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 Δ*cox2* strain. For a cytosol-synthesized Cox2 to restore respiratory growth, it must carry the W56R mutation (cCox2^{W56R}). Nevertheless, only a fraction of cCox2^{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^{W56R}. We generated a strain expressing the mutant protein mtCox2^{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^{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^{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.**

Monitoring Editor
Benjamin S. Glick
University of Chicago

Received: Sep 22, 2017
Revised: Jan 12, 2018
Accepted: Feb 1, 2018

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; Bokori-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; Kyriakouli *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

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E17-09-0560) on February 7, 2018.

*Address correspondence to: Diego González-Halphen (dhalphen@ifc.unam.mx).

Abbreviations used: CcO, cytochrome *c* oxidase; cCox2^{W56R}, cytosol-synthesized Cox2^{W56R} subunit; IM, inner mitochondrial membrane; IMS, intermembrane space; LP, leader peptide; mtCox2, mitochondria-synthesized Cox2 subunit; MTS, mitochondrial targeting signal; OXPHOS, oxidative phosphorylation; TMS, transmembrane segment.

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 (C*c*O) is an indispensable component of OXPHOS that reduces molecular oxygen (O_2) 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 Groott, 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; Torello *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; Torello *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 Sco1/2 and Coa6 copper relay system and is assembled into C*c*O (Rigby *et al.*, 2008; Pacheu-Grau *et al.*, 2015; Ghosh *et al.*, 2016).

Yeast strains lacking the *COX2* gene (Δ*cox2* mutants) are unable to assemble a functional C*c*O and therefore fail to exhibit respiratory growth (Horan *et al.*, 2005), although they can be maintained in fermentative media (Botstein and Fink, 1988). Several unsuccessful efforts have been made to restore respiratory growth of Δ*cox2* mutants by expressing a recoded mitochondrial *COX2* gene from the nucleus after adding a sequence encoding a mitochondrial targeting signal (MTS) to the construct. A strategy involving massive random mutagenesis led to the identification of the point mutation W56R, which allows a cytosol-synthesized yeast Cox2 precursor (cCox2^{W56R}) to complement a Δ*cox2* strain. This Δ*cox2*+cCox2^{W56R} strain grows in nonfermentable carbon sources and exhibits respiratory capacity, although at a lower rate than a wild-type strain (Supekova *et al.*, 2010). Moreover, the strain carrying the allotopically expressed cCox2^{W56R} subunit contains ~40% less C*c*O than the wild-type strain, as judged by in-gel activity of the enzyme and spectroscopic quantitation of cytochromes (Cruz-Torres *et al.*, 2012). The point mutation W56R, located in the first transmembrane segment of the Cox2 protein, diminishes the mean hydrophobicity of the alpha helix, thus allowing the import of the precursor into mitochondria. The need to decrease the hydrophobicity of mitochondrial proteins to allow its allotopic expression has been discussed previously (Popot and de Vitry, 1990; Claros *et al.*, 1995; Bietenhader *et al.*, 2012). Even when the W56R mutation is necessary to allow the allotopic expression of Cox2, the presence of cCox2^{W56R} only partially restores the steady state levels of C*c*O. This could be due either to the mutation per se having a direct effect on C*c*O assembly and stability, or to a limiting step in the biogenesis pathway of the precursor protein cCox2^{W56R}. In this work, we seek to pinpoint which of these two possibilities better explains the lower steady-state levels of C*c*O. First, we generated a yeast strain with a mitochondrial *COX2* gene carrying the W56R substitution (mtCox2^{W56R}) and compared its expression and function with those of a wild-type Cox2. We also addressed the question of how allotopically expressed, cytosol-synthesized cCox2^{W56R} competes for assembly into C*c*O against its mitochondria-synthesized counterpart.

RESULTS

The W56R mutation does not affect the function and stability of Cox2 or the activity of the C*c*O when expressed from the mitochondrial genome

Yeast Δ*cox2* mutants are unable to grow on nonfermentable carbon sources (ethanol/glycerol) but grow similarly to a wild-type strain on glucose (Figure 1A). The Δ*cox2* 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 (cCox2^{W56R}) (Supekova *et al.*, 2010). Allotopically expressed cCox2^{W56R} 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 (Δ*cox2*+cCox2^{W56R}) recovered the ability to grow on ethanol/glycerol, although not to the levels of the wild-type strain (Figure 1A). The Δ*cox2*+cCox2^{W56R} 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 cCox2^{W56R} partially restores respiratory activity as previously documented (Supekova *et al.*, 2010; Cruz-Torres *et al.*, 2012; Elliott *et al.*, 2012). To address the effect of the W56R substitution on C*c*O activity, a strain carrying the corresponding mutation in the mitochondrial gene was generated by biolistic transformation of the mitogenome (mtCox2^{W56R} strain). Just as in the wild-type mtCox2, the mtCox2^{W56R} protein is expected to be cotranslationally inserted into the inner mitochondrial membrane by mitoribosomes, the Oxa1 translocase, and other assembly factors.

The mtCox2^{W56R} strain grew on nonfermentable media at rates comparable to those of the wild type, and thus, higher than those of the Δ*cox2*+cCox2^{W56R} 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 cCox2^{W56R} 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, Δ*cox2*, Δ*cox2*+cCox2^{W56R}, and mtCox2^{W56R} 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 mtCox2^{W56R} mutant (Figure 2A), supporting the observation that the W56R mutation does not seem to interfere with the stability nor with the assembly of the mtCox2^{W56R} protein when it is expressed in the mitochondrion. As expected, no Cox2 band could be detected in the Δ*cox2* mutant (Figure 2A). In contrast, relatively low levels of the mature cCox2^{W56R} subunit were observed in the Δ*cox2*+cCox2^{W56R} transformant, and the majority of the Cox2 signal is observed as a high–molecular weight band, which must

