Mitochondrial versus nuclear gene expression and membrane protein assembly: the case of subunit 2 of yeast cytochrome c oxidase

Diana Rubalcava-Gracia, Miriam Vázquez-Acevedo, Soledad Funes, Xochitl Pérez-Martínez, and Diego González-Halphen*
Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, 04510 Ciudad México, D. F., Mexico

ABSTRACT Deletion of the yeast mitochondrial gene COX2, encoding subunit 2 (mtCox2) of cytochrome c oxidase (CcO), results in a respiratory-incompetent Δcox2 strain. For a cytosol-synthesized Cox2 to restore respiratory growth, it must carry the W56R mutation (cCox2\textsuperscript{W56R}). Nevertheless, only a fraction of cCox2\textsuperscript{W56R} is matured in mitochondria, allowing ∼60% steady-state accumulation of CcO. This can be attributed either to the point mutation or to an inefficient biogenesis of cCox2\textsuperscript{W56R}. We generated a strain expressing the mutant protein mtCox2\textsuperscript{W56R} inside mitochondria which should follow the canonical biogenesis of mitochondria-encoded Cox2. This strain exhibited growth rates, CcO steady-state levels, and CcO activity similar to those of the wild type; therefore, the efficiency of Cox2 biogenesis is the limiting step for successful allotopic expression. Upon coexpression of cCox2\textsuperscript{W56R} and mtCox2, each protein assembled into CcO independently from its genetic origin, resulting in a mixed population of CcO with most complexes containing the mtCox2 version. Notably, the presence of the mtCox2 enhances cCox2\textsuperscript{W56R} incorporation. We provide proof of principle that an allotopically expressed Cox2 may complement a phenotype due to a mutant mitochondrial COX2 gene. These results are relevant to developing a rational design of genes for allotopic expression intended to treat human mitochondrial diseases.

INTRODUCTION The vast majority of eukaryotes contain a mitogenome. The evolutionary constraints that maintain this genetic material in mitochondria have been addressed previously (Adams and Palmer, 2003; Allen, 2015; Björkholm et al., 2015, 2017; Johnston and Williams, 2016). The hydrophobic nature of the proteins encoded in this genome plays a central role in gene retention (Claros et al., 1995; Oca-Cossio et al., 2003; Johnston and Williams, 2016). In the interest of gaining knowledge on why gene retention occurs in mitogenomes and on designing eventual gene therapies for patients with mitochondrial diseases, several attempts to experimentally relocate a mitochondrial gene to the nucleus have been performed using mammalian cell lines, in some cases with moot results (Guy et al., 2002; Manfredi et al., 2002; Ojaimi et al., 2002; Oca-Cossio et al., 2003; Zullo et al., 2005; Bukori-Brown and Holt, 2006; Bonnet et al., 2007; Shimokata et al., 2007; Figueroa-Martínez et al., 2011; Perales-Clemente et al., 2011; Cwerman-Thibault et al., 2015; Boominathan et al., 2016; Björkholm et al., 2017). This strategy, so-called allotopic expression, has also been studied using the yeast Saccharomyces cerevisiae (Banroques et al., 1986; Gearing and Nagley, 1986; Nagley et al., 1988; Law et al., 1990; Sanchirico et al., 1995; Roucou et al., 1999; Supekova et al., 2010; Bietenhader et al., 2012). Allotopic expression of a mitochondrial protein (de Grey, 2000; Kyrakoulis et al., 2008; Tischner and Wenz, 2015) aims to restore the oxidative phosphorylation (OXPHOS) defects of cells by expressing a precursor protein in the cytosol that may be imported into mitochondria, proteolytically matured by mitochondrial matrix.
proteases, integrated into the inner mitochondrial membrane (IM) in a correct topological disposition, modified by the acquisition of prosthetic group(s), if any, and finally assembled into its corresponding OXPHOS complex.

Complex IV or cytochrome c oxidase (CcO) is an indispensable component of OXPHOS that reduces molecular oxygen (O₂) to water, coupled to proton pumping (Wikström, 1977; Yoshikawa et al., 2011). In yeast, it is composed of 11 subunits, three of which are encoded and synthesized inside mitochondria (the core subunits Cox1, Cox2, and Cox3), while eight accessory subunits are nucleus-encoded and imported into the organelle (Mason et al., 1973; Poyton and Groot, 1975; Fontanesi et al., 2008; Stiburek and Zeman, 2010; Mick et al., 2011; Soto et al., 2011; Maréchal et al., 2012). The COX2 gene is generally encoded in the mitochondrial genome (with the exception of a few organisms) along with other genes such as the universally mitochondria-encoded genes COX1 and COB (cytochrome b of complex III; Adams and Palmer, 2003; Burger et al., 2003). In yeast, mitochondrial Cox2 exhibits a 15-amino acid leader peptide (LP), and thus it is synthesized as a preprotein that inserts its first transmembrane segment (TMS) cotranslationally into the inner mitochondrial membrane (Sevarino and Poyton, 1980; Pratje et al., 1983; Clarkson and Poyton, 1989; Torell et al., 1997) through the Oxa1 insertion machinery and with the help of the assembly factors Mba1 and Cox20 (Hell et al., 1997; Herrmann and Bonnefoy, 2004; Fiumera et al., 2007). Furthermore, the 15-residue LP is removed in the mitochondrial intermembrane space (IMS) by the Imp1 protease (Sevarino and Poyton, 1980; Torell et al., 1997). The second TMS is inserted posttranslationally into the IM through Cox18, a membrane-embedded protein known to be specialized in the C-tail translocation of Cox2, along with its associated partners Pnt1 and Mss2 (Saracco and Fox, 2002). Once inserted in the IM, Cox2 acquires two copper molecules as prosthetic groups from the Pnt1 and Mss2 (Saracco and Fox, 2002). Once inserted in the IM, Cox2 acquires two copper molecules as prosthetic groups from the Pnt1 and Mss2 (Saracco and Fox, 2002). Once inserted in the IM, Cox2 acquires two copper molecules as prosthetic groups from the Pnt1 and Mss2 (Saracco and Fox, 2002).

RESULTS

The W56R mutation does not affect the function and stability of Cox2 or the activity of the CoO when expressed from the mitochondrial genome

Yeast Δcox2 mutants are unable to grow on nonfermentable carbon sources (ethanol/glycerol) but grow similarly to a wild-type strain on glucose (Figure 1A). The Δcox2 mutant was transformed with a plasmid encoding Cox2 with the W56R mutation preceded by the MTS of Oxa1 (amino acids 1–47) and the natural 15-amino acid leader peptide of Cox2 (cCox2WT64) (Supkevka et al., 2010). Allotopically expressed cCox2WT64 follows a different biogenesis pathway than its mitochondrial counterpart mtCox2, since it is synthesized in the cytosol and its import is dependent on the TOM/TIM import machinery but independent of the Cox18 translocase (Elliott et al., 2012). As previously described, the complemented strain (Δcox2+cCox2WT64) recovered the ability to grow on ethanol/glycerol, although not to the levels of the wild-type strain (Figure 1A). The Δcox2+cCox2WT64 transformant required more days of culture on solid media than a wild-type strain to exhibit clearly formed colonies (Figure 1A, bottom panels). Thus, the allotopically expressed cCox2WT64 partially restores respiratory activity as previously documented (Supkevka et al., 2010; Cruz-Torres et al., 2012; Elliott et al., 2012). To address the effect of the W56R substitution on CoO activity, a strain carrying the corresponding mutation in the mitochondrial gene was generated by biolistic transformation of the mitogenome (mtCox2WT64) strain. Just as in the wild-type mtCox2, the mtCox2WT64 protein is expected to be cotranslationally inserted into the inner mitochondrial membrane by mitoribosomes, the Oxa1 translocase, and other assembly factors.

