, Pet309 and Mss51 act as translational activators for *COX1* mRNA (FIG. 2), Pet111 is required for *COX2* translation, and Pet54, Pet122, and Pet494 together promote the translation of *COX3* mRNA. While these functions can be separated genetically in *S. cerevisiae* mutants, in wild-type the activators are actually associated with each other and are believed to co-localize the translation of these *COX* mRNAs[28,29]. Indeed, the 5′-UTRs of the *COX2* and *COX3* mRNAs contain topogenic information presumably recognized by their activator proteins[30], which direct the mRNAs to the membrane where subsequent translation occurs.

While little is known about the biochemical mechanism of mitochondrial translational activation, the translational activators of Cox1 have been found to be involved in a regulatory feedback cycle. The *COX1* mRNA activator Pet309 is a member of the pentatricopeptide repeat protein family (PPR) that is often associated with RNA metabolism[31]. Whereas complete loss of Pet309 prevents the translation and stabilization of *COX1* mRNA, mutations in the PPR-motifs selectively affect translation[31,32]. The second *COX1*-specific activator, Mss51, is a multifunctional pioneer protein whose sequence does not contain any known functional domains or motifs. One target of action, shared with Pet309, is the 5′-UTR of the *COX1* mRNA[33,34]. However, Mss51 also has a second target

that maps genetically to the *COX1* open reading frame (ORF) allowing it to promote Cox1 synthesis and/or assembly[35]. Moreover, Mss51 interacts with newly synthesized but unassembled Cox1. Thus, Mss51 appears to interact both with *COX1* mRNA and with Cox1 protein that has not been assembled into the cytochrome oxidase complex. These dual activities of Mss51 allow it to couple synthesis of Cox1 to assembly of cytochrome oxidase.

Complex assembly regulates *COX1* translation

The synthesis of Cox1 by budding yeast mitochondrial ribosomes is reduced by mutations in most other genes that disrupt the assembly of cytochrome oxidase, e.g. mutants affecting Cox2 maturation. This suggests the existence of a regulatory feedback mechanism coupling Cox1 translation to cytochrome oxidase assembly[36]. Such a mechanism would ensure that Cox1, the core platform for cytochrome oxidase assembly, is synthesized at an appropriate rate. This is crucial given that certain unassembled Cox1 species are deleterious to cells since they act as pro-oxidants[37]. The dual activities of Mss51 appear to place it at the nexus of an assembly feedback regulatory system in which the levels of Mss51 available to activate Cox1 synthesis are limited by the amount of Mss51 sequestered by Cox1 protein in cytochrome oxidase assembly intermediate complexes[17,18,33,35,36].

Sequestration of Mss51 into Cox1 complexes

It is unclear whether Mss51 molecules that interact with the *COX1* mRNA 5′-UTR are transferred directly (in *cis*) to nascent Cox1 chains emerging from the associated ribosomes. However, when the *COX2* mRNA 5′-UTR is placed upstream of the *COX1* ORF, Mss51 can still interact with Cox1 in *trans*[33], showing an Mss51 function independent of the 5′-UTR of *COX1* mRNA. In any event, newly synthesized Cox1 also rapidly associates with Cox14 and Coa3 (Cox25), two small inner membrane proteins each with domains that are exposed to both the matrix and the intermembrane space[20,36,38,39]. Newly synthesized Cox1 promotes the dynamic association of Mss51, Cox14, and Coa3 to form a complex with Cox1, which keeps Mss51 in an apparent sequestered active state (MSS51[SA]; FIG. 2). Based on this, it is tempting to speculate that Mss51 in this complex can associate with ribosomes. In the absence of Cox1 synthesis, interactions between Mss51 and Cox14 or Coa3 are not detected, while the interaction between Cox14 and Coa3 is severely reduced, indicating that these factors only associate upon insertion of Cox1 into the membrane[20,33]. Furthermore, Cox14 and Coa3 are both required to ensure a stable interaction between Mss51 and newly synthesized Cox1[20,33]. This association is apparently required to prevent Mss51 from activating Cox1 synthesis, since *cox14Δ* and *coa3Δ* mutants both exhibit hyperactive Cox1 synthesis that is uncoupled from cytochrome oxidase assembly[20,36]. However, Cox1 synthesized in the absence of Cox14 or Coa3 is rapidly degraded before it reaches a state, in which it can act as a pro-oxidant, indicating that these assembly factors stabilize Cox1. The degradation process is likely to involve AAA proteases of the inner membrane[40,41] or the metalloendopeptidase Oma1[16,21], as it has been shown for Coa2-deficient cells, where the pro-oxidant Cox1 is already formed. The necessity for the cell to maintain a pool of unassembled Cox1 for complex assembly on the one hand and to degrade a pro-oxidant form on the other, leads to the attractive idea that different activities for Cox1 degradation may exist that are regulated by its assembly state.

