Cbp3–Cbp6 interacts with the yeast mitochondrial ribosomal tunnel exit and promotes cytochrome *b* synthesis and assembly

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Mitochondria contain their own genetic system to express a small number of hydrophobic polypeptides, including cytochrome *b*, an essential subunit of the *bc₁* complex of the respiratory chain. In this paper, we show in yeast that Cbp3, a *bc₁* complex assembly factor, and Cbp6, a regulator of cytochrome *b* translation, form a complex that associates with the polypeptide tunnel exit of mitochondrial ribosomes and that exhibits two important functions in the biogenesis of cytochrome *b*. On the one hand, the interaction of Cbp3 and Cbp6 with mitochondrial ribosomes is necessary for efficient translation of cytochrome *b* transcript. On the other hand, the Cbp3–Cbp6 complex interacts directly with newly synthesized cytochrome *b* in an assembly intermediate that is not ribosome bound and that contains the assembly factor Cbp4. Our results suggest that synthesis of cytochrome *b* occurs preferentially on those ribosomes that have the Cbp3–Cbp6 complex bound to their tunnel exit, an arrangement that may ensure tight coordination of cytochrome *b* synthesis and assembly.

Introduction

The membrane-embedded complexes driving oxidative phosphorylation in mitochondria consist of subunits that are encoded in either the nuclear or the organellar DNA. Most of these proteins are nuclear encoded; they are synthesized in the cytosol and imported posttranslationally into the organelle (Neupert and Herrmann, 2007; Chacinska et al., 2009). In yeast mitochondria, the genetic system contributes seven hydrophobic translation products that represent the catalytic cores of the complexes (Borst and Grivell, 1978). Their assembly with the nuclear-encoded subunits necessitates complex biogenesis pathways. Over 30 genes have been identified that are obligatory for the formation of cytochrome *c* oxidase (Mick et al., 2011). These genes encode factors required for maturation and translation of *COX* mRNAs as well as proteins that help to insert the redox-active prosthetic groups. Similarly, numerous genes have been identified as being important for biogenesis of ATP synthase and the *bc₁* complex. In the case of the *bc₁* complex,

these factors include three translational activators (Cbs1, Cbs2, and Cbp6) that are mitochondria-specific proteins required for translation of the cytochrome *b* mRNA (*COB* mRNA) and four assembly factors (Cbp3, Cbp4, Bcs1, and Bca1). However, not much is known about how they mediate the formation of a functional *bc₁* complex.

Protein synthesis in mitochondria is performed by organelle-specific ribosomes. These ribosomes developed from the translation system of the bacterial ancestor of mitochondria. Surprisingly little is known about how these ribosomes mediate protein synthesis and how they are organized in the context of respiratory chain assembly. Newly synthesized proteins emerge from the ribosome at the polypeptide tunnel exit, which serves as a docking site for a variety of biogenesis factors. This is well documented in bacteria (Kramer et al., 2009). The interactors of the tunnel exit of bacterial ribosomes can be classified into three different groups, namely (1) processing enzymes like

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peptide deformylase, (2) chaperones like trigger factor, and (3) targeting and membrane insertion components like the signal recognition particle and the SecYEG complex (Ménétret et al., 2000; Kramer et al., 2002; Gu et al., 2003; Bingel-Erlenmeyer et al., 2008). These factors interact with the rim of the polypeptide tunnel exit that is formed by RNA moieties and four conserved proteins, namely L22, L23, L24, and L29. The exact composition and structure of the mitochondrial ribosomal tunnel exit is not known. Cryo-EM reconstruction of mitochondrial ribosomes indicated that their tunnel exits differ substantially from those of their bacterial counterparts (Sharma et al., 2003, 2009). Although homologues of the bacterial ribosomal proteins found at the tunnel exit are conserved in mitochondria, this site also contains proteins found exclusively in these organelles (Gruschke et al., 2010). Hence, this important structure of the ribosome was considerably modified in the course of evolution, implying novel ways to organize translation in mitochondria.

Here, we report that Cbp3, an assembly factor of cytochrome b (Wu and Tzagoloff, 1989), can be cross-linked in isolated mitochondria to Mrpl4, a homologue of the conserved tunnel exit protein L29. This positioning of Cbp3 at the tunnel exit supports an early interaction of Cbp3 with the newly synthesized cytochrome b. Interestingly, Cbp3 forms a complex with Cbp6, a yet ill-defined translational activator of the *COB* mRNA (Dieckmann and Tzagoloff, 1985). By using a combination of yeast genetics and biochemistry, we reveal that the Cbp3–Cbp6 complex has two different functions for biogenesis of cytochrome b. On the one hand, the complex interacts with mitochondrial ribosomes to allow efficient translation of mRNAs containing the 5′ untranslated region (UTR) of the *COB* mRNA. On the other hand, the Cbp3–Cbp6 complex is part of a non–ribosome-bound assembly intermediate of the bc_1 complex that contains newly synthesized cytochrome b and the assembly factor Cbp4. Our data suggest that these two functions of the Cbp3–Cbp6 complex allow coupled synthesis and assembly of cytochrome b, thereby facilitating biogenesis of this central subunit of the bc_1 complex.

Results

Cbp3 binds to mitochondrial ribosomes in proximity to the polypeptide tunnel exit

We recently analyzed the composition of the yeast mitochondrial ribosomal tunnel exit by a chemical cross-linking approach using isolated mitochondria that carried His7-tagged versions of the conserved proteins located at the tunnel exit, namely Mrpl22, Mrp20, Mrpl4, and Mrpl40 (Gruschke et al., 2010). The resulting cross-linking products of these proteins were purified by metal affinity chromatography and analyzed by mass spectrometry. Fig. 1 A shows the formation of several cross-linking products to Mrpl-4His7 upon incubation with m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). Two of these cross-linking products could be clearly identified (Mrpl4-Mba1 and Mrpl4–Mrpl22; Gruschke et al., 2010). Interestingly, in the 74-kD band, we recovered, in addition to fragments of Mrpl4, three different peptides of Cbp3, a protein described to be essential for the assembly of the bc_1 complex of the respiratory chain (Wu and Tzagoloff, 1989).

To confirm that Cbp3 forms a cross-link to Mrpl4, we repeated the experiment using Western blotting for analysis. While the band at 74 kD was absent from mock-treated mitochondria, it appeared after incubation with MBS and could be enriched by metal affinity chromatography from lysates of mitochondria containing Mrpl4His7 (Fig. 1 A). The same band was detected by a Cbp3-specific antibody, confirming that it represents a cross-linking product of Mrpl4 and Cbp3 (Fig. 1 A). To corroborate this result, we performed the cross-linking experiment with mitochondria containing Cbp3His7. Western blotting using antibodies against Mrpl4 proved that the 74-kD band is a cross-linking product of Mrpl4 and Cbp3 (Fig. 1 B).

We next tested whether an interaction of Cbp3 with mitochondrial ribosomes can also be observed by an independent approach. We therefore lysed mitochondria with Triton X-100 and fractionated these lysates by centrifugation through a high density sucrose cushion into supernatant and a ribosome-containing pellet (Fig. 1 C, left). Although the soluble protein aconitase remained in the supernatant fraction, Cbp3 cosedimented in a salt-sensitive fashion with ribosomes (Fig. 1 C, right). The finding that Cbp3 binds almost quantitatively to ribosomes under low salt conditions inspired us to test whether all or only a subset of ribosomes would contain Cbp3. We therefore estimated the relative abundance of Cbp3 over ribosomes by comparing the amounts of Cbp3His7 and Mrpl4His7. The His7 tag did not influence the level of either Cbp3 or Mrpl4 (Fig. 1 D). Western blotting using antibodies against the His7 tag revealed that both proteins are present in similar quantities (Fig. 1 D), suggesting that the levels of Cbp3 are high enough to allow each ribosome to bind one Cbp3. In conclusion, Cbp3 is a novel interactor of the mitochondrial ribosome that binds to the tunnel exit in proximity to Mrpl4.

Cbp3 stabilizes newly synthesized cytochrome *b*

Cbp3, together with Cbp4, Bcs1, and Bca1, is one of four known assembly factors of the bc_1 complex (Nobrega et al., 1992; Crivellone, 1994; Zara et al., 2009; Mathieu et al., 2011), but its molecular function is still unclear. Cbp3 is a highly conserved factor and is present in those bacteria and mitochondria that contain a bc_1 complex. Because of the binding of Cbp3 in proximity to the polypeptide tunnel exit, we asked whether Cbp3 interacts with newly synthesized proteins. Translation products of mitochondria isolated from either a wild-type strain or a strain expressing Cbp3His7 were labeled with [^{35}S]methionine in the presence of the cleavable cross-linker dithiobis(succinimidyl propionate) (DSP; Fig. 2 A). Next, Cbp3His7 was purified, and the radioactive proteins cross-linked to Cbp3His7 were released by incubation with a reducing agent. The newly synthesized cytochrome *b* was enriched on Cbp3His7 (Fig. 2 B, arrow), whereas only background signals were obtained when mitochondria from wild-type cells were used. This indicates that Cbp3 interacts directly with newly synthesized cytochrome *b*.

