The Cbp3–Cbp6 complex coordinates cytochrome *b* synthesis with bc_1 complex assembly in yeast mitochondria

Steffi Gruschke,[1] Katharina Römpler,[1] Markus Hildenbeutel,[1] Kirsten Kehrein,[1] Inge Kühl,[2] Nathalie Bonnefoy,[2] and Martin Ott[1]

[1]Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden
[2]Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, UPR3404, FRC3115, 91198 Gif-sur-Yvette Cedex, France

Respiratory chain complexes in mitochondria are assembled from subunits derived from two genetic systems. For example, the bc_1 complex consists of nine nuclear encoded subunits and the mitochondrially encoded subunit cytochrome *b*. We recently showed that the Cbp3–Cbp6 complex has a dual function for biogenesis of cytochrome *b*: it is both required for efficient synthesis of cytochrome *b* and for protection of the newly synthesized protein from proteolysis. Here, we report that Cbp3–Cbp6 also coordinates cytochrome *b* synthesis with bc_1 complex assembly. We show that newly synthesized cytochrome *b* assembled through a series of four assembly intermediates. Blocking assembly at early and intermediate steps resulted in sequestration of Cbp3–Cbp6 in a cytochrome *b*–containing complex, thereby making Cbp3–Cbp6 unavailable for cytochrome *b* synthesis and thus reducing overall cytochrome *b* levels. This feedback loop regulates protein synthesis at the inner mitochondrial membrane by directly monitoring the efficiency of bc_1 complex assembly.

Introduction

The respiratory chain of mitochondria is composed of subunits that originate from two different genetic systems. Most of the subunits are derived from nuclear genes; they are synthesized in the cytosol and imported into the organelle (Neupert and Herrmann, 2007; Chacinska et al., 2009). Importantly, a handful of hydrophobic membrane proteins that represent core catalytic subunits of respiratory chain complexes are produced by the genetic system of mitochondria. In yeast, mitochondria synthesize cytochrome *b* of the bc_1 complex; Cox1, Cox2, and Cox3 of cytochrome oxidase; and Atp6, Atp8, and Atp9 of the ATP synthase. To allow efficient assembly of the respiratory chain, both genetic systems have to be coordinated to match the quantities of imported, nuclear encoded subunits with those produced in the organelle. In addition, several assembly factors support cofactor acquisition and mediate the stepwise assembly process. In the case of cytochrome oxidase, >20 assembly factors are implicated in these processes (Fontanesi et al., 2006; Mick et al., 2011). In contrast, the knowledge on bc_1 complex assembly is still scarce (Zara et al., 2009a; Smith et al., 2012). Analyses using yeast mutants lacking individual structural subunits have suggested a clear order in the step-wise assembly process (Zara et al., 2004, 2009b). According to these data, the nuclear encoded subunits can be classified into three groups according to their timing of incorporation into the complex (Fig. 1 A). Assembly starts with the insertion of the mitochondrially encoded cytochrome *b* into the inner membrane. Next, the early assembling subunits Qcr7 and Qcr8 are added followed by the intermediate-assembling subunits Cor1, Cor2, cytochrome c_1, and Qcr6. Assembly is completed by the addition of the late-assembling subunits Qcr9, Qcr10, and Rip1. So far, six bc_1 complex assembly factors have been identified (Smith et al., 2012). Cbp3, Cbp4, Cbp6, and Bca1 are assisting early assembly steps, whereas Bcs1 and Mzm1 function later on by mediating Rip1 translocation and assembly (Wu and Tzagoloff, 1989; Nobrega et al., 1992; Crivellone, 1994; Atkinson et al., 2011; Gruschke et al., 2011; Mathieu et al., 2011; Kühl et al., 2012).

We have recently reported that the Cbp3–Cbp6 complex has a dual role in the biogenesis of cytochrome *b* in *Saccharomyces*

Correspondence to Martin Ott: martin.ott@dbb.su.se

Abbreviations used in this paper: BN, blue native; IP, immunoprecipitation; mtDNA, mitochondrial DNA; ORF, open reading frame; *TPI*, *triosephosphate isomerase*; UTR, untranslated region.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 199 No. 1 137–150
www.jcb.org/cgi/doi/10.1083/jcb.201206040

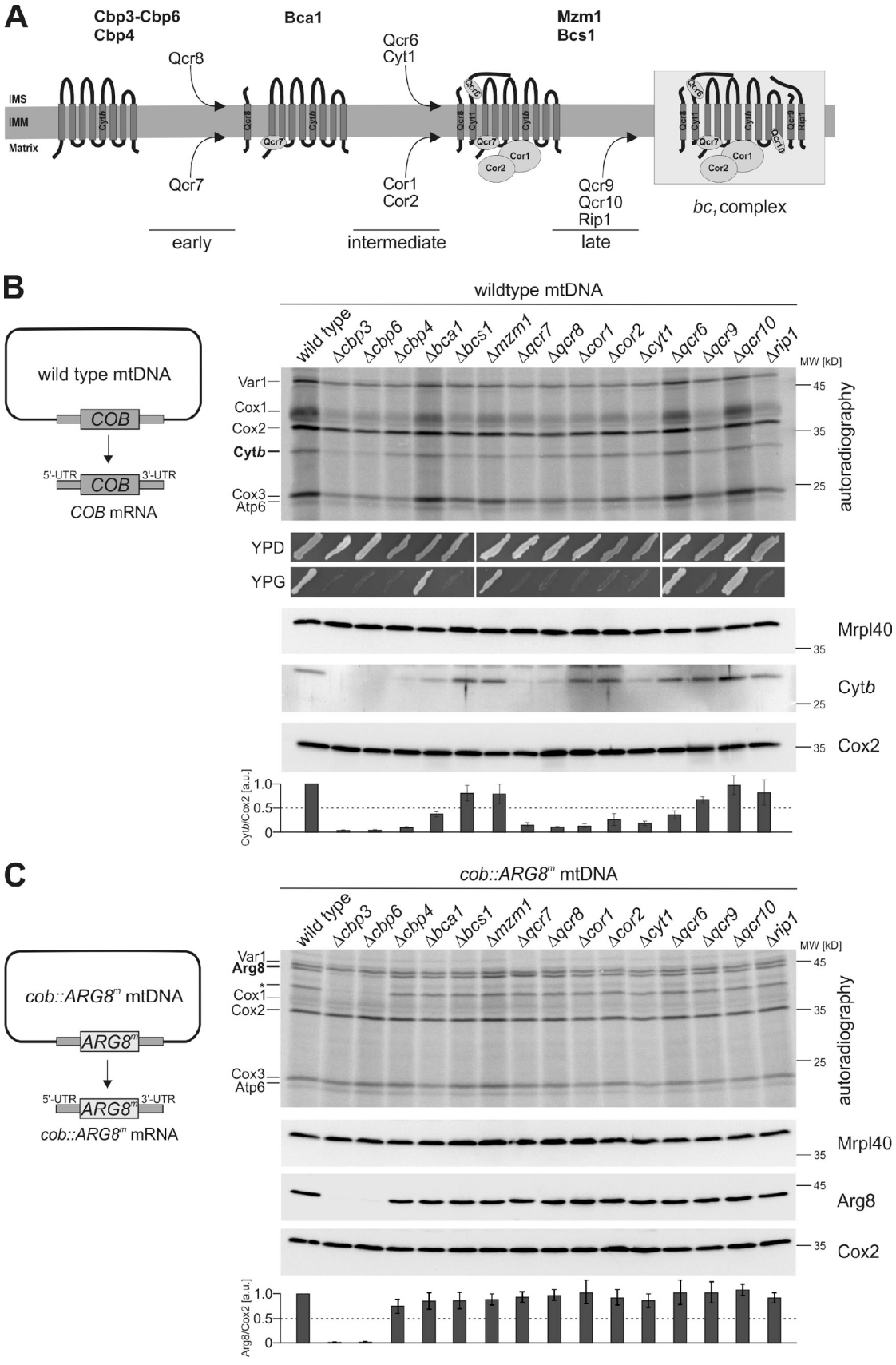

Figure 1. **Blockage of bc_1 complex assembly perturbs cytochrome *b* expression.** (A) Schematic assembly line of the yeast bc_1 complex. Assembly factors are shown in bold. (B) Efficiency of mitochondrial translation and synthesis of cytochrome *b* is perturbed upon bc_1 complex assembly inhibition. Mitochondrial translation products of cells carrying a wild-type mitochondrial genome (left) and deletions of the indicated genes were labeled with [^{35}S]methionine

cerevisiae (Gruschke et al., 2011). On the one hand, interaction of the complex with mitochondrial ribosomes is required for efficient translation of the cytochrome *b* encoding mRNA (*COB*). It is therefore part of the family of translational activators present in mitochondria that promote translation of one dedicated client mRNA (Fox et al., 1988). These factors recognize specific features in the 5′ untranslated region (UTR) of the mRNA and facilitate its translation by an as-yet poorly understood mechanism. On the other hand, the complex directly interacts with the newly synthesized cytochrome *b* to stabilize the protein and promote assembly (Wu and Tzagoloff, 1989; Gruschke et al., 2011). The two roles of the Cbp3–Cbp6 complex on both translation and assembly of cytochrome *b* have striking similarities to the functions of Mss51, a protein involved in biogenesis of cytochrome oxidase (Fontanesi et al., 2008; Mick et al., 2011). Mss51 is required for translation of *COX1* mRNA (Siep et al., 2000) and part of an assembly intermediate containing the newly synthesized Cox1 protein (Perez-Martinez et al., 2003). When assembly of cytochrome oxidase is blocked, Mss51 is sequestered in an assembly intermediate, thus reducing synthesis of Cox1 (Perez-Martinez et al., 2003; Barrientos et al., 2004). By this, the rates of Cox1 production are tuned in respect to cytochrome oxidase assembly.

In the current study, we analyzed assembly of the bc_1 complex and asked how interfering with this process by removal of assembly factors or structural subunits affects expression of cytochrome *b*. Our results reveal four intermediates through which cytochrome *b* assembles. Blockage of assembly after the first, second, and third stage provokes reduced expression of cytochrome *b*, whereas blockage after formation of the fourth intermediate has no effect. In line with a model where sequestration of Cbp3–Cbp6 in the first assembly intermediate reduces cytochrome *b* synthesis, we find that simultaneous overexpression of both proteins overcomes this feedback regulation. We conclude that efficiency of bc_1 complex assembly is sensed by Cbp3–Cbp6 to modulate expression of cytochrome *b*.

Results

Blockage of bc_1 complex assembly perturbs cytochrome *b* expression

Cytochrome *b* is the only subunit of the bc_1 complex encoded in the mitochondrial genome (mtDNA). Currently it is not known whether its expression is modulated in the context of bc_1 complex assembly. To investigate whether this might indeed be the case, we systematically analyzed synthesis of cytochrome *b* in strains lacking structural subunits or assembly factors of the bc_1 complex (Fig. 1 B). Mitochondrial translation products of whole cells were labeled with [^{35}S]methionine, then equal amounts of proteins were extracted, separated on SDS-PAGE, and analyzed by autoradiography and Western blotting. The overall efficiency of mitochondrial translation differed significantly between the strains and coincided with the strains' ability to grow on nonfermentable carbon sources (Fig. 1 B). This confirms previous notions that the metabolic state of the cell directly influences protein synthesis in mitochondria (DeRisi et al., 1997; Ohlmeier et al., 2004). Furthermore, labeling of cytochrome *b* differed even between the strains that cannot respire. This, however, could be explained by three different effects: First, the stability of the newly synthesized protein could be lowered because of a bc_1 complex assembly defect. Second, translation of cytochrome *b* mRNA (*COB* mRNA) could be impaired directly. Third, cytochrome *b* expression could be regulated by a feedback modulation provoked by failure to assemble. To distinguish between these possibilities, we developed a strategy to uncouple translation of *COB* mRNA from assembly of cytochrome *b* by using genetically engineered mitochondrial genomes.