The mtCox2WT64 strain grew on nonfermentable media at rates comparable to those of the wild type, and thus, higher than those of the Δcox2+cCox2WT64 transformant (Figure 1A). A similar result was obtained when growth was followed in liquid, nonfermentable media (Figure 1B). This suggested that the sole presence of the W56R mutation is not detrimental to yeast growth in respiratory media, and also, it reinforced the idea that the allotopically expressed cCox2WT64 must find some impairment either during its import from the cytosol or during its insertion into the right topological position in the IM.

To assess the steady-state levels of the different versions of Cox2, the wild-type, Δcox2, Δcox2+cCox2WT64, and mtCox2WT64 strains were grown in a medium containing galactose as a carbon source. Then a separation of the yeast extracts into cytosolic and mitochondrial fractions was carried out. There were similar levels of mature Cox2 polypeptides in mitochondria isolated from the wild-type strain and the mtCox2WT64 mutant (Figure 2A), supporting the observation that the W56R mutation does not seem to interfere with the stability neither with the assembly of the mtCox2WT64 protein when it is expressed in the mitochondrion. As expected, no Cox2 band could be detected in the Δcox2 mutant (Figure 2A). In contrast, relatively low levels of the mature cCox2WT64 subunit were observed in the Δcox2+cCox2WT64 transformant, and the majority of the Cox2 signal is observed as a high–molecular weight band, which must
correspond to the precursor form of the protein, that is, the form still carrying the MTS (Figure 2A). To confirm the mitochondrial location of the observed Cox2 proteins, mitochondrial and cytosolic fractions were analyzed with antibodies against cytosolic (Hog1) and mitochondrial (Atp2 and Oxa1) protein markers. All wild-type and mutant Cox2 subunits were associated with the mitochondrial fraction (Figure 2A). Surprisingly, that was also the case for the majority of the allotopically expressed cCox2W56R precursor, indicating that although this unprocessed form is mainly localized to mitochondria, it is not available for processing by the matrix proteases. To evaluate if the cCox2W56R precursor was located inside the organelle, mitochondrial fractions from all the strains were subjected to proteinase K (PK) treatment (Figure 2B). Only the cCox2W56R precursor was available for degradation by PK, indicating that at least its immunogenic segment, the C-terminal segment, was exposed to the outside of the outer mitochondrial membrane. This indicates that the cCox2W56R precursor is localized to mitochondria, but it is not completely internalized. As expected, the mature Cox2 bands from the wild-type, Δcox2+cCox2W56R, and mtCox2W56R strains were mostly protected from degradation inside mitochondria (Figure 2B).

To determine whether the interaction of allotopic cCox2W56R with its copper relaying partners is affected, resulting in impaired copper acquisition, we tested the interaction between cCox2W56R and Coa6. This protein interacts with Cox2 and participates in its copper binding; it is also needed to maintain Cox2 protein levels (Ghosh et al., 2014; Pacheu-Grau et al., 2015). Previous studies showed that copper supplementation rescues the respiratory growth of a Δcoa6 mutant (Ghosh et al., 2014). We inferred that if the cCox2W56R interaction with Coa6 is defective, then the supplementation of copper into the medium would bypass this interaction and increase the respiratory growth of the allotopic strain. We did not observe any difference in the respiratory growth of the allotopic strain in the presence or absence of external copper (Figure 2C). We also observed that when cells were incubated at 37°C, the allotopic strain was not able to regain respiratory growth. As previously reported, copper supplementation, but not supplementation with other divalent metals, restores respiratory growth of a Δcoa6 strain. This effect is more clearly visible when cells are incubated at 37°C (Figure 2C). These results indicate that the interaction of cCox2W56R with Coa6 is not affected, suggesting that copper acquisition is not the limiting step that results in diminished assembly of Cox2 into CcO.

To compare the CcO content and activity between the wild-type, Δcox2, Δcox2+cCox2W56R, and mtCox2W56R strains, mitochondria were isolated from cultures grown using galactose as carbon source, solubilized with lauryl maltoside, and subjected to blue native gel electrophoresis (BN–PAGE). CcO activity staining revealed two close bands (IV and IV*) that correspond to the complete CcO complex and to the CcO enzyme lacking subunit Cox6, respectively (Figure 2D, lanes 1, 3, and 4) (Horan et al., 2005; Cruz-Torres et al., 2012). Relatively low CcO activity was observed for the Δcox2+cCox2W56R transformant (Figure 2D, lane 3). As expected, no in-gel CcO activity could be detected in the Δcox2 mutant (Figure 2D, lane 2). In contrast, the in-gel CcO activities of the wild-type strain and the mtCox2W56R mutant were equivalent, suggesting that the W56R mutation does not affect CcO steady-state levels nor impair its activity. In-gel ATPase activity was used as loading control and was roughly equivalent in the four strains (slightly diminished in the Δcox2 mutant; Figure 2D, lanes 5–8).

To explore the formation of supercomplexes in the different strains, isolated mitochondria were also solubilized with the mild
detergent digitonin and subjected to BN–PAGE (Figure 2E). In-gel staining for CcO activity showed the monomeric complex IV, the monomeric complex IV lacking Cox6 subunit (IV*) (Horan et al., 2005), and the II1/I2 and II1/I3 supercomplexes formed by the association of CcO with the bc1 complex. The Δcox2+cCox2W56R transformant exhibited reduced in-gel CcO activity associated with supercomplexes, while the mtCox2W56R mutant exhibited activity comparable to that of the wild-type strain (Figure 2E), indicating that the W56R mutation by itself does not seem to affect either CcO supercomplexes formation or their activity.

Rates of oxygen consumption were also estimated to compare the wild-type strain and the mtCox2W56R mutant (Figure 2F). The specific activities of oxygen uptake measured after the addition of ethanol as substrate were 18.25 and 18.75 nmol O₂/min per 3 × 10⁷ cells for the wild type and the mtCox2W56R mutant, respectively. In contrast, the Δcox2+cCox2W56R transformant had an oxygen consumption rate of 7.05 nmol O₂/min per 3 × 10⁷ cells. In all cases, oxygen uptake was cyanide-sensitive.

Together, these results indicate that the presence of the W56R mutation in the mitochondria-encoded Cox2 does not seem to exert a detrimental effect on the stability and enzymatic activity of this complex. Also, neither protein import nor the acquisition of its prosthetic copper atoms is limiting for cCox2W56R allotopic expression. Therefore, the diminished CcO activity and steady-state levels observed when cCox2W56R is allotopically expressed are more likely due to the presence of obstacles in its biogenesis pathway.