Interestingly, the mitochondrial Hsp70 chaperone, Ssc1, is also associated with Mss51 in both a binary complex comprising Mss51 and Ssc1 as well as in larger complexes that also contain Cox1, Coa3 and Cox14[39,42]. Synthesis of all three mitochondria-encoded cytochrome oxidase subunits was reduced in *ssc1* mutants[43]. However, Ssc1 does not associate with newly synthesized Cox1, but instead with Atp9 and Var1[43]. While it is difficult to imagine Ssc1 playing a role in the folding of the twelve Cox1 transmembrane

domains, it could affect the structure of the C-terminal domain of Cox1, which is exposed to the mitochondrial matrix.

A role of Cox1 in its own regulation was directly shown by generating mutations that truncated the Cox1 C-terminus by 11 or 15 residues[44]. These mutations did not prevent assembly of active cytochrome oxidase. However, they did eliminate assembly feedback regulation of Cox1 synthesis, and weakened the interaction between Mss51 and Cox14 (interaction with Coa3 was not tested). Thus, the C-terminal domain of Cox1 appears to participate in Mss51 sequestration upon its emergence from the ribosome.

Mss51 inactivation by assembly intermediates

The current models for regulation of Cox1 synthesis propose that physical sequestration of Mss51 into assembly intermediate complexes containing newly synthesized Cox1, Cox14, Coa1 and Coa3 prevents it from activating *COX1* mRNA translation. Steps in this important pathway have been inferred by the examination of assembly intermediate complexes in solubilized extracts of wild-type and mutant mitochondria from *S. cerevisiae* using pull-down experiments, blue-native gel electrophoresis and sedimentation[14,17,18,20,42,44]. These data have generated a hypothetical framework for early events, but not a consensus on the pathway (FIG. 2). Furthermore, it is conceivable that in addition to sequestration, Mss51 translational activity may be altered through structural changes.

In the absence of Coa3 or Cox14, Mss51 does not associate with Cox1 or any other factors except Ssc1, and may be fully in a translation-effective form (MSS51^{TE}). Coa1, an additional player in the regulatory cycle, associates with Cox1, Coa3, Cox14 and Mss51[17,18]. MSS51^{SA} accumulates in the absence of Coa1. In *coa1* mutants, most of the detectable Mss51 is in this complex[14,20], but Cox1 synthesis is neither reduced nor subject to normal assembly feedback inhibition[17,18]. This suggests that in a complex lacking Coa1, Mss51 may not be fully inactivated and that binding of Coa1 is required to generate sequestered, translationally inactive Mss51 (MSS51^{SI}). Interestingly, Coa1 is not absolutely required for cytochrome oxidase assembly, since the *coa1Δ* phenotype can be suppressed in an unknown mechanism by adding copper to the growth medium[17], and minute amounts of mature cytochrome oxidase can be formed in the mutant[18].

In the absence of a kinetic analysis of Cox1 biogenesis, the formation of MSS51^{SA} and MSS51^{SI} as distinct pathway intermediates in wild-type *S. cerevisiae* remains hypothetical. However, the currently available data are consistent with such a stage model: at steady state Mss51 complexes that could correspond to MSS51^{TE} and MSS51^{SI} are detectable[14,16,20,42]. Thus, under normal conditions, Mss51 likely exists in different states, the equilibrium of which can be shifted according to the requirements for newly synthesized Cox1.

Connecting Mss51 release to complex assembly

The mechanism that promotes Mss51 release from assembly intermediate complexes remains enigmatic, although data suggest that Shy1 might contribute to this process. The presence of Coa1 in the MSS51^{SI} complex promotes the recruitment of Shy1 to Cox1[14,17,18]. Moreover, a lack of Shy1 traps Mss51 together with Coa1 in the sequestered-inactive state[17]. Shy1, and its homologs in other species, are likely to play a role in the maturation of functional Cox1 through incorporation of heme a_3[14,15,45], either directly[15] or by a chaperone-like activity that maintains Cox1 in an assembly competent state[14,18]. Mutants lacking Shy1 display reduced Cox1 synthesis. This phenotype is partially rescued by the missense mutations *mss51-T167R*, and *mss51-F199I*, or by Mss51 overexpression[36,46]. Moreover, recent studies have shown that overexpression of certain nuclear-encoded Cox-subunits also restores growth of *shy1Δ* mutants on nonfermentable