We first asked whether absence of assembly factors and structural bc_1 complex subunits would directly impair translation of *COB* mRNA and thereby explain the lowered levels of cytochrome *b* in the affected mutants (Fig. 1 B). To be able to analyze this in a setting where stability of the newly synthesized protein is not disturbed by assembly into a respiratory chain complex, we used cells with a mitochondrial genome expressing Arg8 as a reporter for *COB* mRNA translation (Gruschke et al., 2011). To this end, the coding sequence of Arg8 was inserted at the place of the coding sequence of cytochrome *b* (*cob::ARG8*^{m}). As a consequence, Arg8 is produced in mitochondria from an mRNA containing the 5′ UTR of *COB* mRNA that is the target of translational activation (Fig. 1 C). *ARG8* codes for acetylornithine aminotransferase, an enzyme involved in arginine biosynthesis in the matrix. As it is a soluble protein, its stability does not depend on assembly into a respiratory chain complex. When analyzing expression of Arg8 in the mutants, we found that the protein was synthesized efficiently in all strains, with the exception of those lacking *CBP3* or *CBP6* (Fig. 1 C). This showed that apart from Cbp3 and Cbp6, no other assembly factor or structural subunit of the bc_1 complex is directly implicated in translation of mRNAs containing the 5′ UTR of *COB* mRNA.

Ectopically synthesized cytochrome *b* depends on Cbp3–Cbp6 but fails to accumulate to wild-type levels

Next we addressed the question of whether cytochrome *b* synthesis is regulated depending on the efficiency of bc_1 complex

for 15 min in vivo. Proteins were extracted, separated on SDS-PAGE, and analyzed by autoradiography and Western blotting. Membranes were probed with antibodies against Mrpl40 to check for equal loading and with antibodies against Cox2 to estimate expression of another mitochondrially encoded protein. Cells were streaked on plates containing either glucose (YPD) or glycerol (YPG) to score for respiratory growth. The steady-state levels of cytochrome *b* and Cox2 were measured densitometrically, and the Cyt*b*/Cox2 ratio is shown in the diagram. The wild-type ratio was set to 1 and the broken line is drawn at a 50% threshold to qualify as a mutant showing a decreased cytochrome *b* level. Error bars (mean ± SD) are depicted from $n = 3$. a.u., arbitrary units. (C) In the absence of cytochrome *b*, only deletion of *CBP3* or *CBP6* abolishes expression of mRNAs containing the 5′ UTR of *COB*. Mitochondrial translation products of cells harboring the *cob::ARG8*^{m} mitochondrial genome (left) were labeled radioactively and analyzed like described in B. Cox1 is generally only poorly produced when cells are respiratory deficient. The asterisk indicates a degradation product of Arg8. The Arg8/Cox2 ratio was calculated and graphically depicted as in B. $n = 3$.

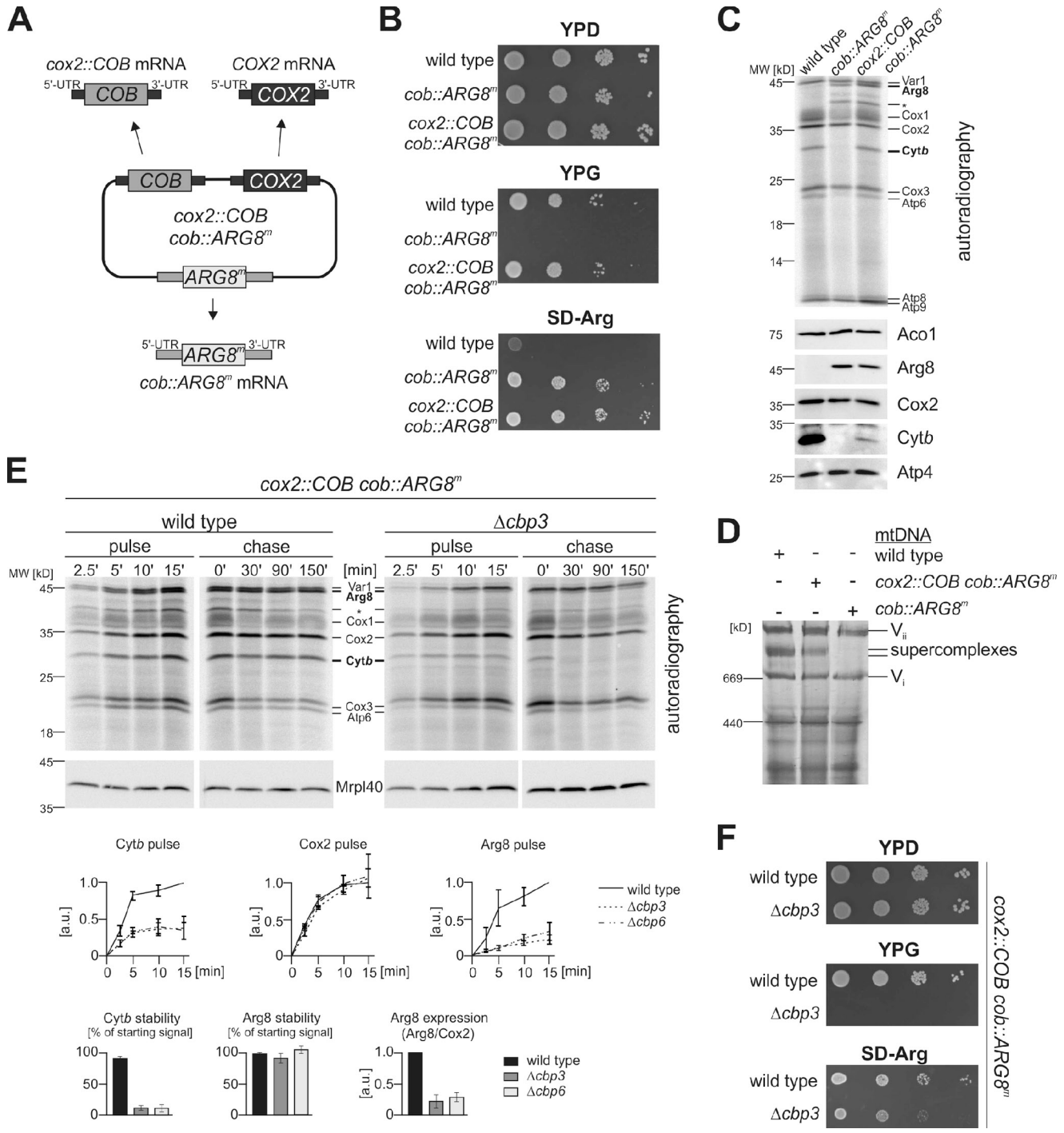

Figure 2. **Ectopically expressed cytochrome *b* depends on Cbp3–Cbp6 but fails to accumulate to wild-type levels.** (A) Scheme of the *cox2::COB cob::ARG8*^{m} mitochondrial genome. The coding sequence of cytochrome *b* was inserted by biolistic transformation and homologous recombination into a non-essential region upstream of *COX2* in the *cob::ARG8*^{m} mitochondrial genome. (B) Ectopically expressed cytochrome *b* can support respiratory growth to a wild-type level. Cells were streaked on plates containing either glucose (YPD) or glycerol (YPG) to score for respiratory growth. Growth on SD-Arg was used to test for expression of Arg8. (C) Ectopically expressed cytochrome *b* is synthesized efficiently but accumulates poorly. Mitochondrial translation products of cells containing the indicated mitochondrial genomes were labeled with [^{35}S]methionine for 15 min in vivo. Proteins were extracted, separated on SDS-PAGE, and analyzed by autoradiography and Western blotting. (D) Supercomplexes are less abundant when cytochrome *b* is expressed ectopically. Mitochondria from cells harboring the indicated mitochondrial genomes were lysed in digitonin and subjected to 1D BN PAGE. The gel was stained with Coomassie brilliant blue. V_i, ATP synthase monomer. V_{ii}, ATP synthase dimer. (E) Ectopically expressed cytochrome *b* requires Cbp3 for stability. Mitochondrial translation products of the indicated cells were pulse-labeled with [^{35}S]methionine for up to 15 min. The fate of newly synthesized proteins was followed for 150 min. Proteins were extracted, separated on SDS-PAGE, and analyzed by autoradiography and Western blotting. The levels of radiolabeled cytochrome *b*, Arg8, and Cox2 during the pulse were measured densitometrically and are shown in the top diagrams. The 15-min signal of the wild type was set to 1. $n = 3$. The bottom graphs depict stability of cytochrome *b* and Arg8 in the strains (calculated as ratio of 150 min/0 min signal) as well as the relative expression level of Arg8 calculated as the Arg8/Cox2 ratio at 150 min (wild-type ratio set to 1). $n = 3$. For the labeling of Δ*cbp6* cells, see Fig. S1. (F) Wild-type and Δ*cbp3* cells containing the *cox2::COB cob::ARG8*^{m} mitochondrial genome were grown on media containing either glucose (YPD and SD-Arg) or glycerol (YPG) as a carbon source. Error bars indicate mean ± SD. For the labeling of Δ*cbp6* cells, see Fig. S1.

assembly. In strains with the *cob::ARG8*^{m} mitochondrial genome, translation of an mRNA containing the *COB* 5′ UTR can be monitored unambiguously. However, this strain is not suitable to analyze a possible feedback-regulated *COB* expression, as no cytochrome *b* is present that can fail to assemble. We therefore generated by biolistic transformation and homologous recombination (Bonnefoy and Fox, 2007) a novel mitochondrial genome that we termed *cox2::COB cob::ARG8*^{m} (Fig. 2 A). This genome lacks some *COX1* introns and contains, in addition to *cob::ARG8*^{m} as a reporter for translation of *COB* mRNA and the *bona fide COX2* gene, a novel gene located upstream of *COX2* that allows synthesizing cytochrome *b* from an mRNA containing the 5′ UTR of *COX2*. Hence, cytochrome *b* synthesis is independent from *COB* mRNA-specific translational regulation. We first scored for growth on media requiring expression of *ARG8*^{m} (SD-Arg) and respiration (YPG) and found that the strain could grow robustly under those conditions (Fig. 2 B). We next tested how Arg8, Cox2, and cytochrome *b* are produced in a strain with the *cox2::COB cob::ARG8*^{m} mitochondrial DNA (mtDNA) and compared synthesis of mitochondrially encoded proteins in this strain to strains harboring either a wild-type or a *cob::ARG8*^{m} mitochondrial genome, which is respiratory deficient because of the lack of cytochrome *b*. The nine proteins encoded in the *cox2::COB cob::ARG8*^{m} genome were robustly synthesized (Fig. 2 C, top). We concluded that the ectopically expressed cytochrome *b* is fully functional and that expression of two different mRNAs bearing the 5′ UTR of *COX2* does not impair growth on nonfermentable carbon sources. However, when we checked levels of respiratory chain subunits that can accumulate at steady-state, significantly reduced amounts of cytochrome *b* were detected, whereas cytochrome oxidase (Cox2) and ATP synthase (Atp4) were only slightly affected or unchanged, respectively (Fig. 2 C, bottom). This decrease in cytochrome *b* was also reflected in lower amounts of supercomplexes formed between bc_1 complex dimers and cytochrome oxidase (Fig. 2 D; Cruciat et al., 2000). A similar reduction of supercomplexes was recently documented for cells lacking Bca1, which indicates that robust growth on the nonfermentable carbon source glycerol does not to require a wild-type level of the bc_1 complex (Mathieu et al., 2011).