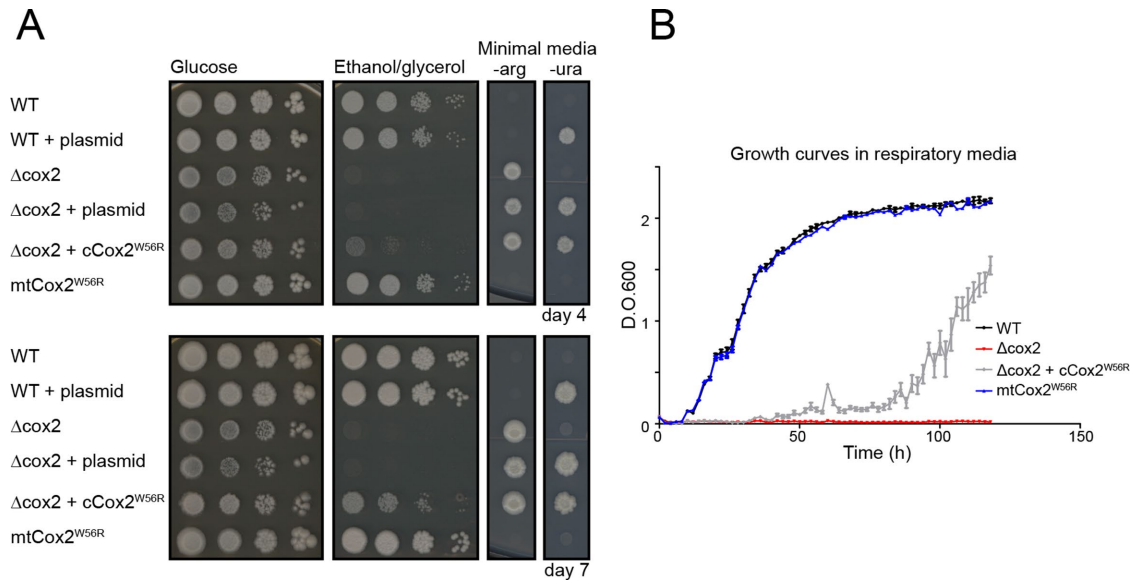

FIGURE 1: The yeast strain carrying the W56R mutation in the mitochondria-synthesized Cox2 subunit grows as a wild-type strain. (A) Tenfold serial dilutions from yeast cultures were spotted on plates containing fermentative (glucose) or nonfermentative (ethanol/glycerol) media. Growth in minimal media lacking arginine (SD-arg) was included to confirm the presence of the *ARG8*^{m} cassette inserted in the mitochondrial *cox2* locus; growth in minimal media lacking uracil (SD-ura) was included to confirm the presence of the pFL61 plasmid. Cells were incubated for 4 d (top panel) or 7 d (bottom panel) at 30°C. The strains were wild type (WT), wild type transformed with empty plasmid (WT + plasmid), the Δ*cox2* mutant (Δ*cox2*), the Δ*cox2* mutant with empty plasmid (Δ*cox2* + plasmid), the allotopic Δ*cox2*+cCox2^{W56R} transformant, and the mutant that gives rise to the mitochondrial mtCox2^{W56R} subunit (mtCox2^{W56R}). (B) Growth curves of the indicated yeast strains in liquid media containing as nonfermentable carbon source a mixture of ethanol plus glycerol. Optical density (O.D.$_{600}$) was recorded every 2 h. The mean and the SD of triplicates are shown for each point.

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 cCox2^{W56R} 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 cCox2^{W56R} precursor was located inside the organelle, mitochondrial fractions from all the strains were subjected to proteinase K (PK) treatment (Figure 2B). Only the cCox2^{W56R} 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 cCox2^{W56R} precursor is localized to mitochondria, but it is not completely internalized. As expected, the mature Cox2 bands from the wild-type, Δ*cox2*+cCox2^{W56R}, and mtCox2^{W56R} strains were mostly protected from degradation inside mitochondria (Figure 2B).

To determine whether the interaction of allotopic cCox2^{W56R} with its copper relaying partners is affected, resulting in impaired copper acquisition, we tested the interaction between cCox2^{W56R} 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 Δ*coa6* mutant (Ghosh *et al.*, 2014). We inferred that if the cCox2^{W56R} 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 Δ*coa6* strain. This effect is more clearly visible when cells are incubated at 37°C (Figure 2C). These results indicate that the interaction of cCox2^{W56R} 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, Δ*cox2*, Δ*cox2*+cCox2^{W56R}, and mtCox2^{W56R} 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 Δ*cox2*+cCox2^{W56R} transformant (Figure 2D, lane 3). As expected, no in-gel CcO activity could be detected in the Δ*cox2* mutant (Figure 2D, lane 2). In contrast, the in-gel CcO activities of the wild-type strain and the mtCox2^{W56R} 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 Δ*cox2* 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 III_2IV_1 and III_2IV_2 supercomplexes formed by the association of CcO with the bc_1 complex. The Δ*cox2*+cCox2^{W56R} transformant exhibited reduced in-gel CcO activity associated with supercomplexes, while the mtCox2^{W56R} 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 mtCox2^{W56R} mutant (Figure 2F). The specific activities of oxygen uptake measured after the addition of ethanol as substrate were 18.25 and 18.75 nmol O_2/min per 3×10^7 cells for the wild type and the mtCox2^{W56R} mutant, respectively. In contrast, the Δ*cox2*+cCox2^{W56R} transformant had an oxygen consumption rate of 7.05 nmol O_2/min per 3×10^7 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 cCox2^{W56R} allotopic expression. Therefore, the diminished CcO activity and steady-state levels observed when cCox2^{W56R} 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 cCox2^{W56R} assemble into CcO in a strain expressing both proteins

