Key within-membrane residues and precursor dosage impact the allotopic expression of yeast subunit II of cytochrome c oxidase

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ABSTRACT Experimentally relocating mitochondrial genes to the nucleus for functional expression (allotopic expression) is a challenging process. The high hydrophobicity of mitochondria-encoded proteins seems to be one of the main factors preventing this allotopic expression. We focused on subunit II of cytochrome c oxidase (Cox2) to study which modifications may enable or improve its allotopic expression in yeast. Cox2 can be imported from the cytosol into mitochondria in the presence of the W56R substitution, which decreases the protein hydrophobicity and allows partial respiratory rescue of a cox2-null strain. We show that the inclusion of a positive charge is more favorable than substitutions that only decrease the hydrophobicity. We also searched for other determinants enabling allotopic expression in yeast by examining the COX2 gene in organisms where it was transferred to the nucleus during evolution. We found that naturally occurring variations at within-membrane residues in the legume Glycine max Cox2 could enable yeast COX2 allotopic expression. We also evidence that directing high doses of allotopically synthesized Cox2 to mitochondria seems to be counterproductive because the subunit aggregates at the mitochondrial surface. Our findings are relevant to the design of allotopic expression strategies and contribute to the understanding of gene retention in organelar genomes.

INTRODUCTION Most mitochondrial proteins are encoded in the nuclear genome, translated by cytosolic ribosomes, and directed to mitochondria. However, migration of mitochondrial genes to the nucleus occurs widely in nature (Adams and Palmer, 2003; Burger et al., 2003; Timmis et al., 2004). These relocated nucleotide sequences acquire more complex regulation and tend to exhibit lower mutation rates (Martin and Herrmann, 1998). Mitochondrial genomes mainly encode subunits of oxidative phosphorylation (OXPHOS) complexes. In general, these proteins are embedded in membranes and are highly hydrophobic, a feature that is considered to be one of the main driving forces for the retention of a genome in mitochondria (Johnston and Williams, 2016).

Experimentally relocating mitochondrial genes to the nucleus holds promise for the development of treatments of mitochondrial diseases (De Grey, 2000; DiMauro et al., 2006; Kyriakouli et al., 2008). A mutation in the mitochondrial genome, yielding a defective protein, could be overcome in principle by the import of a nucleus-encoded functional version of the protein. Multiple efforts have been undertaken to express mitochondria-encoded genes allotopically using yeast or mammalian model systems, but conflicting and inconsistent results call for careful interpretation of results and warrant further experimental testing (Oca-Cossio et al., 2003; Perales-Clemente et al., 2011).

The COX2 gene, encoding subunit 2 (Cox2) of cytochrome c oxidase (CcO), provides an example of natural migration of a mitochondrial gene to the nucleus. This gene is generally mitochondria-encoded, but in a few organisms it is found in the nuclear
Factors that impact allotopic expression

We previously showed that expression of a protein encoded in the nucleus in the mitochondria is a successful strategy (Supekova et al., 2010). We also tested whether naturally occurring mutations allowing the allotopic production of other proteins can be identified. We thus performed a mutagenesis screen of 20,000 alleles (Supekova et al., 2018). We thus performed cellular fractionations to test the cytosolic versus mitochondrial distribution of Cox2WT, Cox2W56K, and Cox2W56Q. Most of the Cox2 precursors were found associated with mitochondria (Figure 1B), indicating that, similarly to Cox2W56K, the limiting step occurs before their processing into mature forms in the mitochondrial matrix. Our results show that other residues at position 56 allow allotopic expression of COX2 and that the inclusion of a positive charge in the first TMS (W56K) is favorable over a substitution that only diminishes hydrophobicity (W56Q).

Mutations that allow the nuclear expression of COX2 in legumes also confer allotopic expression in yeast

We first tested whether other changes at position 56, besides the tryptophan-to-arginine substitution (Supekova et al., 2010) (W56R), can restore the respiratory growth of a cox2-null strain expressing an allotopic version of COX2. We substituted W56 for two residues sharing at least one physicochemical property with arginine: lysine (W56K), which also has a positive charge, and glutamine (W56Q), which has the closest hydrophobicity score to arginine (Calado-Botelho et al., 2011). Upon introduction of the allotopic constructs encoding Cox2W56K or Cox2W56Q into a cox2-null strain, we observed a restoration of respiratory growth. Respiratory growth of the strain with the W56K substitution was comparable to that of the strain carrying the W56R substitution, while the recovery of respiratory competence by Cox2W56K was significantly less (Figure 1A). Hence, the growth behavior of yeast carrying these constructs was Cox2W56K > Cox2W56Q. As a negative control, we included a strain expressing an allotopic wild-type COX2 gene (producing the Cox2WT subunit). As expected, neither this construct producing Cox2WT, nor the empty plasmid, was able to restore respiratory growth in the cox2-null strain (Figure 1A).