To analyze the fate of newly synthesized cytochrome *b* in the absence of Cbp3, we followed the synthesis and stability of mitochondrially encoded proteins in whole cells (Fig. 2 B). To avoid any interference with the complex splicing of mitochondrial
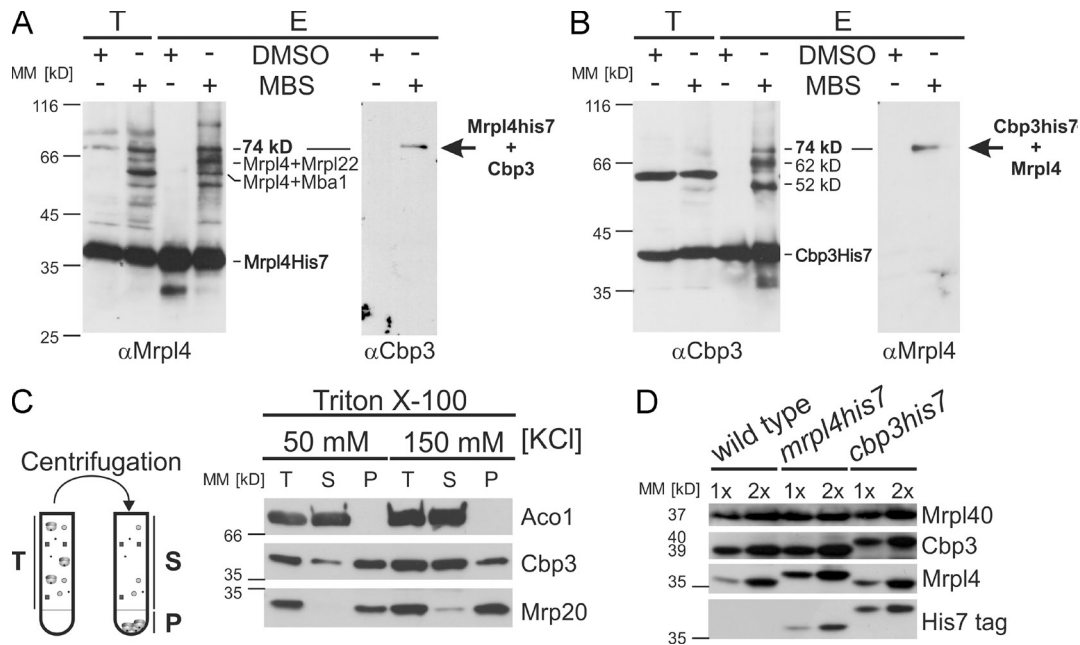
Figure 1. Cbp3 binds to mitochondrial ribosomes in proximity to the polypeptide tunnel exit. (A) Identification of Cbp3 as a cross-linking partner of Mrpl4. Mitochondria containing Mrpl4His7 were incubated with the cross-linker MBS or mock treated (DMSO). Mrpl4His7 and cross-linked proteins were purified on Ni-NTA beads and analyzed by Western blotting with antibodies against Mrpl4 (left) and Cbp3 (right). E, elution after Ni-NTA purification; T, 10% total before Ni-NTA purification. (B) Verification of the cross-link between Mrpl4 and Cbp3. The analysis depicted in A was repeated with mitochondria containing Cbp3His7. Fractions were analyzed by Western blotting using antibodies against Cbp3 (left) and Mrpl4 (right). (C) Cbp3 binds to mitochondrial ribosomes in a salt-sensitive manner. (left) Experimental setup. Ribosomes and co-migrating proteins (P, pellet) are separated from the soluble fraction (S, supernatant) by high-speed centrifugation through a sucrose cushion. (right) Triton X-100 lysates of wild-type mitochondria were fractionated in the presence of 50 or 150 mM KCl. The fractions were analyzed by Western blotting using antibodies against Aco1 (a soluble protein), Mrp20 (a ribosomal component), and Cbp3. T, 100% total before high-speed centrifugation. (D) Cbp3 and ribosomes are present in similar quantities in mitochondria. Increasing amounts of proteins of wild-type, *mrpl4his7*, and *cbp3his7* cells were analyzed by Western blotting using the indicated antibodies. MM, molecular mass.

mRNAs, we used strains carrying intronless mitochondrial genomes. Thus, the mRNAs are present in constant quantities, allowing us to assess their translation independent of the splicing processes. Mitochondrial translation products of wild type or cells lacking *CBP3* were pulse labeled with [^{35}S]methionine. After 15 min, labeling was stopped, and incubation was continued for up to 2.5 h. This approach revealed that both cell types can synthesize all mitochondrial translation products (Fig. 2 C), but cytochrome *b* was less efficiently labeled in Δ*cbp3* cells (Fig. 2 C, right). All newly synthesized proteins in wild-type cells were stable for at least 2.5 h (Fig. 2 D, left). In contrast, newly synthesized cytochrome *b* of the Δ*cbp3* strain was completely degraded after 30 min (Fig. 2 D, right). Atp6 and Cox1 were also affected in this strain, but this did not impair accumulation of ATPase and cytochrome *c* oxidase (Fig. 2 F), indicating that their biogenesis does not depend on Cbp3.

The reduced stability of newly synthesized cytochrome *b* could be either a result of a specific defect caused by the absence of Cbp3 or a result of a general defect in the assembly of cytochrome *b*. We therefore analyzed the fate of mitochondrial translation products in cells impaired in the assembly of the *bc₁* complex. We chose to characterize a Δ*cbp4* strain. Cbp4 is an assembly factor of the *bc₁* complex and interacts with Cbp3 (Kronekova and Rödel, 2005). In contrast to the situation in the absence of Cbp3, the newly synthesized cytochrome *b* is rather stable in Δ*cbp4* cells (Fig. 2 E, right). This indicates that defects in the assembly of the *bc₁* complex do not generally provoke a

rapid degradation of newly synthesized cytochrome *b*. Therefore, it appears that Cbp3 is particularly required to protect newly synthesized cytochrome *b* from proteolytic degradation.

We next assessed the levels of mitochondrial proteins that accumulate in cells lacking Cbp3 or Cbp4. Subunits of cytochrome *c* oxidase (Cox2) and ATPase (Atp4) were not affected in both mutants (Fig. 2 F). Importantly, both mutants failed to accumulate *bc₁* complex subunits (Cyt*b* and Qcr7) to wild-type levels (Fig. 2 F). This indicates a defect in the assembly of the *bc₁* complex that results in degradation of the nonassembled subunits. However, in light of the rapid proteolysis of cytochrome *b* observed only in Δ*cbp3* cells, it appears that Cbp3 and Cbp4 have different functions during the assembly of cytochrome *b*.

Cbp3, but not Cbp4, is critical for efficient translation of the cob::ARG8^m mRNA

The low level of labeling of cytochrome *b* during the pulse kinetics in the Δ*cbp3* mutant (Fig. 2 C) could be explained by either a rapid turnover of the newly synthesized protein or by a direct impairment of translation of its messenger, the *COB* mRNA. To differentiate between both possibilities, we used a system that allows following the synthesis of a reporter protein that does not require a functional *bc₁* complex assembly machinery. Translational control in yeast mitochondria is mainly exerted on the 5' UTRs of the mRNA (Costanzo and Fox, 1988). To analyze whether the absence of Cbp3 or Cbp4 has a direct influence on *COB* mRNA translation, we used a strain with a mitochondrial
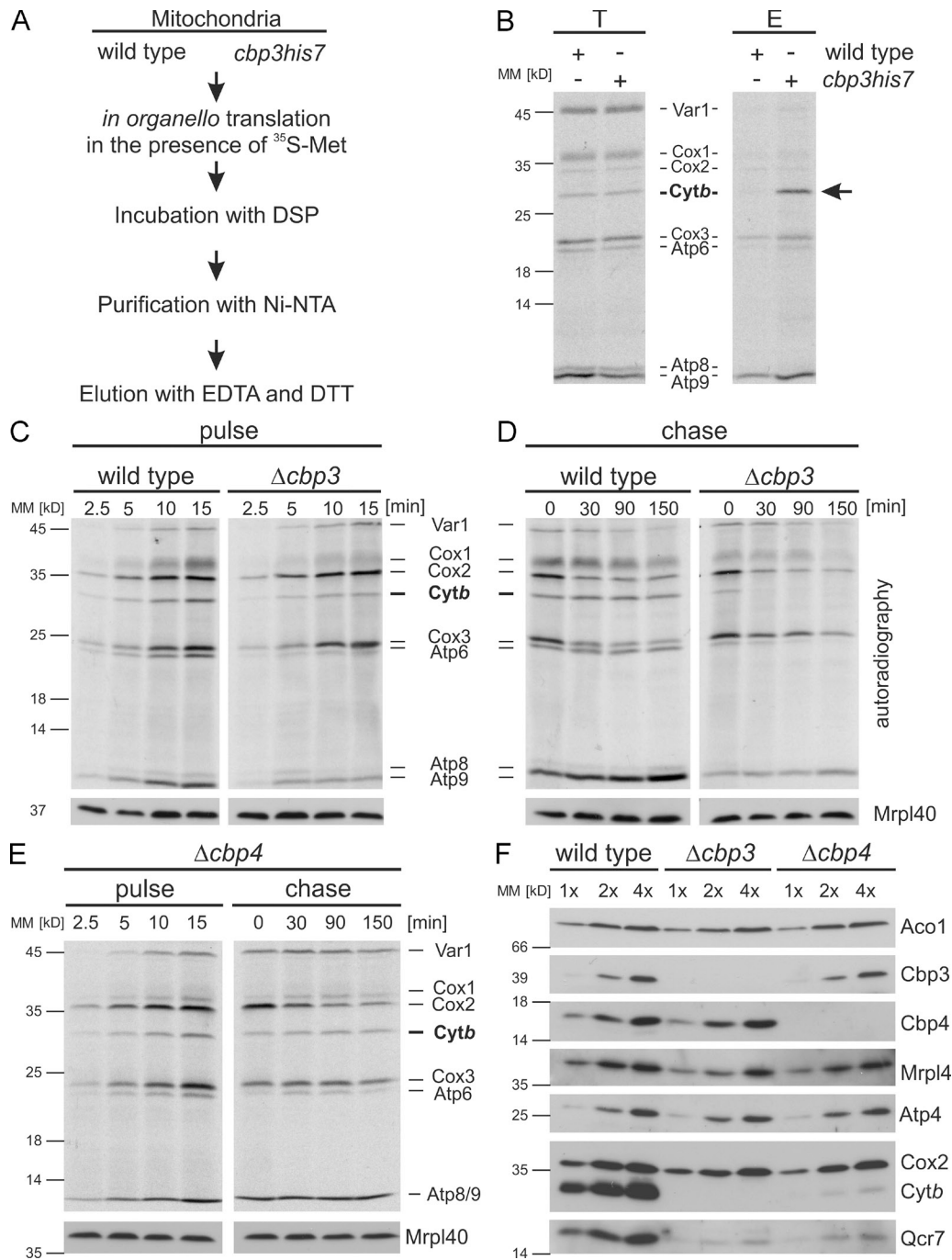
Figure 2. **Cbp3 stabilizes newly synthesized cytochrome *b*.** (A) An experimental procedure to analyze the contact of Cbp3 to mitochondrial translation products. (B) Cbp3 can be cross-linked to newly synthesized cytochrome *b*. Translation products of mitochondria isolated from wild-type or *cbp3his7* cells were labeled with [^{35}S]methionine in the presence of the cleavable cross-linking reagent DSP and purified on Ni-NTA beads. The samples were analyzed by autoradiography. The black arrow indicates the newly synthesized cytochrome *b* that is copurified with Cbp3His7. E, elution after Ni-NTA purification; T, 1% total before Ni-NTA purification. (C) Mitochondrial translation products of wild-type or Δ*cbp3* cells were labeled with [^{35}S]methionine in the presence of cycloheximide to block cytosolic protein synthesis. Samples were taken after the indicated time points and subjected to alkaline lysis followed by SDS-PAGE and autoradiography. A Western blot using anti-Mrpl40 antibody served as a loading control. (D) Newly synthesized cytochrome *b* is destabilized in the absence of Cbp3. Radiolabeled translation products of the indicated strains were followed after the labeling had been stopped by addition of unlabeled methionine. (E) Cytochrome *b* is less efficiently labeled in Δ*cbp4* cells but is stable for up to 2.5 h. The experiments described in C and D were repeated with Δ*cbp4* cells. (F) The absence of Cbp3 and Cbp4 reduces the levels of components of the bc_1 complex. Increasing amounts of proteins of wild-type, Δ*cbp3*, and Δ*cbp4* cells were analyzed by Western blotting using the indicated antibodies. MM, molecular mass.