The mitochondrial wild-type Cox2 protein and the allotopically expressed cCox2W56R assemble into CcO in a strain expressing both proteins

Cytosol-synthesized cCox2W56R faces obstacles during its biogenesis pathway that compromise its processing as well as its presence in functional CcO. If the biogenesis pathway is compromised, it might be that this protein is further affected if it is coexpressed with the endogenous mitochondrial Cox2 whose biogenesis occurs under optimal conditions. Such would be the case of allotopic expression if applied in gene therapy for mitochondrial diseases, where the endogenous, defective protein also assembles into CcO. Moreover, this would be the case in some legume species, where COX transcripts from both the nucleus and mitochondria are detected (Adams et al., 1999). However, it remains unknown if the protein products from both genomes actually assemble into CcO. We therefore asked whether the allotopically expressed cCox2W56R protein competes with the natural Cox2 protein synthesized in yeast mitochondria. To address this question, mtCox2 and cCox2W56R were both expressed in a single strain. To distinguish the mitochondria- and nucleus-encoded Cox2 proteins synthesized in yeast mitochondria. To address this question, mtCox2 and cCox2W56R were both expressed in a single strain. To distinguish the mitochondria- and nucleus-encoded Cox2 subunits by mass spectrometry analysis with 30% coverage (unpublished data).

Overall, we observed that the levels of assembled mitochondria-encoded Cox2-HA were similar in strains where mtCox2 was expressed alone and where both Cox2 versions were expressed (Figure 4A, lanes 6 and 8). The two strains containing allotopic subunits exhibit lower Cox2 levels than the mitochondria-encoded Cox2 in a wild-type strain (Figure 4A, lanes 3 and 4 compared with lane 1); thus, the heterogeneous population of CcO contains more mtCox2 species than cCox2W56R species.

To further explore whether the presence of mtCox2 affects the incorporation of cCox2W56R, or vice versa, we estimated by two-dimensional (2D)-PAGE and Western blot the accumulation of assembled Cox2 subunits with increasing amounts of mitochondrial protein (Figure 5). For the mitochondria-encoded Cox2-HA, the protein levels were similar regardless of the presence or absence of its nucleus-encoded counterpart (Figure 5, A and B). In contrast, the allotopically expressed cCox2W56R could be observed in 50 μg of mitochondria when expressed simultaneously with its mitochondrial counterpart, instead of the minimum of 100 μg when expressed alone (Figure 5, C and D). These levels never reached those of mitochondria-encoded Cox2. If each Cox2 version maintained or increased its accumulation in the presence of its counterpart, we would expect a moderate increase in the total Cox2 levels in a strain expressing both versions. We thus used a strain expressing both Cox2 versions without the HA tag in order to detect the two subunits with the same anti-Cox2 antibody and compared it with a strain only expressing the endogenous Cox2; a moderate increase in Cox2 levels was observed in the strain with both versions present, as determined by band densitometric analysis (Figure 5, E and F).

This observed increase in the levels of assembled cCox2W56R when coexpressed with mtCox2-HA would be expected to elevate...
FIGURE 2: CcO complex activity remains unchanged when the mitochondria-encoded Cox2 subunit contains the W56R mutation. (A) Yeast cytosolic (c) and mitochondrial (m) fractions (100 and 50 µg of protein, respectively) of the indicated strains were separated by SDS-Tricine-PAGE and transferred to nitrocellulose membranes for Western blot analysis with an anti-Cox2 antibody (top panel). Bands corresponding to the mature Cox2 subunits and the cCox2<sup>W56R</sup> precursor (still retaining the MTS of Oxa1) are indicated. Parallel Western blots with antibodies against cytosolic Hog1 and against the mitochondrial proteins Atp2 and Oxa1 served as loading and cell fractionation controls (bottom panels). (B) Mitochondrial fractions from A were treated with 100 µg/ml Proteinase K (PK). The asterisk indicates a partial degradation of Cox2, possibly due to imperfect preparation of the mitochondrial fractions. Bands corresponding to the mature Cox2 subunits are indicated (m), as well as that of the remaining precursor (p). (C) Tenfold serial dilutions from yeast cultures were spotted on fermentative (glucose) or on nonfermentative (ethanol/glycerol) plates in the presence and absence of 5 µM Cu, Co, and Mg bivalent salts at 30 and 37°C. A Δcoa6 strain was included as a positive control for growth rescue by copper supplementation. (D) Isolated mitochondria (250 µg) from the indicated strains were solubilized with lauryl maltoside and separated by BN–PAGE (4–15%). Lanes 1–4, CcO in gel activity; lanes 5–8, ATPase activity used as loading control. Bands corresponding to CcO (IV) and the F<sub>1</sub>F<sub>0</sub>-ATPase (V) are indicated. Band IV* corresponds to CcO lacking the Cox6 subunit (Horan et al., 2005). (E) Mitochondria from D were solubilized with...
Taken together, we conclude that in a strain coexpressing the two Cox2 versions, each is synthesized and assembled independently from its genetic origin; cCox2\(^{W56R}\) assembly is moderately increased in the presence of the endogenous mtCox2; and mtCox2 assembly is similar regardless of the presence or absence of its allotopic counterpart. This results in a slight increase in the overall levels of C\(_{cO}\) complexes, which may contain either mtCox2 or cCox2\(^{W56R}\) subunits, with most of the complexes containing the mitochondria-encoded version.

To test this, we compared the C\(_{cO}\) in-gel activity of the different strains and observed an increase in the C\(_{cO}\) activity for the strain coexpressing both Cox2 versions. In contrast, the ATPase Coomassie staining intensity and in-gel activity were similar in all the strains (Figure 5G). Additionally, we observed an increase of the protein content of the C\(_{cO}\) subunits as judged by Coomassie Blue staining (Figure 5H) and by the immunodetection of assembled Cox1 and Cox3 subunits (Figure 5I, lane 4 compared with lane 2).

FIGURE 3: A strain expressing both the mitochondrial wild-type Cox2 protein (mtCox2) and the allotopic cCox2\(^{W56R}\) precursor grows and exhibits oxygen consumption rates similar to those for a wild-type strain. (A) Tenfold serial dilutions from yeast cultures were spotted on plates containing fermentative (glucose) or nonfermentative (ethanol/glycerol) carbon sources. Growth in minimal media lacking uracil (−ura) was included to confirm the presence of the pFL61 plasmid. Cells were incubated for 2 d (top panel) or 5 d (bottom panel) at 30°C. The assayed strains were wild type (mtCox2), wild type with HA-tagged mitochondrial Cox2 (mtCox2-HA), the Δcox2+cCox2\(^{W56R}\) transformant, and the strain containing both Cox2 versions (mtCox2-HA+cCox2\(^{W56R}\)). (B) Growth curves in liquid media containing the nonfermentable carbon sources ethanol and glycerol of the yeast strains containing wild-type or tagged mitochondrial Cox2 (mtCox2 and mtCox2-HA), the Δcox2 strain, the Δcox2+cCox2\(^{W56R}\) transformant, and the strains containing both the mtCox2 and the cCox2\(^{W56R}\) precursor (mtCox2+cCox2\(^{W56R}\) and mtCox2-HA+cCox2\(^{W56R}\)). Optical density (O.D.\(_{600}\)) was registered every 2 h. The mean and the SD of triplicates are shown for each point. (C) Oxygen consumption of yeast cells. Arrows indicate where 3 × 10^7 cells were added to the oxygen-meter chamber, 50 mM ethanol was added as substrate, and 200 µM sodium cyanide was used to inhibit oxygen consumption. The final volume was 1 ml. The mean and the SD of triplicates are shown for each point.