We next wanted to identify other changes in Cox2 that might enable its allotopic expression in the yeast system. We reasoned that in organisms that naturally encode COX2 in the nucleus, the protein

FIGURE 1: Substituting residues of properties similar to those of arginine in position 56 of allotopic Cox2 restores cellular respiration to a cox2-null strain. (A) Tenfold serial dilutions of yeast liquid cultures from strains transformed with the indicated constructs or with an empty vector (plasmid) were plated on fermentable (glucose), respiratory (ethanol + glycerol), and minimal, selective media (-ura or -arg, for verifying the presence of the transforming plasmid or the mitochondrial genome, respectively). (B) Immunoblots decorated with an anti-Cox2 antibody of cytosolic (c; 100-µg) and mitochondrial (m; 50-µg) fractions of the indicated yeast strains. The Cox2 precursor and mature forms of Cox2 are indicated. Antibodies anti-Atp2 and anti-Oxa1 were used to immunodetect the corresponding mitochondrial markers and anti-Hog1 was used for the cytosolic marker. Squared brackets indicate Cox2 variants expressed from plasmids. All the Cox2 variants contain an MTS from Oxa1.
sequence must carry determinants for import into the mitochondria and subsequent assembly into a functional holoenzyme. For example, the legume *Glycine max* expresses the *COX2* gene from both nuclear and mitochondrial genomes (Adams et al., 1999). Previous research showed that a two-residue substitution within the first TMS of Cox2 is necessary and sufficient for the mitochondrial version of Cox2 to be imported into isolated mitochondria. Accordingly, the nucleus-encoded Cox2 protein could no longer be imported into the organelle when the residues (Q and G) at these two positions were modified to the ones (L and L) present in the mitochondria-encoded Cox2 (Daley et al., 2002b; Figure 2A). The conclusion from this study is that this two-residue substitution was determinant in facilitating the import of Cox2 from the cytosol to the mitochondria.

To test whether this two-residue substitution could confer allo-topic expression in the yeast system, we sought to reproduce the legume Cox2 mutations in the COX2 sequence from yeast. We generated an alignment of the two legume Cox2 subunits with the one from yeast and identified that the key residues conferring import of the legume Cox2 correspond to a valine and a leucine at positions 49 and 51 in the yeast sequence (Figure 2A). We therefore engineered the mutations corresponding to the single and double substitutions V49Q and LS1G in yeast COX2. We observed that only the Cox2V49Q/LS1G variant was able to restore respiratory growth in a cox2-null strain, while the Cox2V49Q and Cox2LS1G variants could not (Figure 2B), demonstrating that both changes are required for successful allotopic expression of COX2. However, the respiratory growth of the Cox2V49Q/LS1G strain was less than that of the Cox2WS6R strain. To test whether the overall decrease in hydrophobicity within the first TMS of Cox2 is sufficient for restoring respiratory growth, we substituted other aliphatic residues at other positions, resulting in the construct Cox2L47Q/V49G. To explore any additive effect, a triple COX2 mutant corresponding to W56R combined with a double substitution (L47Q/V49G/W56R) was also generated. All these constructs could restore the respiratory growth of a cox2-null strain, although to a lesser extent than the construct producing Cox2V49Q/LS1G. We also could not observe any additive effect in the strain producing Cox2V49Q/LS1G/W56R variant. Using in-gel activity staining, we verified that COX activity correlated with the observation that the Cox2WS6R variant exhibits increased respiratory growth compared with that in the variants containing two-residue substitutions (Figure 2C). In contrast, another OXPHOS complex, ATPase, exhibited comparable activity for all strains.

Next, we questioned whether Cox2V49Q/LS1G follows the same bio- genesis pathway as that of allootypically produced Cox2WS6R. In contrast to orthodox Cox2, synthesized in mitochondria, allootypically