Cytosol-synthesized cCox2^{W56R} 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 *COX2* 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 cCox2^{W56R} protein competes with the natural Cox2 protein synthesized in yeast mitochondria. To address this question, mtCox2 and cCox2^{W56R} were both expressed in a single strain. To distinguish the mitochondria- and nucleus-encoded Cox2 subunits, we used a strain where a hemagglutinin tag was fused to the C-terminal end of the mitochondria-encoded Cox2 (mtCox2-HA; Saracco, 2003). This strain coexpressed the cCox2^{W56R} precursor from the nucleus. We also constructed a strain coexpressing the untagged mitochondrial Cox2 (mtCox2) and the cCox2^{W56R} from the nucleus. The presence of the endogenous Cox2 subunits (either wild type or HA-tagged) or the allotopic subunit (carrying the W56R mutation) could then be followed by mass spectrometry and Western blot analysis. While the Δ*cox2*+cCox2^{W56R} transformant grew poorly, the strains expressing both versions of Cox2 (mtCox2+cCox2^{W56R} and mtCox2-HA+cCox2^{W56R}) had growth rates comparable to those of the strains that contained only the mitochondrial versions, mtCox2 or mtCox2-HA (Figure 3, A and B). The oxygen consumption rates of the strains correlated with the growth phenotype: the wild-type strains (mtCox2 and mtCox2-HA) and the mtCox2-HA+cCox2^{W56R} strain behaved indistinguishably and exhibited higher growth rates than the strain expressing only allotopic cCox2^{W56R} (Figure 3C).

To characterize whether Cox2 from different genetic origins compete for assembly into CcO, we looked for these proteins in the assembled CcO complexes of the strain mtCox2-HA+ cCox2^{W56R}. Mitochondria were isolated, solubilized with lauryl maltoside, and resolved by BN–PAGE. The bands corresponding to CcO (bands IV and IV*, identified by in-gel activity) were cut out, further resolved by SDS-Tricine-PAGE, and subjected to Western blot analysis using an anti-yeast Cox2 antibody or an antibody directed against the HA tag. After the membrane was blotted with one antibody, it was stripped and reblotted with the other in order to observe the presence of both Cox2 subunits in the same strain.

As expected, the wild-type strain (mtCox2) and the allotopic strain (Δ*cox2*+cCox2^{W56R}) each revealed one band recognized by the anti-Cox2 antibody (Figure 4A, lanes 1 and 3). The two bands had the same molecular weight, indicating that only the mature version of cCox2^{W56R} is assembled into CcO. The strain carrying mtCox2-HA was recognized by the anti-HA antibody as a band with a higher molecular weight than the untagged Cox2 (Figure 4A, lane 6). We observed that mtCox2-HA cannot be recognized by the anti-Cox2 antibody, probably because the Cox2 antigenic epitope is blocked by the HA tag (Figure 4A, lane 2). In the strain mtCox2-HA+cCox2^{W56R}, Cox2 bands were recognized by the two antibodies (Figure 4A, lanes 4 and 8), indicating that each version can independently assemble into CcO, giving rise to a heterogeneous population of this complex. This was more clearly observed after the membranes were stripped and reblotted with the corresponding antibody (Figure 4B, lanes 4 and 8). Parallel to the immunoblot approach, we also unambiguously identified the two main bands as 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 cCox2^{W56R} species.

To further explore whether the presence of mtCox2 affects the incorporation of cCox2^{W56R}, 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 cCox2^{W56R} 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 cCox2^{W56R} when coexpressed with mtCox2-HA would be expected to elevate

