Cbp3 and Cbp6 are dispensable for synthesis regulation of cytochrome *b* in yeast mitochondria

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Cytochrome *b* (Cyt*b*) is the only mitochondrial encoded subunit from the bc_1 complex. Cbp3 and Cbp6 are chaperones necessary for translation of the *COB* mRNA and Cyt*b* hemylation. Here we demonstrate that their role in translation is dispensable in some laboratory strains, whereas their role in Cyt*b* hemylation seems to be universally conserved. BY4742 yeast requires Cbp3 and Cbp6 for efficient *COB* mRNA translation, whereas the D273-10b strain synthesizes Cyt*b* at wildtype levels in the absence of Cbp3 and Cbp6. Steady-state levels of Cyt*b* are close to wildtype in mutant D273-10b cells, and Cyt*b* forms non-functional, supercomplex-like species with cytochrome *c* oxidase, in which at least core 1, cytochrome c_1, and Rieske iron–sulfur subunits are present. We demonstrated that Cbp3 interacts with the mitochondrial ribosome and with the *COB* mRNA in both BY4742 and D273-10b strains. The polymorphism(s) causing the differential function of Cbp3, Cbp6, and the assembly feedback regulation of Cyt*b* synthesis is of nuclear origin rather than mitochondrial, and Smt1, a *COB* mRNA-binding protein, does not seem to be involved in the observed differential phenotype. Our results indicate that the essential role of Cbp3 and Cbp6 is to assist Cyt*b* hemylation and demonstrate that in the absence of heme *b*, Cyt*b* can form non-functional supercomplexes with cytochrome *c* oxidase. Our observations support that an additional protein or proteins are involved in Cyt*b* synthesis in some yeast strains.

Mitochondrial respiratory complex bc_1 is a dimeric enzyme composed of 10 subunits. Cytochrome *b* (Cyt*b*)[4] is the only subunit encoded in the mitochondrial genome by the *COB* gene. Assembly of the bc_1 complex involves formation of different subcomplexes (1, 2): 1) early stage: subunits Cyt*b*, Qcr7, and Qcr8 interact, forming the first subcomplex (Cyt*b*–Qcr7–Qcr8); 2) intermediate stage: subunits Cyt*b*, Qcr7, Qcr8, Qcr6, cytochrome c_1 (Cytc_1), Cor1, and Cor2 form a 500-kDa subcomplex; dimerization and interaction with the cytochrome *c* oxidase (C*c*O) occurs at this stage (2); and 3) late stage: Rip1, Qcr9, and Qcr10 assemble to form the complete bc_1 complex. Although little is known about regulatory mechanisms in each step of the bc_1 complex assembly, some studies show two important points of regulation, the formation of the subcomplex Cyt*b*–Qcr7–Qcr8 and the assembly of the Rieske iron–sulfur protein Rip1 (3–5). Stability of each subunit in the subcomplex Cyt*b*–Qcr7–Qcr8 depends on the presence of the other two subunits (6), and recently it was demonstrated that synthesis of Cyt*b* is regulated at this early stage of assembly (7).

COB mRNA translation depends on a set of translational activators: Cbs1 and Cbs2, which act on the *COB* mRNA 5′-UTR (8, 9). Cbp1 is involved in maturation, stability (10), and translation of the *COB* mRNA by an unknown mechanism (11). These proteins interact with mitochondrial ribosomes (12, 13). Cbp3 and Cbp6 are chaperones of Cyt*b* that in addition activate *COB* mRNA translation in a cooperative form. These proteins also interact with the tunnel exit of the mitochondrial ribosome, specifically with the ribosomal subunit MrpL4 (14). Cbp3 and Cbp6 interact with newly synthetized Cyt*b*, probably immediately as the peptide exits the mitochondrial ribosome. This interaction is important for addition of heme b_L to Cyt*b*, and it is preserved until Qcr7, Qcr8, the chaperone Cbp4, and the heme b_H assemble with Cyt*b* (15). The model suggests that once Cbp3–Cbp6 dissociate from the newly synthesized Cyt*b*, these chaperones are able to activate additional rounds of *COB* mRNA translation. This idea is supported by the observation that Cyt*b* synthesis is reduced in mutants lacking some of the bc_1 complex subunits, like Qcr7 and Qcr8. In this case, Cbp3 and Cbp6 remain sequestered in a complex with Cyt*b* and are therefore proposed to be impaired for *COB* mRNA translational activation (7).

Feedback assembly regulation of mitochondrial mRNAs translation is a very well-known mechanism in mitochondria. In C*c*O, synthesis of the subunit Cox1 is highly repressed by assembly defects of the complex, and this process is mostly

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This article contains Figs. S1–S4.

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[4] The abbreviations used are: Cyt*b*, cytochrome *b*; C*c*O, cytochrome *c* oxidase; Cytc_1, cytochrome c_1; BN, blue native; HA, hemagglutinin; PVDF, polyvinylidene fluoride.

mediated by the Cox1 translational activator Mss51 (16–18). Synthesis of the mitochondrial-encoded ATP synthase subunits Atp6 and Atp8 decrease by assembly defects on the F1 portion (19). The negative effect on Atp6 and Atp8 synthesis is mediated by Smt1, an integral protein localized in the mitochondrial inner membrane facing the matrix. Smt1 physically interacts with the *ATP8–ATP6* mRNA acting as a translational repressor. Surprisingly, it was shown that Smt1 also interacts with the *COB* mRNA. However, no effect on Cyt*b* synthesis was observed in a Δ*smt1*-null mutant (20).

In this study, we report that Cbp3 and Cbp6 are dispensable for translational activation of Cyt*b* synthesis in the D273-10b yeast strain, whereas in BY4742 cells these chaperones are necessary for Cyt*b* synthesis as previously reported for W303 strain (7, 14). We characterized the role of Cbp3 and Cbp6 in Cyt*b* biogenesis in both strains and studied whether Smt1 was involved in the differential regulation of Cyt*b* synthesis by Cbp3/Cbp6 in the D273-10b and BY4742 strains. Our study demonstrated that the essential role of Cbp3 and Cbp6 is to regulate Cyt*b* hemylation and that a regulatory function in *COB* mRNA translation prevails in some yeast strains. Moreover, non-hemylated Cyt*b* can form non-functional supercomplexes with cytochrome *c* oxidase. Our results provide relevant information about the process of Cyt*b* biogenesis and translational control of the *COB* mRNA.

Results

Cbp3 and Cbp6 are dispensable for Cytb synthesis, but not for bc_1 complex activity in D273-10b lab strains

We were interested in the study of the mechanisms of Cyt*b* synthesis. To see whether assembly defects in bc_1 complex affect Cyt*b* synthesis, we created Δ*qcr7*, Δ*cbp4*, Δ*cbp3*, and Δ*cbp6* mutants in the D273-10b yeast strain. These proteins form the first intermediates at early stages of Cyt*b* assembly and maturation (7, 15). To follow mitochondrial translation, the cells were incubated with [^{35}S]methionine in the presence of cycloheximide, and mitochondrial products were analyzed by SDS-PAGE and autoradiography. Surprisingly, contrary to previous reports (7, 14), depletion of Cbp3 or Cbp6 did not negate Cyt*b* labeling (Fig. 1*A*). Deletion of *CBP4* also showed normal levels of Cyt*b* labeling in agreement with previous reports supporting that this chaperone is not necessary for efficient Cyt*b* synthesis (14). Likewise, the absence of subunit Qcr7 also permitted wildtype levels of Cyt*b* labeling. As expected, cells lacking Cbs1, one of the translational activators of the *COB* mRNA (8), were completely impaired in Cyt*b* synthesis. The majority of experiments regarding Cbp3 and Cbp6 function are based on the W303 strain, so we hypothesized that the difference in the observed phenotype could be due to the strain used. To test this, Cyt*b* synthesis of the same mutants was analyzed in a third yeast strain, BY4742. In these BY4742 mutants, Cyt*b* labeling dramatically decreased in the absence of Cbp3 and Cbp6, as previously reported for W303 nuclear background (7, 14, 15) (Fig. 1*B*). Interestingly, in BY4742 cells Δ*qcr7* and Δ*cbp4* mutations also decreased Cyt*b* labeling, whereas absence of Cbs1 abolished labeling. In the Δ*cbs1* mutant, Cox1 labeling also decreased dramatically, probably because of the presence of introns in the *COX1* gene and the dependence of a *COB* maturase for *COX1* mRNA maturation (21). Even though Cyt*b* [^{35}S]methionine labeling was normal in Δ*cbp3* and Δ*cbp6* mutants in the D273-10b strain, respiratory growth was still compromised (Fig. 1*C*), indicating that in D273-10b cells, Cbp3 and Cbp6 are still necessary for a post-translational step of complex III biogenesis.

Variations in the Cyt*b* [^{35}S]methionine labeling in D273-10b and BY4742 strains might be due to differences in the effect of Cbp3, Cbp6, Qcr7, and Cbp4 on *COB* mRNA translation. Alternatively, it could be due to differences in Cyt*b* instability in the absence of these proteins. To differentiate between these two possibilities, we used the translation reporter gene *ARG8^m*, which has been widely used to study mitochondrial translation. Arg8 is an acetylornithine aminotransferase involved in arginine biosynthesis. It is normally encoded by a nuclear gene and imported into mitochondria using a mitochondrial targeting signal. The mitochondrial encoded Arg8 protein activity is not linked to the respiratory chain function and therefore can be used to assess mitochondrial translation by monitoring growth on media lacking arginine (14, 16, 19, 22). In a strain with the endogenous *ARG8* deleted, we inserted *ARG8^m* in the *COB* locus, maintaining the *COB* untranslated regions and *COB* codons. The precursor *pARG8^m* reporter was fused in frame to the last codon of the *COB* gene to create the construct *COB(1–385)::pARG8^m* (Fig. 1*D*) and followed *ARG8^m* expression by monitoring growth in media lacking arginine. After Western blotting analysis with antibodies against Arg8, we detected the presence of a Cyt*b*-pArg8 fusion protein of the expected size, 90 kDa, as well as a faint band for processed Arg8 (Fig. 1*E*). Cyt*b* was also observed albeit at lower levels as compared with mitochondria carrying wildtype mtDNA in both BY4742 and D273-10b strains. Mitochondrial translation products analysis indicated that processed Cyt*b* is only observed after 40 min of [^{35}S]methionine pulse labeling (Fig. 1*F*). Both strains carrying the *COB(1–385)::pARG8^m* mitochondrial construct supported growth on media lacking arginine (Fig. 1*H* and Fig. S1*A*), as well as respiratory growth, albeit with less efficiency as compared with cells carrying wildtype mitochondrial DNA (Fig. 1*G*). We created Δ*cbp3*, Δ*cbp6*, Δ*qcr7*, Δ*cbp4*, and Δ*cbs1* mutants carrying the *COB(1–385)::pARG8^m* construct in D273-10b and BY4742 strains. With the exception of Δ*cbs1* mutation, the rest of D273-10b mutants showed a robust growth on media lacking arginine (Fig. 1*H*). In contrast, Δ*cbp3* and Δ*cbp6* mutants completely lacked growth capacity in −Arg media in BY4742 cells. This is consistent with previous observations where absence of Cbp3 and Cbp6 reduced −Arg growth of cells carrying a complete *COB* codon replacement with the *ARG8^m* reporter, *cob*Δ*::pARG8^m* (14) (Fig. S1*C*).