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**FIGURE 2:** Mutations that allow the nuclear expression of COX2 in legumes also confer allotopic expression in yeast. (A) Sequence alignments of the first transmembrane segment (TMS1) from the yeast Cox2 (mScCox2) and the mitochondrial and nuclear Cox2 versions of Glycine max, mGmCox2 and nGmCox2, respectively. An asterisk (*) indicates positions that have a single, fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties (scoring $>0.5$ in the Gonnet PAM 250 matrix). A period (.) indicates conservation between groups of weakly similar properties (scoring $<0.5$ in the Gonnet PAM 250 matrix). Key residues that are critical for import of the legume Cox2 from the cytosol are boxed (see text). Two yeast Cox2 constructs were designed based on this alignment: Cox2V49Q/LS1G and Cox2L47Q/V49G. (B) Tenfold serial dilutions of yeast cultures from the indicated strains were spotted on fermentable (glucose), respiratory (ethanol + glycerol), and minimal, selective media (−ura or −arg). (C) In-gel activity staining of complex IV and of complex V as a loading control. Isolated mitochondria (250 µg) from the indicated strains were solubilized with lauryl maltoside and resolved by BN-PAGE. (D) Strains (WT, Δcox2, and Δcox18) were plated on fermentable (glucose), respiratory (lactate), and minimal, selective media (−ura or −arg). Squared brackets indicate Cox2 variants expressed from plasmids. All the Cox2 variants contain an MTS from Oxa1.
FIGURE 3: The allotopically synthesized proteins face the limiting step by aggregating at the mitochondrial periphery. (A) Immunoblots decorated with anti-Cox2 of cytosolic (c; 100-µg) and mitochondrial (m; 50-µg) fractions. Arrows indicate the precursor and mature forms of Cox2. The mitochondrial markers Oxa1 and the cytosolic marker Hog1 were decorated with their corresponding antibodies. (B) Isolated mitochondria (50 µg) were treated with proteinase K (PK) to degrade proteins external to the outer membrane. Parallel samples were preincubated with Triton X-100 to dissolve the membranes and to make all mitochondrial proteins accessible to protease degradation. The asterisk indicates partial degradation of Cox2, possibly due to imperfect mitochondrial preparation. (C) Carbonate extraction separating the membrane extrinsic proteins in the supernatant (S) from the integral membrane proteins in the pellet (P) from yeast expressing the indicated constructs. Atp2 was used as a soluble protein marker and Oxa1 as an integral membrane protein marker. (D) Triton X-100 extraction separating the detergent-solubilized proteins in the supernatant (S) from the (detergent-resistant) aggregated proteins in the pellet (P) from yeast expressing the indicated constructs. Cox3 was used as a solubilized membrane protein marker. Squared brackets indicate Cox2 variants expressed from plasmids. All the Cox2 variants contain an MTS from Oxa1.

Factors that impact allotopic expression

The allotopically synthesized Cox2W56R does not require the Cox18 translocase for the correct insertion of its second TMS into the inner membrane (Elliott et al., 2012), and it was postulated to be released laterally into this membrane directly from the TIM23 complex (Rubalcava-Gracia et al., 2018). We observed that, similarly to the Cox2W56R strain, the Cox2V49Q/L51G variant is able to restore respiration when expressed in a cox18-null (Figure 2D), indicating that the Cox18 translocase is completely dispensable. This suggests that the second TMS of Cox2V49Q/L51G is released laterally into the inner mitochondrial membrane by the TIM23 translocator.

We observed that while the mature forms of Cox2 were protected from degradation upon incubation of mitochondria-enriched fractions with proteinase K (PK), a result indicative of mitochondrial localization, the Cox2 precursors were mostly degraded by the protease (Figure 3A and B). These results may be interpreted in two ways: either the precursors are associated with the external face of the outer mitochondrial membrane or they are retained while in transit through the TOM and TIM23 complexes with their C-termini still exposed to the cytosol (note that our anti-Cox2 antibody recognizes the C-terminus of Cox2). To distinguish between these two possibilities, we performed carbonate and Triton X-100 extractions of the mitochondrial fractions. We found that the precursors are mostly resistant to both carbonate (Figure 3C) and detergent treatments (Figure 3D). As expected, mature Cox2 proteins resist carbonate extraction but are solubilized into the supernatant upon Triton extraction (Figure 3, C and D). These observations suggest that most Cox2 precursors aggregate at the external mitochondrial surface, thus preventing their import.

Taken together, our data show that substitutions at a position different from that of W56
enable the successful allotopic expression of COX and that a major limiting step for importing allotopic Cox2 into mitochondria is its aggregation.

Lowering the dose of allotopic COX2 expression enhances Cox2 import and assembly in CcO

While the use of a multicopy plasmid for allotopic expression of COX2 to ensure successful complementation seemed justified a priori (Supekova et al., 2010), we wondered whether reducing the allotopic expression of COX2 would decrease the rescue of the respiratory defect proportionally. For this purpose, we opted to test allotopic expression under a condition where the same COX2 construct carried in the multicopy plasmid was used as a single copy. To achieve this, we integrated the COX2-expressing construct (under the same phosphoglycerate kinase promoter and terminator present in the multicopy plasmid) in the nuclear genome. Surprisingly, we observed a notable increase in respiratory growth in the strains expressing the nuclear-integrated constructs producing Cox2W56R and Cox2V49Q/L51G in comparison with the strains producing the same Cox2 variants from a multicopy plasmid (Figure 4A). Growth of the yeast containing the single-copy COX2W56R construct was even comparable to that of a wild-type strain, and the strain containing the single-copy COX2V49Q/L51G exhibited more robust respiratory growth than the one with the high-copy COX2W56R. The strain producing Cox2WT did not display any growth in respiratory medium, even at low doses. Immunodetection of Cox2 in mitochondria-enriched fractions of these strains showed that, at low dosage, Cox2W56R and Cox2V49Q/L51G only accumulated in their mature forms at levels similar to those of the WT strain (Figure 4B). As previously observed, at high dosage, Cox2W56R and Cox2V49Q/L51G were mainly observed as precursors, with only a fraction converted to the