medium[47]. Thus, the absence of Shy1 appears to produce a bottleneck in complex assembly that sequesters Mss51, but Shy1 itself may not be necessary for Mss51 release. Interestingly, deletion of the nuclear *COX6* gene caused the greatest reduction in Cox1 synthesis among a set of cytochrome oxidase mutants, as judged by pulse-labeling and by the expression of a fused reporter gene[44]. This suggests that the addition of Cox6, which along with Cox5 is thought to be the first subunit to assemble with newly synthesized yeast and human Cox1[48,49], may be a key step in the ejection of Mss51 from assembling complexes.

COX1 in humans and disease

The malfunction of cytochrome oxidase has severe implications for cellular energy metabolism and causes increased production of reactive oxygen species with a variety of deleterious consequences in humans[50]. A number of severe disorders collectively referred to as mitochondrial encephalomyopathies are caused by cytochrome oxidase deficiency due to mutations in mitochondrial genes coding for structural subunits, or impaired assembly of the complex due to nuclear mutations affecting assembly factors. The high demand of certain tissues such as neurons and muscles for oxidative phosphorylation is apparently related to the tissue specific pathology of the patients.

Several assembly factors functioning in yeast have human orthologs (Supplementary Table 1)[11]. For example, defects in the human copper chaperones Sco1 and Sco2, first identified in yeast, cause a severe cytochrome oxidase deficiency and death shortly after birth[51,52]. Other cytochrome oxidase deficiencies cause Leigh Syndrome, a neurological disorder with bilaterally symmetrical lesions in subcortical brain regions. The most frequent causes of this disorder are mutations affecting the SURF1 protein[53,54] that is associated with early steps of Cox1 biogenesis. However, factors clearly implicated in the translational regulation of Cox1 had not been identified in mammals until very recently.

Since mammalian mitochondrial mRNAs lack significant 5′-UTRs, mechanisms regulating human Cox1 synthesis must differ from those employed in budding yeast. However an activity similar to that of yeast Mss51, whose binding to newly synthesized Cox1 allows continued synthesis and/or assembly[35], could be present in mammals and couple cytochrome oxidase assembly to Cox1 synthesis. While no clear Mss51 ortholog is encoded in mammalian genomes, the mammalian protein ZMYND17, of unknown function, is significantly similar.

In any event, post-transcriptional regulation appears to be a feature of mammalian mitochondrial gene expression, based on studies of human patients with Leigh Syndrome and cytochrome oxidase deficiency. The French Canadian variant of this disease is due to mutations affecting the nuclear gene for *LRPPRC/LRP130* (leucine-rich pentatricopeptide repeat cassette), a mitochondrial protein distantly related to Pet309[55,56]. LRPPRC is an RNA-binding protein[57] that is associated in a mitochondrial ribonucleoprotein with the stem-loop RNA-binding protein SLIRP and, at least, the mitochondria-encoded *COX1* and *COX2* mRNAs[58]. Reduced LRPPRC levels cause lower steady-state levels of most mitochondrially encoded mRNAs and a selective reduction in cytochrome oxidase levels[58]. More recently, analyses of an unrelated Leigh Syndrome patient revealed a specific reduction in Cox1 synthesis due to mutation in a gene named *TACO1*, whose protein product appears to be a *COX1*-specific translational activator in human[59]. The identification of TACO1 highlights the importance of human genetics in identifying missing assembly factors, and shows that similar mechanisms of translation control may regulate the synthesis of respiratory complex subunits in phylogenetically diverse eukaryotes.

Perspectives

Coupling of organellar gene expression at the level of translation to the assembly of organellar gene products into energy-transducing complexes may be a wide-spread phenomenon. In yeast mitochondria, translation of the bicistronic mRNA encoding the hydrophobic Atp6 and Atp8 subunits of the F_0 sector of ATP synthase was recently shown to depend upon the presence of soluble F_1 sector subunits α or β, or a partially assembled complex containing them[60]. While the mechanism of this regulation is unclear, it may prevent the formation of membrane subassemblies containing Atp6 and Atp8, which could dissipate the membrane potential if they are not assembled into a complex with F_1. In chloroplasts of *Chlamydomonas reinhardtii*, translation of organellar encoded mRNAs specifying certain key subunits of photosystem I, photosystem II, and the cytochrome b_6f complex is also coupled to the assembly of those complexes[61]. In parallel to the mechanism proposed for Mss51 function, assembly feedback regulation of cytochrome *f* synthesis depends upon C-terminal residues of cytochrome *f* itself, which could interact with a protein that also regulates translation[62].