genome in which the coding sequence of cytochrome *b* is replaced by the coding sequence of Arg8. Hence, Arg8 is synthesized on mitochondrial ribosomes from an mRNA that contains the 5′ and 3′ UTRs of cytochrome *b* (*COB-ARG8^m-COB*).

Arg8 is a soluble protein of the mitochondrial matrix that is required for biosynthesis of arginine. Because the nuclear version of *ARG8* is deleted in this strain, growth on media lacking arginine (SD-arginine) can be used to score for the ability to
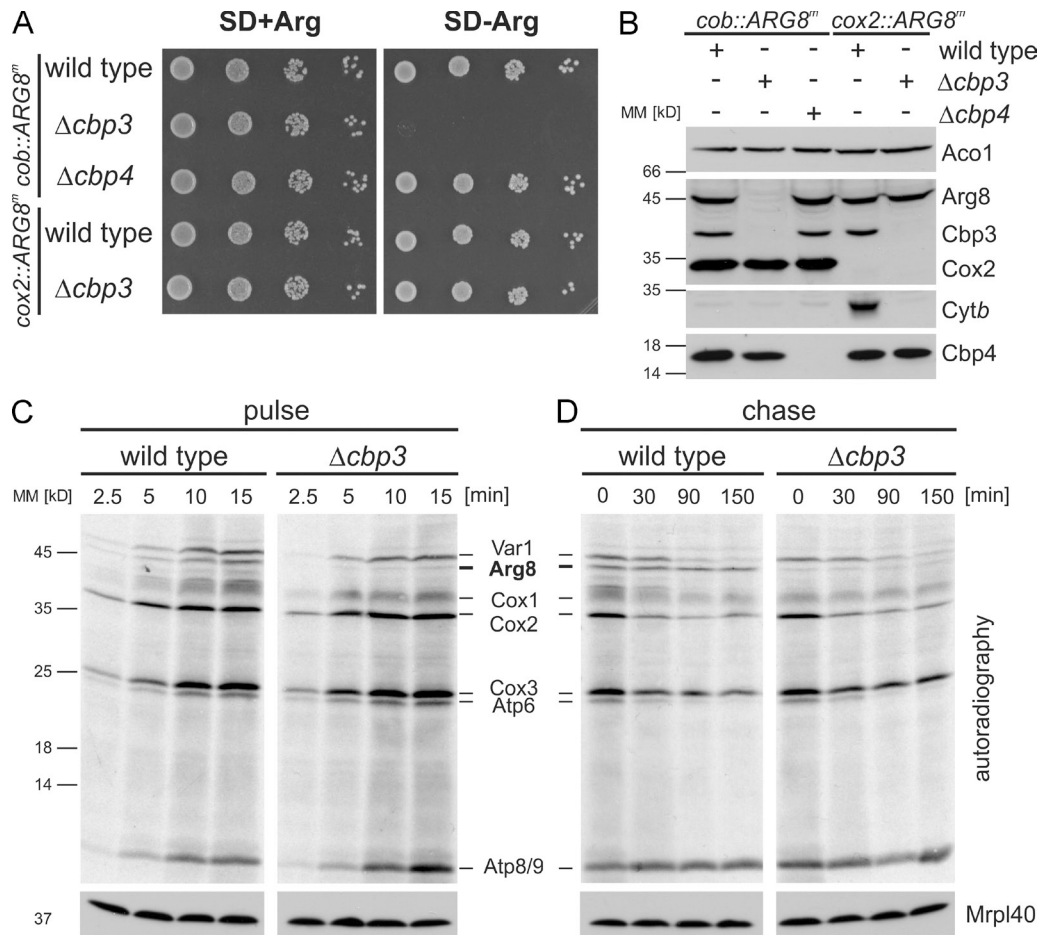
Figure 3. **Cbp3, but not Cbp4, is critical for efficient translation of the *cob::ARG8^m* mRNA.** (A) Growth test of wild-type, Δ*cbp3*, or Δ*cbp4* yeast harboring mitochondrial genomes where a recoded version of Arg8 was inserted into the mitochondrial genome replacing the ORF of Cyt*b* (*cob::ARG8^m*) or Cox2 (*cox2::ARG8^m*). Cells were plated in serial 10-fold dilutions onto media containing or lacking arginine and incubated for 2 d. SD, synthetic medium supplemented with glucose. (B) Western blot analysis of steady-state levels of proteins from strains described in A. (C and D) The absence of Cbp3 in cells harboring the *cob::ARG8^m* mitochondrial genome leads to a strong decrease in the synthesis of Arg8 (C) but does not affect its stability (D). Mitochondrial translation products from *cob::ARG8^m* wild-type or Δ*cbp3* cells were labeled with [^35S]methionine as described in Fig. 2 C. Western blotting using anti-Mrpl40 antibody served as a loading control. MM, molecular mass.

translate the *COB-ARG8^m-COB* mRNA. Deletion of *CBP4* in this background did not impair Arg8 synthesis because the cells could grow similar to the wild type in media with or without arginine (Fig. 3 A). In contrast, the absence of *CBP3* impaired the growth on media lacking arginine (Fig. 3 A), indicating that Cbp3, but not Cbp4, is required for efficient synthesis and accumulation of this reporter (Fig. 3 B).

To check whether Cbp3 is also required for the synthesis of Arg8 when translated from an mRNA containing *COB*-unrelated flanking regions, we deleted *CBP3* in a strain in which the coding sequence of *ARG8^m* was inserted in place of the coding sequence of *COX2* (Bonnefoy and Fox, 2000). Here, deletion of *CBP3* had no influence on growth requiring expression of this gene (Fig. 3 A). Consistent with the growth phenotypes, deletion of *CBP3* in cells with a *cob::ARG8^m* mitochondrial genome provoked a substantial decrease in the steady-state levels of Arg8, whereas there was no effect when cells contained the *cox2::ARG8^m* mitochondrial genome (Fig. 3 B).

To further substantiate this finding, we analyzed mitochondrial protein synthesis in cells containing the *cob::ARG8^m*

mitochondrial genome (Fig. 3 C). In wild-type cells, Arg8 is efficiently made, whereas the absence of Cbp3 leads to a strong decrease in Arg8 production (Fig. 3 C). In contrast to newly synthesized cytochrome *b* that is unstable in the absence of Cbp3 (Fig. 2 D), the small quantities of Arg8 that are synthesized are stable (Fig. 3 D). Collectively, these data indicate that Cbp3 plays an unexpected role in promoting translation of mRNAs that contain the 5′ and 3′ UTRs of *COB* mRNA.