![FIGURE 4: Lowering the expression of allotopic COX2 in a cox2-null strain improves rescue of the respiratory growth.](image-url)

(A) Tenfold serial dilutions of yeast cultures from the indicated strains were plated on fermentable (glucose), respiratory (ethanol + glycerol), and minimal, selective media (-arg or -ura) and in the presence of hygromycin B (+hygB). The letter p represents expression from a multicopy plasmid, and the letter n represents expression from a single copy inserted in the nuclear genome. (B) Cox2 steady-state levels in isolated mitochondria (50 µg) from the indicated strains. Arrows indicate the precursor and mature forms of Cox2. The letters p and n indicate expression levels as in A. (C) Tenfold serial dilutions of yeast cultures from the indicated strains were plated on fermentable (glucose); respiratory (ethanol + glycerol), and minimal, selective media (-arg -ura) and in the presence of hygromycin B (+hygB). The letter c represents expression from a centromeric plasmid. The order of the letters indicates the order in which COX2-expressing constructs were introduced into yeast cells. All the Cox2 variants contain an MTS from Oxa1. (D) Biogenesis model of allotopically synthesized Cox2. On the left, insertion of Cox2 results in its correct topology. On the right, the high hydrophobicity of the first TMS leads the cytosol-synthesized Cox2 to aggregate at the mitochondrial periphery or to be prematurely released by TIM23 into an incorrect topology. TOM: translocase of the outer membrane, TIM: translocase of the inner membrane, MPP: mitochondrial processing peptidase, LP: leader peptide, MTS: mitochondrial targeting sequence, TMS: transmembrane segment.
mature form. In line with the absence of respiratory growth of the strains carrying the COX2WT construct, the Cox2WT protein was observed only as a precursor and, at low dosage, its steady-state level was probably extremely low, as we could not detect it under our experimental conditions (Figure 4B). We observed the same behavior, that is, enhanced respiratory growth, lower accumulation of precursors, and higher levels of mature forms of Cox2, when the same constructs were expressed in few copies from a centromeric plasmid (Figure 4C and unpublished data). To test the importance of high versus low doses of Cox2 for the rescue of respiratory growth, we increased the dosage by producing the high-copy Cox2 variants (p) in a strain already encoding a single-copy Cox2 in the nuclear genome (n). We observed that this strain (p+n) grew in respiratory medium to levels similar to those of the strain expressing Cox2 encoded only in the nucleus (Figure 4C) and verified the presence of the precursors by immunoblotting (unpublished data). Unexpectedly, these increased levels of expression did not show the same growth defect of the high-copy Cox2 strain. However, when we inverted the order of production of allotopic Cox2 variants in the cells by integrating Cox2 into the nuclear genome in a strain already encoding a high-copy Cox2 (p+n), we observed that this strain showed the same level of respiratory growth as the strain expressing a high-copy Cox2 from a plasmid (Figure 4C).

The aforementioned results suggest that, although overexpression of allotopic COX2 constructs is able to restore respiration to cox2-null strains, directing high doses of these proteins to mitochondria at the same time is counterproductive. Because the limiting step resides in the import of allotopic Cox2, low-dose expression seems to have a better outcome in terms of aggregation propensity and recovery of the respiratory growth.

**DISCUSSION**

The experimental relocation of mitochondrial genes to the nucleus has paved the way for the development of therapies intended to treat syndromes caused by mutations in the mitochondrial genome (González-Halphen et al., 2004; Taylor and Turnbull, 2005; Kyrkiouli et al., 2008; Calvo and Mootha, 2010; Gorman et al., 2016). Ongoing research has unveiled several constraining factors that limit allotopic expression (Claro et al., 1995; Oca-Cossio et al., 2003; Figueroa-Martínez et al., 2011; Björkholm et al., 2017). Because most proteins encoded in the mitogenome are integral to the membrane, the high hydrophobicity and the number of TMSs in these proteins seem to be major limiting factors for their import when synthesized in the cytosol (Claro et al., 1995; Johnston and Williams, 2016). In this work we also found that aggregation propensity, which is directly correlated with high hydrophobicity (Chiti et al., 2003; Dobson, 2004), contributes to the efficiency of importability before assembly of allotopic Cox2 into cytochrome c oxidase. Average hydrophobicities for the first TMSs of the different Cox2 variants used in this work are shown in Table 1.