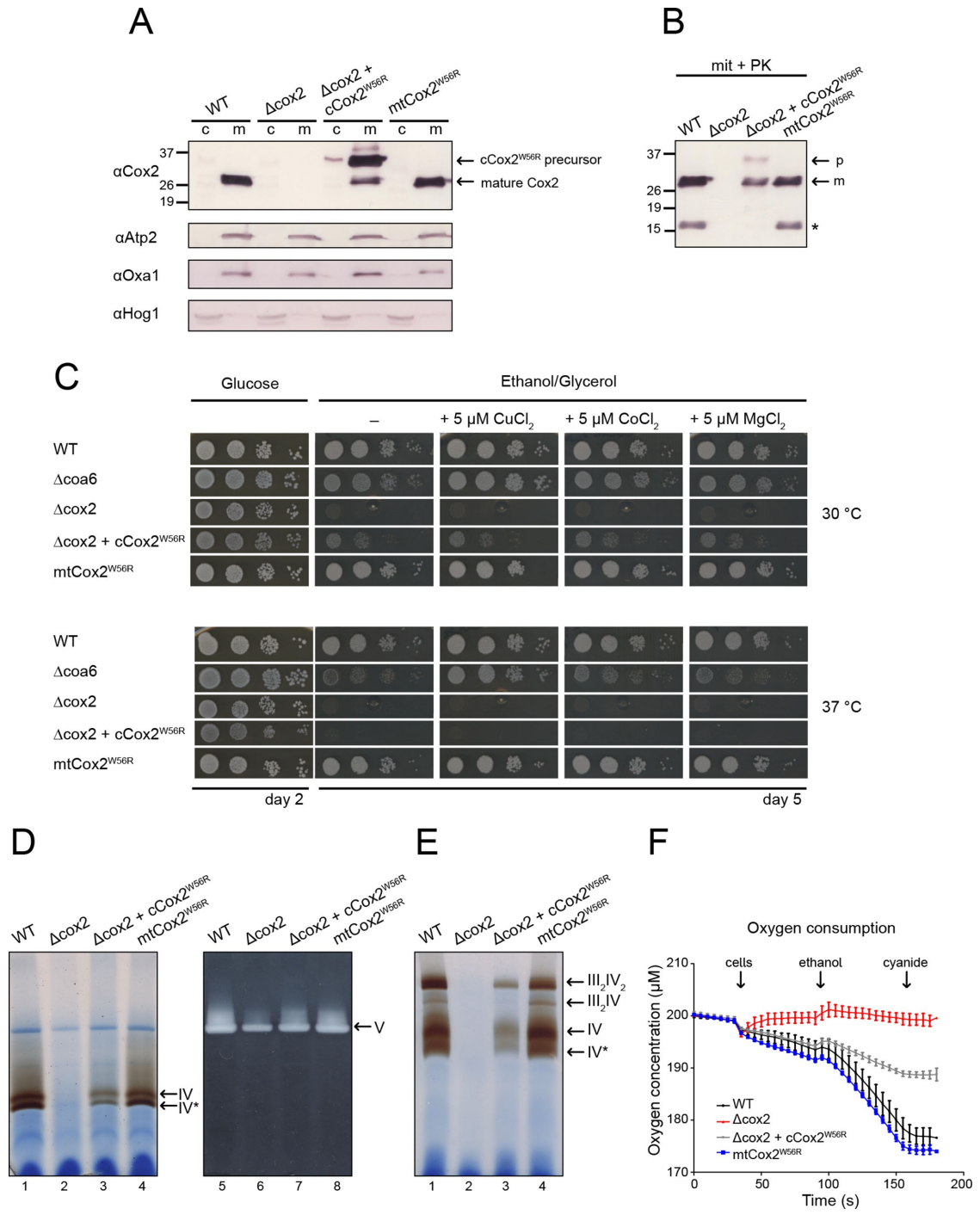

FIGURE 2: CcO complex activity remains unchanged when the mitochondria-encoded Cox2 subunit contains the W56R mutation. (A) Yeast cytosolic (c) and mitochondrial (m) fractions (100 and 50 μg of protein, respectively) of the indicated strains were separated by SDS-Tricine-PAGE and transferred to nitrocellulose membranes for Western blot analysis with an anti-Cox2 antibody (top panel). Bands corresponding to the mature Cox2 subunits and the cCox2^{W56R} 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 Δ*coa6* 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_1Fo-ATPase (V) are indicated. Band IV* corresponds to CcO lacking the Cox6 subunit (Horan *et al.*, 2005). (E) Mitochondria from D were solubilized with

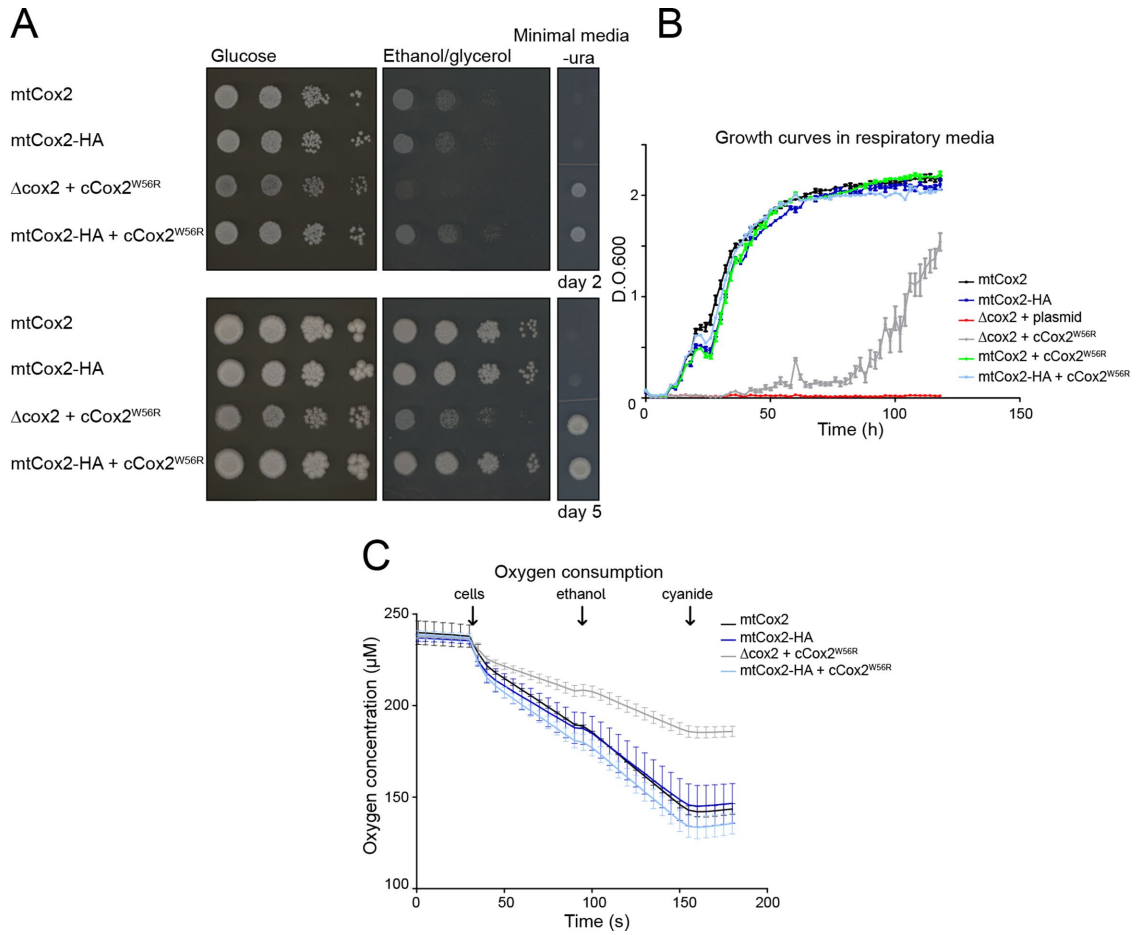

FIGURE 3: A strain expressing both the mitochondrial wild-type Cox2 protein (mtCox2) and the allotopic cCox2^{W56R} precursor grows and exhibits oxygen consumption rates similar to those for a wild-type strain. (A) Tenfold serial dilutions from yeast cultures were spotted on plates containing fermentative (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 Δ*cox2*+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 Δ*cox2* strain, the Δ*cox2*+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 CcO levels also. To test this, we compared the CcO in-gel activity of the different strains and observed an increase in the CcO 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 CcO 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).

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 CcO complexes, which may contain either mtCox2 or cCox2^{W56R} subunits, with most of the complexes containing the mitochondria-encoded version.

digitonin, separated by BN–PAGE (4–15%), and stained for CcO in gel activity. Bands corresponding to CcO and its supercomplexes are indicated. (F) Oxygen consumption of fasted yeast cells. Arrows indicate where 3×10^7 cells were added to the oxygen-meter chamber, 50 mM ethanol was added as substrate, and oxygen uptake was inhibited with 200 μM sodium cyanide. The final volume was 1 ml. The mean and the SD of triplicates are shown for each point.