Cbp3 forms a complex with Cbp6

Because a role of Cbp3 in cytochrome *b* synthesis was not shown before, we asked whether Cbp3 might cooperate with the already identified translational activators of the *COB* mRNA, namely Cbs1, Cbs2, and Cbp6 (Rödel, 1997). To investigate whether one of them would interact with Cbp3, we purified Cbs1, Cbs2, and Cbp6 under native conditions using His7-tagged variants of the proteins (Fig. 4 A). While no Cbp3 was enriched on Cbs1PAHis7 or Cbs2PAHis7, it was efficiently copurified with Cbp6His7. To confirm this interaction, we repeated
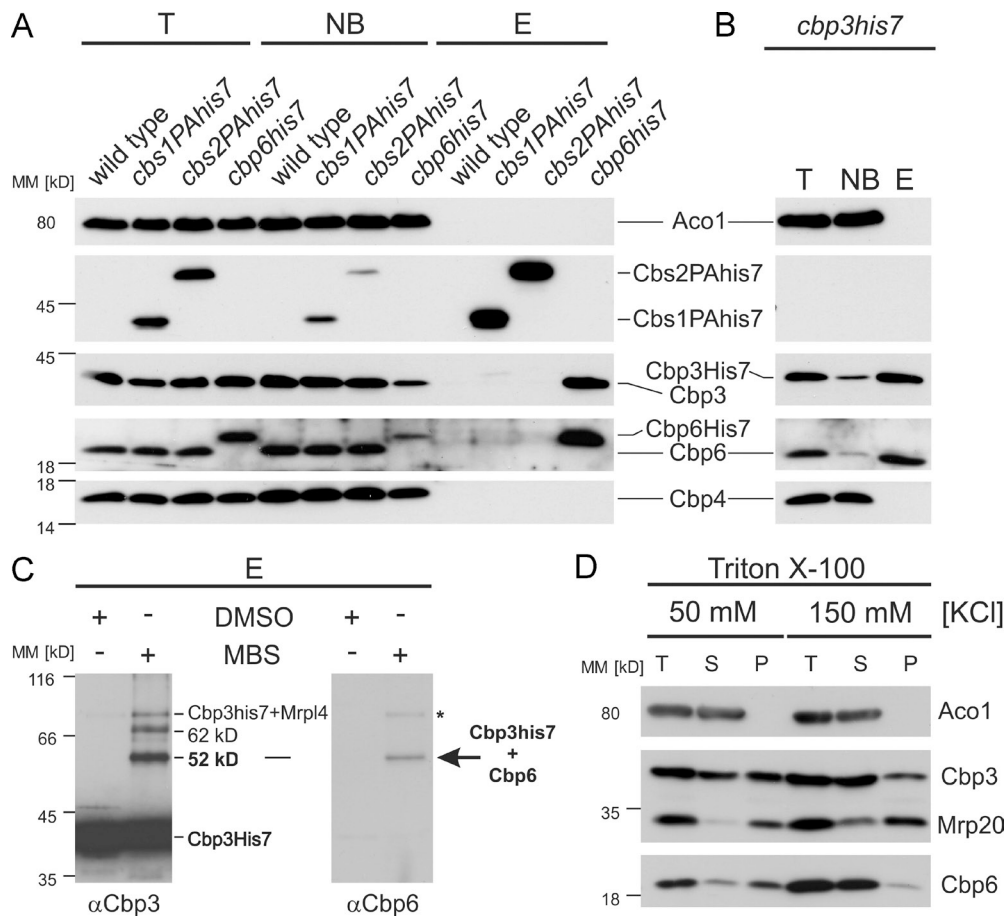
Figure 4. **Cbp3 forms a complex with Cbp6.** (A) Native purification of Cbs1, Cbs2, and Cbp6 complexes. Cbs1 and Cbs2 were equipped with C-terminal ProteinAHis7 tags to allow sensitive detection as well as purification of the proteins via metal affinity chromatography, whereas Cbp6 was expressed with a C-terminal His7 tag. Triton X-100 lysates of the indicated mitochondria were subjected to Ni-NTA chromatography, and the resulting fractions were analyzed by Western blotting. E, elution after Ni-NTA purification; NB, 20% of unbound material after Ni-NTA purification; PAHis7 tag, ProteinA-heptahistidine tag; T, 20% total before Ni-NTA purification. (B) Cbp6 is efficiently purified with Cbp3His7. Native purification of the Cbp3 complex from *cbp3his7* mitochondria was performed as described in A. (C) Cbp3 and Cbp6 can be cross-linked to each other. Mitochondria harboring Cbp3His7 were incubated with the cross-linker MBS or a control (DMSO). Cbp3 and cross-linked proteins were purified as described in Fig. 1 B. The elution fractions were analyzed by Western blotting using antibodies against Cbp3 (left) and Cbp6 (right). The asterisk indicates the cross-link of Mrpl4 to Cbp3His7. (D) Cbp6 co-migrates with mitochondrial ribosomes in a salt-sensitive manner. Triton X-100 lysates of wild-type mitochondria were fractionated as described in Fig. 1 C. MM, molecular mass; P, pellet; S, supernatant; T, 100% total before ribosome fractionation.

the experiment using mitochondria from a strain expressing Cbp3His7 and found that Cbp6 was efficiently copurified (Fig. 4 B). In contrast, Cbp4 did not copurify with the Cbp3–Cbp6 complex under these conditions (Fig. 4, A and B).

Because we could efficiently copurify both Cbp3 and Cbp6 in one complex, we asked whether it was possible to confirm this interaction with an independent approach. We therefore repeated the cross-linking analysis with Cbp3His7 (Fig. 1 B) and decorated the elution fractions with Cbp6 antibodies. This analysis revealed that the band migrating at 52 kD is a cross-linking product of Cbp6 and Cbp3His7 (Fig. 4 C), substantiating that both factors interact with each other in mitochondria. Next, we tested whether Cbp6 co-migrates with Cbp3 and mitochondrial ribosomes upon centrifugation of mitochondrial lysates through a high density sucrose cushion. Similar to Cbp3, Cbp6 cosedimented in a salt-sensitive manner with ribosomes (Fig. 4 D). We therefore concluded that Cbp3 interacts with Cbp6 in a complex that binds to mitochondrial ribosomes.

Synthesis and assembly of cytochrome *b* requires an intact Cbp3–Cbp6 complex

We next deleted *CBP6* in the strain containing the intronless mitochondrial genome and analyzed synthesis and stability of mitochondrially encoded proteins. The absence of Cbp6 provoked defects in both the production and stability of cytochrome *b* (Fig. 5 A) that were indistinguishable from the defects observed in the absence of Cbp3 (Fig. 2, C and D). Next, we deleted *CBP6* in the strain containing the *cob::ARG8^m* mitochondrial genome and found that the absence of *CBP6* reduces growth on media requiring translation of the *COB-ARG8^m-COB* mRNA (Fig. 5 B). Concordantly, synthesis of Arg8 in this strain was significantly reduced (Fig. 5 C), confirming the primary hypothesis that Cbp6 is a translational activator of the *COB* mRNA (Dieckmann and Tzagoloff, 1985).

Because the phenotypes of disruption of either *CBP3* or *CBP6* are strikingly similar, we asked whether both proteins fulfill the same functions or whether they have distinct roles in the biogenesis of cytochrome *b*. Because both proteins interact

Figure 5. **Deletion of *CBP6* provokes a phenotype similar to that of Δ*cbp3* cells.** (A) Cytochrome *b* is destabilized in the absence of Cbp6. Mitochondrial translation products from wild-type or Δ*cbp6* cells were analyzed as described in Fig. 2 (C and D). (B) Cbp6 is critical for efficient translation of a *cob:: ARG8^m* reporter. Cells of wild type, Δ*cbp3*, or Δ*cbp6* harboring the modified mitochondrial genome described in Fig. 3 A were plated in serial 10-fold dilutions onto synthetic media (SD) containing or lacking arginine and incubated for 2 d. (C) The absence of Cbp6 impairs translation of *COB-ARG8^m-COB* mRNA. Mitochondrial translation products of wild-type and Δ*cbp6 cob::ARG8^m* cells were labeled with [^35S]methionine and analyzed by autoradiography. MM, molecular mass.

in one complex, we first tested whether they are required to stabilize each other. When analyzing Cbp3 and Cbp6 in strains in which one of both components was missing, we found that the absence of Cbp3 destabilizes Cbp6 and vice versa (Fig. 6 A). In contrast, both proteins remained stable in the absence of Cbp4 (Fig. 6 A). Moreover, the absence of either Cbp3 or Cbp6 had no influence on the stability of the other *COB* mRNA–specific translational activators Cbs1 and Cbs2 (Fig. 6 B).

To investigate whether increased amounts of Cbp3 or Cbp6 could complement the deletion of *CBP6* and *CBP3*, respectively, we transformed plasmids allowing the overexpression of *CBP3* and *CBP6* into Δ*cbp3* and Δ*cbp6* cells that contain either wild-type (intronless) or *cob::ARG8^m* mitochondrial genomes. Next, cells were scored for expression of cytochrome *b* or the *cob:: ARG8^m* reporter (Fig. 6, C and D). Although the plasmids encoding *CBP3* or *CBP6* could restore accumulation of cytochrome *b* and Arg8 of the respective deletion mutant, overexpression of

either *CBP6* in Δ*cbp3* cells or *CBP3* in Δ*cbp6* had no effect on steady-state levels of cytochrome *b* or Arg8 (Fig. 6, C and D). These observations point to an obligatory shared function of both proteins in synthesis and assembly of cytochrome *b*.

Interaction of Cbp3 and Cbp6 with the ribosome requires formation of a Cbp3–Cbp6 complex

Next, we investigated whether Cbp3 or Cbp6 can interact independently of each other with mitochondrial ribosomes. We therefore lysed mitochondria of these strains with Triton X-100 and tested for co-migration of Cbp3 and Cbp6 with ribosomes. Although in wild-type mitochondria both factors interacted with ribosomes, Cbp3 and Cbp6 were unable to bind to ribosomes in Δ*cbp6* and Δ*cbp3* cells, respectively (Fig. 7 A). We therefore concluded that Cbp3 and Cbp6 can only interact with mitochondrial ribosomes when both proteins form a complex.
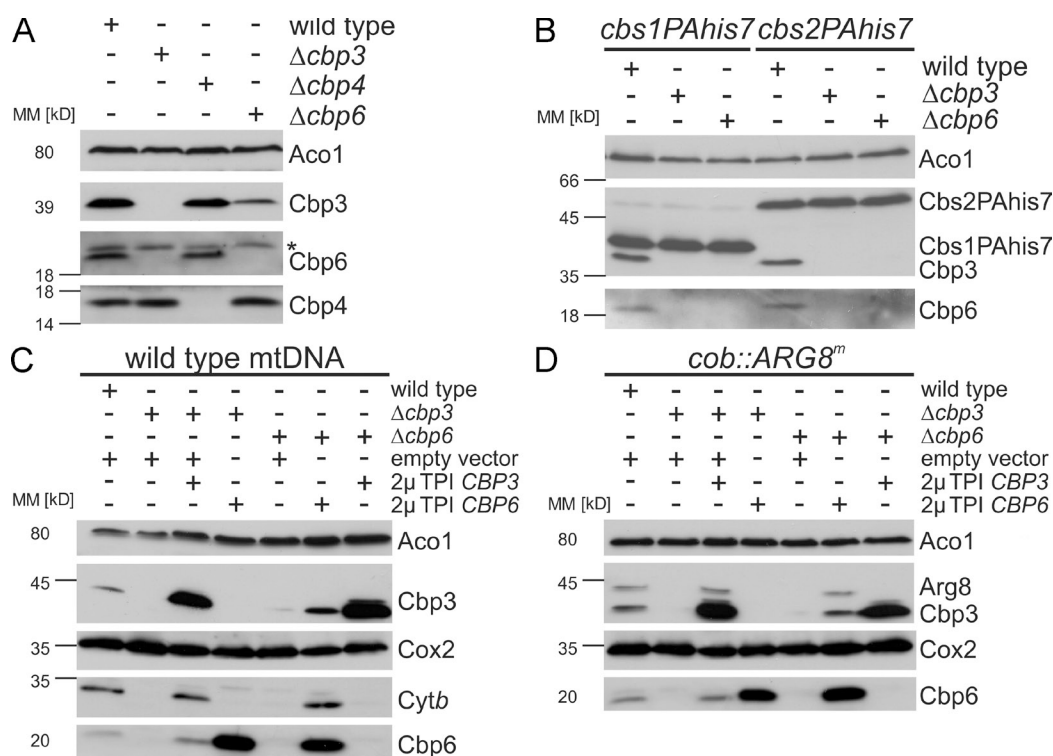
Figure 6. **Cbp3 and Cbp6 act in one complex.** (A) Cbp3 is destabilized when Cbp6 is absent and vice versa. Cell lysates of the indicated yeast strains were analyzed by Western blotting using the indicated antibodies. The asterisk indicates an unspecific cross-reaction of the Cbp6 antibody. (B) The levels of the other translational activators of the *COB* mRNA, Cbs1 and Cbs2, are not affected when *CBP3* or *CBP6* is deleted. Cells expressing ProteinAhis7-tagged variants of Cbs1 or Cbs2 were lysed and analyzed by Western blotting. (C and D) Overexpression of either *CBP3* or *CBP6* cannot complement deletion of the other. The indicated yeast strains harboring the wild-type (C) or the *cob::ARG8^m* mitochondrial DNA (D) were transformed with 2µ plasmids encoding *CBP3* or *CBP6* under control of the *TPI* promoter or an empty vector. Cell lysates were analyzed by Western blotting using the indicated antibodies. MM, molecular mass.