First, we turned our attention to the Cox2 subunit to examine the importance of the residues enabling its allotopic production. Previous work demonstrated that substituting a tryptophan for an arginine decreases the hydrophobicity of the first TMS of Cox2 by adding a positive charge (Supekova et al., 2010). In this study, substitution of a lysine or glutamine in the same position also allowed recovery of the respiratory growth, but residues containing a positive charge (arginine and lysine) yield better rescue than a residue decreasing the hydrophobicity (glutamine). It is possible that Cox2WS56G is poorly inserted into the mitochondrial inner membrane, because the key determinant for importability is the presence of a positive charge (R+ or K+) and not reduction of the hydrophobicity (Q) at this position (von Heijne, 1992; Andersson and von Heijne, 1994) (see Table 1). This view is supported by the fact that while a similar number of Cox2 variant precursors accumulate in the mitochondria, the mature form of Cox2WS56G is significantly less abundant than those of Cox2WS56R and Cox2WS56Q (Figure 1B). This indicates that Cox2WS56G is probably limited in its insertion in the inner membrane because it is more prone to aggregation. That mature Cox2WS56Q and Cox2WS56R accumulate at the same level reinforces the view that a positive charge at this position is the key determinant of Cox2 importation into mitochondria when it originates from the cytosol.

Here, we designed allotopic Cox2 variants in yeast based on naturally evolving alterations in legumes. Notably, we observed that the double substitution reported as key for nuclear expression of Cox2 in Glycine max also allowed the allotopically expressed yeast COX2 to restore respiration to a cox2-null mutant. Similarly to what was observed in in vitro import assays in legumes (Daley et al., 2002b), each single-substitution V49Q or L51G was insufficient to restore respiration. In line with the cytosol-synthesized Cox2 biogenesis model (Daley et al., 2002a; Jiménez-Suárez et al., 2012; Rubalcava-Gracia et al., 2018), the first TMS (TMS1) of Cox2 is transferred to the matrix through the TIM23 translocase, while the second TMS is laterally released into the inner membrane by TIM23 (Figure 4D). As a final step, TMS1 is inserted from the matrix in the inner membrane by Oxa1, positioning Cox2 in its correct topology (Figure 4D). We assume that in the presence of either substitution (V49Q or L51G), the proteins aggregate at the mitochondrial periphery before import because they are too hydrophobic (Figure 4D and Table 1). Accordingly, only the double substitution, which diminishes the overall hydrophobicity of the TMS1 of Cox2, allows its correct insertion into the inner membrane.

It should be mentioned that the legume Cox2 substitutions (V49Q/L51G) are less efficient in conferring respiratory growth than the W56R that was identified through random mutagenesis (Supekova et al., 2010). Indeed, the W56R substitution meets Goldilocks conditions: besides maintaining CoO activity intact (Rubalcava-Gracia et al., 2018), it balances the need to diminish the hydrophobicity of Cox2 TMS1, but at the same time, it maintains the ability of TMS1 to be recognized as a transmembrane domain by the inner membrane translocator Oxa1. Notably, in the mitochondrial Cox2 sequence from Glycine max, the residue corresponding

| Cox2 variant | TMS1 | H |
|-------------|------|---|
| Cox2WT       | IMFYLLVLGLSVSLMTIVMTYS | 0.349 |
| Cox2WS56K    | IMFYLLVLGLVSMLMTIVMTYS | 0.367 |
| Cox2S51G     | IMFYLLVLGQSVSLMTIVMTYS | 0.375 |
| Cox2WS56Q    | IMFYLLVLGLSVSLMTIVMTYS | 0.395 |
| Cox2WS56R    | IMFYLLVLGLVSRMLTIVMTYS | 0.427 |
| Cox2V49Q     | IMFYLLQLGVSMLMTIVMTYS | 0.435 |
| Cox2V49Q/L51G| IMFYLLQLGLVSLMTIVMTYS | 0.461 |
| Cox2V49Q/L51G/G49Q/51G | IMFYLLQLGLVLGVLMTIVMTYS | 0.461 |
| Cox2V56Q/G56R | IMFYLLQLGLLGVSLMTIVMTYS | 0.540 |

Average hydrophobicity for the first transmembrane segment of each Cox2 variant used in this work. The “biological scale” was experimentally obtained and provides a hydrophobicity value for each residue present in the TMS of a model yeast mitochondrial protein (Calado-Botelho et al., 2011). Cox2 variants are ordered by decreasing hydrophobicity (increasing hydrophilicity). Residue substitutions are underlined.
to position 56 contains an arginine, indicating that a positive charge in the first TMS of Cox2 is permissible, at least in some organisms. Production of low levels of Cox2<sup>W56R</sup> results in the accumulation of mature Cox2 at a level similar to that of Cox2<sup>W56R</sup>, suggesting that insertion in the inner membrane occurs to the same extent for both Cox2 variants (Figure 4B). However, the fact that the rescue with Cox2<sup>W56R</sup> is significantly less efficient indicates that the V49Q/ L51G modifications impact the biogenesis of Cox2 or the activity of the yeast enzyme. Furthermore, we observed that the combination of W56R substitution with the V49Q and L51G changes did not further improve respiratory growth, and resulted in lower CoO activity, as observed in Figure 2C. We assume that, despite the fact that these mutations enable respiratory growth, they may also affect CoO activity. Another possibility is that the hydrophobicity of the first TMS of the Cox2<sup>W56R/V49Q/L51G</sup> subunit is lowered to such an extent (Table 1) that it is not promptly recognized as a transmembrane domain and is therefore only partially routed for insertion into the inner membrane by the Oxa1 translocator.