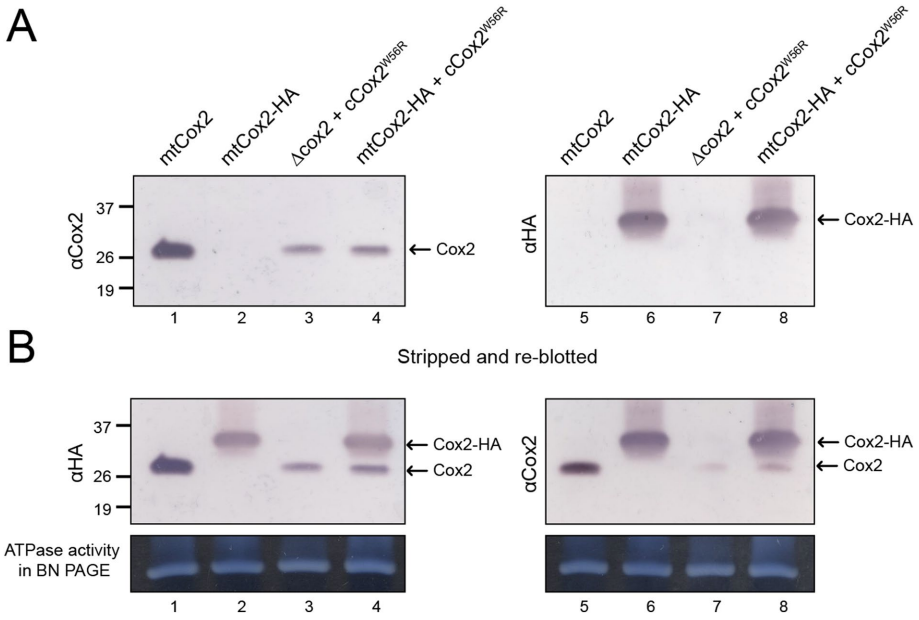

FIGURE 4: Both the nuclear and the mitochondrial Cox2 versions were found assembled into C*c*O. (A) Isolated mitochondria (250 µg) were solubilized with lauryl maltoside and separated by BN–PAGE. Bands corresponding to C*c*O were excised and further separated by SDS–PAGE. The gel was transferred into a nitrocellulose membrane, blocked, and blotted using an anti-Cox2 (lanes 1–4) or an anti-HA (lanes 5–8) antibody. (B) The membranes were stripped and reblotted using an anti-HA (lanes 1–4) or an anti-Cox2 (lanes 5–8) antibody. The stripped membranes do not lose the staining of the first antibody. Bands corresponding to the Cox2 subunits fused to a HA tag (mCox2-HA) and the wild-type Cox2 subunits (mtCox2) are indicated. ATPase in gel activities in the first dimension BN–PAGE were used as loading controls.

DISCUSSION

Allotopic expression is one of the multiple approaches aimed 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 Vincens, 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 Atp8 subunit of mitochondrial ATP synthase (Gearing and Nagley, 1986; Nagley *et al.*, 1988; Law *et al.*, 1990; Roucou *et al.*, 1999). Atp9 and Cox2 required further modifications in their sequences to achieve allotopic expression (Supekova *et al.*, 2010; Bietenhader *et al.*, 2012). For the case of Cox2, the W56R substitution diminished the hydrophobicity of the first transmembrane stretch (TMS1) of the protein, allowing its import into mitochondria (Supekova *et al.*, 2010; Cruz-Torres *et al.*, 2012; Elliott *et al.*, 2012). Inner-membrane protein products of genes that naturally migrated to the nucleus tend to be less hydrophobic than their mitochondria-encoded counterparts (Daley *et al.*, 2002b; Funes *et al.*, 2002; Pérez-Martínez *et al.*, 2000, 2001). All examples of natural, cytosol-expressed Cox2 subunits, that is, those of legumes, chlorophycean algae, and apicomplexan parasites, exhibit low hydrophobicity in their first TMS and slightly increased hydrophobicity in the second TMS, compared with the vast majority of mitochondria-encoded Cox2 subunits (Daley *et al.*, 2002b; Jiménez-Suárez *et al.*, 2012). Successful allotopic expression requires that the protein be internalized into mitochondria, and that it be inserted into the inner membrane in the same topology as the mitochondria-synthesized version, in order to allow it to then follow a normal assembly pathway. The yeast mitochondrial import machinery inserts the allotopic cCox2^{W56R} in the same topology as the endogenous protein but uses a different set of translocases: 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 cCox2^{W56R} 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 cCox2^{W56R} 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 Cox2^{W56R} preprotein in the immunoblots. In this way, the cCox2^{W56R} 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 cCox2^{W56R} seems to be somehow hindered. The majority of cCox2^{W56R} 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 cCox2^{W56R} precursors are correctly processed and inserted in the inner mitochondrial membrane. In addition, copper