Δ*qcr7* BY4742 mutants showed a very weak growth on media lacking arginine, whereas Δ*cbp4* showed a more robust growth on −Arg media (Fig. 1*G*). Growth of the Δ*qcr7* mutant in the absence of supplemental arginine was particularly impaired as compared with Δ*cbp4* and Δ*qcr8* mutants (Fig. S1*B*). However, growth in media lacking arginine was recovered after complete replacement of *COB* codons by *ARG8^m* (Fig. S1*C*), indicating that translation of *COB* mRNA is regulated by the presence of Cyt*b*. These phenotypes are consistent with previous observa-

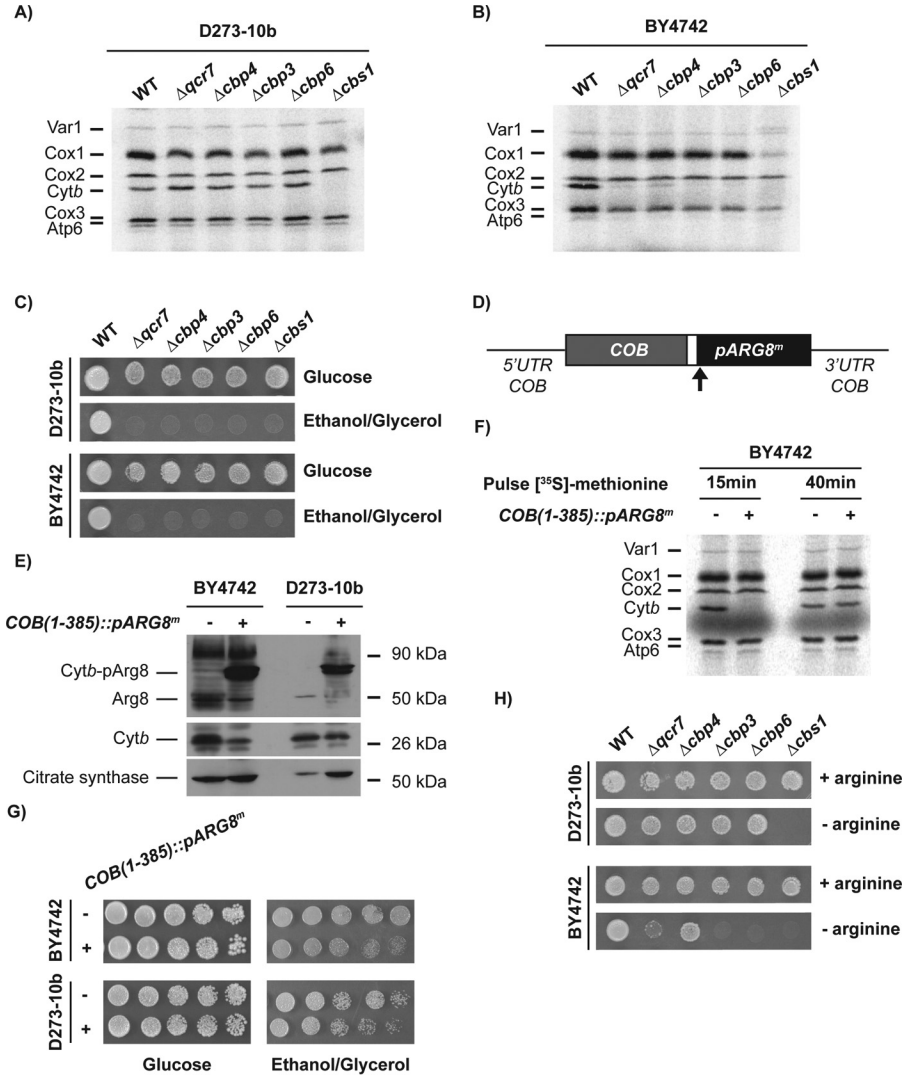

Figure 1. Cbp3 and Cbp6 are dispensable for Cyt*b* synthesis in D273-10b lab strains. *A* and *B*, whole-cell mitochondrial translation products from WT and the indicated mutant strains in the D273-10b (*A*) and BY4742 (*B*) strains were labeled with [^{35}S]methionine in the presence of cycloheximide. The proteins were analyzed by SDS-PAGE and autoradiography. *Cox1*, cytochrome *c* oxidase subunit 1; *Cox2*, cytochrome *c* oxidase subunit 2; *Cox3*, cytochrome *c* oxidase subunit 3; *Atp6*, ATP synthase subunit 6; *Atp8*, subunit 8; *Atp9*, subunit 9; *Var1*, ribosomal subunit Var1. *C*, WT and the indicated mutants from the BY4742 and D273-10b strains were spotted as serial dilutions in rich fermentative (glucose) and respiratory (ethanol/glycerol) media for 3 days at 30 °C. *D*, the *pARG8^m* gene was fused in frame with the complete *COB* codons. The reporter gene contains the Arg8 mitochondrial targeting signal, and the processing site is indicated by an *arrow*. *E*, mitochondria from D273-10b and BY4742 lab strains carrying the *COB(1–352)::pARG8^m*) construct were analyzed by SDS-PAGE and Western blotting using antibodies against Arg8, Cyt*b*, and citrate synthase (as loading control). *F*, cells from the BY4742 lab strain carrying either the WT mitochondrial genome or the *COB(1–352)::pARG8^m* construct were pulse-labeled with [^{35}S]methionine for 15 min or 40 min as in *A*. *G*, respiratory growth of cells carrying the *COB(1–352)::pARG8^m* construct were assessed by 10-fold serial dilutions spotted on YPD or YPEG media and were grown for 3 days at 30 °C. *H*, the indicated mutants from D273-10b and BY4742 lab strains bearing the *COB(1–352)::pARG8^m* construct were spotted on synthetic media in the presence or absence of arginine. 10-fold serial dilutions were grown for 3 days at 30 °C.

tions where different feedback regulation levels of Cyt*b* synthesis occurs in the absence of bc_1 complex subunits and chaperones (7). These results strongly suggest that Cbp3 and Cbp6 are necessary for Cyt*b* synthesis in BY4742 but not in D273-10b. In addition, the assembly feedback regulation of Cyt*b* synthesis by complex III subunits is preserved in BY4742 cells but not in D273-10b cells.

This comparative analysis showed that Cbp3 and Cbp6 are dispensable as translational activators in the D273-10b strain but are still necessary for respiration, probably as chaperones, whereas in the BY4742 lab strain both proteins are necessary to achieve efficient Cyt*b* synthesis. Moreover, our results indicate that assembly feedback regulation of *COB* mRNA translation is present in BY4742 strains, where some subunits and chaperones like Qcr7, Qcr8, and Cbp4 have an important participation in this process.

In D273-10b strain Cytb and Rip1 are stable in the absence of Cbp3 and Cbp6

Previous reports indicate that cells lacking Cbp3, Cbp6, and Qcr7 exhibit high Cyt*b* degradation (6, 23, 24). Because Cbp3, Cbp6, and Qcr7 were dispensable for *COB* mRNA translation in D273-10b strains, we analyzed steady-state levels of Cyt*b* by Western blotting in mutants lacking these proteins. Cyt*b* levels were slightly reduced in Δ*cbp3* and Δ*cbp6* as compared with wildtype in D273-10b cells; however, cells lacking Qcr7 showed

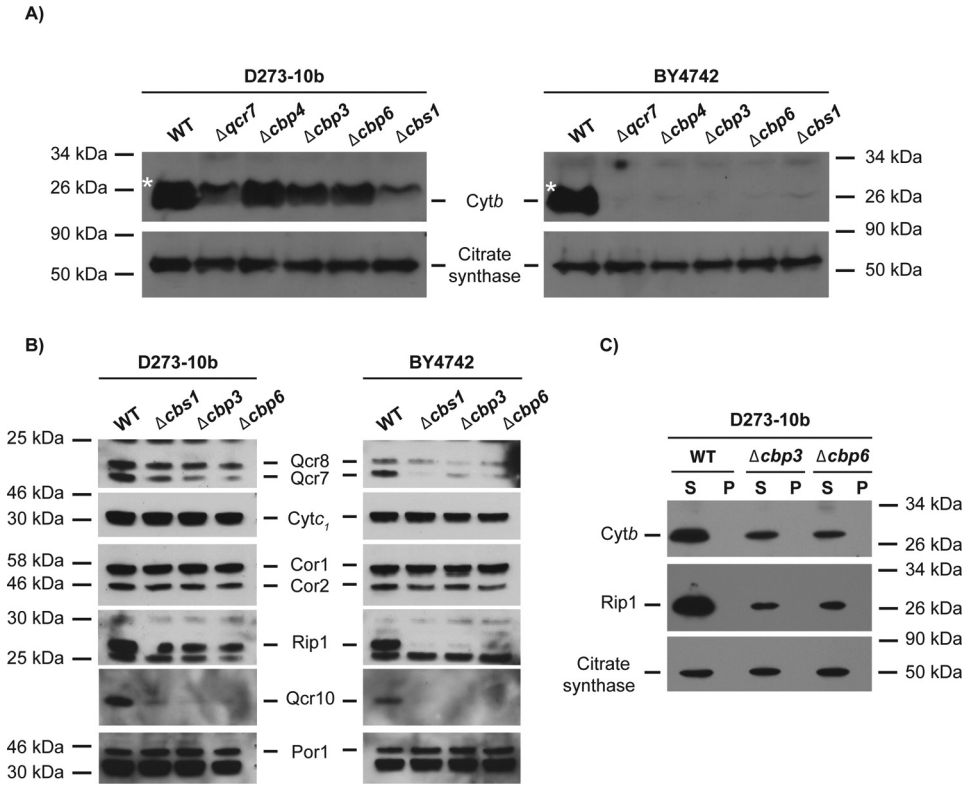

Figure 2. Cyt*b* and Rip1 accumulate in Δ*cbp3* and Δ*cbp6*-null mutants from the D273-10b lab strain. *A*, mitochondrial proteins (20 μg) of the indicated mutants were resolved on a 16% SDS-PAGE in the presence of urea 6 M and transferred to PVDF membrane. Western blotting was carried out with anti-Cyt*b* or anti-citrate synthase (as loading control). The *asterisk* shows an unspecific band. *B*, mitochondrial proteins (20 μg) of D273-10b and BY4742 mutants were analyzed by SDS-PAGE 12% and Western blotting with the indicated antibodies. *C*, isolated mitochondria from WT, Δ*cbp3*, and Δ*cbp6* mutants were first treated with digitonin and sequentially with Triton X-100. After ultracentrifugation, supernatants from both solubilizations were combined. Supernatants (*S*) and pellet (*P*) were loaded on a 12% SDS-PAGE and analyzed by Western blotting using antibodies against Cyt*b*, Rip1, and citrate synthase.

strongly decreased Cyt*b* levels. In contrast, in BY4742 cells, none of the mutants analyzed showed detectable levels of Cyt*b* (Fig. 2*A*).