Our experiments indicate that allotopically expressed proteins face a limiting step by aggregating before their import into mitochondria. When we lowered the COX2 expression levels by integrating the COX2 gene into the nuclear genome, we observed a notable increase in the rescue of the cox2-null strain, which correlated with lower levels of precursor and corresponding enhanced levels of mature Cox2. It is conceivable that excess cytosol-synthesized Cox2 aggregates at the mitochondrial surface, limiting the import of allotopic Cox2 (Figure 4D). These aggregates do not affect the import of other mitochondria-targeted proteins, as the precursors of Oxa1 and Atp2 were not observed to accumulate in our mitochondrial preparations (Figures 1B, 3A, and 4B). We noted that aggregation can also be avoided if large doses of Cox2 are produced in a strain already synthesizing small doses of allotopic Cox2. We explain this observation by the importing competence of cox2-null mitochondria. If large doses of the precursors of Cox2 are directly produced in a cox2-null mutant, most precursors aggregate and only a fraction assemble into CoO, resulting in difficulties in restoring respiration and in building up the electrochemical gradient required for further protein import. At small doses, aggregation of Cox2 is prevented and its assembly into CoO is enhanced, giving rise to an optimal transmembrane gradient that facilitates further import of cytosolic proteins. These results suggest that expression levels of the allotopic gene (from a centromeric plasmid or a high-copy plasmid) define the levels of importability versus aggregation of Cox2. It is also conceivable that cytosolic chaperones and other aggregation-response proteins might play a role in preventing the aggregation of Cox2 precursors.

Aggregation propensity is directly related to hydrophobicity and has been an underinvestigated factor in the study of allotopic expression. Highly hydrophobic proteins imported from the cytosol must avoid aggregating at the mitochondrial periphery to cross both mitochondrial membranes. All reports of allotopic expression in either yeast or cell cultures use high-level expression plasmids to ensure a high accumulation of the desired protein products inside the mitochondria (Guy et al., 2002; Manfredi et al., 2002; Ojaimi et al., 2002; Bokori-Brown and Holt, 2006; Bonnet et al., 2008; Figueroa-Martinez et al., 2011; Cwerman-Thibault et al., 2015; Boominathan et al., 2016). Here, we found that a high level of the allotopically expressed Cox2 protein may be detrimental to the mitochondrial import system.

In summary, we provide a proof of principle that mimicking the modifications found in an organism that naturally transferred its mitochondrial gene to the nucleus can result in successful allotopic expression. This strategy includes expressing a gene with adequate mutations in a low copy number to ensure that moderate protein quantities are produced for mitochondrial import. Our results underscore that dosage, a previously unexplored important parameter, may be critical for allotopic expression in mammals, especially in human cell lines. The relevance of our finding is high, since allotopic expression still represents a promising strategy for developing treatments for patients with mitochondrial diseases. Our data also stress the relevance of studying organisms that naturally and atypically encode hydrophobic mitochondrial OXPHOS proteins in the nuclear genome.

**MATERIALS AND METHODS**

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

The Saccharomyces cerevisiae strains used in this study were derived from the D273-10B parental strain. We used NB40-36A as the wild-type strain (MATα, arg8::hisG, leu2-3, 112, lys2, ura3-52, [rho<sup>+</sup>]), EHW154 as the Δcox2 strain (MATα, arg8::hisG, his3ΔHindIII, leu2-3, 112, lys2, ura3-52, [rho<sup>+</sup>]) cox2-208::ARG8βm, and CAB116 as the Δcox18 strain (MATα, arg8::hisG, his3ΔHindIII, leu2-3,112, lys2, ura3-53, cox18α::kanMX4 [rho<sup>+</sup>]). Cells were grown on YPD as fermentable medium (1% yeast extract, 2% bacteropetone, and 2% dextrose) or YEPG as nonfermentable medium (1% yeast extract, 2% bacteropetone, 3% ethanol, and 3% vol/vol glycerol). Minimal medium was SD or SGal (0.17% yeast nitrogen base [without amino acids and (NH₄)₂SO₄], 0.5% (NH₄)₂SO₄, 2% glucose or galactose supplemented with specific amino acids and nucleotides). All strains were grown at 30°C in liquid (with shaking) or solid medium (containing 1.75% agar). We calculated duplication times by measuring the absorbance of cultures with an initial O.D.<sub>600</sub> of 0.01 every 2 h in a Bioscreen C spectrophotometer (Growth Curves, USA). Dilution series were performed by diluting 10-fold a culture of 0.5 O.D.<sub>600</sub> and plating 3 μl on Petri dishes with the indicated medium.