The defect to accumulate cytochrome *b* could be explained by a problem in the early steps of its biogenesis. We therefore asked whether stability of ectopically expressed cytochrome *b* also depends on the Cbp3–Cbp6 complex. To test this hypothesis, we analyzed in more detail synthesis and turnover of mitochondrially encoded proteins in cells containing the *cox2::COB cob::ARG8*^{m} mitochondrial genome (Fig. 2 E, left). We found that cytochrome *b* and Arg8 were synthesized robustly and that most of the newly synthesized cytochrome *b* was stable. We next deleted *CBP3* or *CBP6* in this strain and found that both synthesis of Arg8 as well as the stability of the newly made cytochrome *b* were strongly affected in the mutants (Fig. 2 E, right; and Fig. S1). Likewise, respiratory growth and growth on media requiring arginine biosynthesis were strongly impaired in the absence of Cbp3 (Fig. 2 F), confirming that the Cbp3–Cbp6 complex has a dual role for biogenesis of cytochrome *b* (Gruschke et al., 2011). Although the reason for the low accumulation of cytochrome *b* in the *cox2::COB cob::ARG8*^{m} strain remains elusive, these results clearly show that ectopically expressed cytochrome *b* is also stabilized by Cbp3–Cbp6 and is fully functional.

Expression of the *cob::ARG8*^{m} reporter is modulated by the efficiency of cytochrome *b* assembly into the bc_1 complex

We next asked whether efficiency of assembly of the bc_1 complex might regulate *COB* mRNA translation. We therefore deleted structural subunits or assembly factors of the bc_1 complex in cells containing the *cox2::COB cob::ARG8*^{m} mitochondrial genome and followed synthesis and accumulation of Arg8 (Fig. 3). Levels of Arg8 differed among the various mutants. To group the mutants, we densitometrically quantified the signals and set a threshold according to which mutants showing 50% signal of the wild-type signal qualified as having decreased *cob::ARG8*^{m} expression. Strikingly, we found that this was the case for a specific subset of mutants. Specifically, Arg8 production was decreased in mutants lacking Cbp3, Cbp6, Cbp4, Bca1, Qcr7, Qcr8, Cor1, Cor2, cytochrome c_1, and Qcr6, whereas expression of *cob::ARG8*^{m} was unaffected by the absence of Bcs1, Mzm1, Qcr9, Qcr10, and Rip1. Interestingly, the first class of mutants affects factors implicated in either early or intermediate steps of bc_1 complex assembly whereas the second class of mutations impairs the late steps. From this we concluded that expression of cytochrome *b* is not generally decreased when a functional bc_1 complex fails to assemble. Instead, only blockage of those steps that occur at an early or intermediate point during assembly influences efficiency of cytochrome *b* synthesis. Our data thus show a direct modulation of cytochrome *b* synthesis in the frame of bc_1 complex assembly.

Distinct early assembly intermediates accumulate in cells where expression of the *cob::ARG8*^{m} reporter is decreased and cytochrome *b* cannot assemble

We next set out to define complexes containing cytochrome *b* in the different mutants with the *cox2::COB cob::ARG8*^{m} mitochondrial genome to understand at which step cytochrome *b* gets blocked during assembly. We isolated mitochondria, lysed them with the mild detergent digitonin, and separated complexes according to their size by 2D blue native (BN)/SDS-PAGE followed by Western blotting analysis of cytochrome *b* (Fig. 4). In wild-type cells with this mitochondrial genome (Fig. 4 A), most of the signal of cytochrome *b* was found at a high molecular mass of ∼750 kD. These complexes reflect the migration behavior of bc_1 complex dimers present in supercomplexes with cytochrome oxidase (Cruciat et al., 2000) because a complex of identical size was also detected with antibodies against Cox2. In addition, a small amount of cytochrome *b* was present in a complex of a smaller size of ∼170 kD. When probing with an antibody against Cbp3, we found that this fraction represents cytochrome *b* bound to the Cbp3–Cbp6 complex. This indicates that this is the major assembly intermediate of cytochrome *b* that is present already in those wild-type cells. In addition, Cbp3 formed two smaller-sized complexes (Fig. 4 A,

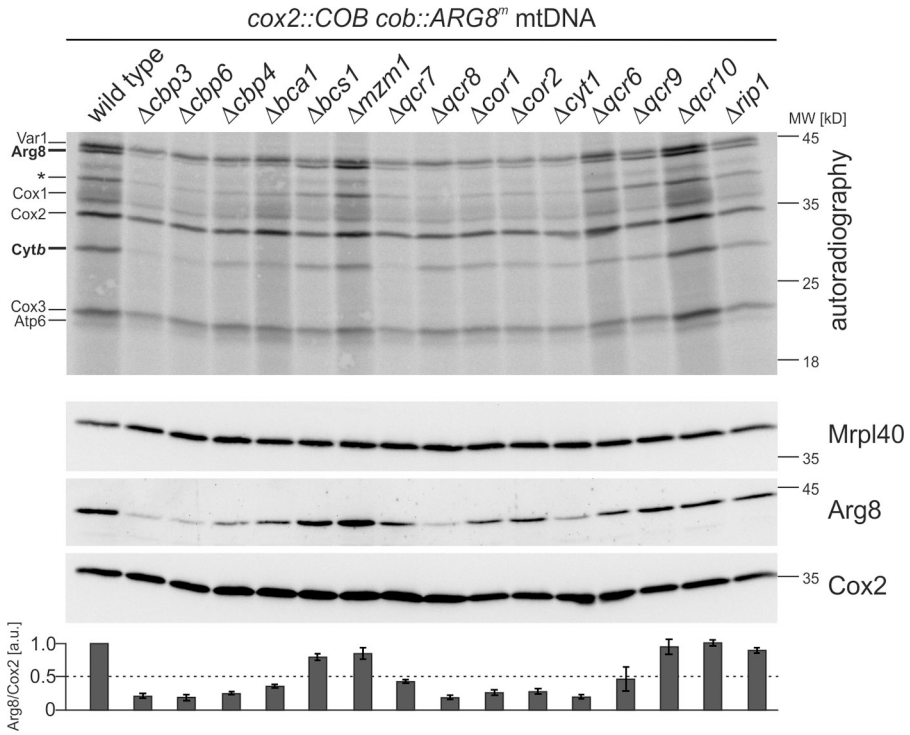

Figure 3. **Expression of *cob::ARG8*^{m} is modulated by the efficiency of bc_1 complex assembly when cytochrome *b* is present.** Mitochondrial translation products of cells with the indicated mutations containing the *cox2::COB cob::ARG8*^{m} mitochondrial genome were labeled with [^{35}S]methionine for 15 min. Proteins were extracted, separated on SDS-PAGE, and analyzed by autoradiography and Western blotting. The asterisk indicates a degradation product of Arg8. The Arg8/Cox2 ratio was calculated and graphically depicted. The wild-type ratio was set to 1. A threshold of 50% of the wild-type signal (broken line) was set to define mutants affected in *cob::ARG8*^{m} expression. $n = 3$. Error bars indicate mean ± SD.

white arrows) that represent the fraction of Cbp3 without cytochrome *b*. Accordingly, these two forms were abundantly present in mitochondria from the *cob::ARG8*^{m} strain that lacks cytochrome *b* (Fig. 4 A). These smaller-sized Cbp3 complexes are monomeric Cbp3 and Cbp3 present in the cytochrome *b*–free Cbp3–Cbp6 complex, which plays a critical role in activation of *COB* mRNA translation (Gruschke et al., 2011). To prove that the 170-kD form of cytochrome *b* is bound to Cbp3, we immunodepleted the digitonin extract with antibodies against Cbp3 and repeated the 2D BN/SDS-PAGE analysis (Fig. 4 B). As expected, this procedure depleted only cytochrome *b* found in the 170 kD complex, whereas cytochrome *b* detected in the 750-kD complex remained unchanged. In summary, Cbp3 is present in different forms: one containing cytochrome *b* and two cytochrome *b*–free forms, later on referred to as free Cbp3.

We then analyzed complexes of cytochrome *b* and Cbp3 in mitochondria lacking individual bc_1 complex subunits (Fig. 4 C). We found that five different complexes of cytochrome *b* could be detected that presumably correspond to distinct assembly intermediates and the fully assembled bc_1 complex in supercomplexes. In those mitochondria affected in early or intermediate steps of bc_1 complex assembly, cytochrome *b* was present in either one (*Δqcr7*), two (*Δqcr8*, *Δcor1*, *Δqcr6*), three (*Δcyt1*), or four (Δcor2) complexes, and the cytochrome *b*–free forms of Cbp3 observed in the corresponding wild type were hardly detectable. In the absence of Qcr7, cytochrome *b* accumulated in the complex of 170 kD that contains Cbp3, whereas disruption of *QCR8* led to the formation of two complexes of cytochrome *b*. The larger (170 kD) of these complexes represented the Cbp3-containing intermediate, whereas the 140 kD complex (Fig. 4 C, black arrows) was of unknown composition. Likewise, an absence of Cor1, Cor2, or cytochrome c_1 led to an accumulation of these two intermediates. Mitochondria lacking cytochrome c_1 or Cor2 additionally accumulated cytochrome *b* in a third intermediate of 370 kD. Furthermore, cells lacking Cor2 could assemble a small portion of cytochrome *b* into the bc_1 complex. The *Δqcr6* mutant is respiratory competent and can therefore assemble the bc_1 complex. Nevertheless, free Cbp3 was hardly detectable in this strain. Collectively, we concluded that in cells that show reduced efficiency of Arg8 translation, cytochrome *b* accumulates in distinct intermediates, one of which contains Cbp3. At the same time, the amounts of the translationally active free Cbp3 are strongly reduced. In contrast, cells affected in the late steps of bc_1 complex assembly (*Δqcr9*, *Δqcr10*, and *Δrip1*) and showing normal Arg8 synthesis accumulated cytochrome *b* in either a 500-kD complex or in a fully assembled form. Like in the corresponding wild type, higher levels of free Cbp3 were present in these cells (Fig. 4 C, white arrows). In summary, we observed that cytochrome *b* is found in four different complexes when assembly of the bc_1 complex is impaired by deletion of structural subunits. In addition, the mutants differ in the complexes formed by Cbp3.

To better understand the composition of the 170-kD and 140-kD intermediates, we analyzed more closely the assembly intermediates of wild type, *Δqcr8*, *Δcyt1*, and *Δrip1* (all with *cox2::COB cob::ARG8*^{m} mtDNA) by 2D BN/SDS-PAGE and Western blotting (Fig. 4 D). In wild-type mitochondria, Cbp3 was found as free Cbp3 and as the 170 kD complex. In addition to cytochrome *b* and Cbp3, the latter complex contained Cbp6 and Cbp4, in line with our previously published observations (Gruschke et al., 2011). Accordingly, Cbp6 and Cbp4 found at 170 kD could also be immunodepleted with antibodies using Cbp3 (Fig. S2). The 170-kD complex appeared to contain an identical set of constituents in *Δqcr8*, *Δcyt1*, and *Δrip1* cells. However, Cbp4 partly shifted to the 140-kD complex in

mitochondria lacking Qcr8 and cytochrome c_1. This 140-kD complex comprises cytochrome *b*, Qcr7, and Cbp4 in *Δqcr8* cells, whereas it additionally contained Qcr8 in *Δcyt1* cells. Interestingly, Cbp3 and Cbp6 were not part of this complex. Thus, upon inhibition of early or intermediate assembly steps, cytochrome *b* accumulates in different intermediates. Because the 140 kD complex contains, in addition to cytochrome *b*, two more nuclear encoded subunits of the bc_1 complex (Qcr7 and Qcr8), it appears likely that this intermediate represents the second subcomplex (intermediate II) after the Cbp3–Cbp6/Cbp4/cytochrome *b* intermediate (intermediate I). Consequently, the 370-kD complex observed in the absence of cytochrome c_1 or Cor2 is the third intermediate (intermediate III), whereas the 500-kD complex present in *Δqcr9* and *Δrip1* cells is the fourth intermediate (intermediate IV) during bc_1 complex assembly (Fig. 4 E).