Because Cyt*b* was stable in mutants lacking Cbp3 or Cbp6 in D273-10b cells, we next evaluated the steady-state levels of other bc_1 complex subunits in these mutants and compared them to protein levels in the BY4742 strain. Subunits like Cytc_1, Cor1, and Cor2 were stable in all mutants from both strains, probably because these three subunits form a stable subcomplex (25) (Fig. 2*B*). Whereas Qcr7, Qcr8, and Rip1 were practically undetectable in BY4742 mutants, these subunits were detected in D273-10b cells lacking Cbp3, Cbp6, and even Cbs1. Interestingly, the Qcr10 subunit was almost absent in all the mutants analyzed in both strains (Fig. 2*B*). This observation suggests that Rip1 is not correctly assembled into the bc_1 complex in the mutants, and therefore Qcr10 stability is compromised, as previously reported (26).

The observed stability of Cyt*b*, which is an early assembly subunit, and of Rip1, a late assembly subunit (25) in the absence of Cbp3 and Cbp6 in the D273-10b strain surprised us, because both mutants are non-respiratory. We asked whether Cyt*b* and Rip1 aberrantly aggregated and became insensitive to mitochondrial protease activity. To test aggregation, we first solubilized mitochondria from Δ*cbp3* and Δ*cbp6* mutants in the D273-10b strain with digitonin and separated pellet from supernatant by ultracentrifugation. The pellet was next solubilized with Triton X-100, and pellet and supernatant fractions were again separated by ultracentrifugation. Aggregates are expected to migrate in the pellet portion of the digitonin–Triton X-100 sequential treatment (5). Western blotting analysis of both fractions showed that Cyt*b* and Rip1 were present in the supernatant fractions, regardless of the presence of Cbp3 and Cbp6 (Fig. 2*C*). This result indicates that at least Cyt*b* and Rip1 subunits are not forming protease-resistant aggregates in the absence of Cbp3 and Cbp6.

Cytb, Rip1, and Cor1 associate into supercomplexes in the absence of Cbp3 and Cbp6 in the D273-10b strain

Because Cyt*b* and Rip1 are stable in D273-10b strains in the absence of Cbp3 and Cbp6, we asked whether these subunits could form high molecular weight complexes related to supercomplexes III_2/IV_2 and III_2/IV. Digitonin-solubilized mitochondria from D273-10b containing the mutations Δ*cbp3*, Δ*cbp6*, Δ*cbp3*/Δ*cbp6*, and Δ*cbs1* were separated by blue native (BN)-PAGE. Western blotting analysis indicated that Cyt*b*, Cor1, and Rip1 co-migrated into III_2/IV_2 and III_2/IV supercomplexes in the wildtype strain (Fig. 3*A*). The Cyt*b*, Cor1, and Rip1 high molecular weight bands were not present after deletion of the cytochrome *c* oxidase subunit *COX1*, where the main species was the dimeric III_2 complex. Cor1 and Rip1 were unable to form the high molecular weight complex after deletion of Cbs1, where Cyt*b* synthesis was abolished. Surprisingly, in the Δ*cbp3* and Δ*cbp6* mutants, and even in the Δ*cbp3*/Δ*cbp6* double mutant, a fraction of Cyt*b*, Cor1, and Rip1 co-migrated in high

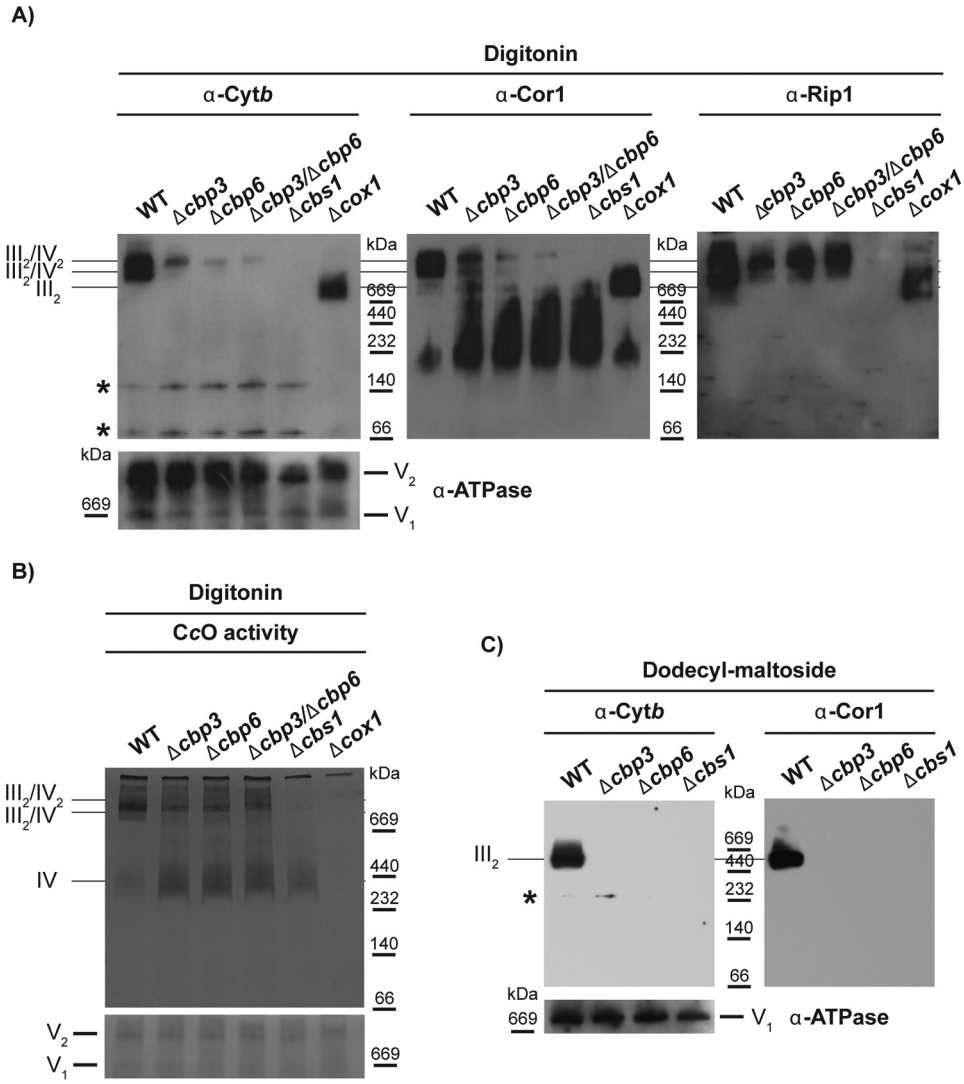

Figure 3. Cyt*b* forms supercomplex-like species in the absence of Cbp3 and Cbp6 in D273-10b lab strains. 200 μg of mitochondrial protein from the indicated mutants were solubilized with digitonin, divided, and then loaded in two different BN-PAGE systems. *A*, one BN-PAGE was analyzed by Western blotting using antibodies against Cyt*b*, Cor1, Rip1, and ATP synthase (as loading control). *Asterisks* indicate unspecific bands not related to Cyt*b*. *B*, the second BN-PAGE was treated with 3,3′-diaminobenzidine and horse Cyt*c* to observe the C*c*O in-gel activity. Coomassie stain is shown as a loading control. *C*, 100 μg of mitochondrial protein were solubilized with dodecyl-maltoside and analyzed by BN-PAGE and Western blotting using antibodies against Cyt*b*, Cor1, and ATP synthase (the monomeric complex, V_1, was used as loading control).

molecular weight complexes with sizes corresponding to supercomplexes III_2/IV_2 and III_2/IV and enriched in III_2/IV_2 complex-like size. In the Δ*cbp3*, Δ*cbp6*, Δ*cbp3*/Δ*cbp6*, and Δ*cbs1*-null mutants, Cor1 migrates in complexes of 232–669 kDa that are absent in the wildtype strain and in the Δ*cox1* strain. These bands probably correspond to the assembly intermediate subcomplex Cor1–Cor2–Cytc_1 (25) (Fig. 3*A*). It calls our attention that most of Rip1 is observed in high mass complexes, suggesting association with supercomplexes, but very little Cor1 fractionates in these high mass bands. To explore the possibility that Rip1 might form another high mass complex perhaps associated with the Bcs1 hexamer (27), we combined a Δ*cpb6* mutation with a deletion of Cox1 (by elimination of Pet309, translational activator of the *COX1* mRNA (28)). In the double mutant, association of Rieske protein, Cor1, and also Cyt*b* with supercomplex-like bands was lost, and only co-migration with a III_2 dimer-like size band was detected (Fig. S2*A*). These observations support that the high-molecular-weight complexes observed in the absence of Cbp3/Cbp6 are related to complex IV and to III/IV supercomplexes. The supercomplex-like band contained active cytochrome *c* oxidase, as revealed by an in-gel activity assay (Fig. 3*B* and Fig. S2*B*). Mitochondria from the Δ*cbs1* mutant presented a ~450-kDa band corresponding to the monomeric C*c*O enzyme, not present in a Δ*cox1*-null mutant. Taken together, these data support the idea that the observed complex in Δ*cbp3*, Δ*cbp6*, and Δ*cbp3*/Δ*cbp6* mutants is related to respiratory supercomplexes, even if the bc_1 complex lacks activity.