We next asked whether the diminished synthesis of cytochrome *b* in *Δcbp3* and *Δcbp6* cells is caused by a destabilization of the *COB* mRNA. Cbp1 is a protein required for the stabilization and translation of the *COB* transcript (Dieckmann et al., 1984; Islas-Osuna et al., 2002) and thus for synthesis of cytochrome *b*. We deleted *CBP1*, *CBP3*, and *CBP6* in cells with an intron-containing mitochondrial genome and analyzed the relative amounts of *COB* mRNA in these cells. The *COB* transcript failed to accumulate in the absence of Cbp1 (Fig. 7 B; Dieckmann et al., 1982), thereby also accounting for the decrease in mature *COX1* mRNA that requires an intron of the *COB* mRNA precursor for maturation (Dhawale et al., 1981; De La Salle et al., 1982). In contrast, the *COB* mRNA is stable in cells lacking Cbp3 or Cbp6, excluding that the defect to synthesize cytochrome *b* in these strains is caused by lower amounts of the mRNA.
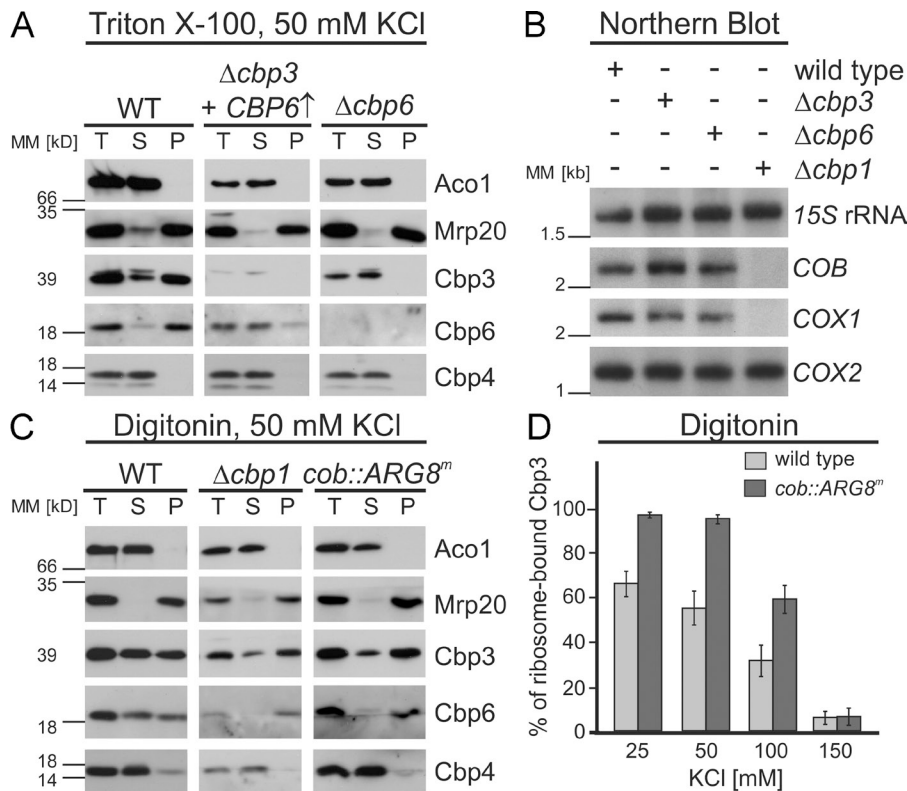
Interaction of the Cbp3–Cbp6 complex with the ribosome is influenced by the presence of cytochrome *b*

Although the Cbp3–Cbp6 complex was almost quantitatively recovered with ribosomes when mitochondria were lysed with Triton X-100 (Fig. 1 C), we observed that a substantial quantity of the complex did not cofractionate with ribosomes when mitochondria were lysed with the mild detergent digitonin (Fig. 7 C). This behavior could reflect a Cbp3–Cbp6 complex

that contains additional proteins and is preserved in digitonin but disrupted by the more stringent detergent Triton X-100. To characterize this digitonin-stable complex in more detail, we asked whether the binding of Cbp3–Cbp6 to ribosomes is altered when cytochrome *b* cannot be produced. We therefore prepared mitochondria from strains lacking *CBP1* (and as a consequence *COB* mRNA) and from a strain carrying the *cob::ARG8^m* mitochondrial genome that cannot synthesize cytochrome *b* but contains an mRNA with the 5′ and 3′ UTRs of *COB*. In contrast to the wild-type situation, the Cbp3–Cbp6 complex was mainly ribosome bound in mitochondria from these two mutants (Fig. 7 C). On the contrary, Cbp4 was mainly found in the soluble fraction of mitochondria from both mutants and the wild type (Fig. 7 C). These results show that the presence of the *COB* mRNA or its translation product cytochrome *b* is not required for binding of the Cbp3–Cbp6 complex to mitochondrial ribosomes.

To further characterize the divergent binding behavior of the Cbp3–Cbp6 complex, we tested the salt sensitivity of Cbp3–Cbp6 co-migration with ribosomes of the wild type and the strain with the *cob::ARG8^m* mitochondrial genome. Increasing the salt concentration from 25 to 150 mM KCl provoked a detachment of Cbp3 from ribosomes in the presence or absence of cytochrome *b* synthesis. Importantly, more Cbp3 was found at the ribosome when cytochrome *b* was absent. Collectively, these data show that the Cbp3–Cbp6 complex interacts with

A Triton X-100, 50 mM KCl

	WT	Δcbp3 + CBP6↑	Δcbp6
MM [kD]	T S P	T S P	T S P

66
35 — Aco1
Mrp20
39 — Cbp3
18 — Cbp6
18
14 — Cbp4

C Digitonin, 50 mM KCl

	WT	Δcbp1	cob::ARG8ᵐ
MM [kD]	T S P	T S P	T S P

66
35 — Aco1
Mrp20
39 — Cbp3
18 — Cbp6
18
14 — Cbp4

B Northern Blot

+	-	-	-	wild type
-	+	-	-	Δcbp3
-	-	+	-	Δcbp6
MM [kb] -	-	-	+	Δcbp1

1.5 — 15S rRNA
2 — COB
2 — COX1
1 — COX2

D Digitonin

□ wild type ■ cob::ARG8ᵐ

y-axis: % of ribosome-bound Cbp3 (0, 20, 40, 60, 80, 100)
x-axis: KCl [mM] (25, 50, 100, 150)

Figure 7. **Interaction of Cbp3 and Cbp6 with the ribosome requires formation of a Cbp3–Cbp6 complex and is influenced by cytochrome b.** (A) Cbp3 and Cbp6 cannot bind to mitochondrial ribosomes in the absence of the other factor. Triton X-100 lysates of mitochondria of the indicated strains were fractionated as described in Fig. 1 C in the presence of 50 mM KCl. Fractions were analyzed by Western blotting. Because Cbp6 is not detectable in the Δcbp3 strain, mitochondria from Δcbp3 cells overexpressing CBP6 were used. (B) COB mRNA is absent in Δcbp1 but not in Δcbp3 and Δcbp6 cells. Total RNA was isolated from mitochondria of the indicated strains and analyzed by Northern blotting. 15S ribosomal RNA served as a loading control. (C) The Cbp3–Cbp6 complex can bind to mitochondrial ribosomes in the absence of cytochrome b but is mainly present in a non–ribosome-bound form in the wild type (WT). Mitochondria of the indicated strains were lysed with digitonin in the presence of 50 mM KCl and fractionated as described in Fig. 1 C. P, pellet; S, supernatant; T, 100% total before ribosome fractionation. (D) Cbp3 is more tightly bound to ribosomes of mitochondria harboring a cob::ARG8ᵐ mitochondrial genome and is released upon treatment with increasing salt concentrations. Mitochondria of wild-type and cob::ARG8ᵐ cells were lysed with digitonin and increasing KCl concentrations as indicated on the x axis and fractionated as in Fig. 1 C. The percentage of Cbp3 that was found in the pellet fraction was determined densitometrically from three independently performed experiments. The error bars represent the standard deviation of these experiments. MM, molecular mass.

ribosomes in a cytochrome b–dependent fashion. Because Cbp3 can directly interact with cytochrome b (Fig. 2 B), it is likely that the non–ribosome-bound form represents an assembly intermediate of the bc₁ complex.

The Cbp3–Cbp6 complex is released from ribosomes upon cytochrome b binding

The finding that the Cbp3–Cbp6 complex also exists in a non–ribosome-bound state inspired us to characterize its composition in the presence or absence of cytochrome b. We therefore purified the complex from mitochondria of cbp3his7 strains containing or lacking CBP1. When the complex was purified from Triton X-100 lysates, Cbp6 was efficiently copurified, and the interaction was not changed by the absence of COB mRNA (Fig. 8 A). Cbp4 was again not purified with Cbp3 from Triton X-100 lysates (Figs. 4 A and 8 A). Next, we purified the complex using digitonin as detergent. Under these conditions, Cbp6, Cbp4, and cytochrome b were purified with Cbp3His7 from wild-type mitochondria (Fig. 8 B). Importantly, Rip1, a subunit of the bc₁ complex, was not copurified, suggesting that the Cbp3–Cbp6–Cbp4–cytochrome b complex reflects an assembly intermediate of the bc₁ complex. However, the absence of COB mRNA provoked a loss of interaction of Cbp4 with the Cbp3His7–Cbp6 complex (Fig. 8 B), indicating that Cbp4 binds to the Cbp3–Cbp6 complex only when cytochrome b can be synthesized.