Newly synthesized cytochrome *b* assembles in a step-wise fashion through intermediates I, II, and III

The ectopically expressed cytochrome *b* does not accumulate as efficiently as in wild-type cells (Fig. 2 B). A possible explanation could be that this cytochrome *b* forms incorrect complexes, thus impairing further assembly. We therefore asked whether the assembly intermediates of the bc_1 complex in cells with the *cox2::COB cob::ARG8^m* mitochondrial genome are identical to those present in cells with a wild-type mitochondrial genome. We addressed this by analyzing the complexes containing cytochrome *b* in wild-type, *Δqcr8*, *Δcyt1*, and *Δrip1* cells with a wild-type mitochondrial genome by 2D BN/SDS-PAGE (Fig. 5 A). We found that the assembly intermediates were identical, irrespective of whether cytochrome *b* was produced from the *cox2::COB* mRNA (Fig. 4 C) or from its authentic mRNA (Fig. 5 A). This shows that the ectopically expressed cytochrome *b* uses the normal assembly path. However, lower levels of free Cbp3 are present in cells containing a wild-type mitochondrial genome compared with cells with the *cox2::COB cob::ARG8^m* mitochondrial genome.

Upon deletion of structural subunits, several complexes of cytochrome *b* accumulate that are not detected in wild-type mitochondria. To answer whether these complexes reflect true assembly intermediates or represent dead-end products, we followed the assembly of newly synthesized cytochrome *b* in isolated wild-type mitochondria (Fig. 5 B). Assembly of cytochrome *b* was facilitated by depletion of mitochondrial translation products through treatment of cells with chloramphenicol before mitochondrial preparation (Rak et al., 2011). As expected, this resulted in a shift of Cbp3 from intermediate I to free Cbp3 (Fig. 5 B, bottom) compared with mitochondria from untreated cells (Fig. 5 A). Next, mitochondrial translation products were labeled with [^{35}S]methionine for 5 min, and the labeling was stopped by addition of puromycin and an excess of unlabeled methionine. Mitochondria were lysed with digitonin immediately and after 5, 15, and 30 min, and cytochrome *b*–containing complexes were analyzed with 2D BN/SDS-PAGE. Directly after the short pulse, most of the newly synthesized cytochrome *b* was present in intermediate I (Fig. 5 B, 0 min). Then, cytochrome *b* was partly shifted from this form to intermediate II and further to intermediate III (Fig. 5 B, 5 and 15 min). After 15 min, a fraction of cytochrome *b* was present in the completely assembled bc_1 complex (Fig. 5 B, 15 and 30 min). We did not observe cytochrome *b* in the form of intermediate IV, which may suggest that this intermediate is rapidly converted to the assembled bc_1 complex. In summary, these results confirm that the intermediates observed in the mutants (Fig. 4 D) are true assembly intermediates of wild-type cytochrome *b*.

Overexpression of Cbp3–Cbp6 allows regaining expression of *cob::ARG8^m* in mutants that fail to assemble cytochrome *b*

We found that when assembly is blocked at early or intermediate steps, cytochrome *b* accumulates in intermediates I, II, and III and that intermediate I contains Cbp3–Cbp6 (Fig. 4). Thus, it is possible that the observed sequestration of Cbp3–Cbp6 in intermediate I and the concomitant decrease in free Cbp3 reduce the efficiency of *COB* expression (Fig. 3). We have previously shown that binding of Cbp3–Cbp6 to the mitochondrial ribosome is required to stimulate translation of the *COB* mRNA (Gruschke et al., 2011). We therefore asked whether the fraction of free Cbp3 correlated with ribosome binding of Cbp3–Cbp6 in mitochondria from wild-type, *Δqcr8*, *Δcyt1*, and *Δrip1* cells carrying the *cox2::COB cob::ARG8^m* mitochondrial genome. Mitochondria were lysed in digitonin and separated into a supernatant and a ribosome-containing pellet by centrifugation through a sucrose cushion (Fig. 6, A and B). In wild-type mitochondria, ∼20% of the Cbp3–Cbp6 complex was bound to the ribosome. In contrast, significantly less Cbp3–Cbp6 was ribosome-bound in mitochondria from the *Δqcr8* and *Δcyt1* strain, whereas more Cbp3–Cbp6 was ribosome-bound in the *Δrip1* strain.

These results confirmed that in strains where cytochrome *b* accumulates at the level of intermediate I, II, or III (Fig. 4), less of the Cbp3–Cbp6 complex is ribosome-bound and therefore not available to stimulate *COB* translation. This raised the question of whether increased levels of Cbp3–Cbp6 would rescue expression of *cob::ARG8^m*. We transformed wild-type, *Δqcr8*, *Δcyt1*, and *Δrip1* cells carrying the *cox2::COB cob::ARG8^m* mitochondrial genome with either empty plasmids or plasmids allowing the simultaneous overexpression of *CBP3* and *CBP6*. In these strains, we analyzed synthesis of mitochondrial translation products and accumulation of the proteins by autoradiography and Western blotting (Fig. 6 C). Strikingly, overexpression of *CBP3* and *CBP6* resulted in increased Arg8 synthesis in the *Δqcr8* and *Δcyt1* mutant, giving rise to elevated steady-state levels of the protein. Likewise, cytochrome *b* accumulated to a slightly higher level in both mutants. This was confirmed by analysis of complexes that are formed by Cbp3 in the cells overexpressing the Cbp3–Cbp6 complex (Fig. 6 D). In wild-type and *Δrip1* cells, overexpression of the complex resulted in increased levels of free Cbp3, whereas intermediate I was rather constant. In contrast, higher levels of the Cbp3–Cbp6 complex in cells lacking Qcr8 or cytochrome c_1 lead to a relative increase in intermediate I. This likely accounts for the increased amount of steady-state cytochrome *b*

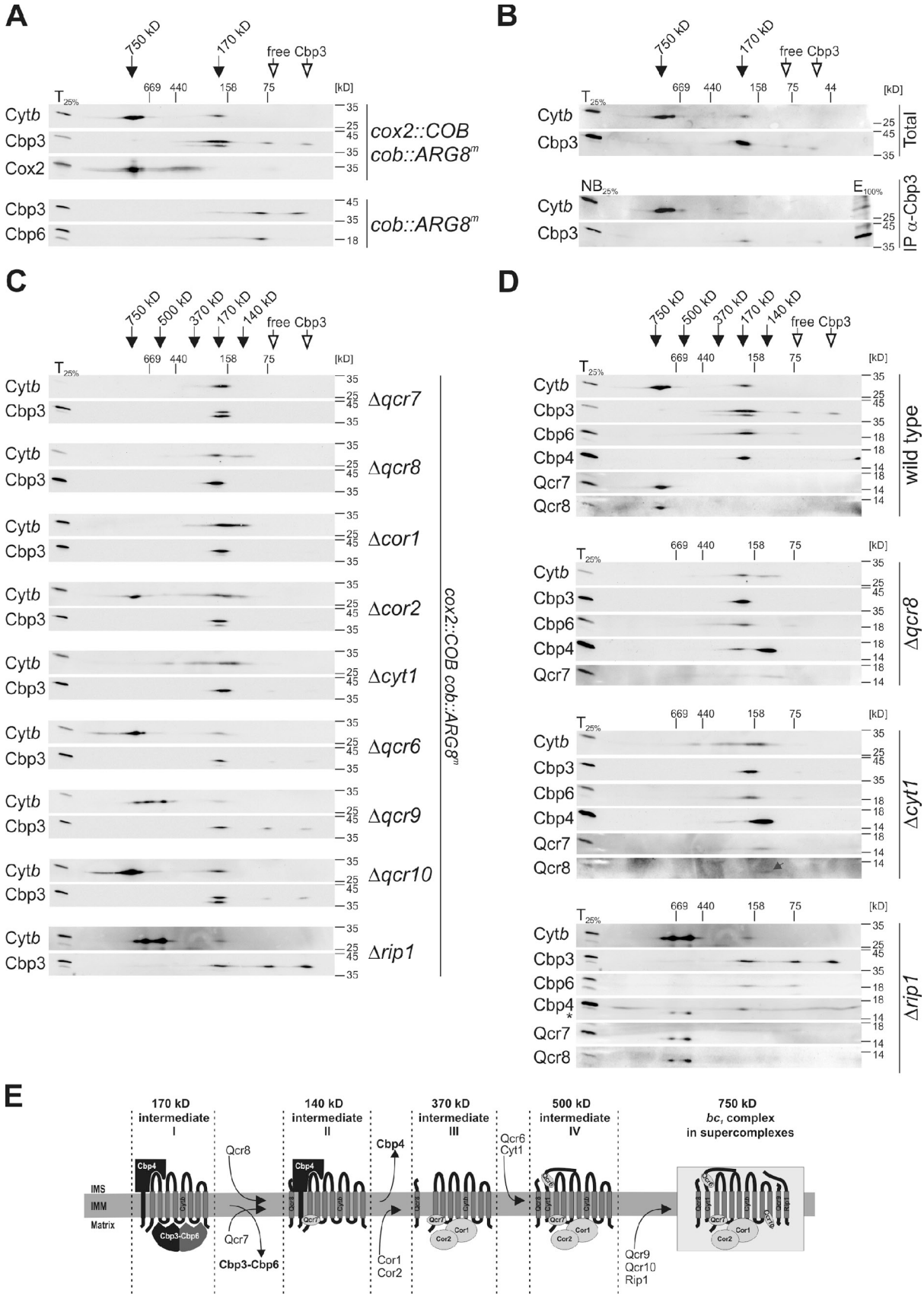

Figure 4. **Distinct early assembly intermediates accumulate in cells where expression of the *cob::ARG8*^{m} reporter is decreased and cytochrome *b* cannot assemble.** (A) Mitochondria of strains carrying the *cox2::COB cob::ARG8*^{m} or the *cob::ARG8*^{m} mitochondrial genome were lysed in digitonin, separated on 2D BN/SDS-PAGE, and analyzed by Western blotting with antibodies against cytochrome *b*, Cbp3, Cbp6, or Cox2. Black arrows indicate the complexes