Solubilization of mitochondria with dodecyl-maltoside usually separates the III–IV associations and maintains individual IV and III_2 complexes (29). Thus, mitochondria from D273-10b strains carrying the Δ*cbp3*, Δ*cbp6*, Δ*cbp3*/Δ*cbp6*, or Δ*cbs1* mutations were solubilized with dodecyl-maltoside and analyzed by BN-PAGE. Only wildtype mitochondria maintained Cyt*b* and Cor1 in the ~670-kDa band corresponding to dimeric complex III (Fig. 3*C*), whereas Δ*cbp3*, Δ*cbp6*, Δ*cbp3*/Δ*cbp6*, or

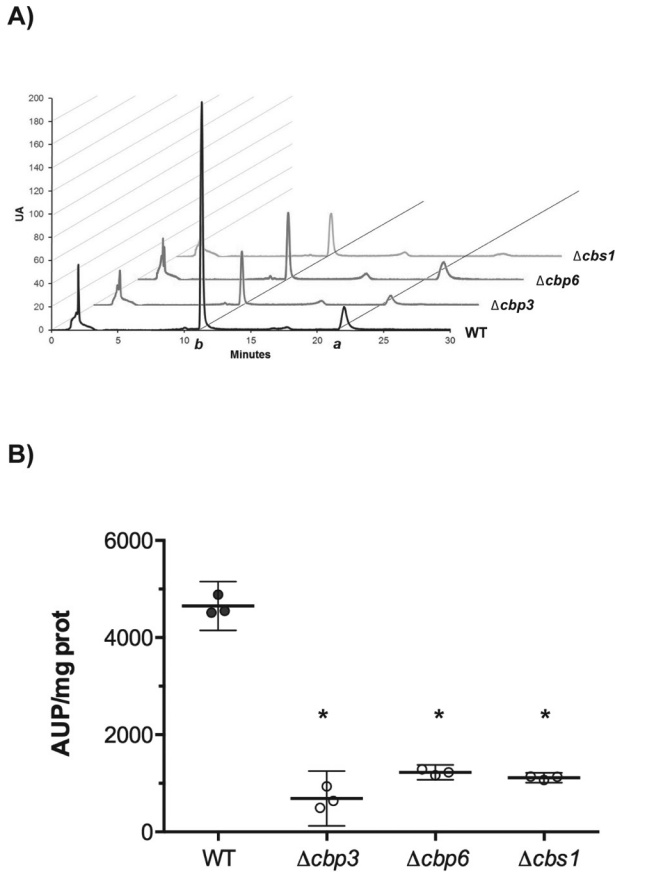

Figure 4. Cbp3 and Cbp6 are still necessary for Cyt*b* hemylation in the D273-10b lab strain. Mitochondrial proteins (700 μg) from the indicated mutants were treated with acidic acetone (1% HCl), clarified, and then separated by reverse-phase HPLC. *A*, retention time peaks of heme *b* (*peak b*) and heme *a* (*peak a*) are represented by arbitrary units (*UA*). *B*, the area under the peak (*AUP*)/mg protein was calculated by the mean of two biological and one technical replicate ($n = 3$). Confidence interval (95%) is represented by *error bars*. *, $p < 0.0001$ *versus* WT.

Δ*cbs1* mitochondria showed undetectable levels of this band. These data indicate that in the absence of Cbp3 and Cbp6, the bc_1 complex subunits Cyt*b* and Cor1 are not forming a stable complex III. Moreover, these data suggest that association with the C*c*O is necessary to maintain the interaction of bc_1 complex subunits.

Cbp3 and Cbp6 are still necessary for Cytb hemylation in the D273-10b strain

Even though Cyt*b* synthesis proceeds and aberrant supercomplexes form in the absence of Cbp3 and Cbp6, these chaperones are still necessary for respiration. In W303 lab strain, it was observed that Cbp3 and Cbp6 are necessary for heme *b* assembly into the Cyt*b* subunit (15). We asked whether Cbp3 and Cbp6 were still necessary for Cyt*b* hemylation in the D273-10b lab strain. Hemes were extracted from purified mitochondria by incubation with acidic acetone (1% HCl), and the extract was analyzed by reverse phase HPLC. We used hemin as a control to identify heme *b*, with a retention time of 12 min (data not shown). HPLC analysis showed a dramatic decrease of the total heme *b* present in Δ*cbp3* and Δ*cbp6* mutants (Fig. 4). This level was similar to the one observed in the absence of Cbs1, used as negative control because Cyt*b* is completely absent in this strain (8). Our results indicate that even when Cbp3 and Cbp6 are not necessary for *COB* mRNA translation in D273-10b strains, they are still necessary for Cyt*b* hemylation.

Cbp3 interacts with the COB mRNA and with the mitochondrial ribosome in both D273-10b and BY4742 strains

Cbp3 and Cbp6 interact with the mitochondrial ribosome tunnel exit in W303 lab strains (14). Thus, we investigated whether Cbp3 associates with the mitoribosome in BY4742 strains, in which this protein is necessary for Cyt*b* synthesis, and in D273-10b strains, in which Cbp3 is dispensable for Cyt*b* synthesis. To detect Cbp3, we fused a hemagglutinin (HA) epitope to the C-terminal end of Cbp3. The construction expressing this protein was expressed from a centromeric plasmid and transformed into Δ*cbp3* cells from either BY4742 or D273-10b strains. The presence of the HA epitope on Cbp3 did not disrupt respiratory growth of otherwise wildtype cells (Fig. S3). Mitochondria was digitonin-solubilized in the presence of 10 mM Mg^+, a condition in which both ribosomal subunits are associated. The extracts were loaded on a discontinuous sucrose gradient (20–40%) and ultracentrifuged. Six fractions were collected and analyzed by SDS-PAGE and Western blotting. In both strains, BY4742 and D273-10b, Cbp3–HA co-migrated with Mrp20 (protein from the large ribosomal subunit) and with Mrp51 (protein from the small ribosomal subunit) (Fig. 5, *A* and *B*), indicating that in both strains Cbp3–HA interacts with the mitoribosome.

Considering that Cbp3 is necessary for efficient Cyt*b* synthesis in W303 (14) and BY4742 lab strains (this study), we asked whether this protein physically interacts with the *COB* mRNA. Mitochondria from BY4742 and D273-10b strains expressing the *CBP3-HA* or *CBP3* gene were solubilized with dodecylmaltoside. The mitochondrial extract was immunoprecipitated using antibodies against the HA epitope, and RNA was isolated and analyzed by reverse transcription-PCR (30). For cDNA synthesis and PCR amplification, we used primers specific for *COB* and also for *VAR1* (used as a negative control because translation of this mRNA is independent of Cbp3). After HA immunoprecipitation, Western blotting analysis showed that Cbp3 was efficiently immunoprecipitated in both BY4742 and D273-10b mitochondria (Fig. 5*C*). First strand cDNA and PCR amplification indicated that in both strains Cbp3 physically interacts with the *COB* mRNA (Fig. 5*D*).

The polymorphism(s) between D273-10b and BY4742 producing differential function of Cbp3, Cbp6, and Qcr7 in Cytb synthesis has a nuclear origin and is not present on Smt1

The observed difference on the role of Cbp3, Cbp6, Qcr7, and Cbp4 in Cyt*b* synthesis regulation between the two lab strains suggests the existence of polymorphism(s) producing these distinct phenotypes. This polymorphism(s) could locate either on nuclear or in mitochondrial genes. To investigate this, we exchanged the mitochondrial genomes between the two lab strains, creating two different cybrid strains: D273-10b with the BY4742 mtDNA and BY4742 with the D273-10b mtDNA. Next, by whole-cell [^{35}S]methionine labeling in the presence of cycloheximide, we analyzed the effect of Δ*qcr7*, Δ*cbp3*, Δ*cbp6*, and Δ*cbp4* mutations on Cyt*b* synthesis. We included a Δ*qcr10*

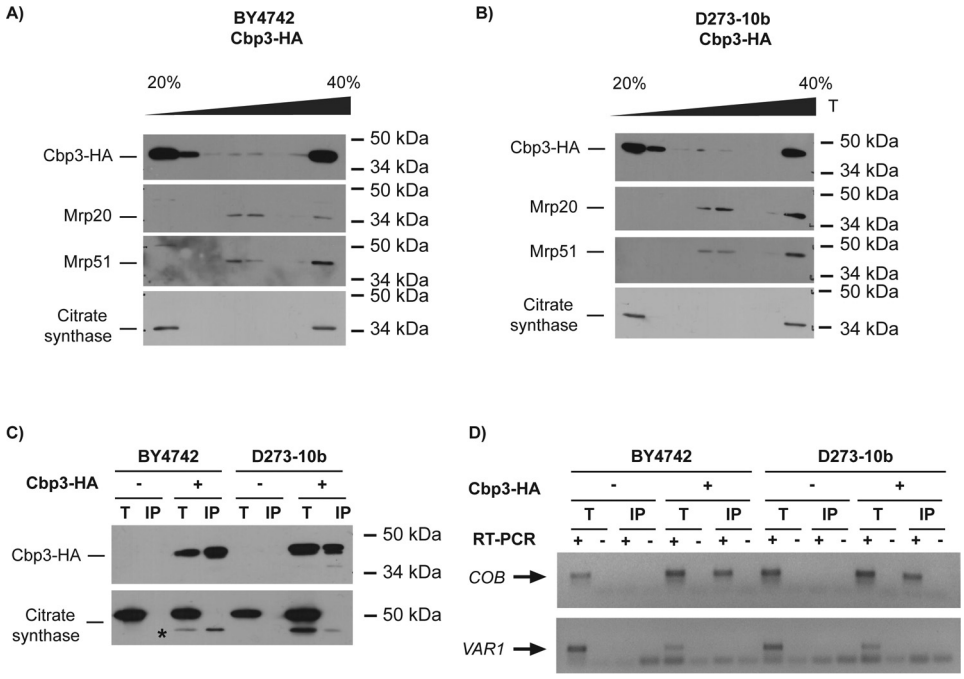

Figure 5. Cbp3 and Cbp6 interact with the mitochondrial ribosome and with the *COB* mRNA in both D273-10b and BY4742 lab strains. *A* and *B*, 500 μg of isolated mitochondria from BY4742 (*A*) and D273-10b (*B*) cells expressing *CBP3-HA* from a low-copy plasmid were solubilized with digitonin, clarified, and loaded into a discontinuous sucrose gradient (20–40%) for ultracentrifugation. 1/10 of the clarified portion was taken as a total control (*T*). Proteins from seven fractions were TCA-precipitated and analyzed by SDS-PAGE (12%) and Western blotting. Cbp3–HA was detected with an antibody against the HA epitope. Antibodies against Mrp20 and Mrp51 were used as markers for large and small ribosomal subunits, respectively. Citrate synthase was used as a control for a non-ribosomal mitochondrial protein. *C*, 500 μg from isolated mitochondria were solubilized with dodecyl maltoside, and Cbp3–HA or untagged Cbp3 were subjected to immunoprecipitation with an antibody against HA. Immunoprecipitate (*IP*, one-third) and total (*T*, 5% of the mitochondrial extract used for immunoprecipitation) fractions were separated by SDS-PAGE and analyzed by Western blotting with antibodies against HA and citrate synthase (as a negative control for interaction). The *asterisk* corresponds to the previous HA immunoblot analysis of Cbp3–HA. *D*, RNA was extracted from the total (*T*) and immunoprecipitate (*IP*) fractions. Each fraction was divided in two, and cDNA was prepared in the presence (+) or absence (−) of reverse transcriptase (*RT*) using primers for the *COB* and *VAR1* 5′-UTRs. The (−) *RT lanes* represent a negative control for DNA contamination. The PCR products were run on agarose gel, and the gel pictures were color-inverted.

mutant as control of a subunit whose absence has no effect on Cyt*b* synthesis (7, 31). D273-10b mutants with BY4742 mtDNA showed Cyt*b* labeling pattern as a D273-10b strain, where Δ*qcr7*, Δ*cbp3*, and Δ*cbp6* mutants exhibited wildtype Cyt*b* labeling (Fig. 6*A*). Δ*cbp4* and Δ*qcr10* cells also labeled Cyt*b* as wildtype. In concordance, steady-state levels of Cyt*b* in the mutants are close to wildtype levels (Fig. 6*B*). BY4742 mutants with D273-10b mtDNA behaved as BY4742 cells: the absence of Cbp3, Cbp6, and Qcr7 dramatically reduced Cyt*b* labeling, whereas the absence of Cbp4 reduced only mildly Cyt*b* labeling (Fig. 6*C*). In agreement, steady-state levels of Cyt*b* in these mutants were also reduced (Fig. 6*D*). These results suggest that the polymorphism(s) between D273-10b and BY4742 is located in a nuclear gene or genes.