The net C\(_{cO}\) levels also. To test this, we compared the C\(_{cO}\) in-gel activity of the different strains and observed an increase in the C\(_{cO}\) activity for the strain coexpressing both Cox2 versions. In contrast, the ATPase Coomassie staining intensity and in-gel activity were similar in all the strains (Figure 5G). Additionally, we observed an increase of the protein content of the C\(_{cO}\) subunits as judged by Coomassie Blue staining (Figure 5H) and by the immunodetection of assembled Cox1 and Cox3 subunits (Figure 5I, lane 4 compared with lane 2).
DISCUSSION

Allotopic expression is one of the multiple approaches aimed to towards the treatment of mitochondrial syndromes (DiMauro et al., 2006; Kyriakouli et al., 2008; Tischner and Wenz, 2015). Each mitochondria-encoded protein exhibits different characteristics, and the approach to their allotopic expression varies in each case; however, the hydrophobicity of the polypeptides is considered to be one of the limiting constraints for successful allotopic expression. With the notable exception of the ribosomal protein Var1, yeast mitochondria-encoded inner membrane proteins tend to be more hydrophobic than nucleus-encoded inner membrane proteins, indicating that genes encoding hydrophilic products migrate to, and functionally integrate into, the nucleus more favorably (Johnston and Williams, 2016). Thus, the high hydrophobicity profiles of some inner-membrane proteins impose an obstacle for the functional relocalization of their corresponding mitochondrial genes to the nucleus, either by natural migration or by experimental design (Popot and de Vitry, 1990; Claros et al., 1995; Claros and Vicens, 1996; Daley et al., 2002b; Ojaimi et al., 2002; Adams and Palmer, 2003; Oca-Cossio et al., 2003; González-Halphen et al., 2004; Figueroa-Martínez et al., 2011; Björkholm et al., 2015; Neupert, 2015; Johnston and Williams, 2016). Yeast has been widely used as a model organism to assay allotopic expression and has allowed the complementation of several strains carrying defects in mitochondrial genes. Successful attempts to express recoded mitochondrial genes from the nucleus with an added MTS and without any further modification are the bI4 maturase (Banroques et al., 1986); the Var1 ribosomal protein (Sanchirico et al., 1995); and the Atg8 subunit of mitochondrial ATP synthase (Gearing and Nagley, 1986; Nagley et al., 1988; Law et al., 1990; Roucou et al., 1999). Atg8 and Cox2 required further modifications in their sequences to achieve allotopic expression while it still requires the participation of Oxa1 and Cox20 in the biogenesis route, Cox18 was shown to be dispensable (Elliott et al., 2012). We propose that the TIM23 translocon is involved in the allotopic cCox2W56R insertion, given its dual ability to distribute proteins using either one of two pathways: the conservative or the stop-transfer sorting (Bohnert et al., 2010). Thus, the allotopic cCox2W56R that gets properly inserted must translocate its first TMS through TIM23 into the matrix (via the conservative pathway), while the more hydrophobic second TMS is retained within the same translocase and laterally inserted into the inner membrane (via the stop-transfer pathway), therefore allowing it to bypass Cox18 participation (Elliott et al., 2012; Figure 6, right-hand side). Then the MTS is processed by the MPP protease in the matrix while still leaving the cCox2’s 15-residue leader peptide (LP) at the N-terminal end; this sequence promotes the first TMS to be inserted into the inner membrane through Oxa1, allowing the N-terminus of the protein to be exposed to the IMS. The LP is then removed by the Imp1 protease that resides in the IMS (He and Fox, 1997; Hell et al., 1998). This last processing step occurs very efficiently, since we cannot detect the LP-containing cCox2W56R preprotein in the immunoblots. In this way, the cCox2W56R subunit ends up exhibiting the same topology in the membrane as its conventional, mitochondria-synthesized Cox2 counterpart, with both N- and C-termini exposed toward the IMS (Figure 6).

Despite all the above, the biogenesis route followed by cCox2W56R seems to be somehow hindered. The majority of cCox2W56R was found associated to mitochondria although still exposed to the cytosol, as suggested by its degradation when external proteinase K was added. Nevertheless, not all the allotopically expressed cCox2W56R precursors are correctly processed and inserted in the inner mitochondrial membrane. In addition, copper...
FIGURE 5: Assembled steady-state Cox2 levels in a strain where only one COX2 gene version is expressed, either mitochondria- or nucleus-encoded, compared with a strain where both versions are present. For panels A–F, increasing amounts of mitochondrial proteins were loaded in the first dimension and total assembled Cox3 protein and ATPase Coomassie stains in the first dimension blue native electrophoresis (BN–PAGE) were used as loading controls.
(A) Mitochondria-encoded mtCox2-HA detected using an anti-HA antibody in a strain where it is expressed alone (left-hand side) and in a strain where it is accompanied by the allotopic cCox2<sup>W56R</sup> (right-hand side). (B) Densitometry quantification for the raw volume of bands in A as normalized to the ATPase levels in the Coomassie stain of the first-dimension electrophoresis (BN–PAGE). (C) Allotopic cCox2<sup>W56R</sup> detected using an anti-Cox2 antibody in a strain where it is expressed alone (left-hand side) and in a strain where it is accompanied by its mitochondria-synthesized counterpart (right-hand side). (D) Densitometry quantification for the raw volume of bands in C as normalized to the ATPase levels in the Coomassie stain of the BN–PAGE. (E) Total assembled Cox2 protein levels in a strain where an untagged mitochondria-encoded Cox2 is expressed alone (left-hand side) and in a strain where it is accompanied by its allotopically expressed counterpart (right-hand side). (F) Densitometry quantification for the raw volume of bands in E as normalized to the ATPase levels in the Coomassie stain of the BN–PAGE. Mitochondria were separated by 2D-PAGE as in Figure 4. (G) Isolated mitochondria (250 µg) from the indicated strains were solubilized with lauryl maltoside, separated by BN–PAGE, and stained for CcO in-gel activity. Complex V (ATPase) activity and Coomassie stain were used as loading controls. (H) Bands corresponding to the CcO complex were excised from a BN–PAGE gel and subjected to SDS-Tricine-PAGE. A resulting Coomassie blue–stained gel of the resolved CcO polypeptides is shown. (I) Western blot of a gel as in H shows the abundance of subunits Cox1, Cox2, and Cox3 as recognized by the corresponding antibodies. The membrane was stripped and rebotted in the order anti-Cox3, anti-Cox2, anti-Cox1. The Cox2 antibody recognizes Cox2 only without the HA-tag.
acquisition prior to assembly does not seem to be limiting in the allotopic strain. Thus, the limiting step resides in the correct insertion of cCox2W56R into the inner membrane. We consider a scenario where the cCox2W56R precursors that are not correctly inserted might be prematurely released into the inner membrane, most probably because their first TMS, although exhibiting a diminished hydrophobicity, is still hydrophobic enough to have difficulty in crossing the inner membrane freely thorough TIM23. Being in the limit between prohibitive and permissive hydrophobicity values, TIM23 may fully translocate the first TMS of some cCox2 precursors, but will also release some of these first TMS into the membrane laterally (Mokranjac and Neupert, 2010; Calado-Botelho et al., 2011). Thus, a population of cCox2W56R precursors will end up with the incorrect topology N-terminus exposed to the mitochondrial matrix and the C-terminus facing the IMS or the cytosol. These improperly assembled cCox2W56R polypeptides should be recognized as abnormal by mitochondrial proteases such as i-AAA and readily degraded (Figure 6) (Bietenhader et al., 2012; Gerdes et al., 2012). On the other hand, when Cox2W56R is expressed in the mitochondrion, the W56R substitution behaves as a silent mutation, since it does not seem to affect growth curves, respiration rates, or CcO accumulation. We thus suggest that the allotopic expression of cCox2W56R is limited not because the W56R mutation affects the assembly of cCox2W56R into CcO, nor because the
substitution has a detrimental effect on CcO activity or stability, but because the translocation of the first TMS (most probably through TIM23) is a process that occurs inefficiently and at a low rate. This explains the observed high accumulation of the cCox2\textsuperscript{W56R} precursor in the mitochondrial fraction and the lower overall accumulation of CcO\textsuperscript{W56R} in yeast mitochondria.