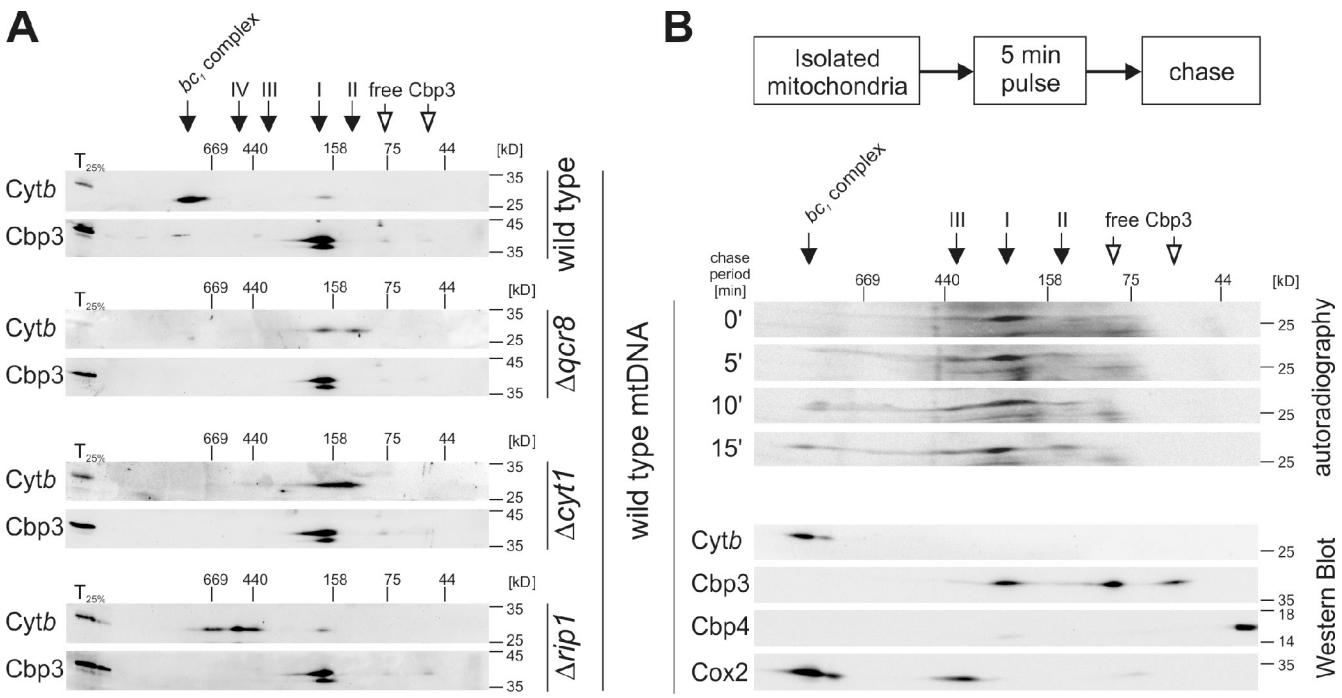

Figure 5. **Newly synthesized cytochrome *b* assembles in a step-wise fashion through intermediates I, II, and III.** (A) Cytochrome *b* produced from its authentic mRNA forms the same assembly intermediates as ectopically expressed cytochrome *b*. Mitochondria of the indicated strains with wild-type mtDNA were lysed in digitonin, separated on 2D BN/SDS-PAGE, and analyzed by Western blotting. $T_{25\%}$, 25% of the material used for 2D BN/SDS-PAGE. (B) Assembly pathway of newly synthesized cytochrome *b*. Wild-type cells were grown in the presence of 4 mg/ml chloramphenicol to deplete mitochondrial translation products and to increase the pool of nuclear encoded subunits, thereby allowing efficient assembly of cytochrome *b*. Mitochondria were isolated, mitochondrial translation products were radiolabeled, and complexes of cytochrome *b* were analyzed after the indicated time points by 2D BN/SDS-PAGE followed by autoradiography (for all time points) and Western blotting (for time point 0 min). The nomenclature of the intermediates is identical to Fig. 4 E.

in these cells (Fig. 6 C). Importantly, the amounts of free Cbp3 increased as well, indicating that more of the translationally active form of the Cbp3–Cbp6 complex is present in the organelle, allowing increased expression of *cob::ARG8*^{m} in these strains (Fig. 6 C). Collectively, our data show that blocking specifically the early or intermediate steps in the assembly of cytochrome *b* decreases synthesis of cytochrome *b*. This feedback modulation of *COB* expression is likely caused by sequestration of Cbp3–Cbp6 in an assembly intermediate, thereby preventing Cbp3–Cbp6 from binding the ribosome and stimulating new rounds of cytochrome *b* synthesis. This organization allows modulating expression of *COB* in the context of cytochrome *b* assembly to ensure an efficient coordination of synthesis and assembly.

Discussion

In this study, we investigated the early steps in the biogenesis of the mitochondrially encoded cytochrome *b*. Analyses of yeast mutants disrupted in structural subunits of the bc_1 complex have previously provided evidence on the general pathway of bc_1 complex assembly (Zara et al., 2004, 2009a,b). Here, by analyzing dynamics of assembly and the presence of assembly factors within the intermediates, we were able to significantly refine the model of how the early steps of cytochrome *b* biogenesis are organized (Fig. 7). Our results suggest the following scheme: translation of *COB* mRNA requires the two translational activators Cbs1 and Cbs2 (Rödel, 1986), which act in a yet ill-defined step in translation. In addition to these

in which cytochrome *b* is present. White arrows indicate the cytochrome *b*–free form of Cbp3: one representing the Cbp3–Cbp6 complex (left) and one representing the monomeric Cbp3 (right). $T_{25\%}$, 25% of the material used for 2D BN/SDS PAGE. The top two panels of data (Cyt*b* and Cbp3 for *cox::COB cob::ARG8*^{m}) in A are presented again in D of this figure and labeled "wild type." (B) Mitochondria carrying the *cox2::COB cob::ARG8*^{m} mtDNA were lysed in digitonin, an immunoprecipitation (IP) using an antibody against Cbp3 was performed, and total (top panels) and unbound (bottom panels) fractions were analyzed by 2D BN/SDS-PAGE and Western blotting. For a control of bound proteins, the SDS-eluted fraction ($E_{100\%}$) after the IP was loaded onto the 2D SDS-PAGE and analyzed by Western blotting. $T_{25\%}$, 25% of the mitochondrial lysate before the IP that was used for 2D BN/SDS-PAGE. $NB_{25\%}$, 25% of the unbound material after the IP that was used for 2D BN/SDS-PAGE. These data are presented again in Fig. S2. (C) Mitochondria of the indicated mutants that harbored the *cox2::COB cob::ARG8*^{m} mitochondrial genome were analyzed as in A. The data for strains *Δqcr8*, *Δcyt1*, and *Δrip1* in C are presented again in D of this figure. (D) Mitochondria from wild type, *Δqcr8*, *Δcyt1*, and *Δrip1* were analyzed by 2D BN/SDS-PAGE and Western blotting with the indicated antibodies. The strains correspond to the ones in A and C. The Cyt*b* and Cbp3 data for strains *Δqcr8*, *Δcyt1*, and *Δrip1* are the same data presented in C, and the Cyt*b* and Cbp3 data for wild-type are the same data presented in A. The gray arrow in the *Δcyt1* strain points to the Qcr8 signal as the protein is hardly detectable in this mutant. The asterisk at the lower signal in the Cbp4 blot of the *Δrip1* strain corresponds to residual signal of the Qcr7 antibody, with which the membrane was probed before. (E) Schematic drawing of intermediates formed during cytochrome *b* assembly into the bc_1 complex. The exact composition of intermediate III (present in cells lacking cytochrome c_1 or Cor2) is at present not known.

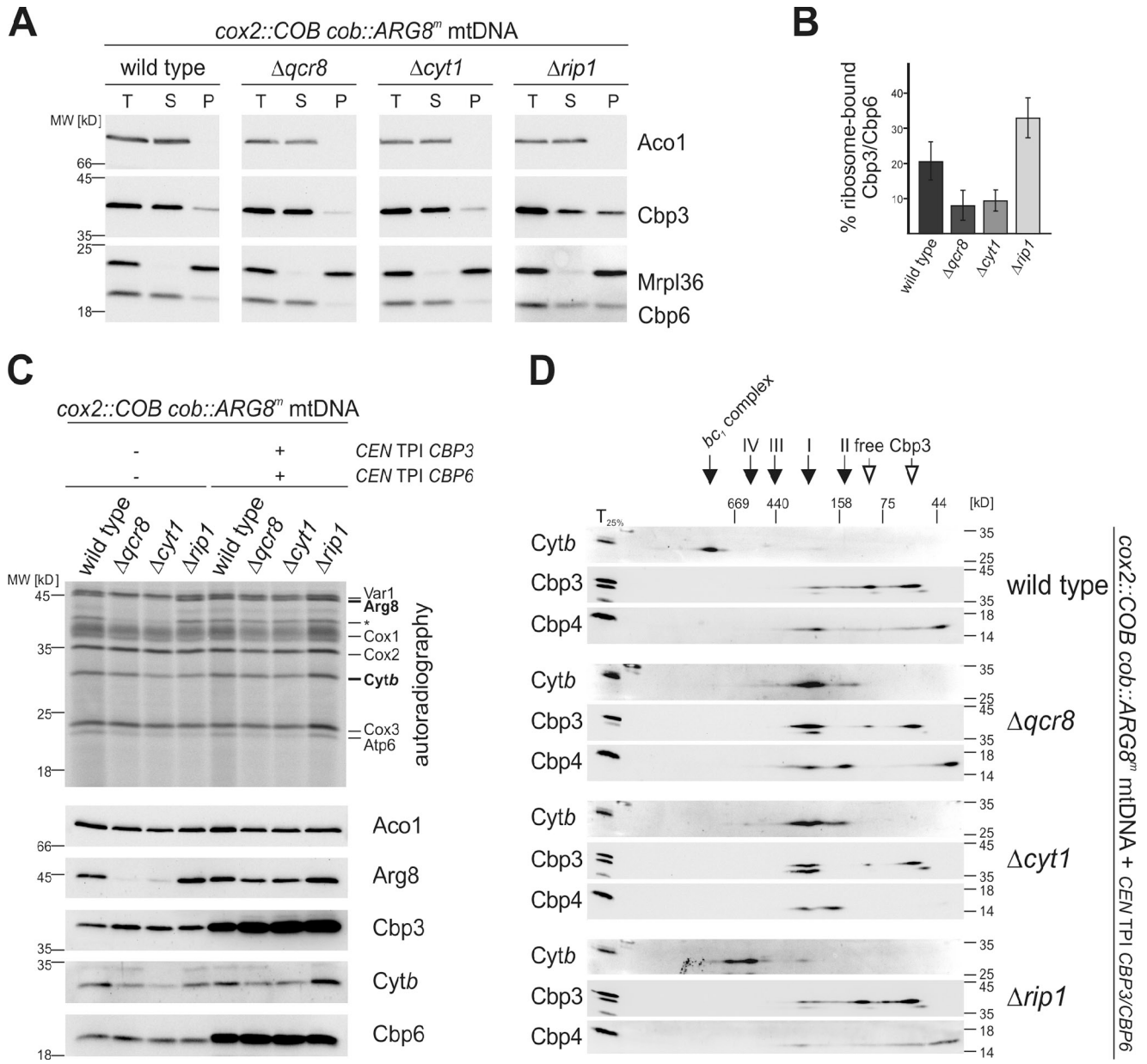

Figure 6. **Overexpression of Cbp3–Cbp6 allows regaining expression of *cob::ARG8*^{m} in cells where cytochrome *b* assembly is disturbed at early or intermediate steps.** (A) Less Cbp3–Cbp6 is ribosome-bound in *Δqcr8* and *Δcyt1* cells. Indicated mitochondria harboring the *cox2::COB cob::ARG8*^{m} mitochondrial genome were lysed in digitonin and fractionated by centrifugation through a high-density sucrose cushion. The fractions were analyzed by Western blotting and quantified using densitometry (B). *n* = 3, error bars are depicted. T, 100% total before centrifugation. S, supernatant after centrifugation. P, ribosome-containing pellet after centrifugation. Error bars indicate mean ± SD. (C) Simultaneous overexpression of *CBP3* and *CBP6* restores expression of the *ARG8*^{m} reporter in *Δqcr8* and *Δcyt1* cells. The indicated cells harboring the *cox2::COB cob::ARG8*^{m} mitochondrial genome were transformed with either empty plasmids or plasmids allowing the overexpression of *CBP3* and *CBP6* from single copy (*CEN*) plasmids with the strong *TPI* promoter. Mitochondrial translation products of these strains were radiolabeled and analyzed by autoradiography and Western blotting. The asterisk indicates a degradation product of Arg8. (D) Overexpression of *CBP3* and *CBP6* increases the cytochrome *b*–free pool of the Cbp3–Cbp6 complex. Mitochondria from the strains used in C overexpressing *CBP3* and *CBP6* were isolated and analyzed as in Fig. 4 A. The nomenclature of the intermediates is identical to Fig. 4 E.