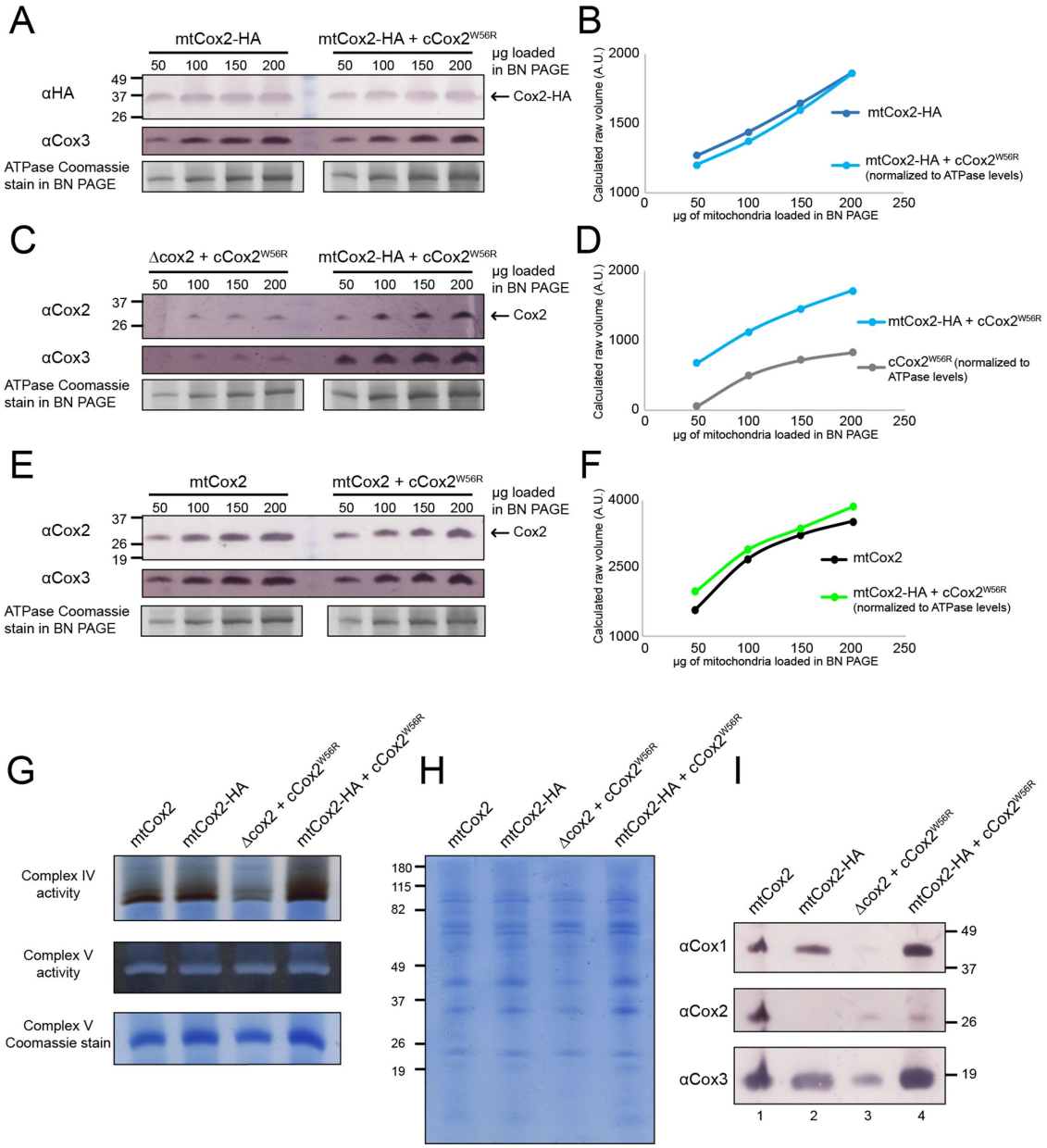

FIGURE 5: Assembled steady-state Cox2 levels in a strain where only one *COX2* gene version is expressed, either mitochondria- or nucleus-encoded, compared with a strain where both versions are present. For panels A–F, increasing amounts of mitochondrial proteins were loaded in the first dimension and 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^{W56R} (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^{W56R} 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 C*c*O in-gel activity. Complex V (ATPase) activity and Coomassie stain were used as loading controls. (H) Bands corresponding to the C*c*O complex were excised from a BN–PAGE gel and subjected to SDS-Tricine-PAGE. A resulting Coomassie blue–stained gel of the resolved C*c*O 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 reblotted in the order anti-Cox3, anti-Cox2, anti-Cox1. The Cox2 antibody recognizes Cox2 only without the HA-tag.