The yeast allotopic COX2<sup>K65R</sup> gene was recoded for expression from the nucleus and a sequence encoding the MTS of Oxa1 was added at the 5' end, as previously described (Supekova et al., 2010). The construct was chemically synthesized (GeneScript, Piscataway, NJ) and cloned at the NotI site in the pFL61 vector, which contains a phosphoglycerate kinase (PGK) promoter and terminator (Minet et al., 1992).

Additional COX2 mutations (WT, W56K, W56Q, V49Q, L51G, V49Q/L51G, L47Q/V49G, L47Q/V49G/W56R) were constructed using the pFL61 plasmid containing the COX2<sup>K65R</sup> insert via site-directed mutagenesis, according to the manufacturer's instructions (QuikChange II site-directed mutagenesis kit, Agilent Technologies). The presence of the site-directed mutations was confirmed by DNA sequencing. For the expression of the constructs from centromeric plasmids, the fragments containing the COX2 constructs flanked by the PGK promoter and terminator were cloned from pFL61 into pRS306H upon digestion with HindIII and Smal restriction enzymes. Nuclear localization was achieved by integration transformation at the URA3 nuclear locus using the pRS306H plasmid and hygromycin B resistance as a selection (Taxis and Knop, 2006).

Yeast cells were transformed in the presence of lithium acetate and salmon sperm DNA as described (Gietz and Schiestl, 2007). At least 12 transformed individual colonies were grown in Petri dishes containing fresh SD medium and then were replica-plated into YEPG to ensure that the selected colonies had respiratory growth similar to the rest of the transformants.
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 harvested at logarithmic growth phase (O.D. 0.6–1.0), washed with H2O at 4500 rpm in a Beckman Coulter JA-25.5 rotor (2500 × g) for 5 min, resuspended in TD buffer (100 mM Tris, 10 mM dithiothreitol), 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 KH2PO4 [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 mM sorbitol, 10 mM Tris [pH 7.4], 1 mM EDTA, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride [PMSF]) using a homogenizer and pestle (30 strokes). Samples were transferred to 1.5-ml tubes and centrifuged at 4500 rpm in a microcentrifuge (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 frozen and stored at −70°C until use.

Proteinase K protection assays
For protease treatment of mitochondria, 50 µg of mitochondria was gently resuspended in 100 µl of SH buffer and incubated at 20°C for 30 min in the presence of 100 µg/ml Proteinase K; parallel reactions were made in the presence of 1% Triton X-100. PMSF was added to all suspensions (at 4 mM final concentration), which were then incubated on ice for 10 min and centrifuged at 14,000 rpm (18,400 × g) for 10 min. Most of the supernatant in each tube was discarded; the pellet was resuspended with 4 µl of 6X loading buffer (375 mM Tris-HCl [pH 6.8], 6% SDS, 48% glycerol, 9% β-mercaptoethanol) + 4 mM PMSF and incubated for 1 min in boiling water before being loaded onto 12% SDS-tricine-PAGE gels.

Sodium carbonate and Triton X-100 extractions
Integral membrane proteins were separated from peripheral or soluble proteins by carbonate extraction. Aggregated proteins were separated from membrane proteins by Triton X-100 extraction. Briefly, 50 µg of mitochondria was incubated with 100 µl of cold 0.1 M Na2CO3 (pH 11.0) or with 9.4 µl of 1% Triton X-100 (2 mg Triton X-100/mg protein) for 30 min on ice. The samples were centrifuged at 90,000 × g in a TLA 55 rotor for 30 min at 4°C. Supernatants and pellets were incubated with 500 µl of 12.5% TCA for 15 min on ice, followed by centrifugation for 15 min at 14,000 rpm (18,400 × g) at 4°C. Samples were washed by adding 500 µl of cold 100% acetone, centrifuged for 10 min at 14,000 rpm (18,400 × g) at 4°C, and dried for ∼10 min at room temperature. The dried pellets were resuspended in 15 µl of 1X loading buffer and incubated for 1 min in boiling water before loading onto 12% SDS-tricine-PAGE gels.

Gel electrophoresis and in-gel enzymatic activities
Denaturing gel electrophoresis was carried out via SDS-tricine-polyacrylamide gels with 12% acrylamide (Schagger, 1994a). Sample preparation and blue native polyacrylamide gel electrophoresis (BN-PAGE) were carried out as described (Schagger, 1994b). Briefly, mitochondria were washed twice in 250 mM sorbitol, 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 amino-caproic acid, 50 mM Bis-Tris, pH 7.0) and 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 forCcO activity (Wittig et al., 2007; Wittig and Schägger, 2007) and ATPase activity (Zerbetto et al., 1997; Wittig and Schägger, 2005). Gels were stained with Coomassie solution (50% ethanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue G) or otherwise transferred and then subjected to immunodetection.