Unfortunately, many attempts to identify the polymorphism(s) associated with the differential phenotype have failed. One candidate was the nuclear gene *SMT1*. This gene has three polymorphisms: A → G, T → C, and A → G at positions 400, 644, and 668 of the *orf*, respectively. These polymorphisms change three amino acids in D273-10b with respect to BY4742 and W303: methionine 134 to valine, phenylalanine 206 to serine, and histidine 223 to arginine (data obtained from the *Saccharomyces* genome database). Smt1 was found to interact with the *COB* mRNA and to act as a translational repressor of the *ATP8–ATP6* mRNA (20). To study whether Smt1 was involved in the differential regulation of Cyt*b* synthesis by Cbp3 and Cbp6, we first tested whether in the BY4742 strain Smt1 was acting as a translational repressor of the *COB* mRNA as was demonstrated for the *ATP8–ATP6* mRNA (20). In this case, we expected that double mutants Δ*cbp3* or Δ*cbp6* combined with Δ*smt1* resulted in wildtype levels of Cyt*b* synthesis. Whole cell [^{35}S]methionine labeling in the presence of cycloheximide showed that Cyt*b* synthesis had normal levels in the Δ*smt1* mutant. However, in the Δ*cbp6*/Δ*smt1* double mutant, Cyt*b* synthesis was not recovered in comparison to labeling of a Δ*cbp6* mutant (Fig. 7*A*). These data were confirmed by analyzing growth on media lacking arginine of the same strains in Fig. 7*A* but carrying the *COB(1–385)::ARG8^{m}* construct in the mitochondrial genome. Neither the single mutant Δ*cbp6* nor the double mutant Δ*cbp6*/Δ*smt1* was able to grow on media lacking arginine (Fig. 7*B*). Because these experiments tested the effect of the absence of Smt1, we next asked whether the presence of Smt1 expressing the D273-10b gene would compensate Cyt*b* synthesis in the BY4742 Δ*cbp6* mutant. Thus, we cloned *SMT1* from D273-10b lab strain in low-copy and high-copy yeast expression plasmids and transformed them into BY4742 cells carrying the Δ*cbp6* mutation. *In vivo* translation assays indicated that Cyt*b* synthesis was not recovered after overexpression of *SMT1* carrying the D273-10b lab strain polymorphisms (Fig. 7*C*). Together, these results suggest that Smt1 is not related to the role of Cbp3 and Cbp6 in Cyt*b* synthesis. Because Smt1 physically interacts, directly or indirectly, with

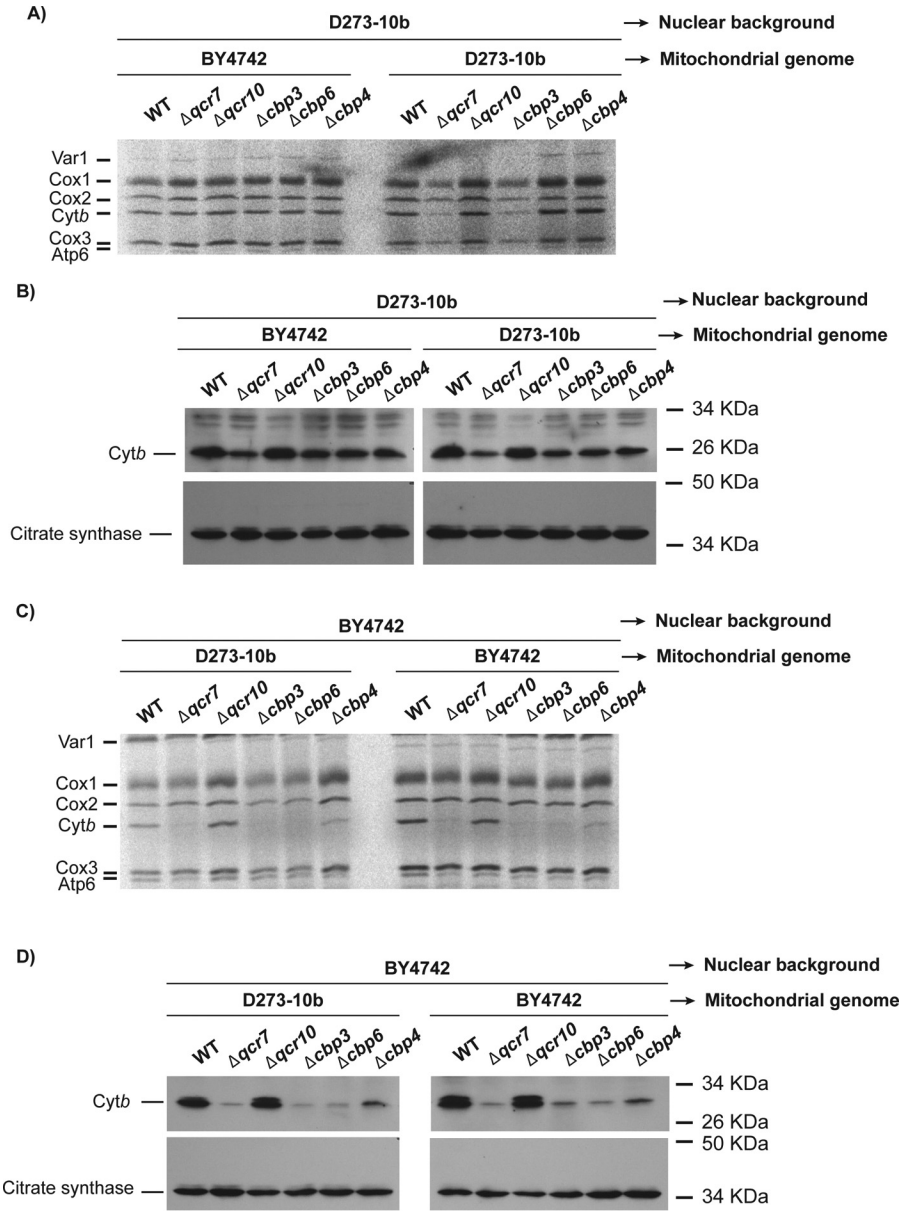

Figure 6. The polymorphism(s) associated with the differential phenotype of Δ*cbp3* and Δ*cbp6* mutants between D273-10b and BY4742 lab strains is located in the nuclear genome. *A*, mitochondrial DNA from BY4742 was introduced in rho0, D273-10b cells by cytoduction. These cells were labeled with [^{35}S]methionine in the presence of cycloheximide, and the proteins were analyzed by SDS-PAGE and autoradiography. *B*, mitochondria (20 μg) from the same strains as in *A* were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. *C*, mitochondrial DNA from D273-10b was introduced in rho0, BY4742 cells by cytoduction. The cells were [^{35}S]methionine-labeled in the presence of cycloheximide, and proteins were analyzed as in *A*. *D*, mitochondria (20 μg) from the same strains in *C* were analyzed by SDS-PAGE and Western blotting with antibodies against Cyt*b* and citrate synthase.

the *COB* mRNA, it must have a different role in Cyt*b* biogenesis.

Discussion

An essential step on bc_1 complex biogenesis is assembly of the only mitochondrial encoded subunit, Cyt*b*, where chaperones and translational activators of the *COB* mRNA coordinate to form the first Cyt*b* assembly intermediaries (7). Cbp3 and Cbp6 were described as specific translational activators of the *COB* mRNA (14). Moreover, Cbp3 and Cbp6 act as chaperones necessary for respiratory growth (14, 23, 32), and it is proposed that they sense the assembly state of Cyt*b* on early stages and coordinate synthesis and assembly (7, 14). In the course of our studies to understand the function of Cbp3 and Cbp6, we observed that these proteins have a different role in Cyt*b* biogenesis depending on the yeast lab strain we used. Cbp3 and Cbp6 are necessary for Cyt*b* synthesis in the BY4742 strain, whereas in D273-10b lab strain, these proteins are dispensable for translational regulation. However, in both strains Cbp3 and Cbp6 are necessary for respiratory growth and Cyt*b* hemylation.