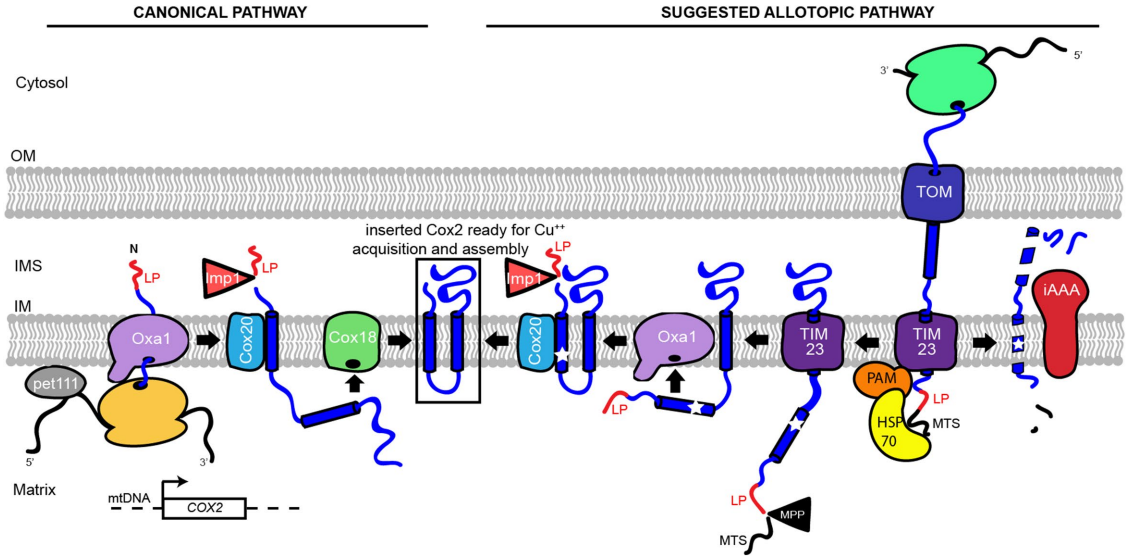

FIGURE 6: Proposed biogenesis routes for mitochondria-encoded Cox2 and for nucleus-encoded cCox2^{W56R}. The left-hand side of the scheme shows the classical biogenesis route of Cox2 from the mitochondrial matrix. In this route, Pet111 translationally activates the yeast mitochondria-encoded Cox2 subunit's mRNA (Green-Willms *et al.*, 2001). This subunit is synthesized by mitoribosomes as a precursor containing an N-terminal leader peptide (LP) of 15 residues (Sevarino and Poyton, 1980) that promotes its insertion into the membrane through the Oxa1 machinery (Torello *et al.*, 1997). The LP is processed by the intermembrane space (IMS) protease Imp1 following translocation of the first TMS of Cox2 into the mitochondrial inner membrane (IM) by Oxa1. Processing is assisted by the integral inner membrane protein Cox20 (Elliott *et al.*, 2012). The C-terminal domain of Cox2, containing the second TMS, is exported posttranslationally by the Cox18 translocase (Saracco and Fox, 2002; Fiumera *et al.*, 2007; Bonnefoy *et al.*, 2009). The mature Cox2 subunit that reached its final topology, with both the N- and C-termini facing the IMS (Yoshikawa *et al.*, 1998), is depicted inside a box. The right-hand side of the scheme illustrates the suggested biogenesis pathway of the allotopic cCox2^{W56R} subunit; the white star represents the W56R point mutation. On the allotopic route, the protein is synthesized in cytosolic ribosomes and, most probably, cotranslationally imported by the TOM import machinery. The W56R mutation will allow full translocation of the first TMS to the matrix through TIM23, albeit at a low rate. The MPP protease will process the experimentally added MTS of Oxa1, leaving the 15-residue leader peptide of yeast Cox2 intact. On one hand, the N-terminal part of the mature Cox2 would be translocated back through the IM in an exportlike reaction, probably mediated by the LP of Cox2^{W56R} with the assistance of Oxa1 and Cox20. On the other hand, TIM23 may then recognize a stop transfer signal within the second TMS and will insert it laterally into the IM (Neupert and Herrmann, 2007). The allotopically expressed cCox2^{W56R} subunit will thus achieve the same functional N-out, C-out topology typical of the Cox2^{WT} subunit. This mature allotopically expressed cCox2^{W56R} subunit is biochemically indistinguishable from its Cox2 counterpart except for the presence of the W56R point mutation (Cruz-Torres *et al.*, 2012). The represented import mechanism of the allotopically expressed Cox2^{W56R} subunit is similar to those proposed for the naturally nucleus-encoded Cox2 precursor of soybean (Daley *et al.*, 2002a) and for the cytosol-synthesized Cox2A precursor of the colorless alga *Polytomella* sp. (Jiménez-Suárez *et al.*, 2012). As the majority of the cCox2^{W56R} proteins are found in the precursor form, we suggest that while their first TMS is retained in TIM23, their MTS is recognized by the PAM and Hsp70 machinery and is unavailable for processing by the MPP protease. A fraction of cCox2^{W56R} may be prematurely released into the IM (extreme right-hand side of the scheme), since the first TMS of cCox2^{W56R} is still hydrophobic enough to be laterally inserted into the IM by TIM23. This gives rise to an improperly inserted population of cCox2^{W56R} with the topology N-terminus in, C-terminus out, which should be degraded readily by quality control mitochondrial proteases such as i-AAA (Bietenhader *et al.*, 2012; Gerdes *et al.*, 2012).

acquisition prior to assembly does not seem to be limiting in the allotopic strain. Thus, the limiting step resides in the correct insertion of cCox2^{W56R} into the inner membrane. We consider a scenario where the cCox2^{W56R} 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^{W56R} 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 cCox2^{W56R} 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 cCox2^{W56R} 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 Cox2^{W56R} 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 *cc*O accumulation. We thus suggest that the allotopic expression of cCox2^{W56R} is limited not because the W56R mutation affects the assembly of cCox2^{W56R} into *cc*O, nor because the

substitution has a detrimental effect on C*c*O 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^{W56R} precursor in the mitochondrial fraction and the lower overall accumulation of C*c*O^{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^{W56R}. However, these new changes could start affecting the assembly, activity, and stability of the whole C*c*O 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 Δ*cox2* 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 C*c*O complex.

Legumes provide an illustrative example of a recent transfer of the *COX2* gene to the nucleus; it possibly occurred once in the Phaseolae linage 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 C*c*O complexes were not explored. We predict, on the basis of the results obtained here, that in some legume species there will be a heterogeneous C*c*O 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 complex population: the cytosol-synthesized version and the mitochondria-expressed version carrying the deleterious mutation (or a wild-type version, given the heteroplasmy in mammalian mitochondria). In this work, when both Cox2 protein versions were coexpressed, the final steady state levels of Cox2 in the C*c*O 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^{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^{W56R} to efficiently enter the assembly pipeline of the C*c*O complex. C*c*O 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 C*c*O accumulation levels, since its synthesis is required for the overall progress of the C*c*O 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 C*c*O (Ott and Herrmann, 2010; Mick *et al.*, 2011). Furthermore, stalled assembly of the C*c*O complex results in selective degradation of the Cox1 subunit (Khalimonchuk *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^{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^{W56R} is enhanced (Figure 7).

We consider the observations here described relevant to the understanding of C*c*O 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 $(NH_4)_2SO_4$], 0.5% $(NH_4)_2SO_4$, 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 $CuCl_2$, $CoCl_2$, or $MgCl_2$.