two factors, Cbp3–Cbp6 has to bind to the tunnel exit of the mitochondrial ribosome to allow efficient synthesis of cytochrome *b* (Gruschke et al., 2011). By this, Cbp3–Cbp6 is optimally positioned to bind the newly synthesized protein, and it is this interaction that releases Cbp3–Cbp6 from the ribosome (Gruschke et al., 2011). Once released, the assembly factor Cbp4 is recruited (Gruschke et al., 2011) to form the first intermediate in the assembly line of cytochrome *b*. Importantly, this intermediate I is the main reservoir for unassembled cytochrome *b* and accumulates to easily detectable amounts in wild-type mitochondria. Moreover, most of the Cbp3–Cbp6 is present in this intermediate, whereas only a small quantity of Cbp3–Cbp6 is present as a cytochrome *b*–free form. Attachment of the early assembling subunits Qcr7 and Qcr8 to intermediate I provokes release of Cbp3–Cbp6, whereas Cbp4 remains bound and is therefore the assembly factor present in intermediate II. Binding of Cor1 and Cor2 to cytochrome *b* releases Cbp4, and intermediate III is formed. In the next step, the second

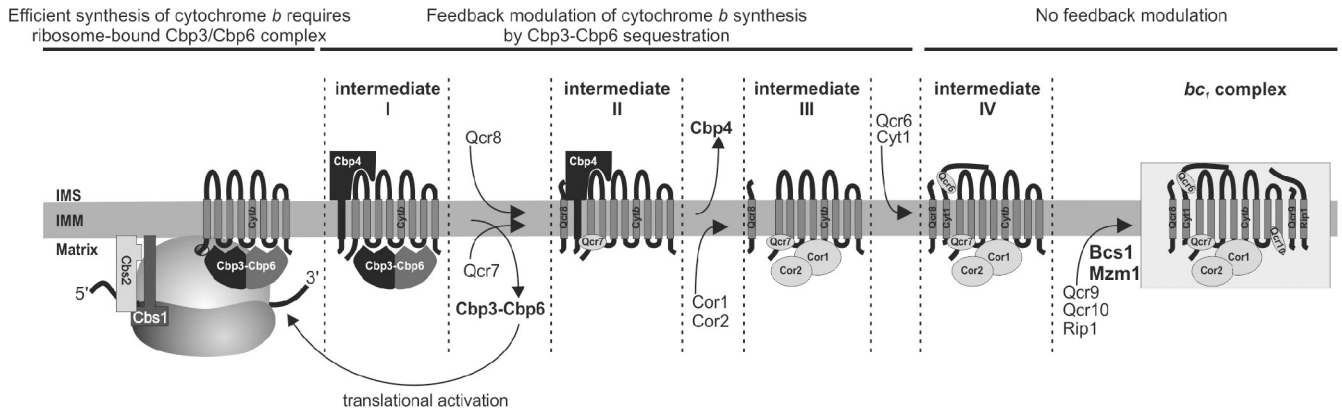

Figure 7. **Model of assembly of cytochrome *b* and the feedback loop modulating *COB* expression.** Biogenesis of cytochrome *b* can be divided into several steps. First, cytochrome *b* is synthesized on membrane-associated ribosomes that have the Cbp3–Cbp6 complex bound to the tunnel exit. For this step, the other translational activators Cbs1 and Cbs2 are also required. Next, the fully synthesized cytochrome *b* interacts with Cbp3–Cbp6 and is released from the ribosome. Cbp4 is then recruited to this complex, giving rise to assembly intermediate I. Qcr7 and Qcr8 are added to cytochrome *b* to form intermediate II, which provokes release of Cbp3–Cbp6 from cytochrome *b*. Next, the intermediate assembling subunits Cor1, Cor2, cytochrome c_1, and Qcr6 are added to form intermediate III and IV. The addition of Qcr9, Qcr10, and Rip1 completes assembly of the bc_1 complex. When formation of intermediate IV is compromised, intermediates I, II, and III pile up. Through this process, Cbp3–Cbp6 is sequestered in intermediate I and is no longer available to activate further rounds of *COB* expression.

catalytically active subunit, cytochrome c_1, and the structural subunit Qcr6 are added, giving rise to intermediate IV, previously described as the 500-kD complex (Zara et al., 2007). To this complex, the third catalytically active subunit, the Fe/S protein Rip1, and two accessory peptides (Qcr9 and Qcr10) are added to complete assembly of the bc_1 complex. In these late steps, two factors act on the newly imported Rip1 protein. First, Mzm1 stabilizes the Fe/S cluster in Rip1 and potentially supports its association with the AAA protein Bcs1 (Atkinson et al., 2011). Bcs1 then transports the folded Fe/S-containing Rip1 across the inner mitochondrial membrane and facilitates its integration into the bc_1 complex (Cruciat et al., 1999; Wagener et al., 2011).

The early steps of cytochrome *b* biogenesis are specifically organized to allow very efficient interaction of the newly synthesized cytochrome *b* with its dedicated assembly factor Cbp3–Cbp6 (Gruschke et al., 2011). Because *COB* mRNA is only translated efficiently when Cbp3–Cbp6 is bound to the ribosomal tunnel exit, it is ensured that the newly synthesized cytocrhome *b* can rapidly interact with Cbp3–Cbp6. Here, we found that this organization is not strictly required for cytochrome *b* assembly. When cytochrome *b* is synthesized from an mRNA containing the 5′ UTR of *COX2*, the ectopically expressed protein interacts with Cbp3–Cbp6 and can be fed into the bc_1 complex assembly line, resulting in respiratory chain activities that allow wild-type growth on respiratory media. Although produced at rates similar to the wild-type protein, significantly less cytochrome *b* can accumulate. The molecular reason for this is currently not known. However, the newly synthesized cytochrome *b* can pass through all the normal steps of bc_1 complex assembly. It is therefore tempting to speculate that ectopically expressed cytochrome *b* has a problem very early in its biogenesis. We observed that when cytochrome *b* is synthesized from its authentic mRNA, lower levels of free Cbp3 are detectable compared with the case where cytochrome *b* is expressed ectopically. This suggests that Cbp3–Cbp6 interacts most efficiently with cytochrome *b* in the situation where cytochrome *b* is produced from its authentic mRNA. By this, intermediate I would be readily formed, resulting in lower level of free Cbp3 in the wild type.

Importantly, we provide evidence here that the Cbp3–Cbp6 complex plays a key role in regulating cytochrome *b* expression in the context of bc_1 complex assembly. Specifically, we find that when assembly of the bc_1 complex is blocked at early or intermediate steps, expression of cytochrome *b* is reduced whereas blockage at later points has no influence on cytochrome *b* synthesis (Fig. 7). We analyzed the behavior of the Cbp3–Cbp6 complex and found that it can be present in two forms: first, it is part of intermediate I of the bc_1 complex assembly line and, second, it can be found in a form lacking cytochrome *b* that is implicated in activation of *COB* mRNA translation. Consistently, the latter form is well detectable in the wild type and mutants where late-assembling subunits are missing. In contrast, in mutants affected in cytochrome *b* expression because of feedback modulation, the translationally active free Cbp3–Cbp6 is almost absent. Blockage of intermediate assembly steps leads to the accumulation of cytochrome *b* in intermediate II that does not contain Cbp3–Cbp6. The increased population of intermediate I in such mutants could be explained by a queuing of assembly intermediates. Increasing the amount of Cbp3 and Cbp6 by overexpression results in higher levels of free Cbp3 and a concomitant increase in translational activation of *COB* mRNA. This clearly demonstrates that Cbp3–Cbp6 is sequestered in an assembly intermediate and that this sequestration modulates cytochrome *b* expression.

The regulatory feedback modulating synthesis of cytochrome *b* is similar to the case of Cox1. The *COX1* mRNA requires two translational activators, namely Pet309 and Mss51 (Decoster et al., 1990; Manthey and McEwen, 1995). Similar to Cbp3–Cbp6, Mss51 is a part of assembly intermediates, and accumulation of these intermediates restricts *COX1* expression by sequestration of Mss51 (Perez-Martinez et al., 2003; Barrientos

et al., 2004; Fontanesi et al., 2008; Mick et al., 2011). Likewise, overexpression of *MSS51* suppresses this effect (Barrientos et al., 2002). However, sequestration appears not to be the only mode of regulating *COX1* expression. In mutants lacking the assembly factor Coa1, Mss51 is also sequestered in an assembly intermediate, but *COX1* expression is not affected (Pierrel et al., 2007; Mick et al., 2011). This indicates that translational regulation by Mss51 could use additional, yet unknown mechanisms. A higher level of complexity might also be found in the case of Cbp3–Cbp6. Support for such a notion comes from the analysis of *Δqcr6* cells that also show reduced expression of *COB* but no accumulation of intermediate II, as observed in cells lacking Qcr7, Qcr8, Cor1, Cor2, and cytochrome c_1. The exact molecular mechanisms by which Mss51 and Cbp3–Cbp6 activate translation of their target mRNAs are not known. Given that free Cbp3 interacts with the mitochondrial ribosome to activate translation, it is tempting to speculate that Mss51, directly or indirectly, interacts with the translation machinery in a similar way.

In summary, our study shows that expression of cytochrome *b* is modulated depending on the efficiency of bc_1 complex assembly. In the case of cytochrome oxidase and ATP synthase, a regulation of synthesis of mitochondrially encoded subunits in the frame of complex assembly has previously been demonstrated (Barrientos et al., 2004; Rak and Tzagoloff, 2009). Our work shows that this is also the case for the bc_1 complex and thus provides the missing part to demonstrate that such regulatory circuits are a common feature for biogenesis of respiratory chain complexes of dual genetic origin. Because these feedback loops have now been demonstrated in yeast mitochondria, it will be exciting for future research to define whether similar ways to regulate translation also exist in mammalian mitochondria.

Materials and methods

Yeast strains and growth media

All strains used in this study were isogenic to the wild-type strain W303. The strains contained various mitochondrial genomes (intronless, *cob::ARG8^m*, and *cox2::COB cob::ARG8^m* with a wild-type *COX2* gene) and harbored an *arg8::HIS3* mutation in the nuclear genome. *CBP3*, *CBP6*, *CBP4*, *BCA1*, *BCS1*, *MZM1*, *QCR7*, *QCR8*, *COR1*, *COR2*, *CYT1*, *QCR6*, *QCR9*, *QCR10*, and *RIP1* were disrupted using a Kanamycin resistance cassette (Table S1). Plasmids overexpressing *CBP3* or *CBP6* were constructed by inserting the *CBP3* open reading frame (ORF) between the NcoI and HindIII sites of pYX132 (*CEN* plasmid, *triosephosphate isomerase* [*TPI*] promoter, *TRP1* selectable marker) or the *CBP6* ORF between the NcoI and SalI sites of pYX142 (*CEN* plasmid, *TPI* promoter, *LEU2* selectable marker), respectively, and transformed simultaneously into yeast cells. Yeast cultures were grown at 30°C in minimal medium (0.17% yeast nitrogen base and 0.5% ammonium sulfate) or YP medium (1% yeast extract and 2% peptone) supplemented with 2% dextrose, 2% galactose, or 2% glycerol.