The dual function of Cbp3 and Cbp6 in Cyt*b* synthesis/assembly was first observed in W303 yeast lab strains (14). It was not surprising that BY4742 lab strains displayed a similar phenotype as W303 cells, because both strains share a more recent common ancestor than with the D273-10b strain (33, 34),

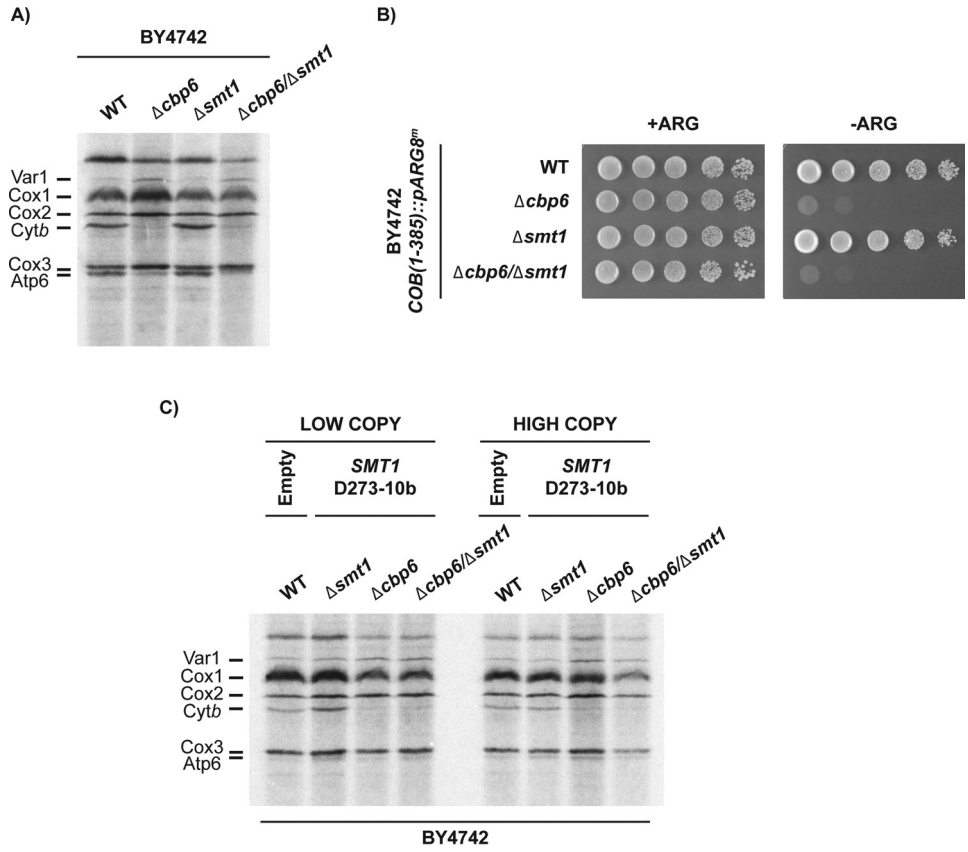

Figure 7. SMT1 is not involved in the differential regulation of Cyt*b* synthesis between BY4742 and D273-10b lab strains. *A*, mitochondrial translation products from the WT, Δ*cbp6*, Δ*smt1*, and Δ*cbp6*/Δ*smt1*-null mutants were labeled with [^{35}S]methionine in the presence of cycloheximide. Proteins were analyzed by SDS-PAGE and autoradiography. *B*, D273-10b cells carrying the *COB(1–352)::pARG8*^{m} mitochondrial construct were grown on 10-fold serial dilutions on complete media (+*ARG*) or media lacking arginine (−*ARG*) for 3 days at 30 °C. *C*, BY4742 cells carrying the D273-10b *SMT1* gene expressed form low-copy or high-copy plasmids were [^{35}S]methionine pulse-labeled, and mitochondrial translation products were analyzed as in *A*. Wildtype cells were transformed with empty plasmid as indicated.

which arose from a different lineage (35). Previous reports also observed that in D273-10b lab strains Cbp3 and Cbp6 were only involved in bc_1 complex assembly rather than in Cyt*b* synthesis regulation (23, 32). Moreover, the homologs of Cbp3 and Cbp6 in *Schizosaccharomyces pombe* are only involved in the bc_1 complex assembly, but not in Cyt*b* synthesis (24). These observations suggest that the role of Cbp3 and Cbp6 in *COB* mRNA translational regulation was acquired at a very recent event, after the divergence of *Saccharomyces cerevisiae* D273-10b strain from W303 and BY4742 strains.

Even though *COB* mRNA translation occurs normally in D273-10b strain in the absence of Cbp3 and Cbp6, cells are incapable of supporting respiratory growth. This could be explained because these chaperones are still required for Cyt*b* hemylation in the D273-10b lab strain. Accordingly, Hildenbeutel *et al.* (15) reported that Cbp3–Cbp6 coordinate apocytochrome *b* hemylation. These chaperones interact with a heme-free Cyt*b* intermediate, as well as with a Cyt*b* protein carrying heme b_L. This result shows that the function of Cbp3 and Cbp6 as chaperones for Cyt*b* hemylation is well-conserved in different *S. cerevisiae* laboratory strains. The exact mechanism by which heme *b* assembles into Cyt*b* is still unknown, the heme *b* could be assembled from the mitochondrial matrix or from the inner membrane (36), and interaction of Cbp3 and Cbp6 with newly synthesized Cyt*b* may keep it in an "accessible" conformation to be hemylated. Moreover, association of Cbp3 and Cbp6 with the ribosomal tunnel exit might be important for this process (15). Accordingly, we observed that even when Cbp3 is dispensable for translation in D273-10b cells, this chaperone interacts with the mitochondrial ribosome in both the BY4742 and in the D273-10b strains, as previously reported for W303 cells (14). This interaction could be important for early interaction with Cyt*b* or for efficient communication between the translational machinery and assembly of Cyt*b*. In this translation/assembly coordination process, Rrf1 and Mif3 might also be involved as previously proposed (37).

We also observed that in D273-10b cells, Cyt*b* interacts with cytochrome *c* oxidase to form non-functional supercomplexes in the absence of Cbp3 and Cbp6. These supercomplexes contain at least Cyt*b*, Core 1, and the Rieske iron–sulfur subunits. Several reports show that supercomplexes formation can occur at early steps of *Cc*O and bc_1 complex assembly (2, 38, 39). Moreover, *Cc*O subunit Cox2 can form a 230-kDa subcomplex with the early assembly intermediate composed of Cyt*b*, Qcr7, and Qcr8 (2). These data support a model in which bc_1 complex interacts with *Cc*O at early assembly stages and suggest that this interaction is independent from Cyt*b* hemylation and Cbp3/Cbp6. Alternatively, Cbp3/Cbp6 might participate on regulation of Cyt*b* incorporation into supercomplexes, explaining

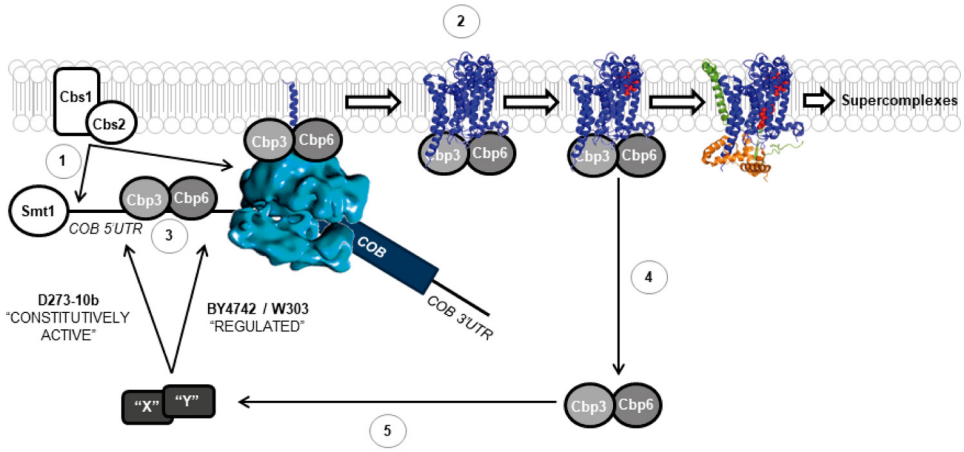

Figure 8. Model showing a mechanism for the differential regulation of Cyt*b* synthesis by Cbp3 and Cbp6 in distinct laboratory yeast strains. Translational activation of the *COB* mRNA depends on Cbs1 and Cbs2 (8, 9) (1). Cbp3 and Cbp6 interact with the ribosomal tunnel exit (14) and with newly synthesized Cyt*b* to promote hemylation/assembly (hemes in *red*) (7, 15) (2). Cbp3 interacts with the *COB* 5′-UTR mRNA (this work), probably to promote efficient translation (3). These interactions are present in both D273-10b and BY4742 lab strains. Cyt*b* assembly proceeds to form the first intermediary, containing Qcr7 (*orange*) and Qcr8 (*green*) subunits, and then Cbp3/6 release from the Cyt*b* complex (7). We propose the existence of additional factors X and Y, which in turn become active in BY4742 and W303 lab strains, allowing an efficient *COB* mRNA translation (5). Absence of Cbp3/6 prevents factors X and Y from becoming active, and thereby Cyt*b* synthesis is reduced. In contrast, in D273-10b strain factors X and Y are constitutively active, even in the absence of Cbp3 and Cbp6. In addition, Smt1 physically interacts with the *COB* mRNA to carry over an unknown function that does not seem to be related to Cbp3/Cbp6 (20). Tertiary and quaternary structures were taken from the Protein Data Bank (code 1EZV).

why in the absence of these chaperones, non-functional supercomplexes form.

How is Cyt*b* synthesis differentially regulated by Cbp3/Cbp6 in D273-10b *versus* BY4742/W303 strains? We hypothesize that there are additional proteins participating in the Cbp3/Cbp6-dependent translational regulation observed in BY4742 and W303 lab strains. This function may arise from polymorphism(s) affecting the protein(s) sequence and therefore activity. Here we showed that the differential role of Cbp3/Cbp6 comes from polymorphisms in the nuclear genome rather than in the mitochondrial DNA. This polymorphism(s) confers a dominant phenotype to D273-10b cells over BY4742 cells, meaning that diploids resulting from the cross of D273-10b and BY4741 have normal levels of Cyt*b* synthesis in the absence of Cbp3 and Cbp6 (Fig. S4). Moreover, tetrad analysis of diploid, *CBP3* heterozygous cells carrying the *COB(1–385)::ARG8*^{m} suggested that there are two nuclear genes involved in the observed differential phenotype of Δ*cbp3* mutants (data not shown).

We propose some modifications to the current model for Cyt*b* synthesis and assembly (40). Translational activation of the *COB* mRNA depends on Cbs1 and Cbs2, which act on the *COB* mRNA 5′-UTR and also interact with the mitochondrial ribosome (8, 12) (Fig. 8, *part 1*). Cbp3 and Cbp6 interact with the ribosomal tunnel exit (7) and with the Cyt*b* peptide probably to facilitate Cyt*b* hemylation (15) and/or assembly (Fig. 8, *part 2*). We also demonstrated that Cbp3 physically interacts with the *COB* mRNA. This interaction could be direct or mediated by another protein or by the ribosome (Fig. 8, *part 3*). Interestingly, the Cbp3-*COB* mRNA association was observed in both lab strains, suggesting that this interaction alone is not sufficient for *COB* mRNA translational regulation. Once Cyt*b* assembly proceeds to form the first intermediaries containing Qcr7 and Qcr8 subunits (and Cbp4; not shown), then Cbp3/Cbp6 release from the Cyt*b* complex (7) (Fig. 8, *part 4*). Protein "X" (or perhaps proteins X and Y) becomes active only upon release of Cbp3/Cbp6 from the Cyt*b* complex in BY4742 and W303 lab strains to allow efficient *COB* mRNA translation (Fig. 8, *part 5*). In these lab strains, absence of Cbp3/Cbp6 prevents the factors X and Y from becoming active, and therefore Cyt*b* synthesis is reduced. In contrast, in D273-10b lab strain factors X and Y are constitutively active, even in the absence of Cbp3 and Cbp6, possibly because of a loss of communication with these chaperones. Thus, Cyt*b* synthesis is normal even in the absence of Cbp3/Cbp6.