More detailed understanding of assembly feedback control in the mitochondria of model organisms and humans may emerge from further study of the sequences in which assembly intermediates form, their precise compositions and structure, and the interaction dynamics of their constituents. Clearly, a more biochemical understanding will require the development of a true mRNA-dependent *in vitro* translation system derived from mitochondria (as opposed to translation in isolated intact organelles).

An important health-related goal is to elucidate control mechanisms that operate in human mitochondria. The identification of as-yet-undiscovered nuclear-encoded components may emerge from large-scale gene inactivation studies in cultured cells. The study of their mitochondria-encoded regulatory targets would clearly be advanced by the development of methods to genetically transform and alter animal mitochondrial genomes in a directed fashion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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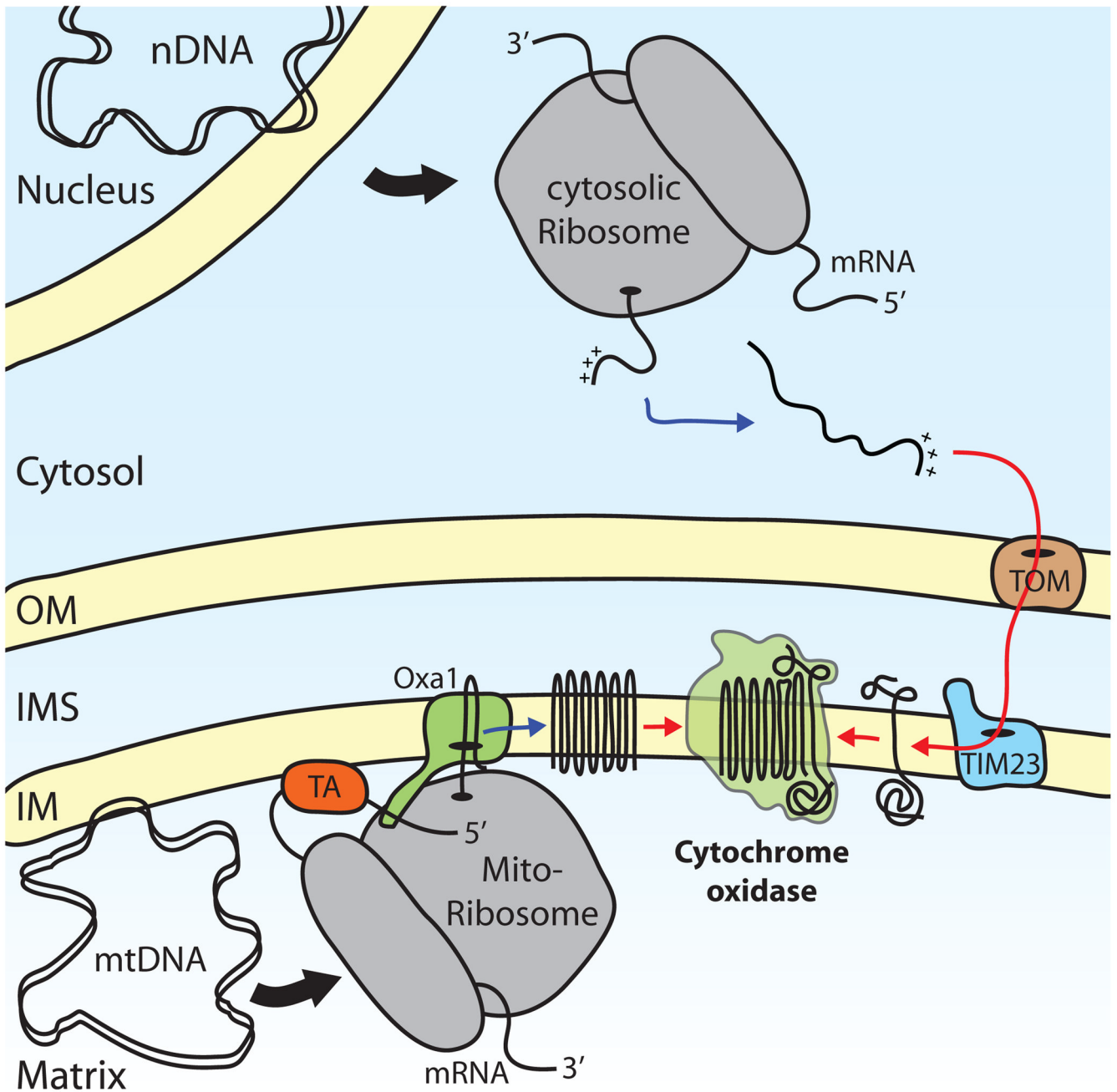
Figure 1. Dual origin of cytochrome oxidase subunits