Construction of the cox2::COB cob::ARG8^m mitochondrial genome

Biolistic transformation (Bonnefoy and Fox, 2007) was used to manipulate the mitochondrial DNA of *S. cerevisiae* to relocate the *COB* ORF under the control of *COX2* regulatory sequences upstream of the *COX2* gene. This was done in a strain where the *ARG8^m* reporter replaced the bona fide *COB* gene (YTE31; Ding et al., 2008), a gift from T. Fox (Cornell University, Ithaca, NY). First, two silent mutations, $G_{291}A$ and $T_{657}C$, were introduced into the 1,158-nucleotide intronless *S. cerevisiae COB* ORF to eliminate internal NdeI and EcoRI sites. Restriction sites were also created on each side of this recoded *COB* gene: NdeI at the 5′ end by adding CAT upstream of the ATG initiation codon, and XhoI at the 3′ end by fusing CTCAGA immediately after the stop codon. These sites were used to flank the *COB* recoded ORF with the *COX2* regulatory regions. At the 5′ end, a 76-bp sequence was added composed of an EcoRI site fused to 70 bp of the region upstream of the *COX2* gene (positions −73 to −4 relative to the ATG): this *COX2* region contains the promoter and most of the sequence specifying the *COX2* 5′ UTR. At the 3′ end of the recoded *COB* gene, the 119 bp located immediately downstream of the *COX2* stop codon fused to an EcoRI site were added after the XhoI site: these 119 bp contain the dodecamer signal for mRNA processing. This insert was cloned in the forward orientation into the engineered EcoRI site upstream of *COX2* in pPT24-wt, a derivative of pPT24 (Thorsness and Fox, 1993), where the *COX2-RM220* mutation (Fox, 1979) has been corrected to wild type by site-directed mutagenesis. The final plasmid pPT24-wt-COB thus contains ∼200 bp of authentic mitochondrial DNA, the chimeric *cox2::COB* gene, and 2.3 kb of mitochondrial DNA including the full *COX2* ORF 300 bp downstream of the chimeric *COB* gene. The plasmid pPT24-wt-COB was bombarded into the *rho° kar1-1* strain DFS160 (Steele et al., 1996), and positive mitochondrial transformants were detected by mating with the tester strain NB160 (Bonnefoy and Fox, 2000). Positive clones were subcloned several times and retested by crossing with NB160 to yield the stable synthetic *rho*$^-$ NB376. This strain was crossed with YTE31 that contains a *cob::ARG8^m* construct in place of the normal *COB* gene and lacks at least the *COX1* intron *ai4* that is dependent on the *COB* maturase *bi4* for its excision (Ding et al., 2008). As expected, the resulting diploid NB378 could grow on glycerol and on media lacking arginine. After sporulation and tetrad dissection, NB378 *kar1-1* spores were selected that contain the final *cox2::COB* construct integrated into a *cob::ARG8^m COX1-Δi rho+* genome. These spores were used to transfer the new mitochondrial genome into various recipient strains by cytoduction (Bonnefoy and Fox, 2007).

Isolation of mitochondria

Yeast cells were grown to mid-exponential phase (OD_{595nm} = 1.3) and harvested by centrifugation (3,000 *g*, 5 min). After washing once with distilled water, the cell pellet was resuspended (2 ml/g cell wet weight) in MP1 buffer (0.1 M Tris-base and 10 mM dithiothreitol) and incubated for 10 min at 30°C. Cells were harvested, washed once with 1.2 M sorbitol, resuspended (6.7 ml/g cell wet weight) in MP2 buffer (20 mM KP_i pH7.4, 0.6 M sorbitol, and 3 mg/g cell wet weight zymolyase 20T [Seikagaku Corporation]), and incubated with shaking for 1 h at 30°C for spheroplastation. Spheroplasts were harvested (3,000 *g*, 5 min, 4°C) and resuspended (13.4 ml/g 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]). The following steps were performed on ice and centrifugations were performed at 4°C. After homogenizing the spheroplast suspension twice by 10–15 strokes of a Teflon plunger in a tight fitting homogenizer (Sartorius AG), the homogenate was centrifuged at 3,000 *g* for 5 min. Centrifugation was repeated once and mitochondria were then harvested by centrifugation at 17,000 *g* for 12 min. The pellet was resuspended in SH buffer (0.6 M sorbitol and 20 mM Hepes, pH 7.4) to a final concentration of 10 mg/ml. Mitochondria were snap-frozen in liquid nitrogen.

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 0.15 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 the addition of 6 µCi [^{35}S]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 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 taking samples after the indicated time points, which were lysed and TCA precipitated. Pellets were resolved in sample buffer, subjected to SDS-PAGE on 16:0.2% acrylamide/bisacrylamide gels, and analyzed by autoradiography and Western blotting.

Fractionation of mitochondrial lysates

Isolated mitochondria (300 µg) were lysed for 30 min on ice in lysis buffer containing 1% digitonin, 50 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, T) and the rest was under-layered 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 (S) was then collected and the ribosome-containing pellet (P) 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 bc_1 complex assembly intermediates by 1D BN PAGE and 2D BN/SDS-PAGE

Isolated mitochondria (250 µg) were lysed in BN PAGE solubilization buffer (1% digitonin, 50 mM NaCl, 50 mM imidazole, pH 7, 1 mM EDTA, 1 mM PMSF, 2 mM aminohexanoic acid, and 10% glycerol) for 30 min on ice. After a clarifying spin for 10 min at 25,000 *g* at 4°C, 25% of the lysate was taken as a total and the rest was loaded onto a linear (4–13%) BN polyacrylamide gradient gel. Electrophoresis was performed at 4°C. After one third of the run, the cathode buffer (50 mM Tricine, 7.5 mM imidazole, pH unadjusted, and 0.02% Coomasie G-250) was replaced by cathode buffer B/10 (50 mM Tricine and 7.5 mM imidazole, pH unadjusted; Wittig et al., 2006). For 1D BN PAGE analysis, the gel was stained with Coomassie brilliant blue. For the 2D SDS PAGE, gel slices from the first dimension were incubated in SDS running buffer containing 1% SDS, incubated for 20 min at 37°C, and placed vertically onto a SDS polyacrylamide gel. Separated protein complexes were analyzed by Western blotting.

Immunodepletion of bc_1 complex assembly intermediates

Isolated mitochondria (500 µg) were lysed in solubilization buffer (1% digitonin, 50 mM KCl, 50 mM imidazole, pH 7.4, 20 mM Hepes/KOH pH 7.4, 1 mM EDTA, 1 mM PMSF, 2 mM aminohexanoic acid, and 10% glycerol) for 30 min on ice. The lysate was cleared at 25,000 *g* at 4°C for 10 min. Antibodies against Cbp3 were incubated with protein A–Sepharose beads and added to half of the mitochondrial lysate to immunoprecipitate Cbp3-containing complexes. The unbound material (NB) as well as the other half of the lysate (T) was subjected to 2D BN/SDS-PAGE. 25% of the samples were loaded directly on the SDS-PAGE and 75% of the samples were used for the 1D BN PAGE. 2D BN/SDS-PAGE was performed as described in the previous paragraph. The bound material after the immunoprecipitation (E) was eluted from the washed beads with SDS sample buffer and analyzed by SDS PAGE.

Assembly kinetics of the bc_1 complex

To facilitate the assembly of newly synthesized cytochrome *b*, nuclear encoded proteins were accumulated in mitochondria before their isolation. To this end, mitochondrial translation was blocked by adding chloramphenicol to a final concentration of 4 mg/ml to yeast cultures 2 h before isolation of mitochondria. To label mitochondrial translation products, 800 µg of chloramphenicol pretreated mitochondria were incubated in 800 µl of in organello buffer (20 mM Hepes/KOH, pH 7.4, 15 mM potassium phosphate buffer, pH 7.4, 0.6 M sorbitol, 150 mM KCl, 12.66 mM $MgSO_4$, 12.15 µg/ml amino acid mix [without Tyr, Cys, or Met], 66.66 µM cysteine, 12.13 µg/ml tyrosine, 7.5 mM phosphoenolpyruvate, 6 mM ATP, 0.75 mM GTP, 5 mM α-Ketoglutarate, and 10 µg/ml pyruvate kinase) at 30°C. After 2 min, mitochondrial translation was initiated by adding 80 µCi [^{35}S]methionine and stopped 5 min later by adding nonradioactive methionine to a final concentration of 10 mM and puromycin to a final concentration of 80 µM. Assembly of newly synthesized mitochondrially encoded proteins was allowed to continue at 30°C. After a chase period of 0, 5, 15, or 30 min, 25% of the initial reaction was harvested and diluted 1:5 in ice-cold SH buffer (20 mM Hepes/KOH, pH 7.4, and 0.6 M sorbitol). Mitochondria were reisolated by centrifugation at 16,000 *g* at 4°C for 10 min and lysed in 50 µl of BN PAGE solubilization buffer (1% digitonin, 50 mM NaCl, 50 mM imidazole, pH 7, 1 mM EDTA, 1 mM PMSF, 2 mM aminohexanoic acid, and 10% glycerol) for 30 min on ice. Lysates were clarified by centrifugation at 25,000 *g* for 10 min at 4°C and analyzed by 2D BN/SDS-PAGE.

Miscellaneous

Antibodies against Qcr7, Qcr8, and cytochrome *b* were obtained by immunizing rabbits with purified MBP-Qcr8, MBP-Qcr7, and peptide HGSSNPLGITGNLDRIPMHSYFI (amino acids 204–226 of cytochrome *b* coding sequence), respectively. The antibody against Arg8 was a gift from T. Fox (Cornell University). Signals from autoradiography or Western blotting were quantified with ImageJ software.

Online supplemental material

Fig. S1 shows that cells carrying the *cox2::COB cob::ARG8^m* mitochondrial genome and lacking Cbp6 show the same phenotype as the corresponding *Δcbp3* mutant. Fig. S2 shows that the 170 kD complex (intermediate I) is composed of Cbp3, Cbp6, Cbp4, and cytochrome *b*, and can be specifically depleted using an antibody against Cbp3. Table S1 shows a summary of the yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201206040/DC1.

We are grateful to Tom Fox (Cornell University) for generously sharing materials. We would like to thank Alex Tzagoloff (Columbia University) for helpful discussions, Martin Jung (University of Homburg, Germany) for help with antibody production, and Claes Andreasson (Stockholm University, Sweden) for critical reading of the manuscript. We thank Mauricette Gaisne for the correction of the *COX2-RM220* mutation present in the original pPT24 plasmid.

This work was supported by the Swedish Research Council (VR), the Center for Biomembrane Research (CBR) at Stockholm University, the German Research Council (research unit 967), the Stiftung Rheinland-Pfalz fuer Innovation, Germany, and by the Agence Nationale pour la Recherche (ANR) JCJC06-0163. Exchange between our laboratories was supported by PROCOPE2010 from the French ministry and the German Academic Exchange Service (DAAD), Germany. I. Kühl was supported by ANR and by a grant from the Fondation pour la Recherche Médicale (FDT20091217787), France. K. Kehrein was a recipient of a predoctoral fellowship from the Carl Zeiss foundation, Germany, and M. Hildenbeutel is supported by a post-doctoral stipend from the Wenner Gren-Foundation, Stockholm, Sweden.