Next, we aimed to identify the topology of the Cbp3–Cbp6 complex containing cytochrome b and Cbp4. We first extracted mitochondrial membranes with either high salt or sodium carbonate. The Cbp3–Cbp6 complex and Cbp4 could not be extracted from the membrane with high salt (Fig. 8 C, left). When membranes were extracted with alkaline solution, Cbp3 was recovered in the soluble fraction, whereas Cbp4 and Cbp6 remained in the pellet, indicating that both proteins interact more tightly with the membrane than Cbp3 (Fig. 8 C, right). Cbp6 does not contain a predictable transmembrane segment, whereas Cbp4 has such a domain close to the N terminus followed by a large hydrophilic region. We next asked how Cbp4 and Cbp6 might be oriented in the membrane. To answer this, we exposed mitochondria or mitoplasts (mitochondria whose outer membranes are ruptured by hypotonic swelling) to proteinase K treatment. Cbp3 and Cbp6 were protected from proteolytic degradation (Fig. 8 D), demonstrating that both proteins are located in the mitochondrial matrix. Cbp4 was degraded in mitoplasts, showing that large parts of the protein are present in the intermembrane space (Fig. 8 D, right).

The presence of Cbp4 in the cytochrome b–containing Cbp3–Cbp6 complex inspired us to test whether addition of Cbp4 might induce release of the Cbp3–Cbp6 complex from ribosomes. We therefore first checked whether an interaction of Cbp3 with cytochrome b occurs in the absence of Cbp4.
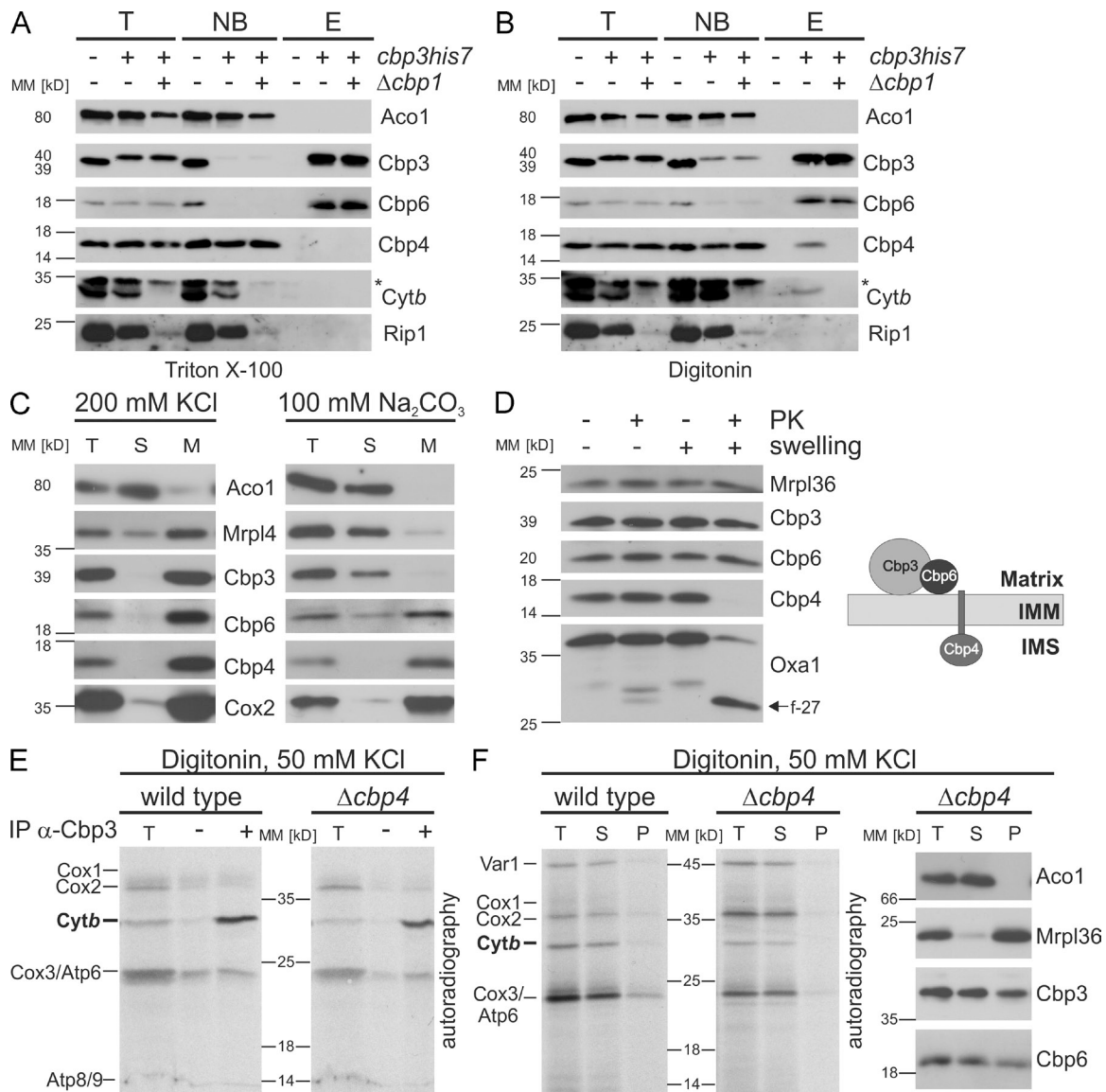
Figure 8. **The Cbp3–Cbp6 complex is released from ribosomes upon cytochrome *b* binding.** (A and B) Cbp4 interacts with the Cbp3–Cbp6 complex only in the presence of cytochrome *b*. Mitochondria of the indicated strains were lysed with Triton X-100 (A) or digitonin (B), and Cbp3His7 was purified under native conditions as described in Fig. 4 A. The asterisks indicate a nonspecific cross-reaction of the antibody against cytochrome *b*. E, elution after Ni-NTA purification; NB, 20% of unbound material after Ni-NTA purification; T, 20% total before Ni-NTA purification. (C) Cbp6 and Cbp4 are tightly associated with the inner mitochondrial membrane (IMM). Mitochondrial membranes were extracted with high salt (left) or carbonate (right) as described in the Materials and methods section. M, membrane fraction. (D) Cbp3 and Cbp6 are located in the mitochondrial matrix, whereas Cbp4 faces the intermembrane space (IMS). Wild-type mitochondria were incubated in isoosmotic or hypoosmotic buffers (swelling) and treated with proteinase K (PK) or left untreated. The arrow points to a fragment of Oxa1 (f-27) that confirms efficient swelling. (right) A model of the topology of Cbp3, Cbp6, and Cbp4. (E) The Cbp3–Cbp6 complex can bind newly synthesized cytochrome *b* in the absence of Cbp4. Translation products from wild-type and Δ*cbp4* mitochondria were labeled with [^{35}S]methionine. The organelles were reisolated, lysed with digitonin, and subjected to coimmunoprecipitation with antibodies against Cbp3 or preimmune serum. The samples were analyzed by autoradiography. T, 5% total before immunoprecipitation (IP). (F) The Cbp3–Cbp6 complex is mainly present in a non–ribosome-bound state in the absence of Cbp4, and newly synthesized cytochrome *b* is liberated from ribosomes independent of Cbp4. Translation products from wild-type and Δ*cbp4* mitochondria were labeled with [^{35}S]methionine. Mitochondria were reisolated, lysed with digitonin, and fractionated as described in Fig. 1 C in the presence of 50 mM KCl. Samples were analyzed by autoradiography (left) and Western blotting using the indicated antibodies (right). MM, molecular mass; P, pellet; S, supernatant.

Translation products of mitochondria from wild-type and Δ*cbp4* cells were labeled with [^{35}S]methionine. Next, mitochondria were lysed with digitonin, and proteins associated with Cbp3 were precipitated with antibodies against Cbp3. This approach revealed that the Cbp3–Cbp6 complex interacts with newly synthesized cytochrome *b* even in the absence of Cbp4 (Fig. 8 E). This newly synthesized cytochrome *b* was

similarly released from ribosomes in the presence or absence of Cbp4 (Fig. 8 F, left). Moreover, when we checked for ribosome co-migration of the Cbp3–Cbp6 complex in digitonin lysates, we found that a comparable fraction of the complex was present in the supernatant of mitochondria from Δ*cbp4* and wild-type cells (Figs. 7 C and 8 F, right). We therefore conclude that it is the binding of cytochrome *b* to the
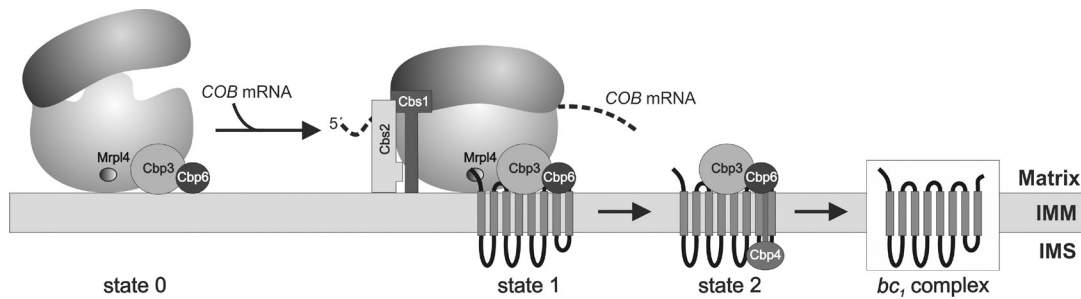
Figure 9. Hypothetical model for the role of the Cbp3–Cbp6 complex during biogenesis of cytochrome b. In the absence of an mRNA, the Cbp3–Cbp6 complex is bound to mitochondrial ribosomes (state 0). *COB* mRNA binds to the ribosome, and translation can only be initiated efficiently when the Cbp3–Cbp6 complex is ribosome bound (state 1). Cbp3 and Cbp6 then interact with the newly synthesized cytochrome *b.* Cbp4 is recruited (state 2), and this complex mediates the assembly of cytochrome *b* into a functional *bc₁* complex. IMM, inner mitochondrial membrane; IMS, intermembrane space.