Respiratory chain subunits are encoded by both nuclear DNA (nDNA), such as Cox5 and mitochondrial DNA (mtDNA), such as Cox1. Nuclear-encoded proteins are translated on cytosolic ribosomes as precursor proteins that contain information that targets them for mitochondria (+++). Precursors are recognized and transported across the mitochondrial membranes by the translocase of the outer membrane (TOM) complex and the translocase of the inner membrane (TIM23) complex, which cooperate in this process. mtDNA is associated with the inner mitochondrial membrane (IM) and its transcription, as well as translation, occurs in close proximity. Mitochondrial ribosomes translate mRNAs that are bound to the membrane by specific translational activators (TA), such as Pet309 in the case

of the mitochondria-encoded Cox1. The translocase Oxa1 inserts nascent polypeptides in a co-translational manner into the membrane and helps these proteins to attain their correct topology. After the insertion of both mitochondria- and nuclear-encoded proteins into the membrane, these subunits can assemble into a functional complex. (OM, outer mitochondrial membrane; IMS, intermembrane space)
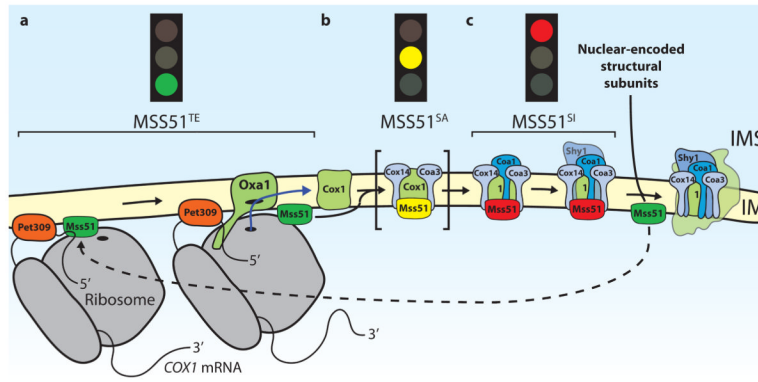
Figure 2. Mechanistic model for the translational regulation of Cox1

Mss51 regulates the translation of Cox1 by interacting with Cox1 mRNA, and also Cox1 protein that has not yet been assembled into the mature cytochrome oxidase complex. **a**| Cox1 is synthesized by mitochondrial ribosomes upon activation by the translational activators Pet309 and Mss51. Here Mss51 is in a translation-effective state (MSS51^{TE}). Cox1 is inserted into the inner mitochondrial membrane (IM) co-translationally by the Oxa1 translocase. **b**| Mss51 is also required for Cox1 translation independently of the 5′UTR. It interacts with newly synthesized Cox1 and the transmembrane proteins Cox14 and Coa3 (cytochrome oxidase assembly), which promote the Cox1–Mss51 interaction. In this sequestered active state (MSS51^{SA}), which has been observed in cells lacking the assembly factor Coa1, Mss51 may be able to initiate further rounds of Cox1 synthesis on mitochondrial ribosomes (not shown) or it might be loosely associated with this complex so that some Mss51 can be released to activate translation. **c**| The association of Coa1 with this complex in wild type cells converts Mss51 to a sequestered inactive state (MSS51^{SI}), which prevents it from activating translation. Moreover, Coa1 association promotes the binding of Shy1 to Cox1, which might positively regulate the insertion of the heme cofactor into Cox1. (It is currently debated whether Shy1 and Mss51 exist in the same complex with Cox1, since different experimental approaches lead to opposite results.) Subsequent addition of further, nuclear-encoded subunits of cytochrome oxidase, such as Cox6, leads to the release of Mss51 from the assembly intermediates, which allows further rounds of Cox1 synthesis. The mitochondrial Hsp70 Ssc1, which has been suggested to associate with Mss51 in the 'TE' and in the 'SI' states is not shown.