One could conclude from the above that allotopic expression of the Cox2 subunit could be resolved simply by further diminishing the hydrophobicity of the first TMS by the inclusion of more polar residues, allowing TIM23 to fully and efficiently translocate the first TMS of cCox2\textsuperscript{W56R}. However, these new changes could start affecting the assembly, activity, and stability of the whole CcO complex. A delicate balance between a low hydrophobicity that allows the effective translocation of the first TMS through TIM23, and a high enough hydrophobicity to be recognized as a TMS by the membrane insertion machinery (Oxa1), and therefore to be introduced with the right topology into the inner mitochondrial membrane, is required. This also explains why, of all the possible mutations that could be obtained by a massive, random mutagenesis, only the W56R mutation allowed substantial respiratory growth of a Δccox2 mutant (Supekova et al., 2010). We find it an interesting challenge to establish which other mutation or combination of mutations would be required to successfully reach the balance between readily inserting the allotopic Cox2 protein into the inner membrane and still allowing full activity of the CcO complex.

Legumes provide an illustrative example of a recent transfer of the COX2 gene to the nucleus; it possibly occurred once in the Phaseolae lineage and resulted in different intermediate stages of the transfer process. Some legumes display intact copies of both nuclear and mitochondrial COX2, and their transcripts could be detected for both versions (Adams et al., 1999). However, the translation of the Cox2 proteins and their assembly into CcO complexes were not explored. We predict, on the basis of the results obtained here, that in some legume species there will be a heterogeneous CcO population, with some complexes containing the cytosol-synthesized Cox2 subunit and the majority the mitochondrial version.

In some cases, the approach of using allotopic expression as a genetic therapy for mitochondrial diseases also implies having two versions of the same protein assembled into an OXPHOS COX2 complex population: the cytosol-synthesized version and the mitochondria-expressed version carrying the deleterious mutation (or a wild-type version, given the heteroplasm in mammalian mitochondria). In this work, when both Cox2 protein versions were coexpressed, the final steady state levels of Cox2 in the CcO populations were mostly of mitochondrial origin and only a fraction of cytosolic origin. This suggests that even in the face of successful allotopic expression, the mitochondrial synthesis and assembly of proteins is much more efficient and contributes primarily to the final accumulation levels of a given OXPHOS complex. Therefore, in a best-case scenario, the allotopic protein will contribute, alleviating the activity of the OXPHOS complex in a relatively modest proportion, but most probably it could still attain the minimum requirements to significantly improve the respiratory activity. To our knowledge, in the known cases of allotopic expression reported in the literature, no efforts were made to analyze the extent to which the two protein versions competed for assembly. Our data provide proof of concept that the allotopic expression of an engineered gene may successfully complement a phenotype due to a mutation in the mitogenome.

Notably, we observed a moderate increase in the levels of the assembled cCox2\textsuperscript{W56R} allotopic protein when coexpressed with the endogenous mtCox2 than when expressed alone. We hypothesize that the presence of mtCox2 allows the newly inserted cCox2\textsuperscript{W56R} to efficiently enter the assembly pipeline of the CcO complex. CcO biogenesis is complex and highly regulated and each mitochondria-encoded subunit is proposed to form modules with different cytosolic subunits (McStay et al., 2013). The Cox1 subunit plays a central role in determining the CcO accumulation levels, since its synthesis is required for the overall progress of the CcO assembly line. This subunit is autoregulated by following a retro-feedback loop: after its cotranslational insertion, the Cox1 subunit sequesters Mss51, its translational activator, thus preventing it from activating the synthesis of more Cox1 subunits. For Mss51 to be liberated, Cox1 needs to acquire its cofactors and continue to assemble into a functional CcO (Ott and Herrmann, 2010; Mick et al., 2011). Furthermore, stalled assembly of the CcO complex results in selective degradation of the Cox1 subunit (Khalmouchuk et al., 2012). These regulatory mechanisms adjust the level of Cox1 synthesis to the amounts that can be successfully assembled. The Cox2 and Cox3 modules then assemble with the Cox1 module.

We propose that when the allotopic cCox2\textsuperscript{W56R} is the only version present in the cell, it has low-efficiency assembly, leading to lower levels of the Cox1 module (Figure 7). Indeed, in this context, synthesis of Cox1 and steady state levels are decreased (Cruz-Torres et al., 2012). When the mitochondria-encoded Cox2 is coexpressed, and since this protein is naturally assembled with high efficiency, this leads to higher accumulation of the Cox1 module, and therefore the assembly of cCox2\textsuperscript{W56R} is enhanced (Figure 7).

We consider the observations here described relevant to the understanding of CcO biogenesis, of the conditions imposed for a functional transfer of mitochondrial genes to the nucleus, and of the requirements for a rational design of genes for allotopic expression intended for the development of gene therapies for human mitochondrial diseases.

**MATERIALS AND METHODS**

**Strains, gene constructs, and culture conditions**

All *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown on fermentable media, YPD or YPGal (1% yeast extract, 2% bactopeptone, and 2% dextrose or galactose). The nonfermentable medium was YPEG (1% yeast extract, 2% bactopeptone, 3% ethanol, and 3% [vol/vol] glycerol). Minimal media were SD or SGal (0.17% yeast nitrogen base [without amino acids and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}], 0.5% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and 2% dextrose or galactose supplemented with specific amino acids and nucleotides). All strains were grown at 30°C in liquid (with shaking) or solid media (containing 1.75% agar). Absorbance at 600 nm was measured every 2 h in a Bioscreen C spectrophotometer ( Growth Curves, USA). For metal supplementation experiments, growth media contained 5 μM final concentration of CuCl\textsubscript{2}, CoCl\textsubscript{2}, or MgCl\textsubscript{2}.

The *S. cerevisiae* COX2 gene encoding a cytosol-synthesized precursor protein with the W56R point mutation (cCox2\textsuperscript{W56R}) was recorded for expression from the nucleus. A region encoding the Oxa1 MTS was added at the 5’ end, as previously described (Supekova et al., 2010). The construct was chemically synthesized (GeneScript, Piscataway, NJ) and cloned in the pFL61 vector, which contains a phosphoglycerate kinase promoter, a kind gift from Patrice Hamel (Ohio University, Columbus, OH). Yeast cells were transformed in the presence of lithium acetate and salmon sperm DNA as described (Gietz and Schiestl, 2007).

The COX2 mitochondrial gene expressing the protein with the W56R mutation (mtCox2\textsuperscript{W56R}) was constructed using site-directed mutagenesis (QuickChange II site-directed mutagenesis kit, Agilent Technologies) on the pJM2 plasmid (Mulero and Fox, 1993). This plasmid
haploids. Correct integration of the mtCox2<sup>W56R</sup> construct into mtDNA was confirmed by PCR and DNA sequencing.