The identity of proteins X and Y remains to be elucidated. Laboratory *S. cerevisiae* strains contain polymorphisms, introduced mutations, and genetic markers that confer differential phenotypes on mitochondrial function (41, 42). Chaperones that are currently known to be involved in Cyt*b* biogenesis were discarded (Cbs1, Cbs2, Cbp1, and Cbp4), because sequence comparisons did not show any changes between D273-10b and BY4742/W303 strains. A candidate was Smt1, which physically interacts with the *COB* mRNA (20) and has three amino acid changes in the D273-10b protein as compared with BY4742 and W303. However, our results did not support that Smt1 has a regulatory role of Cyt*b* synthesis that is related to Cbp3 and Cbp6. Other previously identified alleles affecting mitochondrial function that might be candidates are *HAP1* and *MRM1*(42). However, none of these genes are likely to be involved in the differential function of Cbp3/Cbp6 that we observe. Hap1, a heme-responsive transcriptional factor of genes involved in electron transfer reactions (43) has a C-terminal end mutation in some S288c-derived strains that modifies respiratory and oxygen metabolism (44). However, although BY4742 has the *hap1* mutation, W303 and D273-10b have wildtype *HAP1*, ruling out the possibility that *HAP1* could be involved in the Cbp3/Cbp6 differential function we observed between D273-10b and BY4742/W303 strains. Mutations on the promoter of *MRM1*, encoding a mitochondrial methyltransferase, are associated with respiratory defects in some lab

strains (42, 45). However, this gene is wildtype in W303, D273-10b, and BY4742 strains.

Experimental procedures

Yeast strains and genetic methods

S. cerevisiae D273-10b (ATCC24657), BY4742 (ATCC4040004), and BY4741 (ATCC4040002) strains used in this study are listed on Table 1. Genetic methods and media were as previously described (46, 47). Strains were cultured in complete fermentable media containing 1% yeast extract, 2% Bacto-peptone, and 2% glucose (YPD) or 2% galactose (YPGal), or in synthetic complete media containing 0.67% yeast nitrogen base and 2% glucose and lacking uracil or the indicated amino acids. Nuclear deletion constructs with *LEU2*, *URA3*, *HIS3MX6*, or *KanMX4* cassettes were made by PCR. Correct integration of the different constructs into the nuclear genome was confirmed by PCR. The *CBP3-HA* construct, including 320 and 352 bp of the *CBP3* 5′- and 3′-UTR, respectively, was amplified by fusion PCR (48). This product was ligated into NotI sites of the yeast expression plasmid pRS416.

Mitochondrial transformation

The construct *COB(1–385)::pARG8^m* was made by PCR amplification of three fragments: one included the last 351 bp of the *COB orf* and another covered the first 470 bp of the *ARG8^m orf* encoding the Arg8 mitochondrial targeting signal (22). These two products were used as templates for fusion PCR (48). The fusion product was cut with XhoI and EcoRI and cloned into equally digested pBluescript SK(+) vector to obtain plasmid pYCV55. The third PCR product included the complete *ARG8^m* orf and 625pb of *COB* 3′-UTR; this product was cloned in pYCV55 digested with NcoI and EcoRI. The resulting plasmid, pYCV56, was transformed by high-velocity microprojectile bombardment into the rho0 strain NAB69 (49). Transformants were selected by their ability to rescue growth in media lacking arginine when mated with the strain NB71 (49). Transformants with the *COB(1–385)::ARG8^m* plasmid were mated with XPM201 (derived from D273-10b lab strain) (50) and AGG26 (derived from BY4742 lab strain), and haploid cytoductants were selected for their ability to grow in media lacking arginine. Correct integration of the *COB(1–385)::ARG8^m* constructs into mtDNA was confirmed by PCR and DNA sequencing.

Analysis of mitochondrial proteins

Mitochondria were isolated from cells grown in YPGal media until late log phase. The cells were disrupted with glass beads or by zymolyase 20T treatment as described (51). The proteins were resolved by SDS-PAGE on 12% gels (52) or 16% in the presence of 6 M urea (53), transferred to a polyvinylidene fluoride (PVDF) membrane and detected by immunoblotting with horseradish peroxidase–conjugated antibodies to HA (Roche) or the indicated rabbit polyclonal antibodies: anti-Qcr7, anti-Qcr8, anti-Cor1, anti-Cor2, anti-Rip1, and anti-Qcr10 (Vicenzo Zara); anti-Rip1, anti-Cor1, and anti-Mrp20 (Rosemary Stuart); anti-citrate synthase and anti-Mrp51 (Thomas D. Fox); and anti-Cyt*b*. Secondary goat IgG anti-mouse or anti-rabbit (Santa Cruz Biotechnology) conjugated to horseradish peroxidase was detected with the Pierce ECL (Thermo Scientific) or Immobilon™ Western Chemiluminescent HRP Substrate (Millipore). All loaded proteins were normalized by protein quantification using the Lowry method (54).

Protein aggregation analysis

Protein aggregation analysis was performed as described previously (5). Samples (100 μg) of mitochondrial protein were washed with 250 mM sorbitol, 50 mM Bis-Tris and lysed with 1% digitonin, 30 mM Tris, pH 7.4, 200 mM KCl, 5 mM EDTA, and 0.5 mM PMSF for 30 min on ice. After ultracentrifugation at 4 °C for 30 min at 100,000 × *g* in a TLA 120.2 rotor, supernatant was recovered into a new tube (S1). The resultant pellet was lysed with 1% Triton, 30 mM Tris, pH 7.4, 200 mM KCl, 5 mM EDTA, and 0.5 mM PMSF for 5 min on ice. Pellet (P) and supernatant (S2) were separated by ultracentrifugation at 4 °C for 30 min at 100,000 × *g* in a TLA 120.2 rotor. Supernatants S1 and S2 were mixed (S) and treated with TCA for protein precipitation.

Heme b analysis

Total heme extraction was performed as previously described (55). Samples (700 μg) of Histodenz (Sigma) purified mitochondrial protein were treated with 300 μl acidic acetone (3% HCl) and incubated for 5 min at room temperature. Supernatant was recovered after centrifugation for 5 min at 16,200 × *g*. Supernatant was mixed with 1% trifluoroacetic acid in a proportion 1:1. HPLC analysis was performed as described (56) in a Beckman HPLC unit with System Gold. Supernatant was injected onto a 4.6- by 250-μm SunFire C_{18} 5-mm column (Waters). Hemes were eluted from the column at a flow rate of 1 ml/min using a 0–100% gradient of acetonitrile containing 1% of trifluoroacetic acid. Elution of heme compounds was monitored at 400 nm. Purified heme *b* (Sigma) was used as standard to determine elution time. For heme *b* analysis, two different mitochondria purifications were used as biological replicates with one technical replicate. For each biological replicate, heme *b* extractions and HPLC analysis were made with one technical replicate ($n = 3$). Statistical analysis was made by one-way analysis of variance followed by Bonferroni post hoc (GraphPad Prism software, version 6.0). Two-sided adjusted p values for multiple comparisons are presented.

Synthesis of mitochondrial proteins

In vivo labeling of cells in the presence of [^{35}S]methionine and cycloheximide was performed as previously described (16). After 15 or 40 min of pulse labeling, the cells were chilled on ice and disrupted by vortexing with glass beads to obtain mitochondria by centrifugation. Mitochondrial proteins were resolved on a 16% polyacrylamide gel, transferred to a PVDF membrane, and analyzed with a Typhoon 8600 phosphorimaging device (GE Healthcare).

Blue native electrophoresis

BN-PAGE was performed as described previously (29). Samples (100 μg) of mitochondrial protein were washed with 250 mM sorbitol, 50 mM Bis-Tris and lysed with 750 mM aminocaproic acid, 50 mM Bis-Tris, and either digitonin or *n*-dodecyl

Table 1

Yeast strains used in this study

The mitochondrial genome is indicated in parentheses.