Submitted: 11 June 2012
Accepted: 23 August 2012

References

Atkinson, A., P. Smith, J.L. Fox, T.Z. Cui, O. Khalimonchuk, and D.R. Winge. 2011. The LYR protein Mzm1 functions in the insertion of the Rieske Fe/S protein in yeast mitochondria. *Mol. Cell. Biol.* 31:3988–3996. http://dx.doi.org/10.1128/MCB.05673-11

Barrientos, A., D. Korr, and A. Tzagoloff. 2002. Shy1p is necessary for full expression of mitochondrial *COX1* in the yeast model of Leigh's syndrome. *EMBO J.* 21:43–52. http://dx.doi.org/10.1093/emboj/21.1.43

Barrientos, A., A. Zambrano, and A. Tzagoloff. 2004. Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in *Saccharomyces cerevisiae*. *EMBO J.* 23:3472–3482. http://dx.doi.org/10.1038/sj.emboj.7600358

Bonnefoy, N., and T.D. Fox. 2000. *In vivo* analysis of mutated initiation codons in the mitochondrial *COX2* gene of *Saccharomyces cerevisiae* fused to the reporter gene *ARG8^m* reveals lack of downstream reinitiation. *Mol. Gen. Genet.* 262:1036–1046. http://dx.doi.org/10.1007/PL00008646

Bonnefoy, N., and T.D. Fox. 2007. Directed alteration of *Saccharomyces cerevisiae* mitochondrial DNA by biolistic transformation and homologous recombination. *Methods Mol. Biol.* 372:153–166. http://dx.doi.org/10.1007/978-1-59745-365-3_11

Chacinska, A., C.M. Koehler, D. Milenkovic, T. Lithgow, and N. Pfanner. 2009. Importing mitochondrial proteins: machineries and mechanisms. *Cell*. 138:628–644. http://dx.doi.org/10.1016/j.cell.2009.08.005

Crivellone, M.D. 1994. Characterization of *CBP4*, a new gene essential for the expression of ubiquinol-cytochrome *c* reductase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 269:21284–21292.

Cruciat, C.M., K. Hell, H. Fölsch, W. Neupert, and R.A. Stuart. 1999. Bcs1p, an AAA-family member, is a chaperone for the assembly of the cytochrome *bc(1)* complex. *EMBO J.* 18:5226–5233. http://dx.doi.org/10.1093/emboj/18.19.5226

Cruciat, C.M., S. Brunner, F. Baumann, W. Neupert, and R.A. Stuart. 2000. The cytochrome bc_1 and cytochrome *c* oxidase complexes associate to form a single supracomplex in yeast mitochondria. *J. Biol. Chem.* 275:18093–18098. http://dx.doi.org/10.1074/jbc.M001901200

Decoster, E., M. Simon, D. Hatat, and G. Faye. 1990. The *MSS51* gene product is required for the translation of the *COX1* mRNA in yeast mitochondria. *Mol. Gen. Genet.* 224:111–118. http://dx.doi.org/10.1007/BF00259457

DeRisi, J.L., V.R. Iyer, and P.O. Brown. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*. 278:680–686. http://dx.doi.org/10.1126/science.278.5338.680

Ding, M.G., C.A. Butler, S.A. Saracco, T.D. Fox, F. Godard, J.P. di Rago, and B.L. Trumpower. 2008. Introduction of cytochrome *b* mutations in *Saccharomyces cerevisiae* by a method that allows selection for both functional and non-functional cytochrome *b* proteins. *Biochim. Biophys. Acta*. 1777:1147–1156. http://dx.doi.org/10.1016/j.bbabio.2008.04.029

Fontanesi, F., I.C. Soto, D. Horn, and A. Barrientos. 2006. Assembly of mitochondrial cytochrome *c*-oxidase, a complicated and highly regulated cellular process. *Am. J. Physiol. Cell Physiol.* 291:C1129–C1147. http://dx.doi.org/10.1152/ajpcell.00233.2006

Fontanesi, F., I.C. Soto, and A. Barrientos. 2008. Cytochrome *c* oxidase biogenesis: new levels of regulation. *IUBMB Life*. 60:557–568. http://dx.doi.org/10.1002/iub.86

Fox, T.D. 1979. Five TGA "stop" codons occur within the translated sequence of the yeast mitochondrial gene for cytochrome *c* oxidase subunit II. *Proc. Natl. Acad. Sci. USA*. 76:6534–6538. http://dx.doi.org/10.1073/pnas.76.12.6534

Fox, T.D., M.C. Costanzo, C.A. Strick, D.L. Marykwas, E.C. Seaver, and J.K. Rosenthal. 1988. Translational regulation of mitochondrial gene expression by nuclear genes of *Saccharomyces cerevisiae*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 319:97–105. http://dx.doi.org/10.1098/rstb.1988.0034

Gruschke, S., K. Kehrein, K. Römpler, K. Gröne, L. Israel, A. Imhof, J.M. Herrmann, and M. Ott. 2011. Cbp3-Cbp6 interacts with the yeast mitochondrial ribosomal tunnel exit and promotes cytochrome *b* synthesis and assembly. *J. Cell Biol.* 193:1101–1114. http://dx.doi.org/10.1083/jcb.201103132

Kühl, I., T.D. Fox, and N. Bonnefoy. 2012. *Schizosaccharomyces pombe* homologs of the *Saccharomyces cerevisiae* mitochondrial proteins Cbp6 and Mss51 function at a post-translational step of respiratory complex biogenesis. *Mitochondrion*. 12:381–390. http://dx.doi.org/10.1016/j.mito.2012.02.002

Manthey, G.M., and J.E. McEwen. 1995. The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae*. *EMBO J.* 14:4031–4043.

Mathieu, L., S. Marsy, Y. Saint-Georges, C. Jacq, and G. Dujardin. 2011. A transcriptome screen in yeast identifies a novel assembly factor for the mitochondrial complex III. *Mitochondrion*. 11:391–396. http://dx.doi.org/10.1016/j.mito.2010.12.002

Mick, D.U., T.D. Fox, and P. Rehling. 2011. Inventory control: cytochrome *c* oxidase assembly regulates mitochondrial translation. *Nat. Rev. Mol. Cell Biol.* 12:14–20. http://dx.doi.org/10.1038/nrm3029

Neupert, W., and J.M. Herrmann. 2007. Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* 76:723–749. http://dx.doi.org/10.1146/annurev.biochem.76.052705.163409

Nobrega, F.G., M.P. Nobrega, and A. Tzagoloff. 1992. *BCS1*, a novel gene required for the expression of functional Rieske iron-sulfur protein in *Saccharomyces cerevisiae*. *EMBO J.* 11:3821–3829.

Ohlmeier, S., A.J. Kastaniotis, J.K. Hiltunen, and U. Bergmann. 2004. The yeast mitochondrial proteome, a study of fermentative and respiratory growth. *J. Biol. Chem.* 279:3956–3979. http://dx.doi.org/10.1074/jbc.M310160200

Perez-Martinez, X., S.A. Broadley, and T.D. Fox. 2003. Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p. *EMBO J.* 22:5951–5961. http://dx.doi.org/10.1093/emboj/cdg566

Pierrel, F., M.L. Bestwick, P.A. Cobine, O. Khalimonchuk, J.A. Cricco, and D.R. Winge. 2007. Coa1 links the Mss51 post-translational function to Cox1 cofactor insertion in cytochrome *c* oxidase assembly. *EMBO J.* 26:4335–4346. http://dx.doi.org/10.1038/sj.emboj.7601861

Prestele, M., F. Vogel, A.S. Reichert, J.M. Herrmann, and M. Ott. 2009. Mrpl36 is important for generation of assembly competent proteins during mitochondrial translation. *Mol. Biol. Cell*. 20:2615–2625. http://dx.doi.org/10.1091/mbc.E08-12-1162

Rak, M., and A. Tzagoloff. 2009. F1-dependent translation of mitochondrially encoded Atp6p and Atp8p subunits of yeast ATP synthase. *Proc. Natl. Acad. Sci. USA*. 106:18509–18514. http://dx.doi.org/10.1073/pnas.0910351106

Rak, M., S. Gokova, and A. Tzagoloff. 2011. Modular assembly of yeast mitochondrial ATP synthase. *EMBO J.* 30:920–930. http://dx.doi.org/10.1038/emboj.2010.364

Rödel, G. 1986. Two yeast nuclear genes, *CBS1* and *CBS2*, are required for translation of mitochondrial transcripts bearing the 5′-untranslated *COB* leader. *Curr. Genet.* 11:41–45. http://dx.doi.org/10.1007/BF00389424

Siep, M., K. van Oosterum, H. Neufeglise, H. van der Spek, and L.A. Grivell. 2000. Mss51p, a putative translational activator of cytochrome *c* oxidase subunit-1 (*COX1*) mRNA, is required for synthesis of Cox1p in *Saccharomyces cerevisiae*. *Curr. Genet.* 37:213–220. http://dx.doi.org/10.1007/s002940050522

Smith, P.M., J.L. Fox, and D.R. Winge. 2012. Biogenesis of the cytochrome bc(1) complex and role of assembly factors. *Biochim. Biophys. Acta*. 1817:276–286. http://dx.doi.org/10.1016/j.bbabio.2011.11.009

Steele, D.F., C.A. Butler, and T.D. Fox. 1996. Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. *Proc. Natl. Acad. Sci. USA*. 93:5253–5257. http://dx.doi.org/10.1073/pnas.93.11.5253

Thorsness, P.E., and T.D. Fox. 1993. Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. *Genetics*. 134:21–28.

Wagener, N., M. Ackermann, S. Funes, and W. Neupert. 2011. A pathway of protein translocation in mitochondria mediated by the AAA-ATPase Bcs1. *Mol. Cell*. 44:191–202. http://dx.doi.org/10.1016/j.molcel.2011.07.036

Wittig, I., H.P. Braun, and H. Schägger. 2006. Blue native PAGE. *Nat. Protoc.* 1:418–428. http://dx.doi.org/10.1038/nprot.2006.62

Wu, M., and A. Tzagoloff. 1989. Identification and characterization of a new gene (*CBP3*) required for the expression of yeast coenzyme QH2-cytochrome *c* reductase. *J. Biol. Chem.* 264:11122–11130.

Zara, V., I. Palmisano, L. Conte, and B.L. Trumpower. 2004. Further insights into the assembly of the yeast cytochrome bc_1 complex based on analysis of single and double deletion mutants lacking supernumerary subunits and cytochrome *b*. *Eur. J. Biochem.* 271:1209–1218. http://dx.doi.org/10.1111/j.1432-1033.2004.04024.x

Zara, V., L. Conte, and B.L. Trumpower. 2007. Identification and characterization of cytochrome $bc(_1)$ subcomplexes in mitochondria from yeast with single and double deletions of genes encoding cytochrome $bc(_1)$ subunits. *FEBS J.* 274:4526–4539. http://dx.doi.org/10.1111/j.1742-4658.2007.05982.x

Zara, V., L. Conte, and B.L. Trumpower. 2009a. Biogenesis of the yeast cytochrome bc_1 complex. *Biochim. Biophys. Acta*. 1793:89–96. http://dx.doi.org/10.1016/j.bbamcr.2008.04.011

Zara, V., L. Conte, and B.L. Trumpower. 2009b. Evidence that the assembly of the yeast cytochrome bc_1 complex involves the formation of a large core structure in the inner mitochondrial membrane. *FEBS J.* 276:1900–1914. http://dx.doi.org/10.1111/j.1742-4658.2009.06916.x