Isolation of yeast mitochondria
Mitochondria were isolated from 100–200 ml of liquid SGal cultures as previously reported (Herrmann et al., 1994). Briefly, cells were transformed by high-velocity micro projectile bombardment into the ρ<sup>0</sup> strain DFS160 (Bonnefoy and Fox, 2007). Transformants were selected by their ability to rescue respiratory growth when mated with the strain SCS188 carrying a partial deletion of cox2. Transformants with the mtCox2<sup>W56R</sup> plasmid were mated with the EHW154 strain and cytoductants were selected for their ability to grow in YPEG as shown in Figure 7.

**TABLE 1:** Strains used in this work.

| Strain   | Genotype                                                                 | Source/reference              |
|----------|---------------------------------------------------------------------------|-------------------------------|
| NB40-36A | D273-10B; MATα, lys2, arg8::hisG, ura3-52, leu2-3, 112, [rho+]             | Thomas D. Fox, Cornell University |
| EHW154   | D273-10B; MATα, lys2, arg8::hisG, ura3-52, leu2-3, 112, his3-ΔHindIII, [rho+] cox2-208::ARG8m | Thomas D. Fox, Cornell University |
| DFS160   | D273-10B; MATα, arg8Δ::ura3, ura3-52, leu2Δ, ade2-101, kar1-1, [rho<sup>0</sup>] | Thomas D. Fox, Cornell University |
| SCS188A  | D273-10B; MATα, ade2-101, ura3-52, kar1-1, [rho+] cox2-75c                  | Thomas D. Fox, Cornell University |
| SCS101   | D273-10B; MATα, ura3-52, leu2-3,112, lys2, his3-ΔHindIII, arg8Δ::hisG, [rho+] COX2::3xHA | Saracco, 2003 |
| DRG101   | D273-10B; MATα, lys2, arg8::hisG, ura3-52, leu2-3, 112, his3-ΔHindIII, [rho+] COX2W56R | This study |
| YMR245W  | BY4741; MATα, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, coa6-Δ51-315               | Winzeler et al., 1999          |
harvested in the logarithmic growth phase (O.D.\textsubscript{600} 1.2–1.5), washed with H\textsubscript{2}O at 4500 rpm (2500 × g) for 5 min, resuspended in TD buffer (100 mM Tris, 10 mM \textit{d}-cithiothreitol), and centrifuged at 4500 rpm (2500 × g) for 5 min. The cells were resuspended in 10 ml of zymolase buffer (1.2 mM sorbitol, 20 mM KH\textsubscript{2}PO\textsubscript{4} [pH 7.4], 3–5 mg zymolase/g of wet weight), incubated for 1 h in a shaker at 30°C, and centrifuged at 4500 rpm (2500 × g) for 5 min at 4°C. The pellet was resuspended in 1 ml of Dounce buffer (0.6 M sorbitol, 10 mM Tris [pH 7.4], 1 mM EDTA, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride) using a homogenizer and pestle (30 strokes). Samples were transferred to 1.5-ml tubes and centrifuged at 4500 rpm (2000 × g) for 5 min at 4°C. The supernatants were transferred to new tubes and centrifuged at 12,000 rpm (13,500 × g) for 10 min at 4°C. The supernatant (cytosolic fraction) was collected in new tubes and the pellet (mitochondrial fraction) was resuspended in 100 µl of SH buffer (0.6 M sorbitol, 20 mM HEPES, pH 7.4). Protein concentration was determined by a modified Lowry method (Markwell et al., 1978). Mitochondria and cytosolic fractions were shock frozen in liquid N\textsubscript{2} and stored at –70°C until further use.

Oxygen uptake measurements

The rate of oxygen consumption was measured in whole cells using an oxygen meter, Model 782 (Warner/Strathkelvin Instruments), with a Clark-type electrode in a 1-ml water-jacketed chamber at 30°C (Estabrook, 1967), and data were analyzed using the 782 Oxygen System software (Warner/Strathkelvin Instruments). The assay contained 20 mM MES pH 6.0 (triethanolamine) and 50 mM ethanol as substrate. Where indicated, 200 µM NaCN was added to inhibit CcO activity. The cells were washed once with H\textsubscript{2}O prior to the measurements or, where indicated, were washed and fasted overnight in H\textsubscript{2}O. The samples were normalized by the number of cells.

Gel electrophoresis and in-gel enzymatic activities

Denaturing gel electrophoresis was carried out in a SDS-tricine-PAGE system (Schägger, 1994a). Sample preparation and blue native PAGE (BN–PAGE) were carried out as described (Schägger, 1994b). Briefly, mitochondria were washed in 250 mM sorbitol and 50 mM Bis-Tris (pH 7.0) and centrifuged at 12,000 rpm (13,500 × g) for 10 min at 4°C. The pellet was resuspended in sample buffer (750 mM aminocaproic acid, 50 mM Bis-Tris, pH 7.0), solubilized with 2 g of lauryl maltoside per g of protein for 30 min with gentle stirring, and centrifuged at 13,200 rpm (16,400 × g) at 4°C for 12 min. The supernatants were loaded on 5–15% polyacrylamide gradient gels. The stacking gel contained 4% (wt/vol) polyacrylamide. In-gel activities were carried out following established procedures for CcO activity (Wittig and Schägger, 2007; Wittig et al., 2007) and ATPase activity (Zerbetto et al., 1999). Further electrophoretic separation of the CcO complex was performed by excising the band corresponding to this complex (identified by in-gel activity of a parallel lane) and loading it into SDS-tricine-PAGE. Gels were stained with Cooamassie solution (50% ethanol, 10% acetic acid, 0.1% Cooamassie Brilliant Blue G) or transferred and then subjected to immunodetection.

Immunooassays

From SDS-tricine-PAGE, proteins were electrotransferred onto a nitrocellulose Trans-Blot membrane (Bio-Rad) for immune detection. Membranes were washed, blocked, and independently incubated for 8 h with the following antibodies: anti-Cox1 antibody at a 1:3300 dilution (MitoSciences), anti-Cox2 antibody at a 1:7500 dilution (Invitrogen; Molecular Probes), anti-Cox3 antibody at a 1:15,000 dilution (Molecular Probes), anti-Oxa1 antibody at a 1:10000 dilution, anti-Hog1 antibody at a 1:2000 dilution (Santa Cruz Biotechnology), anti-HA antibody at a 1:15,000 dilution (Sigma Aldrich), and anti-Atp2 antibody at a 1:50,000 dilution. Alkaline phosphatase (ALP)– conjugated immunoglobulin Gs (1:15,000 for 2 h) were used as secondary antibodies. Insoluble black–purple precipitates on bands were formed upon addition of nitro blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyl phosphate p-toluidine salt. Images of the immune-decorated polypeptide bands were captured in a HP Scanjet G4050. To carry out a second Western blot reaction with a different primary antibody, membranes were stripped by incubation for 45 min at 50°C in the presence of 2% SDS, 62.5 mM Tris-HCl, pH 6.8, and 100 mM β-mercaptoethanol. Band intensity was measured by densitometric analysis using the GelAnalyzer 2010a freeware.

Tandem mass spectrometry

After SDS-tricine PAGE and Cooamassie staining, bands of interest were sent for mass spectroscopy identification (LC/ESI-MS/MS) at the Protein Biochemistry Laboratory at Texas A&M University.