Strain	Nuclear (mitochondrial) genotype	Reference/source
NB40-36a	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112*, D273-10B (ρ^+)	Ref. 16
DAU1	*Matα,ade2, ura3Δ*, D273-10b (ρ^+)	Ref. 58
XPM201	*Matα, arg8::hisG, leu2-3,112, lys2, ura3-52, D273-10b* (ρ^+, $\Delta\Sigma ai$)[b]	Ref. 50
NAB69	*Mata, ade2-101, arg8-delta::hisG, ura3-52, kar1-1* (ρ^0)	Ref. 49
NB71	*Matα, ade2-101, ura3-52, leu2-delta, arg8-delta::URA3, kar1-1*(ρ^+, *cox3Δ::ARG8^m-1*)	Ref. 49
BY4742	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0*, BY4742 (ρ^+)	YKO Matx Strain Collection–Glycerol Stocks (Open Biosystems
BY4741	*Mata, his3-delta1, leu2-delta0, met15-delta0, ura3-delta0*, BY4741 (ρ^+)	YKO Matx Strain Collection–Glycerol Stocks (Open Biosystems
Δ*qcr7*	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, qcr7::KANMX4*, BY4742 (ρ^+)	YKO Matx Strain Collection–Glycerol Stocks (Open Biosystems)
Δ*qcr10*	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, qcr10::KANMX4*, BY4742 (ρ^+)	YKO Matx Strain Collection–Glycerol Stocks (Open Biosystems)
Δ*cbp4*	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, cbp4::KANMX4*, BY4742 (ρ^+)	YKO Matx Strain Collection–Glycerol Stocks (Open Biosystems)
Δ*smt1*	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, smt1::KANMX4*, BY4742 (ρ^+)	YKO Matx Strain Collection–Glycerol Stocks (Open Biosystems)
Δ*cbp3*	*Mata, his3-delta1, leu2-delta0, met15-delta0, ura3-delta0, cbp3::KANMX4*,BY4741 (ρ^+)	Collection–Glycerol Stocks (Open Biosystems)
AGG24	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112*, D273-10B (ρ^+, BY4742 mtDNA)[a]	This study
AGG25	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp3::KANMX4*, D273-10B (ρ^+, BY4742 mtDNA)	This study
AGG28	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp4::KANMX4*, D273-10B (ρ^+, BY4742 mtDNA)	This study
AGG29	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, qcr7::KANMX4*, D273-10B (ρ^+, BY4742 mtDNA)	This study
AGG30	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, qcr10::KANMX4*, D273-10B (ρ^+, BY4742 mtDNA)	This study
AGG33	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3*, BY4742 (ρ^+, $\Delta\Sigma$ai[b], *COB(1-352)::pARG8^m*)	This study
AGG34	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, qcr7::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai^b$, *COB(1-352)::pARG8^m*)	This study
AGG35	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, qcr8::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG36	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp3::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG37	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp4::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG38	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3*, BY4742 (ρ^+, NB40-36a mtDNA)	This study
AGG39	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, qcr7::KANMX4*, BY4742 (ρ^+, NB40-36 mtDNA)	This study
AGG40	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, qcr10::KANMX4*, BY4742 (ρ^+, NB40-36 mtDNA)	This study
AGG41	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp3::KANX4*, BY4742 (ρ^+, NB40-36 mtDNA)	This study
AGG42	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp6::LEU2*, BY4742 (ρ^+, NB40-36 mtDNA)	This study
AGG43	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, qcr7::KANMX4*, D273-10B (ρ^+)	This study
AGG46	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp3::KANMX4*, D273-10B (ρ^+)	This study
AGG47	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp4::KANMX4*, D273-10B (ρ^+)	This study
AGG48	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112*, D273-10B (ρ^+, $\Delta\Sigma ai$, *COB(--352)::pARG8^m*)	This study
AGG56	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp6::LEU2*, D273-10B (ρ^+)	This study
AGG57	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp6::LEU2*, D273-10B (ρ^+, BY4742 mtDNA)	This study
AGG58	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, cbp3::KANMX4*, BY4742 (ρ^+)	This study
AGG59	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, cbp6::LEU2*, BY4742 (ρ^+)	This study
AGG60	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp4::KANMX4*, BY4742 (ρ^+, NB40-36a mtDNA)	This study
AGG61	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp3::KANMX4, cbp6::LEU2*, D273-10B (ρ^+)	This study
AGG62	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp6::LEU2*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG63	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, qcr7::KANMX4*, D273-10B (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG65	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp3::KANMX4*, D273-10B (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG66	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp6::LEU2*, D273-10B (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG67	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp4::KANMX4*, D273-10B (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG68	*Mata, his3-delta1, leu2-delta0, met15-delta0, ura3-delta0 qcr7::KANMX4*, BY4741 (ρ^+)	This study
AGG69	*Mata, his3-delta1, leu2-delta0, met15-delta0, ura3-delta0 cbp6::LEU2*, BY4741 (ρ^+)	This study
AGG70	*Mata, his3-delta1, leu2-delta0, met15-delta0, ura3-delta0 cbp4::KANMX4*, BY4741 (ρ^+)	This study
AGG71	*Matα/a, lys2$^{+/-}$, arg8::hisG$^{+/-}$, ura3-52/ura3-delta0, leu2-3,112/leu2-delta0, met15-delta0$^{-/+}$, his-delta1$^{-/+}$*, D273-10b/BY4741 (ρ^+)[c]	This study
AGG72	*Matα/a, lys2$^{+/-}$, arg8::hisG$^{+/-}$, ura3-52/ura3-delta0, leu2-3,112/leu2-delta0, met15-delta0$^{-/+}$, his-delta1$^{-/+}$, qcr7::KANMX4$^{+/+}$*, D273-10b/BY4741 (ρ^+)	This study
AGG73	*Matα/a, lys2$^{+/-}$, arg8::hisG$^{+/-}$, ura3-52/ura3-delta0, leu2-3,112/leu2-delta0, met15-delta0$^{-/+}$, his-delta1$^{-/+}$,cbp6::LEU2$^{+/+}$*, D273-10b/BY4741 (ρ^+)	This study
AGG74	*Matα/a, lys2$^{+/-}$, arg8::hisG$^{+/-}$, ura3-52/ura3-delta0, leu2-3,112/leu2-delta0, met15-delta0$^{-/+}$, his-delta1$^{-/+}$, cbp4::KANMX4$^{+/+}$*, D273-10b/BY4741 (ρ^+)	This study
AGG75	*Matα, lys2, arg8::hisG, ura3-52, leu2-3,112, cbs1::URA3*, D273-10B (ρ^+, $\Delta\Sigma$ai[b], *COB(1-352)::pARG8^m*)	This study
AGG76	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbs1::HIS3MX6*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG77	*Matα, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, cbs1::URA3*, BY4742 (ρ^+)	This study

Table 1—*continued*

Strain	Nuclear (mitochondrial) genotype	Reference/source
AGG78	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, cbs1::URA3*, D273-10B (ρ^+)	This study
AGG87	*Mata/a, lys2$^{+/-}$, arg8::hisG$^{+/-}$, ura3-52/ura3-delta0, leu2-3,112/leu2-delta0, met15-delta0$^{-/+}$, his-delta1$^{-/+}$, cbp3::KANMX4$^{+/+}$*, D273-10b/BY4741 (ρ^+)	This study
AGG89	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, smt1::KANMX4, cbp6::LEU2*, BY4742 (ρ^+)	This study
AGG91	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, smt1::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG92	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, smt1::KANMX4, cbp6::LEU2*, BY4742 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
AGG93	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3,qcr7::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
AGG95	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3,cbp4::KANMX4* BY4742 (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
AGG96	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp3::KANMX4*, BY4742 (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
AGG97	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, qcr7::KANMX4*, D273-10B (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
AGG99	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp4::KANMX4*, D273-10B (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
AGG100	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp3::KANMX4* D273-10B (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
AGG101	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, pet309::URA3, cpb6::LEU2*, D273-10B[a] (ρ^+)	This study
AGG102	*Mata/Mata, lys2$^{+/-}$, arg8::hisG$^{+/-}$, ura3-52$^{+/[minus]}$*], *leu2-3,112$^{+/+}$, ade2 $^{-/+}$, his3-11,15$^{-/+}$, trp1-1$^{-/+}$, ura3-1$^{-/+}$, cbp3::KANMX4$^{+/-}$*, D273-10B/W303 (ρ^+, $\Delta\Sigma ai$, *COB(1-352)::pARG8^m*)	This study
DFM2	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3*, BY4742 (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	Daniel Flores-Mireles
DFM5	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112*, D273-10B (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	Daniel Flores-Mireles
YC140	*Mata, ade2, ura3-delta, MSS51-3XHA, pet309::URA3*, D273-10b (ρ^+, *cox1-delta*)	This study
YC166	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, cbs1::URA3*, D273-10B (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
YC173	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbp6::LEU2*, BY4742 (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
YC174	*Mata, lys2, arg8::hisG, ura3-52, leu2-3,112, cbp6::LEU2*, D273-10B (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study
YC175	*Mata, his3-delta1, leu2-delta0, lys2-delta0, ura3-delta0, arg8::URA3, cbs1::HIS3MX6*, BY4742 (ρ^+, $\Delta\Sigma ai$, *cobΔ::ARG8^m*)	This study

[a] mtDNA refers to mitochondrial genome.
[b] $\Delta\Sigma ai$ refers to the intronless *COX1* gene.
[c] +/− corresponds presence (+) or absence (−) of the allele in both chromosomes in the diploid strain.

β-D-maltoside on a protein detergent relation of 2:1 and 1:2, respectively, for 15 min (*n*-dodecyl β-D-maltoside) or 30 min (digitonin) on ice. Mitochondrial extracts were cleared at 16,200 × *g* for 12 min, and the supernatants were mixed with 2.5 μl of 5% Coomassie solution (750 mM aminocaproic acid, 50 mM Bis-Tris). Extracts were loaded on a 5% to 12% polyacrylamide gel and transferred to a PVDF membrane. The proteins were detected by immunoblotting with the indicated antibodies. In-gel *Cc*O activity was performed after BN-PAGE using 0.04% diaminobenzidine (Sigma–Aldrich) and 0.02% of horse heart cytochrome *c* (Sigma–Aldrich) in phosphate buffer, pH 7.4 (57).

RNA immunoprecipitation assay

This technique was performed as previously described (30). Mitochondria (500 μg) were lysed with 0.7% *n*-dodecyl β-D-maltoside, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, RNaseOUT (Invitrogen), and Minicomplete protease inhibitors (Roche). The solubilized fractions were incubated with an anti-HA antibody coupled to protein A-Sepharose (GE Healthcare). Immunoprecipitates were washed twice with 500 μl of lysis buffer and twice with 1 ml of 20 mM HEPES-KOH, pH 7.4, and then resuspended in 150 μl of the same buffer. One-third of the precipitate fractions were saved for Western blotting analysis, and the remainder was used for RNA extraction. RNA from total and immunoprecipitated fractions was extracted by incubation with TRIzol® reagent (Invitrogen). 20 ng of RNA were treated with 1 unit of DNase I (Invitrogen). The first strand of cDNAs were prepared by the addition of primers for *COB* or *VAR1* in the presence of SuperScript III reverse transcriptase (Invitrogen). The resulting cDNAs were used as PCR templates to amplify *COB* or *VAR1* 5′-UTRs. Note that under these conditions, RT-PCRs are not quantitative.

Sucrose fractionation of mitochondrial lysates

Samples (500 μg) of mitochondrial protein were lysed with 1% digitonin, 10 mM MgOAc, 50 mM NaCl, 20 mM HEPES-KOH, pH 7.4, and 1 mM PMSF for 30 min on ice and clarified by centrifugation at 16,200 × *g* for 10 min. Supernatants were loaded into a discontinuous sucrose gradient of 40, 30, and 20% containing 0.1% digitonin, 10 mM MgOAc, 20 mM DTT, 10 mM Tris-HCl, pH 7.4, and 0.5 mM PMSF. Sucrose gradients were ultracentrifuged at 145,000 × *g* in a SW-55Ti rotor for 2 h at 4 °C. Fractions of 600 μl were taken and TCA-treated for protein precipitation. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected by immunoblotting with the indicated antibodies.

Imaging and figure edition

For better visualization, all figures from pictures, scanning, or phosphorimaging were adjusted in contrast and brightness using the Adobe Photoshop software. Pictures of serial dilutions, agarose gels, *in gel* activity, and Coomassie Blue gels from Figs. 1 (*G* and *H*), 3*B*, 6*D*, and 7*B* and Figs. S1 (*A*–*C*), S2 (*A* and *B*), and S3 were taken by a camera. *In vivo* labeling from Figs. 1 (*A*, *B*, and *F*), 6 (*A* and *C*), and 7 (*A* and *C*) and Fig. S4 (*A* and *B*) was visualized by exposure to a storage phosphor screen and then scanned in a Typhoon FLA700 or Typhoon 9400 (GE

Healthcare). Western blotting images from Figs. 1*E*, 2 (*A* and *B*), 2*C*, 3 (*A* and *C*), 5 (*A*–*C*), and 6 (*B* and *D*) and Fig. S2*A* were visualized by exposure to a film and then scanned in a scanner Color LaserJet Pro MFP M477fmw (HP).

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