The *S. cerevisiae COX2* gene encoding a cytosol-synthesized precursor protein with the W56R point mutation (cCox2^{W56R}) was recoded 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^{W56R}) was constructed using site-directed mutagenesis (QuikChange II site-directed mutagenesis kit, Agilent Technologies) on the pJM2 plasmid (Mulero and Fox, 1993). This plasmid

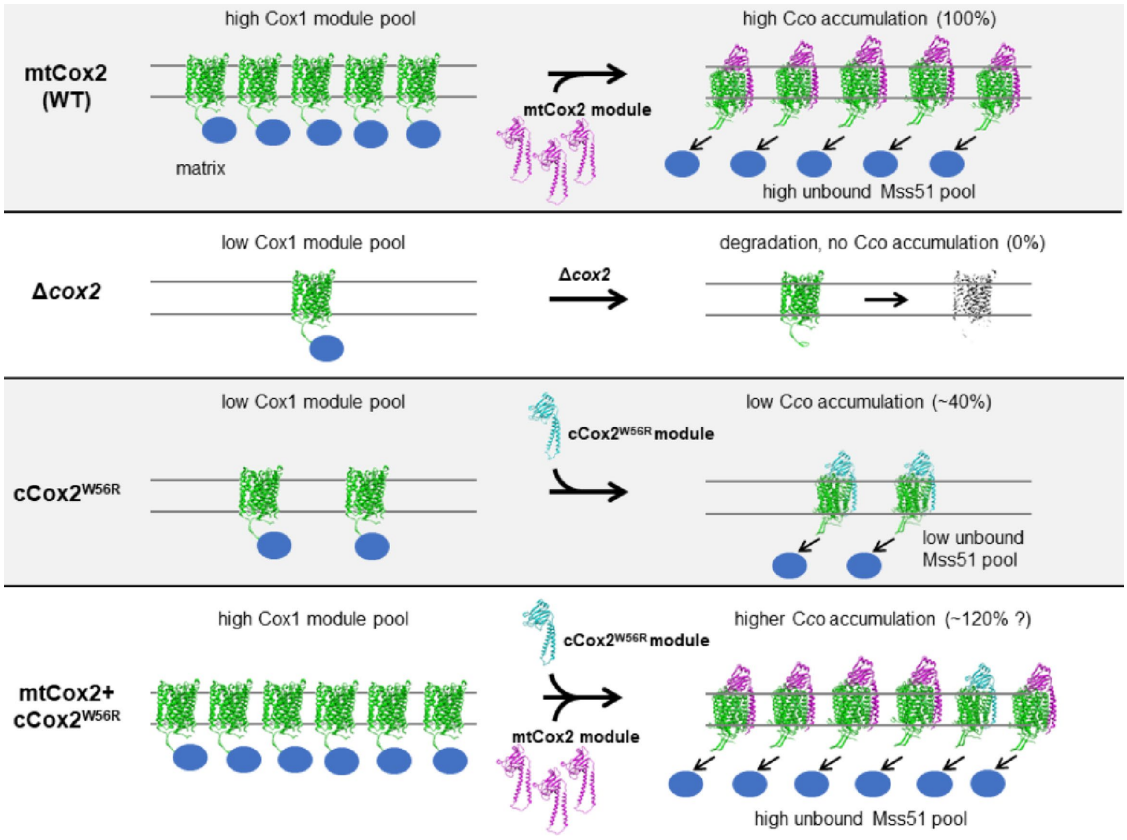

FIGURE 7: Cox1 synthesis is regulated and determines its further assembly into CcO. For canonical CcO assembly in a wild-type strain, the synthesized Cox2 (mtCox2) module is incorporated into the Cox1 module (McStay *et al.*, 2013), promoting the release of Mss51 (blue circles), the translational activator of Cox1, and thus enabling more Cox1 synthesis (Perez-Martinez *et al.*, 2009). In a Δ*cox2* strain, unassembled Cox1 module is readily degraded (Khalimonchuk *et al.*, 2012), resulting in no CcO accumulation taking place. In the allotopic strain, the mature cCox2^{W56R} levels are limiting. This would result in low incorporation of the cCox2^{W56R} assembly module with the Cox1 module, a decreased release of Mss51, and relatively low CcO levels. In a strain where both Cox2 versions are expressed, the total Cox2 protein levels are increased. The presence of mitochondria-encoded Cox2 allows more efficient Cox1 synthesis, enhancing the assembly of more cCox2^{W56R} modules into Cox1 modules. This would result in a higher accumulation of CcO.

was transformed by high-velocity micro projectile bombardment into the ρ^0 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^{W56R} plasmid were mated with the EHW154 strain and cytoductants were selected for their ability to grow in YPEG as haploids. Correct integration of the mtCox2^{W56R} 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

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; MATa, *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^0]	Thomas D. Fox, Cornell University
SCS188A	D273-10B; MATa, *ade2-101, ura3-52, kar1-1,* [rho+] *cox2-75c*	Thomas D. Fox, Cornell University
SCS101	D273-10B; MATa, *ura3-52, leu2-3,112, lys2, his3-ΔHindIII, arg8-Δ::hisG,* [rho+] *COX2::3xHA*	Saracco, 2003
DRG101	D273-10B; MATa, *lys2, arg8::hisG, ura3-52, leu2-3, 112,his3-ΔHindIII,* [rho+] *COX2W56R*	This study
YMR245W	BY4741; MATa, his3Δ1, leu2Δ0, met15Δ*0, ura3Δ0,* coa6-Δ*51-315*	Winzeler *et al.*, 1999

TABLE 1: Strains used in this work.

harvested in the logarithmic growth phase ($O.D._{600}$ 1.2–1.5), washed with H_2O at 4500 rpm (2500 × *g*) for 5 min, resuspended in TD buffer (100 mM Tris, 10 mM DL-dithiothreitol), and centrifuged at 4500 rpm (2500 × *g*) for 5 min. The cells were resuspended in 10 ml of zymoliase buffer (1.2 mM sorbitol, 20 mM KH_2PO_4 [pH 7.4], 3–5 mg zymoliase/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_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 C*c*O activity. The cells were washed once with H_2O prior to the measurements or, where indicated, were washed and fasted overnight in H_2O. 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 C*c*O activity (Wittig and Schägger, 2007; Wittig *et al.*, 2007) and ATPase activity (Zerbetto *et al.*, 1997; Wittig and Schägger, 2005). Further electrophoretic separation of the C*c*O 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 Coomassie solution (50% ethanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue G) or transferred and then subjected to immunodetection.

Immunoassays

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 1:1000 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 Coomassie 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 IN202715 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).

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