ACKNOWLEDGMENTS

We thank Thomas D. Fox (Cornell University) for most of the strains used in this project and Johannes M. Herrmann (University of Kaiserslautern) for the kind gift of the Oxa1 antibody. We are also grateful to Rodolfo García-Villegas and Yolanda Camacho-Villasana for their expert assistance in the mitochondrial transformation technique. We also thank Larry Dangott (Protein Biochemistry Laboratory at Texas A&M University) for carrying out the mass spectrometry analysis. This work was partially supported by Grants 239219 and 279125 from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico) and IN 208917 from the Dirección General de Asuntos del Personal Académico (DGAPA-UNAM, Mexico) to D.G.-H.; CONACyT-CB 237344 and DGAPA-UNAM IN20715 to S.F.; and DGAPA-UNAM IN208711-3 to X.P.-M. CONACyT also supported with a fellowship the studies of D.R.-G. in the Biomedical Sciences Ph.D. program (Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México).

REFERENCES

Adams K, Palmer JD (2003). Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Mol Phylogenet Evol 29, 380–395.

Adams KL, Song K, Roesler PG, Nugent JM, Doyle JL, Doyle JJ, Palmer JD (1999). Intracellular gene transfer in action: dual transcription and multiple silencings of nuclear and mitochondrial cox2 genes in legumes. Proc Natl Acad Sci USA 96, 13863–13868.

Allen JF (2015). Why chloroplasts and mitochondria retain their own genomes and genetic systems: colocation for redox regulation of gene expression. Proc Natl Acad Sci USA 112, 10231–10238.

Banroques J, Delahodde A, Jacq C (1986). A mitochondrial RNA maturase gene transferred to the yeast nucleus can control mitochondrial mRNA splicing. Cell 46, 837–844.

Bietenhader M, Marts A, Tetaud E, Aiyar RS, Selmeh CH, Kucharzyk R, Clauder-Münster S, Giraud M-F, Godard M, Godard F, Salin B, et al. (2012). Experimental relocation of the mitochondrial ATP9 gene to the nucleus reveals forces underlying mitochondrial genome evolution. PLoS Genet 8, e1002876.

Björkholm P, Ernst AM, Hagström E, Andersson SGE (2017). Why mitochondria need a genome revisited. FEBS Lett 591, 65–75.

Björkholm P, Harish A, Hagström E, Ernst AM, Andersson SGE (2015). Mitochondrial genomes are retained by selective constraints on protein targeting. Proc Natl Acad Sci USA 112, 10154–10161.

Bohnert M, Rehling P, Guiard B, Herrmann JM, Pfanner N, van der Laan M (2010). Cooperation of stop-transfer and conservative sorting mechanisms in mitochondrial protein transport. Curr Biol 20, 1227–1232.

Bokori-Brown M, Holt IJ (2006). Expression of algal nuclear ATP synthase subunit 6 in human cells results in protein targeting to mitochondria but no assembly into ATP synthase. Rejuvenation Res 9, 455–469.
Bonneyo F, Niumera HL, Dujardin G, Fox TD (2009). Roles of Ox1-related inner-membrane translocases in assembly of respiratory chain complexes. Biochim Biophys Acta 1793, 60–70.

Bonneyo F, Fox TD (2007). Directed alteration of Saccharomyces cerevisiae mitochondrial DNA by bioilastic transformation and homologous recombination. Methods Mol Biol 372, 153–166.

Bonnet C, Baltinbacher V, Ellouze S, Augus T, Bénit P, Forster V, Rustin P, Sahel JA, Corral-Debrinski M (2007). Allotopic mtRNA mobilization to the mitochondrial surface rescues respiratory chain defects in fibroblasts harboring mitochondrial DNA Mutations Affecting Complex I or V Subunits. Rejuvenation Res 10, 127–144.

Bonmaanoh A, Vanhooser S, Basisty N, Powers K, Crampton AL, Wang X, Friedricks N, Schilling B, Brand MD, O’Connor MS (2016). Stable nuclear expression of ATP8 and ATP6 genes rescues a mtDNA Complex V null mutant. Nucleic Acids Res 44, 9342–9357.

Bottstein D, Fink GR (1988). Yeast: an experimental organism for modern biologists. Science 240, 1439–1443.

Burger G, Gray MW, Lang BF (2003). Mitochondrial genomes: anything goes. Trends Genet 19, 709–716.

Caliò-Botelho S, Osterberg M, Reichert AS, Yamano K, Björkholm P, Endo T, von Heijne G, Kim H (2011). TIM23-mediated insertion of transmembrane α-helices into the mitochondrial inner membrane. EMBO J 30, 1003–1011.

Clarkson GHD, Poyton RO (1989). A role for membrane potential in the biogenesis of cytochrome c oxidase subunit 11, a mitochondrial gene product. J Biol Chem 264, 1011–1014.

Clark MG, Perea J, Samatey FA, Popot JL, Jacq C (1995). Limitations to in vivo import of hydrophobic proteins into yeast mitochondria. The case of a cytoplasmically synthesized apocytochrome b. Eur J Biochem 771, 762–771.

Claros MG, Vincens P (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem 240, 779–786.

Cruz-Tones V, Vázquez-Acevedo M, García-Villegas R, Pérez-Martínez X, Mendoza-Hernández G, González-Halpden D (2012). The cytosol-synthesized subunit II (Cox2) precursor with the point mutation W56R is correctly processed in yeast mitochondria to rescue cytochrome c oxidase. Biochim Biophys Acta 1817, 2128–2139.

Cwerman-Thibault H, Augustin C, Lechauve C, Ayache J, Ellouze S, Sahel J-A, Corral-Debrinski M (2015). Nuclear expression of mitochondrial ND4 leads to the protein assembling in complex I and prevents optic atrophy and visual loss. Mol Ther Methods Clin Dev 2, 15003.

Daley DO, Adams KL, Clifton R, Whelan J (2002a). Gene transfer from mitochondrion to nucleus: novel mechanisms for gene activation from Cox2. Plant J 30, 11–21.

Daley DO, Clifton RP, Whelan J (2002b). Intracellular gene transfer: reduced hydrophobicity facilitates gene transfer for subunit 2 of cytochrome c oxidase. Prog Natl Acad Sci USA 99, 10510–10515.

de Grey AD (2000). Mitochondrial gene therapy: an arena for the biomedical use of inteins. Trends Biotechnol 18, 394–399.

DiMauro S, Hirano M, Schon EA (2006). Approaches to the treatment of mitochondrial diseases. Muscle Nerve 34, 265–283.

Daley DO, Adams KL, Clifton R, Whelan J (2002a). Gene transfer from mitochondrion to nucleus: novel mechanisms for gene activation from Cox2. Plant J 30, 11–21.

DiMauro S, Hirano M, Schon EA (2006). Approaches to the treatment of mitochondrial diseases. Muscle Nerve 34, 265–283.

Elliot LE, Saraco SA, Fox TD (2012). Multiple roles of the Cox20 chaperone in assembly of Saccharomyces cerevisiae cytochrome c oxidase. Genetics 190, 559–567.

Estabrook RW (1967). Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol 10, 41–47.