Immunodetection
From SDS-tricine-PAGE, proteins were electrotransferred onto a nitrocellulose Trans-Blot membrane (Bio-Rad) for immunodetection. Membranes were washed, blocked, and independently incubated for 4 h with the following antibodies: anti-Cox2 antibody at a 1:9000 dilution (Invitrogen; Molecular Probes), anti-Oxa1 antibody at a 1:1000 dilution, anti-Hog1 antibody at a 1:2000 dilution (Santa Cruz Biotechnology), and anti-Atp2 antibody at a 1:50,000 dilution. Alkaline phosphatase-conjugated IgGs (1:15,000 for 2 h) were used as secondary antibodies. Insoluble black–purple precipitates were formed upon addition of nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3’-indolyl phosphate p-toluidine salt. Images of the immunodecorated polypeptide bands were captured in an HP Scanjet G4050. For immunodetection on previously probed membranes using 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.

In silico protein sequence analysis
A TMS1 of 24 residues was considered for the yeast Cox2. The hydrophobicity calculations were performed using the “biological” hydrophobicity scale (Calado-Botelho et al., 2011).

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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, Roessler 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.
Andersson H, von Heijne G (1994). Membrane protein topology: effects of delta mu H+ on the translocation of charged residues explain the "posi-
tive inside" rule. EMBO J 13, 2267–2272.
Björkholm P, Ernst AM, Hagström E, Andersson SGE (2017). Why mitochondrial need a genome revisited. FEBS Lett 591, 65–75.
Bokari-Brown M, Holt UJ (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.
Bonnet C, Augustin S, Ellouze S, Bénit P, Bouaita A, Rustin P, Sahel J-A, Corral-Debrinski M (2008). The optimized algic expression of ND1 or ND4 genes restores respiratory chain complex I activity in fibroblasts harboring mutations in these genes. Biochim Biophys Acta 1783, 1707–1717.
Bonamaison A, Vanhooser S, Basisty N, Powers K, Crampton AL, Wang X, Friedricks S, Schilling B, Brand MD, O’Connor MS (2016). Stable nuclear expression of ATP8 and ATP6 genes rescues a mDNA complex V null mutant. Nucleic Acids Res 44, 9342–9357.
Burger G, Gray MW, Lang BF (2003). Mitochondrial genomes: anything goes. Trends Genet 19, 709–716.
Camacho-Mateos S, Osterberg M, Reichert AS, Yamano K, Björkholm P, Endo T, von Heijne G, Kim H (2011). TIM23-mediated insertion of transmembrane helices into the mitochondrial inner membrane. EMBO J 30, 1003–1011.
Calvo SE, Mootha VK (2010). The mitochondrial proteome and human disease. Annu Rev Genomics Hum Genet 11, 25–44.
Chit F, Stefani M, Taddei N, Ramponi G, Dobson CM (2003). Rationalization of the effects of mutations on peptide and protein aggregation rates. Nature 424, 805–808.
Claro MG, Perea J, Shu Y, Samatey FA, Popot JL, Jacq C (1995). Limitations to in vivo import of hydrophilic proteins into yeast mitochondria. The case of a cytoplasmically synthesized apocytochrome b. Eur J Biochem 771, 762–771.
Cruz-Tones V, Vázquez-Acevedo M, García-Villegas R, Pérez-Martínez X, Mendoza-Hernández G, González-Halphen D (2012). The cytosol-synthesized subunit II (Cox2) precursor with the point mutation W56R is correctly processed in yeast mitochondria to rescue cytochrome oxidase. Biochim Biophys Acta 1818, 2128–2139.
Cwerman-Thibault H, Augustin S, Lechauve C, Ayache J, Ellouze S, Sahel J-A, Augustin S, Bonnet C, Bénit P, Bouaita A, Rustin P, Sahel J-A, van Heijne G (1994). Membrane protein topology: effects of mutations on peptide and protein aggregation rates. Nature 369, 1050–1053.
Daley DO, Adams KL, Clifton R, Qualmann S, Millar AH, Palmer JD, Pratje E, Cwerman-Thibault H, Augustin S, Lechauve C, Ayache J, Ellouze S, Sahel J-A, Augustin S, Bonnet C, Bénit P, Bouaita A, Rustin P, Sahel J-A, van Heijne G (1994). Membrane protein topology: effects of mutations on peptide and protein aggregation rates. Nature 369, 1050–1053.
Daley DO, Clifton R, Whelan J (2002b). Intracellular gene transfer: reduced efficiency and visual loss. Mol Ther—Methods Clin Dev 2, 15003.
Daley DO, 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 R, Whelan J (2002b). Intracellular gene transfer: reduced hydrophobility facilitates gene transfer for subunit 2 of cytochrome c oxidase. Trends Biotechnol 18, 394–399.
Dávalos V, Dabbeni-Sala F (1997). Quantification of muscle mitochondrial DNA. Biochim Biophys Acta 1307, 31–35.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.
Diego JJ, Davidson E, King MP, González-Halphen D (2011). What limits the possibility of transmembrane protein, to the nucleus. Nat Genet 30, 394–399.