Cbp3–Cbp6 complex (but not the interaction of Cbp4 with the Cbp3–Cbp6–cytochrome *b* complex) that triggers release of the complex from the ribosome.

Discussion

In this study, we have shown that the Cbp3–Cbp6 complex is a novel ligand of the tunnel exit of the yeast mitochondrial ribosome with a dual role in the biogenesis of cytochrome *b.* Our data support a working model (Fig. 9) in which dynamic interactions of this complex with the ribosome and newly synthesized cytochrome *b* allow a coordinated synthesis and assembly of this central component of the *bc₁* complex. The Cbp3–Cbp6 complex can bind to the mitochondrial ribosome in the absence of cytochrome *b* synthesis (Fig. 9, state 0), and ribosome binding is required for efficient translation of the *COB* mRNA (Fig. 9, state 1). Upon synthesis of cytochrome *b,* the Cbp3–Cbp6 complex interacts with the newly synthesized protein, and this provokes liberation of the complex from the ribosome (Fig. 9, state 2). By binding to the tunnel exit of mitochondrial ribosomes, the Cbp3–Cbp6 complex is well positioned to allow an efficient interaction with the newly synthesized cytochrome *b.* This interaction of Cbp3–Cbp6 with cytochrome *b* is important to protect the newly synthesized polypeptide from proteolytic degradation and to promote its assembly. Interestingly, Cbp4 is part of the Cbp3–Cbp6–cytochrome *b* complex. Cbp4 exposes a large soluble domain into the intermembrane space. It is therefore possible that Cbp4 is part of an assembly step that acts on cytochrome *b* from the intermembrane space side of the inner membrane.

In addition to an involvement in assembly of the *bc₁* complex, the Cbp3–Cbp6 complex plays an important role in promoting synthesis of cytochrome *b.* The Cbp3–Cbp6 complex shares the latter function with Cbs1 and Cbs2, two factors acting on the 5′ UTR of the *COB* mRNA (Rödel, 1986; Rödel and Fox, 1987). Although the binding sites of Cbs1 and Cbs2 on the ribosome (Krause-Buchholz et al., 2004, 2005) are still enigmatic, we show here that the Cbp3–Cbp6 complex binds in the vicinity of the tunnel exit. This interaction with the ribosome is important to allow efficient synthesis of cytochrome *b.* At present, we can only speculate about how binding of the complex to the tunnel exit influences cytochrome *b* synthesis. A possible scenario is that the long 5′ UTR of the *COB* mRNA contacts the

Cbp3–Cbp6 complex. Consequently, such a contact might be necessary for efficient initiation of translation.

To date, not much is known about the molecular functions of translational activators in mitochondria. Genetic evidence suggests that at least some of them are implicated in initiation (Green-Willms et al., 1998; Nouet et al., 2007; Williams et al., 2007), in line with reported interactions of these factors with the small ribosomal subunit (McMullin et al., 1990; Haffter et al., 1991; Williams et al., 2005, 2007). In addition, recent studies showed that translational activators can regulate protein synthesis in the context of respiratory chain assembly, and feedback loops have been identified for both Cox1 (Perez-Martinez et al., 2003; Barrientos et al., 2004) and Atp6 (Rak and Tzagoloff, 2009). This is best understood for the case of Cox1 synthesis that specifically requires two translational activators, Pet309 and Mss51 (Mick et al., 2011). Apart from activating translation of the *COX1* mRNA, Mss51 also mediates assembly of the newly synthesized Cox1. In case of a blocked cytochrome *c* oxidase assembly, Mss51 is sequestered in an assembly intermediate and is therefore not available to activate a new round of *COX1* translation. By this mechanism, synthesis of Cox1 is adjusted to levels that can successfully be incorporated into the enzyme.

The Cbp3–Cbp6 complex might work in a similar way to Mss51 because it promotes both synthesis and assembly of cytochrome *b.* Its impact on cytochrome *b* synthesis is nevertheless different than the situation of *COX1* translation because, here, deletion of Mss51 results in a complete block of Cox1 synthesis (Perez-Martinez et al., 2003), whereas translation of *COB* mRNA can still proceed in the absence of Cbp3 and Cbp6, albeit at reduced levels. Further work will reveal whether synthesis of cytochrome *b* is influenced by the efficiency of *bc₁* complex assembly.

The tunnel exit of cytoplasmic ribosomes is the site where a variety of general biogenesis factors accept the newly synthesized proteins for further maturation (Kramer et al., 2009). To do so, these factors interact with a conserved set of proteins located at the rim of the tunnel exit. Homologues of these bacterial ribosomal proteins are also found in mitochondrial ribosomes, and it was exciting to see that Mrpl4, the homologue of the bacterial L29 protein, can be cross-linked to Cbp3. In contrast to the interaction partners of the tunnel exit of cytoplasmic ribosomes, the Cbp3–Cbp6 complex is specifically required for the biogenesis

of cytochrome *b* and therefore for only one translation product. The finding that a product-specific biogenesis factor interacts with the tunnel exit of mitochondrial ribosomes might suggest that synthesis of distinct translation products in mitochondria requires a specialized organization for each encoded protein. Such a specialization on specific translation products might be possible because of the extremely low number of different proteins that are synthesized in mitochondria. It will be exciting for future research to unravel the molecular mechanisms by which mitochondria synthesize these proteins.

Materials and methods

Yeast strains and growth media

All strains used in this study (Table S1) were isogenic to either the wild-type strain YPH499 (Sikorski and Hieter, 1989) or W303. The strains carrying the intronless or the *cob::ARG8[m]* mitochondrial genome were contributed by A. Tzagoloff (Columbia University, New York, NY), and the strain carrying the *cox2::ARG8[m]* mitochondrial genome was contributed by N. Bonnefoy (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). His7- or ProteinAHis7-tagged variants of Mrpl4, Cbp3, Cbp6, Cbs1, and Cbs2 were generated by replacing the stop codons of the endogenous ORFs by a sequence encoding a heptahistidine or ProteinA-heptahistidine tag using a HIS3 selection cassette (Lafontaine and Tollervey, 1996). *CBP3*, *CBP6*, *CBP4*, and *CBP1* were disrupted with a Kanamycin resistance cassette. Yeast cultures were grown at 30°C in lactate medium and YP (1% yeast extract and 2% peptone) medium supplemented with 2% dextrose, 2% galactose, or 2% glycerol.

Isolation of mitochondria

Yeast cells were grown to midexponential phase (OD_{595nm} = 1.3), harvested by centrifugation (3,000 *g* for 5 min), washed once with distilled water, resuspended (2 ml/g of cell weight) in MP1 buffer (0.1 M Tris base and 10 mM dithiothreitol), and incubated for 10 min at 30°C. Cells were then washed once with 1.2 M sorbitol, resuspended (6.7 ml/g of cell wet weight) in MP2 buffer (20 mM KP_i, pH 7.4, 0.6 M sorbitol, and 3 mg/g of cell wet weight zymolyase 20T [Seikagaku Biobusiness]), and incubated, shaking for 1 h at 30°C to digest the cell wall (spheroplastation). Spheroplasts were harvested (3,000 *g* for 5 min at 4°C) and resuspended (13.4 ml/g of cell wet weight) in homogenization buffer (10 mM Tris, pH 7.4, 0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF, and 0.2% Albumin bovine Fraction V, fatty acid free [Serva]). All subsequent steps were performed on ice; centrifugations were performed at 4°C. The spheroplast suspension was homogenized in two portions by 10–15 strokes of a Teflon plunger in a tight-fitting homogenizer (Sartorius Stedim Biotech S.A.). The homogenate was centrifuged at 3,000 *g* for 5 min, and the supernatants were combined. Centrifugation was repeated until no residual cell debris was visible. Then mitochondria were harvested by centrifugation at 17,000 *g* for 12 min. The pellet was resuspended in isotonic buffer (0.6 M sorbitol and 20 mM Hepes, pH 7.4) to give a final concentration of 10 mg/ml.

Construction of a mitochondrial genome with the *COB* coding sequence replaced by *ARG8[m]*

The *cob::ARG8[m]* mitochondrial genome was constructed by A. Tzagoloff using PCR amplification of the 5′ and 3′ UTR of *COB* using the primer pairs 5′-GGCGAATTCGATATCATTAATATTAATATAATCGTC-3′/5′-GGCG GATCCTGATTTTCTAAATGCCATATTATTATT-3′ and 5′-GGCGGATCCCG-GTAGAGTTAATAAATAATATAT-3′/5′-GGGTCTAGAGATTCTATAATAATTAT-GCTTTATG-3′. The two fragments were amplified from mitochondrial DNA of the respiratory-competent haploid strain MR6 (Rak et al., 2007) as the template. The PCR products were digested with EcoRI–BamHI and XbaI–BamHI, respectively, and ligated to the EcoRI and XbaI sites of pJM2 (Steele et al., 1996). The resultant plasmid was termed pCOB/ST3. *ARG8[m]* was amplified using the primer pair 5′-GGCGGATCCTTCAAAA-GATATTTATCATCAAC-3′/5′-GGCGGATCCTTAAGCATATACAGC-3′ and cloned into the BamHI site of pCOB/ST3. The resultant plasmid pCOB/ST5 was introduced into the *kar1-1* strain αDFS160ρ⁰ by biolistic transformation (Bonnefoy and Fox, 2007) with the PDS-1000/He particle delivery system (Bio-Rad Laboratories). Transformants were selected for their ability to rescue the *cox2* mutation of M9-94/A3 (Tzagoloff et al., 1975). A transformant verified to have acquired the modified *cob::ARG8[m]* gene (αDFS160/COB/ST5) was crossed to the *ARG8*-deficient mutant MRSI⁰, a derivative of MR6 and SDC22 (Duvezin-Caubet et al., 2003; Rak et al., 2007) that had previously been cytoduced with an intronless mitochondrial genome from strain MCC109 (a gift from P. Perlman, University of Texas Southwestern Medical Center, Dallas, TX). Cytoductants in which the coding sequence of *COB* had been replaced by that of *ARG8[m]* were identified by their ability to grow on media lacking arginine and by lack of respiratory growth.