Figueueroa-Martínez F, Vázquez-Martínez S, van Lis R, Pérez-Martínez X, Vázquez-Acevedo M, King MP, González-Halpden D (2002). The typically mitochrondrial DNA-encoded ATP6 subunit of the F1F0-ATPase is encoded by a nuclear gene in Chlamydomonas reinhardtii. J Biol Chem 277, 6051–6058.

Gerdes F, Matsuta T, Langer T (2012). Mitochondrial AAA proteases—towards a molecular understanding of membrane-bound proteolytic machines. Biochim Biophys Acta 1823, 49–55.

Ghosh A, Pratt AT, Soma S, Theriault SG, Griffin AT, Trivedi PP, Gohil VM (2016). Mitochondrial disease genes COA6, COX6B and SCO2 have overlapping roles in COX2 biogenesis. Hum Mol Genet 25, 660–671.

Ghosh A, Trivedi PP, Timbalia JA, Griffin AT, Rahn JJ, Chan SSL, Gohil VM (2014). Copper supplementation restores cytochrome c oxidase assembly defect in a mitochondrial disease model of COA6 deficiency. Hum Mol Genet 23, 3596–3606.

Gietz RD, Schiestl RH (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2, 35–37.

González-Halpden D, Funes S, Pérez-Martínez X, Reyes-Prieto A, Claros MG, Trivedi PP, Timbalia JA, Griffin AT, Rahn JJ, Chan SSL, Gohil VM (2014). Copper supplementation restores cytochrome c oxidase assembly defect in a mitochondrial disease model of COA6 deficiency. Hum Mol Genet 23, 3596–3606.

Gietz RD, Schiestl RH (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2, 35–37.

González-Halpden D, Funes S, Pérez-Martínez X, Reyes-Prieto A, Claros MG, Trivedi PP, Timbalia JA, Griffin AT, Rahn JJ, Chan SSL, Gohil VM (2014). Copper supplementation restores cytochrome c oxidase assembly defect in a mitochondrial disease model of COA6 deficiency. Hum Mol Genet 23, 3596–3606.

Gietz RD, Schiestl RH (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2, 35–37.

González-Halpden D, Funes S, Pérez-Martínez X, Reyes-Prieto A, Claros MG, Trivedi PP, Timbalia JA, Griffin AT, Rahn JJ, Chan SSL, Gohil VM (2014). Copper supplementation restores cytochrome c oxidase assembly defect in a mitochondrial disease model of COA6 deficiency. Hum Mol Genet 23, 3596–3606.

Gheti R, Schiestl RH (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2, 35–37.

González-Halpden D, Funes S, Pérez-Martínez X, Reyes-Prieto A, Claros MG, Trivedi PP, Timbalia JA, Griffin AT, Rahn JJ, Chan SSL, Gohil VM (2014). Copper supplementation restores cytochrome c oxidase assembly defect in a mitochondrial disease model of COA6 deficiency. Hum Mol Genet 23, 3596–3606.

Gietze J, Qi X, Pollitt F, Schon EA, Manfredi G, Carelli V, Martinuzzi A, Hauwaw LW, Lewis AW (2002). Rescuing of a mitochondrial deficiency caused by a heteroplasmy in human cells stably expressing a cytochrome c oxidase subunit II (Cox2) cDNA. Biochim Biophys Acta 1793, 60–70.
Mokranjac D, Neupert W (2010). The many faces of the mitochondrial TIM23 complex. Biochim Biophys Acta 1797, 1045–1054.

Mulero JJ, Fox TD (1993). Alteration of the Saccharomyces cerevisiae COX2 mRNA 5’-untranslated leader by mitochondrial gene replacement and functional interaction with the translational activator protein PET111. Mol Biol Cell 4, 1327–1335.

Nagley P, Farrell LB, Gearing DP, Nero D, Meltzer S, Devenish RJ (1988). Assembly of functional proton-translocating ATPase complex in yeast mitochondria with cytoplasmically synthesized subunit 8, a polypeptide normally encoded within the organelle. Proc Natl Acad Sci USA 85, 2091–2095.

Neupert W (2015). A perspective on transport of proteins into mitochondria: a myriad of open questions. J Mol Biol 427, 1135–1158.

Neupert W, Herrmann JM (2007). Translocation of proteins into mitochondria. Annu Rev Biochem 76, 723–749.

Oca-Cossio J, Kenyon L, Hao H, Moraes CT (2003). Limitations of allotypic expression of mitochondrial genes in mammalian cells. Genetics 165, 707–720.

Ojaimi J, Pan J, Santra S, Snell WJ, Schon EA (2002). An algal nucleus-encoded subunit of mitochondrial ATP synthase rescues a defect in the analogous human mitochondrial-encoded subunit. Mol Biol Cell 13, 3836–3844.

Ott M, Herrmann JM (2010). Co-translational membrane insertion of mitochondrially encoded proteins. Biochim Biophys Acta 1803, 767–775.

Pacheu-Grau D, Bareth B, Dudek J, Junis L, Vogtle FN, Wissel M, Leary SC, Dennerlein S, Rehling P, Deckers M (2015). Cooperation between COA6 and SCO2 in COX2 maturation during cytochrome c oxidase assembly links two mitochondrial cardiomyopathies. Cell Metab 21, 823–833.

Pérez-Martínez X, Vazquez-Acevedo M, Funes S, Claros MG, Antaramian A, Vazquez-Acevedo M, Funes S, Tolkunova E, Pérez-Martos A, Pérez-Martínez X, Fernández-Silva P, Acín-Pérez R, Pérez-Martos A, Pacheu-Grau D, Bareth B, Dudek J, Juris L, Vögtle FN, Wissel M, Leary SC, Ott M, Herrmann JM (2010). Co-translational membrane insertion of mitochondrial subunit II of cytochrome c oxidase in Chlamydomonad algae is a heterodimer encoded by two independent nuclear genes. J Biol Chem 276, 11302–11309.

Pérez-Martínez X, Butler CA, Shingu-Vázquez M, Fox TD (2009). Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in Saccharomyces cerevisiae mitochondria. Mol Biol Cell 20, 4371–4380.

Pérez-Martínez X, Vazquez-Acevedo M, Tolkunova E, Funes S, Claros MG, Davidson E, King MP, González-Halpchen D (2001). Subunit II of cytochrome c oxidase in Chlamydomonad algae is a heterodimer encoded by two independent nuclear genes. J Biol Chem 276, 11302–11309.

Pérez-Martínez X, Butler CA, Shingu-Vázquez M, Fox TD (2009). Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in Saccharomyces cerevisiae mitochondria. Mol Biol Cell 20, 4371–4380.

Pérez-Martínez X, Vazquez-Acevedo M, Tolkunova E, Funes S, Claros MG, Davidson E, King MP, González-Halpchen D (2001). Subunit II of cytochrome c oxidase in Chlamydomonad algae is a heterodimer encoded by two independent nuclear genes. J Biol Chem 276, 11302–11309.

Pérez-Martínez X, Butler CA, Shingu-Vázquez M, Fox TD (2009). Dual functions of Mss51 couple synthesis of Cox1 to assembly of cytochrome c oxidase in Saccharomyces cerevisiae mitochondria. Mol Biol Cell 20, 4371–4380.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.

Pratle E, Tagliabue A, González-Halpchen D (2000). Unusual location of a functional interaction with the translational activator protein PET111. Nature 266, 271–273.

Peyron RO, Groot GSP (1975). Biosynthesis of polypeptides of cytochrome c oxidase in isolated mitochondria. Nature 266, 271–273.