Cross-linking and denaturing purification

Mitochondria from yeast strains expressing His7-tagged proteins were incubated in isotonic buffer (0.6 M sorbitol and 20 mM Hepes, pH 7.4). The membrane-permeable, noncleavable cross-linker MBS was dissolved in DMSO and used at a final concentration of 200 µM. DMSO without cross-linker served as a vehicle control. Cross-linking was performed at 25°C for 45 min. For analysis of cross-linking to newly synthesized proteins encoded in the mitochondrial genome, in organello translation was performed at 30°C as described in the Labeling of mitochondrial translation products in organello section. The membrane-permeable, cleavable cross-linker DSP was added 10 min after addition of [³⁵S]methionine, and labeling was allowed to go on for another 30 min. Cross-linking was stopped by addition of 100 mM Tris/HCl, pH 8.0, and 100 mM β-mercaptoethanol and incubating the samples for 10 min at 25°C. Mitochondria were then reisolated by centrifugation at 25,000 *g* for 10 min at 4°C and for purification of His7-tagged proteins on nickel–nitrilotriacetic acid (Ni-NTA) beads lysed in 50 µl of 1% SDS and briefly heated at 98°C. Next, the sample was adjusted to 1 ml with binding buffer (1% Triton X-100, 300 mM NaCl, 20 mM imidazole, pH 7.4, and 20 mM KP_i, pH 7.4), and the His-tagged proteins and their cross-linking partners were purified on Ni-NTA beads.

Ni-NTA purification was performed by incubating the respective samples with 10 µl Ni-NTA beads for 2 h at 4°C. The beads were then washed three times with binding buffer and eluted with binding buffer containing 500 mM imidazole, pH 7.4. In the case of DSP, the cross-linker was cleaved by 100 mM DTT for 30 min at 37°C. The samples were then separated via SDS-PAGE and analyzed by Western blotting and immunodetection or autoradiography.

Native purification of Cbs1, Cbs2, Cbp6, or Cbp3

Mitochondria from the wild type or mutants expressing ProteinAHis7-tagged Cbs1 or Cbs2 or His7-tagged Cbp6 or Cbp3 (1 mg each) were lysed for 30 min in 1 ml of buffer containing 1% Triton X-100 or 1% digitonin, 150 mM KCl, 20 mM Hepes/KOH, pH 7.4, 1 mM PMSF, 1× Complete Protease Inhibitor mix, 20 mM imidazole, pH 7.4, and 20% glycerol. Tagged Cbs1, Cbs2, Cbp6, or Cbp3 and complexed proteins were purified by incubating the respective samples with 10 µl Ni-NTA beads tumbling for 3 h at 4°C. The beads were then washed three times with lysis buffer containing 0.1% detergent and were eluted with lysis buffer containing 0.1% detergent and 500 mM imidazole, pH 7.4. Alternatively, Cbp3 was purified from mitochondrial digitonin lysates by immunoprecipitation using serum against Cbp3 or preimmune serum (as a negative control) and Protein A–Sepharose beads. When interactions with newly synthesized mitochondrially encoded proteins were analyzed, in organello translation was performed at 30°C as described in the Labeling of mitochondrial translation products in organello section, radioactive labeling was stopped by adding 8 mM of unlabeled methionine, mitochondria were reisolated, and immunoprecipitation of Cbp3 was performed. Beads were washed as previously described and an additional time with 20 mM Hepes/ KOH, pH 7.4. Bound proteins were eluted with sample buffer. Samples were separated via SDS-PAGE and analyzed by Western blotting and/ or autoradiography.

Labeling of mitochondrial translation products in vivo

Cells were grown on minimal medium containing 2% galactose, 20 µg/ml arginine, methionine, threonine, and tyrosine, 30 µg/ml isoleucine, 50 µg/ml phenylalanine, 100 µg/ml valine, and 0.1% glucose (Prestele et al., 2009). A cell amount corresponding to an optical density (OD_{595nm}) of 0.5 was collected, washed twice with minimal medium containing 2% galactose, and incubated at 30°C for 10 min in the same buffer supplemented with 0.12 mg/ml of all amino acids except methionine. After inhibiting cytosolic protein synthesis with 0.15 mg/ml cycloheximide, labeling of mitochondrial proteins was started by addition of 6 µCi [³⁵S]methionine. Pulse labeling was stopped after the indicated time points by addition of lysis buffer (1.85 M NaOH and 1.1 M β-mercaptoethanol) containing 8 mM of unlabeled methionine. After incubation for 10 min on ice, proteins were precipitated with 14% TCA. The stability of mitochondrial translation products

was chased by stopping a 15-min labeling reaction by the addition of 8 mM of unlabeled methionine and by taking samples after the indicated time points, which were lysed and TCA precipitated as previously described. Pellets were resolved in sample buffer, subjected to SDS-PAGE on 16/0.2% acrylamide/bisacrylamide gels, and analyzed by autoradiography and Western blotting.

Labeling of mitochondrial translation products in organello
Mitochondria were resuspended at a final concentration of 1 mg/ml in translation buffer (0.6 M sorbitol, 150 mM KCl, 15 mM KP_i, pH 7.4, 20 mM Hepes, pH 7.4, 12.67 mM $MgCl_2$, 4 mM ATP, 0.5 mM GTP, 5 mM phosphoenolpyruvate, 5 mM α-ketoglutarate, 12.13 µg/ml alanine, arginine, aspartic acid, asparagine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine, 66.67 µM cysteine, and 10 µg/ml pyruvate kinase). After 5 min of incubation at 30°C, 4 µCi [^{35}S]methionine/100 µg mitochondria was added, and incubation was pursued for 20 min. Labeling of mitochondrial translation products was stopped by the addition of unlabeled methionine to a final concentration of 8 mM, and mitochondria were reisolated by centrifugation (10,000 g at 4°C for 10 min).

Fractionation of mitochondrial lysates
300 µg of isolated mitochondria was lysed for 30 min on ice in lysis buffer containing 1% Triton X-100 or 1% digitonin, 50 or 150 mM KCl, 1× Complete Protease Inhibitor mix, 0.5 mM $MgCl_2$, and 20 mM Hepes/KOH, pH 7.4. After a clarifying spin for 10 min at 25,000 g at 4°C, one half of the extract was precipitated with 12% TCA (100% total), and the rest was underlayered with 50 µl sucrose solution (1.2 M sucrose and 20 mM Hepes/KOH, pH 7.4) and centrifuged for 105 min at 200,000 g in a TLA100 rotor (Beckman Coulter) at 4°C. The supernatant was then collected, and the ribosome-containing pellet was resuspended in lysis buffer. Proteins of both fractions were precipitated with 12% TCA. The resulting pellets were dissolved in sample buffer, separated by SDS-PAGE, and analyzed by Western blotting.

Analysis of mitochondrial transcripts by Northern blotting
For RNA isolation, mitochondria were resuspended in AE buffer (50 mM sodium acetate, pH 5.3, and 10 mM EDTA, pH 8) and lysed by addition of 1% SDS. After addition of an equal volume of phenol, pH 4.5–5, the mixture was briefly heated (65°C), rapidly chilled on ice, and stored at −80°C until the phenol was frozen. Next, the aqueous and phenolic phase was separated by centrifugation (13,000 g for 5 min), and the aqueous phase was collected, mixed with an equal amount of phenol/chloroform/isoamylalcohol solution (25:24:1), and centrifuged again. The RNA was precipitated from the aqueous phase in the presence of 0.3 M sodium acetate, pH 5.3, and isopropanol (2 vol) by incubation at −20°C for at least 20 min and centrifugation at 16,100 g for 5 min. The RNA pellet was washed once with 80% ethanol, dried, and resuspended in diethylpyrocarbonate-H_2O. For Northern blotting analysis, 2 µg mitochondrial RNA of each strain was separated on a denaturing 1.2% formaldehyde-agarose gel, washed with diethylpyrocarbonate-H_2O and TBE (45 mM Tris, 45 mM borate, and 1 mM EDTA), and subsequently transferred to a nylon membrane. After blotting, the membrane was briefly washed in 2× SSC (0.3 M NaCl and 30 mM Na_3-citrate, pH 7.0) and dried overnight. Before hybridization, RNA was UV cross-linked and prehybridized with Church buffer (1 mM EDTA, 7% SDS, 1% BSA, and 0.25 M NaP_i, pH 7.2). RNA was hybridized with gene-specific ^{32}P-labeled probes overnight and analyzed by autoradiography.

Analysis of the topology of Cbp3, Cbp6, and Cbp4
Alkaline extraction of proteins was performed from mitochondria resuspended in 0.1 M Na_2CO_3 and incubated for 30 min, shaking at 4°C. Membranes and associated proteins were separated by centrifugation at 30,000 g for 45 min. Proteins from the resulting supernatant were precipitated with 12% TCA. Membrane pellets were directly resuspended in sample buffer. Fractions were analyzed by SDS-PAGE followed by Western blotting.

Alternatively, membrane-spanning and -associated proteins were fractionated from soluble proteins by salt extraction. Mitochondria were resuspended in buffer containing 200 mM KCl and 20 mM Hepes/KOH, pH 7.4, and subjected to three cycles of freeze thawing in liquid nitrogen and 37°C. Membranes were collected by centrifugation, and the fractions were further processed as described for the alkaline extraction procedure.

To analyze the submitochondrial localization of proteins, mitochondria were either resuspended in isoosmotic (600 mM sorbitol and 20 mM Hepes/KOH, pH 7.4) or hypoosmotic (20 mM Hepes/KOH, pH 7.4) buffer to generate mitoplasts. Mitochondria or mitoplasts were

then incubated with 100 µg/ml proteinase K for 30 min on ice or left untreated. The protease was inhibited by the addition of 1 mM PMSF, and mitochondria (or mitoplasts) were reisolated, washed three times with a buffer containing 600 mM sorbitol, 80 mM KCl, 20 mM Hepes/KOH, pH 7.4, and 1 mM PMSF, resuspended in sample buffer, and subjected to SDS-PAGE and Western blotting.

Miscellaneous
The antibody against the His7 tag was purchased from QIAGEN, and the antibody against the ProteinA tag was purchased from Sigma-Aldrich. Antibodies against Cbp3, Cbp4, and Cbp6 were obtained by immunizing rabbits with purified mature Cbp3His6, Cbp4(61–171), and MBP-Cbp6, respectively. The Arg8 antibody was a gift from T. Fox (Cornell University, Ithaca, NY).

Online supplemental material
Table S1 contains a summary of the yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201103132/DC1.

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